Physical activity counteracts tumor cell growth in colon carcinoma C26-injected muscles: an interim report

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Abstract

Skeletal muscle tissue is a rare site of tumor metastasis but is the main target of the degenerative processes occurring in cancer-associated cachexia syndrome. Beneficial effects of physical activity in counteracting cancer-related muscle wasting have been described in the last decades. Recently it has been shown that, in tumor xeno-transplanted mouse models, physical activity is able to directly affect tumor growth by modulating inflammatory responses in the tumor mass microenvironment. Here, we investigated the effect of physical activity on tumor cell growth in colon carcinoma C26 cells injected tibialis anterior muscles of BALB/c mice. Histological analyses revealed that 4 days of voluntary wheel running significantly counteracts tumor cell growth in C26-injected muscles compared to the non-injected sedentary controls. Since striated skeletal muscle tissue is the site of voluntary contraction, our results confirm that physical activity can also directly counteract tumor cell growth in a metabolically active tissue that is usually not a target for metastasis.

Key Words: cancer cachexia, colon carcinoma C26 cells, tumor cell growth, muscle inflammation, voluntary running

Cancer cachexia is a multifactorial catabolic syndrome associated with tumor progression. It is characterized by progressive muscle degeneration mediated by an imbalance between protein synthesis and protein degradation in favor of an increased rate of skeletal muscle proteolysis. This chronic wasting condition is mainly sustained by high levels of circulating pro-inflammatory cytokines and tumor-released factors (e.g., interleukins 1β and 6 (IL1β, IL6), tumor necrosis factor alpha (TNF-α) and proteolysis inducing factor (PIF)), which specifically trigger the proteolysis of muscle proteins. To date, no specific cures exist for cancer cachexia and the pharmacological strategies commonly adopted aim to treat the cancer itself, rather than targeting degenerative molecular mechanisms at the muscle site.

Recently, there is an emerging interest in the possible effects of physical activity on the incidence, prognosis and treatment of cancer. A wide range of beneficial effects mediated by physical exercise in cancer cachexia have been reported in both murine and human studies. Epidemiological studies showed that physical activity has a pivotal role in reducing tumor incidence, improving responsiveness to chemotherapy and, in general, ameliorating cancer patients’ quality of life. In this view, physical exercise has been proposed as an adjuvant therapy in cancer, including breast and colon cancers. A recent report described a causal relationship between physical activity and tumor growth inhibition. Pedersen et al. showed that voluntary running suppresses tumor growth by remodeling the inflammatory background in the tumor microenvironment in several tumor models. Specifically, high circulating epinephrine levels due to voluntary running increase NK cell tumor infiltration which in turn impairs tumor growth. Although many studies address the beneficial effects of physical exercise in cancer cachexia, colon carcinoma C26 cells, tumor cell growth, muscle inflammation, voluntary running
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**Table 1. Physical activity. Values are means ± SEM of 3 independent experiments per group**

|            | Total DST (Km) | Speed at day 4 (Km/h) | Average | Max  |
|------------|----------------|-----------------------|---------|------|
|            |                |                       |         |      |
| Ctr WR     | 18.06 ± 3.52   | 1.3 ± 0.07            | 2.5 ± 0.09 |      |
| C26 WR     | 19.55 ± 2.60   | 1.4 ± 0.06            | 2.5 ± 0.04 |      |
|            |                |                       | 15:57:47 ± 02:17:12 |      |
|            |                |                       | 14:55:29 ± 01:54:17 |      |

exercise at multi-organ and multi-systemic levels,\textsuperscript{18} it is still not clear why skeletal muscle tissue, which is a main tissue target of pathophysiological changes occurring in cancer cachexia,\textsuperscript{12,15} is refractory to tumor cell colonization and metastasis.\textsuperscript{19-21}

Here, we performed histological analyses of *tibialis anterior* (TA) muscles from mice injected with colon carcinoma C26 cells to test the effects of physical activity on tumor cell growth specifically at the contraction site. Our results showed a significant reduction of tumor cell growth in muscles from physically active mice compared with the sedentary ones. These data confirm the ability of physical exercise to counteract tumor cell growth.

**Materials and Methods**

**Mice**

Nine 8-weeks-old male BALB/c mice were used for this study. To study the effects of physical activity on tumor cell growth, 1x10\textsuperscript{5} murine colon carcinoma C26 cells were re-suspended in PBS (50µl) and injected in *tibialis anterior* (TA) muscles. TA from the contralateral leg was injected with the same volume of PBS as control in each mouse. Mice were housed in standard conditions with day/night cycles of 12 hours, received water and food *ad libitum*, and were euthanized 4 days after tumor injection.

