Exercise effects on lung tumor metastases and in vitro alveolar macrophage antitumor cytotoxicity

J. M. Davis, M. L. Kohut, D. A. Jackson, L. H. Colbert, E. P. Mayer, and A. Ghaffar
Department of Exercise Science, School of Public Health; and Department of Microbiology and Immunology, School of Medicine, University of South Carolina, Columbia, South Carolina 29208

EVIDENCE THAT PHYSICAL ACTIVITY may protect against cancer has accumulated over the past few years, and a number of reviews on this topic have been published (5, 14, 28, 30). Several studies reported that the incidence of all-cancer mortality is inversely associated with physical activity (5, 22). An inverse relationship also appears to exist between physical activity and site-specific cancers such as that of the breast and colon. However, the strength of the association at several sites remains questionable, and the amount of activity required to reduce the risk of cancer has not been established. Animal models of tumor development typically demonstrate that exercise inhibits tumorigenesis (1, 6, 11, 21, 26, 32), although a few exceptions to this general finding have been reported (7, 33, 34). The intensity and duration of exercise as well as the timing of exercise in relation to tumor administration varied in these investigations, and it is possible that this accounts for the difference in results. It has also been suggested that the probability of observing a protective effect of exercise increases as intensity and duration increase (32).

The role of exercise in tumor metastases, perhaps the most devastating aspect of cancer, has not been widely studied. The lung is one of the major sites of metastases. Results from the few studies focusing on exercise and metastases showed that a single bout of swimming in rats increased lung metastases twofold (4), whereas 8–9 wk of exercise training either decreased or had no effect on pulmonary metastases (12, 16, 17). Variations in the type, duration, and intensity of exercise as well as the species of animal used make firm conclusions difficult to draw. Further characterization of exercise and the metastatic process is warranted in view of the fact that the primary cause of death of cancer patients is therapy-resistant metastases.

The mechanisms underlying exercise and subsequent tumor development have not yet been identified. Modulation of immune function may be one mechanism responsible for altered tumor growth associated with exercise. Effector cells of the innate immune system such as the macrophage or Natural killer (NK) cell are cytotoxic to a number of tumor cells in vitro as well as in vivo (2, 9). During and/or after a bout of exercise, alterations in the functions of these cells have been reported (12, 17, 18, 36, 37). Few studies have simultaneously examined the effects of exercise on both metastases and immune function. However, one recent investigation reported a decrease in lung metastases, although no change in splenic NK cell activity was observed (16). In another study by the same group, no change in lung metastases occurred as a result of chronic exercise, even though NK activity was increased (17). Therefore, a systematic characterization of other potential immune effectors and metastatic spread in response to several doses of exercise may provide important insight into those mechanisms mediating the postulated inverse association between physical exercise and cancer.

The purpose of this study was to determine whether an acute bout of exercise can alter pulmonary metastases of B16 melanoma and alveolar macrophage antitumor function. Because some of the literature in this area suggests that there may be a critical dose of exercise necessary to observe an effect on tumorigenesis, both low and high doses of exercise were used.

METHODS

Animals. Pathogen-free male C57BL/6 mice, 6–8 wk of age, purchased from Harlan Labs (Madison, WI) were used in all experiments. Animals were acclimated to the animal facility for ~3 days. Animals were housed five per cage and maintained on a 12:12-h light-dark cycle in a low-stress environment (22°C, 50% relative humidity, low noise). All mice were fed Purina Chow and received water ad libitum.

Exercise. All mice were acclimated to treadmill running for a 3-day period at a speed of 1–12 m/min for 10 min/day. Mice were then randomly assigned to one of the following exercise or control groups: Ex-30, Ex-F, Con, or Ex-F-24 h. Ex-30 mice were killed immediately after exercise or 8 h later. Alveolar macrophages from Ex-F was lower than Con after exercise and, to a lesser extent, 8 h later. In Ex-30, this effect was only observed immediately after exercise. The data suggest that prolonged exercise has a protective effect on lung tumor metastases and enhances alveolar macrophage antitumor cytotoxicity.

B16 melanoma; cancer; mice; immunity; running
run for a 30-min period at 18 m/min on a 5% grade, which is estimated to elicit 55–65% maximum oxygen consumption (V\textsubscript{O2max}). Ex-F mice ran the first 30 min at 18 m/min on a 5% grade, and thereafter the treadmill speed was increased 3 m/min every 30 min until fatigue, which occurred at ~3 h. The exercise intensity at fatigue was estimated to be 68–78% V\textsubscript{O2max}. Con mice remained in lanes directly above the treadmill and were exposed to the same handling and noise stress without actually running. In the second metastasis experiment, 20 mice were assigned to Con, with 10 killed after 30 min and 10 killed at 2–3 h. No effect of death time on tumor foci was noted, and so the data for all 20 mice were combined. For the high-dose tumor experiment, an Ex-F24 h group was included. Ex-F24 h mice also ran to the point of fatigue; however, the exercise was completed 24 h before any experimental manipulations. Fatigue was defined as that point at which several minutes of gentle prodding with the hand was no longer a sufficient stimulus to encourage mice to keep pace with the treadmill speed. Electric shock was never used in these experiments because mice respond well to gentle hand prodding. After one acute bout of exercise or control treatment, mice were either killed for cell collection or injected with tumor cells and returned to the animal facility for 7–10 days.

Tumor metastasis. The tumor cell line B16F1 melanoma (ATCC no. CRL 6323; American Type Culture Collection, Rockville, MD), which is syngeneic to the C57BL/6 mouse strain, was used in all experiments. Tumor cells were maintained in RPMI media (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum (Environmental Diagnostics, Burlington, NC). The adherent B16 cells were removed from tissue culture flaskse by incubating them for 10 min with trypsin-EDTA (GIBCO BRL). Cells were harvested by centrifugation, washed once, and adjusted to concentrations ranging from 5 x 10\textsuperscript{5} to 5 x 10\textsuperscript{6} cells/ml. To assess the development of pulmonary metastases after exercise, 0.2 ml of B16 melanoma cells was injected into a tail vein. Two concentrations of tumor cells were tested, a high dose (5 x 10\textsuperscript{6} cells/ml) and a low dose (5 x 10\textsuperscript{5} cells/ml). The Ex-30, Ex-F, and Con groups of mice were injected with tumor cells 30 min after their respective exercise or control treatments. Mice in the Ex-F24 h group were killed 24 h after completing the exercise session. After tumors were injected into the tail vein, mice were returned to their cages and remained in the animal facility. Mice receiving the low dose of tumor cells were killed 7 days later, whereas mice receiving the low dose of tumor cells were killed 10 days after tumor inoculation. The lungs were removed at death and stained with Bouin's fixative. The number of tumor foci (pulmonary metastases) on the surface of the lungs was counted under a dissecting microscope by an investigator blinded to the treatment groups.

Radiolabeled B16 melanoma cells were injected to assess the pattern of tumor cell arrest in response to exercise. The B16 cells were labeled by incubating them with sodium chromate ([\textsuperscript{51}Cr]) (ICN Biomedicals, specific activity 292 mCi/mg Cr) for 1 h and then washing the cells several times to remove any excess label. Ex-30, Ex-F, and Con mice were injected with the radiolabeled B16 cells 15 min postexercise. Again, 0.2 ml of cells at a concentration of 1 x 10\textsuperscript{5} cells/ml (2 x 10\textsuperscript{5} cells/mouse) were injected into the lateral tail vein. Thirty minutes after the tumor injection, all mice were killed, and lungs, liver, spleen, and blood were collected from each animal. These organs were weighed and then placed in a gamma counter to determine the percentage of radiolabeled cells localized in each organ. In an additional experiment, Ex-F and Con mice were killed at several time points postinoculation (30 min, 4 h, and 16 h) to examine the time course of tumor cell retention within the lung.