All the mice used in this study were treated in accordance with ARRIVE guidelines and following the three R’s rule of Replacement, Reduction and Refinement principles.\textsuperscript{22} Protocols adopted in the study have been approved by the animal experimentation ethics committee of KU Leuven, Belgium.

**Exercise protocol**

Mice were randomly assigned to three experimental groups: (i) C26-injected mice hosted in standard cages (sedentary control group, C26 REST), (ii) non-injected running mice hosted in wheel-equipped cages (running control group, Ctr WR) and (iii) C26-injected running mice hosted in wheel-equipped cages (C26 WR). Cages were prepared as previously described.\textsuperscript{23} Briefly, one cage per mouse was used and all wheels were supplied with a cycle computer in order to record physical activity data (i.e. daily distance, total distance, average and maximum speed and time spent on the wheel). Mice in the running groups were hosted in wheel-equipped cages starting from the day before tumor cell injection, to familiarize the animals with the use of the new environmental stimulus (i.e. wheel) until the day of sacrifice (4 days after tumor cells injection).

**Histology**

TA muscles were dissected, weighed, embedded in tissue freezing medium (Leica, Wetzlar, GE), frozen in liquid nitrogen-cooled isopentane and stored at -80°C. Muscle cryosections of 7 µm thickness were obtained using a cryostat (Leica Biosystems). For histological analysis, the sections were stained with Hematoxylin and Eosin (H&E, Sigma-Aldrich) using a standard method.

![Fig 1](image-url). Voluntary wheel running exercise. (A) Mice from running groups were hosted in wheel-equipped cages. (B) non-injected running mice (Ctr WR) and C26-injected running mice (C26 WR) covered comparable daily distance until the end of experiment, 4 days. Data are means ± SEM of 3 independent experiments.
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Table 2. Body and muscle weight.

|                  | Body weight (BW) | Muscle weight/BW (mg/gr) |
|------------------|------------------|--------------------------|
|                  | Initial          | Final                    | C26     | PBS     |
| C26 REST         | 26.47 ± 0.97     | 25.13 ± 1.04             | 2.96 ± 0.06* | 2.38 ± 0.03 |
| C26 WR           | 24.93 ± 0.29     | 23.17 ± 0.27             | 2.67 ± 0.11 | 2.43 ± 0.09 |

Values are means ± SEM of 3 independent experiments per group.
TA C26 REST versus TA PBS REST: *p < 0.05 by 1way ANOVA.

Immunofluorescence
Transverse cryosections were fixed in 4% paraformaldehyde for 10 min at room temperature (RT) and permeabilized in 1% bovine serum albumin (BSA)/0.2% Triton in Phosphate-Buffered Saline solution (PBS) for 30 min. After incubation with 1% BSA in PBS (30 min), samples were incubated with a goat polyclonal anti-Ki67 Ab (Sc-78, Santa Cruz) (1:100 in BSA), followed by incubation with anti-goat Alexa fluor 488 conjugated Ab (Molecular Probes, Eugene, OR) (1:500 in BSA). Macrophage staining: samples were incubated with a rat anti-mouse F4/80 (Bio Rad MCA 497G), followed by incubation with anti-mouse Alexa fluor 594 conjugated Ab (Molecular Probes, Eugene, OR). IgG-immunostaining was performed by incubation with anti-mouse Alexa fluor 594 conjugated Ab (Molecular Probes, Eugene, OR) (1:500 in BSA) for 45 min at RT. Nuclei were stained for 5 min with 0.5 μg/ml Hoechst 33342 (Sigma).

Morphometric analysis
Morphometric analysis was performed on pictures from H&E staining. Three different cross-sectional areas of each muscle were analyzed. Muscle and tumor areas were calculated as relative percentages. ImageJ Software (National Institutes of Health, Bethesda, MD, USA) was used to perform this analysis.

Statistics
All quantitative data are presented as mean ± standard error of mean (SEM) of three independent experiments. Statistical analysis was performed using Student’s t-test or one-way and two-way ANOVA followed by Bonferroni post-hoc testing using GraphPad Prism 5 software (GraphPad Software, San Diego, CA). A p-value less than 0.05 was considered to be statistically significant.

Results
Voluntary running activity showed no significant differences in terms of daily distance, total distance, average running speed and total running time (Fig. 1 B and Table 1) between non-injected and C26-injected mice. These data indicate that the amount of tumor cells injected in the muscles did not affect voluntary physical activity of the mice.
By histological analyses we evaluated the effects of

![Fig 2. Size and muscle weight analyses.](image)
(A) In C26 REST mice (B1.4, B1.5 and B1.6) C26-injected TA muscles appear to be larger than the contralateral PBS-injected muscles compared with those derived from C26 WR (B1.1, B1.2 and B1.3). Dashed yellow lines help the comparisons. (B) Muscle weight analysis confirmed the differences observed in (A). Error bars are shown as means ± SEM of 3 independent experiments; ** p = 0.01 by 2way ANOVA.
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physical activity on tumor growth 4 days after tumor cell injection in TA muscles from mice at rest and under voluntary running conditions. Since the study was performed using a small number of animals per experimental group (n = 3), we tracked samples from every mouse in order to display the consistency of the

Fig 3. Histological features of areas containing tumor cells in C26-injected muscles. C26-injected muscles from mice at rest and running conditions developed tumor areas characterized by a high number of cells/area. Tumor cells had bigger nuclei (yellow arrowheads), compared with the infiltrating cells observed in the tumor areas (yellow arrows) or myonuclei (white arrows). Right up and down panels represents magnification areas from yellow rectangles of left panels. Bars: bottom left = 100 μm, bottom right = 200 μm.

Fig 4. H&E staining of C26-injected muscles. (A) H&E staining of representative transverse cryosections obtained by photomicrographs reconstruction. Dashed black lines represents areas of tumor cell growth. (B) Morphometric analysis of tumor cell growth. Bar = 0.5 mm. Error bars are shown as means ± SEM of 3 independent experiments.
results. Specifically, mice from the sedentary injected-group (C26 REST) are indicated in the figures as B1.4, B1.5, B1.6, while mice from voluntary running injected-group (C26 WR) are indicated as B1.1, B1.2, and B1.3, according with the experimental recording system adopted in our laboratory. At the end-point (sacrifice day), C26-injected muscles from mice at rest were bigger than the PBS-injected contralateral ones, while no significant differences were found between C26 and PBS-injected muscles from the exercised group (Fig. 2 A). Muscle weight analysis confirmed these findings (p < 0.01, Fig. 2 B, table 2).

Next, by H&E staining, we identified areas containing tumor cells in C26-injected muscles (Fig. 3 A and 4 A). Regions containing tumor cells were characterized by a high number of cells per area. Tumor cells had larger nuclei compared to the myonuclei and mononuclear cells observed in the tumor areas, as Fig. 5. Tumor cell growth in C26-injected muscles. (A) Representative images of tumor cell growth areas. Green, Ki-67 positive nuclei, dashed yellow lines represent muscle fibers, blue Hoechst. (B) Tumor cell growth analysis. Error bars are shown as means ± SEM of 3 independent experiments; *p < 0.05 compared with Student’s t-test.

Fig 6. Inflammatory background. (A) Representative images of local inflammation in control and tumor cells-injected muscles. Red, mouse IgG, blue Hoechst. Bar = 100 μm. (B) Spleen weight analysis between control, C26 REST and C26 WR mice. Error bars are shown as means ± SEM of 3 independent experiments.
shown in figure 3 A. Moreover, the staining for F4/80 revealed only a limited amount of macrophage cells within the tumor areas (Fig. 3 B). To investigate the nature of the differences in size and weight of injected muscles between non-exercised and running mice, we analyzed the tumor areas developed in muscles (Fig. 4 A). Specifically, cross-sectional area (CSA) analysis of C26-injected muscles showed that tumor cells expanded at the site of injection. No single cells were found far from the side of injection (Fig. 3 A and 4 A).

Morphometric analysis showed that in C26-injected muscles from sedentary mice the tumor cells represent 7.9% ± 1.2 of the total muscle CSA, while in muscles from the C26-injected running group the average of tumor cells-covered area was 5.1% ± 1.3/CSA (Fig. 4 B). These results indicate a trend of reduced tumor growth in the running mice. Since tumor cells are characterized by high proliferative rates, we further investigated the effects of physical activity on tumor cell growth by analyzing the cells expressing Ki-67, a known marker of cellular proliferation (Fig. 5 A and B).24 This approach allowed us to better determine the extent and the impact of tumor cells growth in the muscle tissue, without the contribution of other cells (e.g. inflammatory infiltrate), which were limited compared to the number of tumor cells in C26-injected muscles (Fig. 3 B). Quantitative analysis of Ki-67 positive cells from tumor areas showed a significant reduction of tumor cell proliferation in muscles of running mice compared to those derived from sedentary mice (p < 0.05, Fig. 5 B).