In vitro alveolar macrophage cytotoxicity. After one bout of exercise, Ex-30, Ex-F, and Con mice were killed at two time points: immediately postexercise and 8 h postexercise. The lungs were removed and lavaged with 10–15 ml of RPMI. The lung lavage cells were then washed, and contaminating red blood cells were lysed with Tris ammonium chloride. Alveolar cells were seeded in 96-well flat-bottom microtiter plates at several concentrations and maintained at 37°C and 5% CO\textsubscript{2} for 3 h to allow macrophages to adhere to the plate. Nonadherent cells were then removed by gentle washing. B16 cells (5 x 10\textsuperscript{5}) were then added to each well and to several control wells without macrophages. The plate was then incubated for 48 h at 37°C and 5% CO\textsubscript{2}. At this time, 0.25 μCi of [\textsuperscript{3}H]thymidine (Tdr) (ICN Biomedicals, specific activity 6.7 Ci/mmol) was added to each well. The plates were incubated for 16 h, and the amount of Tdr incorporated was determined by scintillation counting.

Tdr uptake in the wells containing only the B16 tumors were used as control values. This uptake measured "uninhibited" growth of the tumor cells; therefore any Tdr uptake less than this represents cytostasis or cytolyis of the tumor cells by the macrophages. Percent cytotoxicity was calculated as follows: (Tdr uptake in test well/Tdr uptake in control well) x 100. Macrophages do not proliferate and incorporate Tdr in this assay. Therefore, tumor cell growth is quantified by measuring Tdr uptake. This assay measures both macrophage-mediated tumor growth inhibition (cytostasis) and cytolysis, because lysed cells do not incorporate Tdr.

Statistical analysis. All data were analyzed using the SAS statistical package (SAS Institute, Cary, NC). Lung tumor foci, localization of radiolabeled cells, and macrophage cytotoxicities were analyzed by one-way ANOVA (P < 0.05). Radioactivity (counts/min) in the localization experiment with three time points was analyzed using a two-way ANOVA (P < 0.05). Post hoc comparisons between groups were made using t-tests with the Bonferroni adjustment, with statistical significance set at P < 0.017 and P < 0.008 for the experiments with three and four groups, respectively. P values between 0.05 and the adjusted value were treated as trends.

RESULTS

Exercise and pulmonary metastases. The number of lung tumor foci was compared among all groups to determine whether exercise could alter the development of pulmonary tumor metastases. A difference in the number of lung tumor foci was observed among groups (P < 0.05, Fig. 1). Ex-F mice had significantly fewer tumors than mice in either the Con or Ex-30 groups. The same effect was observed after the injection of either a high (Fig. 1A) or low dose (Fig. 1B) of tumor cells. Mice exercised 24 h before tumor injection (Ex-F24 h) showed a trend (P < 0.04) toward fewer tumor foci than the Con mice (Fig. 1B). There was no difference between the number of foci in Ex-30 and Con mice. A single bout of exercise until the point of volitional fatigue appears to decrease the number of pulmonary metastases.

Tumor cell retention. It has been suggested that alterations in circulatory patterns known to occur during exercise might alter the pattern of tumor cell arrest. To address this issue, we compared the number of radiolabeled B16 melanoma cells that settled in various organs when injected 15 min after exercise. The
The large majority of B16 cells settled in the lung (75%), a small portion settled in the liver (5%), and a very small number of cells were found in the spleen (1%) and blood (1%). There was no difference in the pattern of tumor cell arrest among the three groups: Con, Ex-30, and Ex-F (Fig. 2). These findings show that the large majority of cells arrest in the lung, and alterations in circulating patterns occurring during exercise do not change the pattern of tumor cell arrest.

To examine the time course of tumor cell retention in the lungs, the amount of radiolabel remaining in the lungs at several time points postinjection was measured in Ex-F and Con mice. There were no differences in retention between the two groups (Fig. 3) at any time point (P > 0.05). There was significantly (P < 0.05) less radiolabel in the lungs at both 4 and 16 h compared with that immediately postexercise.

Effect of exercise on alveolar macrophage cytotoxicity in vitro. Alveolar macrophage cytotoxicity, which reflects the ability of the macrophage to destroy tumor cells and/or limit tumor growth, was also measured. A change in the ability of the alveolar macrophage to destroy tumor cells could potentially account for the decrease in tumor metastases. Alveolar macrophage cytotoxicity was measured immediately postexercise and at 8 h postexercise. Alveolar macrophages from Ex-F mice had significantly enhanced cytotoxicity compared with Con mice immediately postexercise at two effector:target ratios (40:1, P = 0.0001 and 20:1, P = 0.0001) but not at the 80:1 effector:target ratio (P = 0.059) (Fig. 4A). The Ex-30 group also showed a significant increase in cytotoxicity compared with Con at the 20:1 ratio (P = 0.006). Eight hours postexercise, a difference between the groups was no longer present (Fig. 4C), with the exception of enhanced cytotoxicity in the Ex-F group vs. Con at one effector:target ratio (20:1, P = 0.01).

This difference in macrophage cytotoxicity is probably not due to exercise effects on macrophage adherence. Cell counts of adhered macrophages were within 5% for the exercise and control conditions (data not shown). Previous work from our lab has also demonstrated a lack of exercise effects on the adherence of peritoneal macrophages (36).

**DISCUSSION**

Previous studies have demonstrated that prolonged intense exercise can inhibit the growth of a primary tumor (1, 11, 24, 26). Results from this study reveal that a single bout of prolonged exercise can also alter the metastatic spread of injected tumor cells. This effect was observed after a treadmill run to the point of volitional fatigue (~3 h) but failed to occur after only 30
min of exercise. Although it has been reported that the exercise protocol used in our Ex-30 treatment should elicit \( \sim 84\% \text{VO}_2\text{max} \) in mice (8), it is more likely closer to 55–65% \text{VO}_2\text{max} based on data on \text{VO}_2\text{max} of mice and other rodents from Taylor (31). This is in contrast to our fatigue protocol (Ex-F), in which the intensity increased from 55–65% \text{VO}_2\text{max} during the first 30 min to \( \sim 68–78\% \text{VO}_2\text{max} \) at fatigue. Therefore, it is possible that a critical exercise intensity and duration is required to induce protective mechanisms that limit lung metastases of the B16 melanoma. This also appears to be true for induction of mammary carcinogenesis (34). However, a systematic evaluation of exercise duration and intensity parameters is necessary before firm conclusions can be drawn.

To our knowledge, this is the first report demonstrating that a single bout of exercise results in decreased lung metastases. These findings appear to conflict with another published report on metastatic spread after a single bout of swimming stress, in which Ben-Eliyahu et al. (4) suggested that a single session of intermittent swim exercise enhanced the metastatic spread of mammary tumor cells. This apparent discrepancy may be accounted for by the use of different tumors and animal species, but it is more likely related to the different protocols used in each experiment. Rats in the Ben-Eliyahu experiments were not acclimated to the swimming protocol, and extreme psychological stress associated with the fear of drowning (weights were placed on the rats’ tails) was clearly a confounding factor. In contrast, we spent several days acclimating the mice to the treadmill to reduce additive stress associated with this novel environment. In addition, in our studies control animals were kept in simulated treadmill lanes directly over the runners and exposed to similar handling and noise stressors, whereas control animals in the study by Ben-Eliyahu remained undisturbed in their cages.

A decrease in the initial arrest of circulating tumor cells without a change in the survival rate of these cells could potentially explain the reduction in metastases observed in the Ex-F group. Blood flow patterns are known to be altered in response to exercise and may account for changes in tumor cell arrest. Glucocorticoid injection before intravenous injection of B16 tumor cells actually enhanced pulmonary metastases by increasing the initial arrest of tumor emboli in pulmonary capillaries (10), and the dose of exercise used in our study has been shown to elevate corticosteroid levels (data not shown). For these reasons, the question of whether exercise alters the initial arrest pattern and subsequent metastases was important to address. Our results demonstrated that the initial arrest of tumor cells within the lungs does not appear to be altered by physical exercise. At 30 min postinjection \( \sim 75\% \) of the radiolabel remained in the lungs of all three groups (Con, Ex-F, and Ex-30; Fig. 2). It is therefore likely that a change in the pattern of initial tumor cell arrest cannot explain the reduction of metastases observed in the Ex-F group.