Because tumor development is normally associated with an inflammatory response in the target tissue, we also analyzed the inflammatory state of injected muscles (Fig. 6). Immunoglobulin staining revealed a high inflammatory background in C26-injected muscles, while a basal level of IgG was found in both PBS-injected and healthy control muscles (Fig. 6 A). These observations indicate that tumor cells induced local inflammation. Next, we measured the weight of the spleen, an organ sensitive to chronic inflammatory conditions including cancer. Quantitative analysis showed a comparable weight of the spleen between C26-injected and non-injected mice (Fig. 6 B). These data, together with the analysis of body weight of non-exercised tumor-injected mice, which showed no changes in body weight between the day of tumor cells injection and the end of experiment (Table 2), confirmed that tumor cells mediated at local level the muscle changes without any further contribution due to the presence of a chronic systemic inflammation.

Discussion

A general consensus has been reached in the last years concerning the beneficial effects of physical exercise in the prevention, management and treatment of chronic degenerative conditions, including cancer.5,11,12 Currently, public health offices recommend regular physical exercise for a healthy lifestyle.6

In cancer epidemiology, physical activity is associated with a reduced incidence, a better prognosis, an increased responsiveness to therapy and a general improvement in patients’ quality of life.11 These evidences result from clinical studies performed on specific tumor types, including breast, colon and pancreatic cancers.11 The effects of physical activity on tumor growth have long been debated.25,26 Interestingly, a recent study showed that voluntary running exercise leads to a remodeling of immune cells colonizing the tumor microenvironment of tumor-transplanted mice.17 In particular, researchers showed that physical activity negatively affects tumor growth by increasing the recruitment of NK cells within the tumor mass.19

Skeletal muscle tissue is the main target of the pathophysiological changes occurring both in early,27-29 and in advanced stages of cancer progression, such as loss of muscle mass and function.30,31 The elevated systemic inflammation originating from the tumor is a pivotal mediator of muscle wasting observed in cancer patients.2 The inflammation also affects other organs and tissues (e.g. fat tissue), a condition known as cancer cachexia syndrome. Thus, according to data reported in the literature, there is a strong correlation between tumor progression and immune response.32,34 In this picture, the elevated inflammatory background can mask direct beneficial effects of physical activity on tumor cells growth and the molecular mechanisms involved.

Here, we analyzed the direct effects of physical activity on tumor cell growth in a chronic systemic inflammation-independent context. Specifically, we adopted voluntary running as a form of exercise compatible with advanced stages of tumor progression, as emerged by clinical studies performed in cancer patients.15 We injected C26 colon carcinoma cells in TA muscles of BALB/c mice, to directly determine the effects of muscle contraction on their growth. Muscle is the most represented and metabolically active tissue in the body, and its capillary bed is certainly extremely extended. Thus, it could theoretically represent a suitable site for tumor development. However, clinical reports only rarely document events of tumor metastasis occurring in skeletal muscles. So far, tumor metastasis in muscle tissue have been reported only in a few cases of laryngeal squamous carcinoma,35-37 lung cancer,38 papillary thyroid cancer,39 bladder cancer,40 and gastrointestinal stromal tumor.41,42 Our data show that muscles injected with C26 cells develop tumor cells expansion after 4 days of cell implantation, suggesting that muscle tissue per se is a permissive environment for tumor cell growth. Histological analyses revealed that a total of ~15 hours of voluntary wheel running (table 1), which is considered a low-intensity43,44 and aerobic12 exercise, accumulated over a period of 4 days leads to a
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significant reduction of tumor cell growth compared with muscles from sedentary mice. Interestingly, we injected tumor cells in muscles of healthy mice and samples were analyzed 4 days later, so the observed tumour cell growth was independent of the systemic inflammation usually reported after longer periods in tumor bearing mouse models. This aspect was confirmed by weight analysis of the spleen, that revealed no differences between healthy and C26 muscle-injected mice. On the contrary, an increased local inflammatory background was observed in C26-injected muscles from both sedentary and running mice, compared with non-injected and PBS-injected muscles. Overall, our results indicate that, although the muscle microenvironment is generally unfavorable for growth of metastatic cancer cells, its refractoriness can be modulated by exercise. The observations of this study confirmed that physical activity acts as negative regulator for tumor cells growth in skeletal muscle tissue. However, further analyses are needed to better investigate the relationship between tumor-mediated local inflammation, tumor dissemination and physical activity. Whether the inflammatory, reparative and myoregenerative responses that may occur in muscle during wheel running\(^{57-29,45-47}\) may have direct effects on the metastatic processes remain to be investigated.

Contributions
EB conceived and supervised the project. EB and CH performed the experiments and optimized the methodology. TV, KK and SDS helped the elaboration of the methodology and the prototype characterization. EB wrote the manuscript and PH helped in revising it.

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Conflict of Interest
The authors declare no potential conflict of interests.

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