Enhancement of the immune response after exercise is a potential mechanism that may account for the reduction in tumor burden. Various host effector cells including NK cells, lymphokine activated killer cells, and macrophages can kill a broad spectrum of tumor cells and do not require prior antigen priming to exert their cytotoxic effects. Both macrophages and NK cells are thought to play a role in limiting metastases by altering the early steps of implantation and growth of secondary foci (2, 19). In particular, the ability to control metastases of various tumor cells has been attributed to both NK cells (2, 3) and alveolar macrophages (9, 27, 29). The NK cell has been implicated as an important antitumor effector cell in vivo after chronic exercise (13, 17, 18). However, given the fact that macrophages also play an important role in limiting metastasis involving B16 melanoma (9), the mechanisms of exercise effects on tumor metastases in this model should not be limited to NK cells.

In fact, there are several reasons to suggest that the NK cell may not be solely responsible for the exercise effects observed in this experiment. For example, an acute bout of exhaustive exercise has been shown to suppress murine splenic NK activity (23). Secondly, B16 melanoma cells in culture appear to be relatively resistant to NK activity unless primed with interferon (20, 27, 38). Finally, our tumor cell retention data (Fig. 3) show that there were no differences in tumor cell clearance between Con and Ex-F groups over a 16-h period after injection of radiolabeled tumor cells. This argues against an important role for the NK cell because NK cell antitumor activity is believed to be rapid in vitro (within 4 h), and the clearance of tumor cells from the lungs within 4 h after intravenous injection has been used as a measure of NK activity in vivo (3, 25). Indeed, others have used this method to support a role for NK cells in the enhanced clearance of CIRAS3 (18) or YAC-1 (13) tumor cells from the lungs after exercise. However, it should be noted that when anti-asialo GM1 antibody was injected to inhibit NK activity, it did not completely eliminate the enhanced
clearance in the exercised mice in comparison to control mice, suggesting that other mechanisms may be responsible for this effect.

In this study, we proposed that the alveolar macrophage may play an important role in the apparent protective effect of exercise on tumor metastasis. Our results demonstrate that prolonged exercise (Ex-F) increased alveolar macrophage cytotoxicity above Con immediately postexercise and that this effect was still apparent, although to a lesser extent, at 8 h postexercise. The effect in Ex-30 appeared to be less strong than Ex-F immediately postexercise and was not present at 8 h postexercise. Although the reduction in pulmonary metastasis cannot be directly related to the alveolar macrophage cytotoxicity, it is possible that rather long-lasting effects of macrophage activation might be responsible for the observed reduction in metastases. This might include direct inhibition of infiltration and growth of the tumor cells as well as the release of antitumor and immunostimulatory cytokines (35). However, we do not have any information regarding alveolar macrophage function at any time point between 8 h postexercise and 10 days postexercise (time of death for lung metastases enumeration), which makes it difficult to make any strong conclusions about the contribution of alveolar macrophages. Perhaps additional experiments involving the administration of relatively specific macrophage toxins such as silica or carrageenan before exercise may provide further insights into the role of the macrophage in this exercise-metastases model.

Analyses of the potential cellular mechanisms leading to exercise-induced increases in macrophage activity were not examined in this study. However, the results from prior studies suggest that exercise-induced elevation of peritoneal macrophage cytotoxicity may be mediated in part by tumor necrosis factor (36) or through the release of reactive nitrogen intermediates (37). It is possible that exercise may act to enhance tumor necrosis factor or reactive nitrogen intermediates in alveolar macrophages as well, and these products may increase antitumor function.

The findings from this study clearly show that the number of tumor metastases resulting from an intravenous injection of B16 melanoma cells is decreased after an acute bout of exercise to fatigue. Furthermore, there is an enhancement of antitumor cytotoxicity by alveolar macrophages from animals that have undergone an acute bout of exercise. A direct cause and effect relationship between these two observations cannot be implied. Further studies that directly address the role of the alveolar macrophage in vivo may provide a better understanding of this relationship.

Address for reprint requests: J. M. Davis, Dep. of Exercise Science, Univ. of South Carolina, Columbia, SC 29208.

Received 23 Jun 1997; accepted in final form 30 Jan 1998.

REFERENCES


