Pergamon

European Journal of Cancer Vol. 31A, Nos 13/14, pp. 2309–2312, 1995

0959-8049/95 $9.50+0.00

0959-8049(95)00441-6

Original Paper

Tumour Purine Nucleotides and Cell Proliferation in Response to Exercise in Rats

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Voluntary physical exercise can delay the onset of anorexia and cachexia in tumour-bearing rats. A substrate deviation in the host which has been hypothesised as tumour burden is reduced despite an increase in food intake. Therefore, we determined the levels of purine nucleotides, the energy charge and the cell division rate in tumours from exercising animals in the postexercise period. Tumour content of purine nucleotides was analysed by HPLC. Tumour cell kinetics was studied by flow cytometry after incorporation of bromodeoxyuridine (BrdU) into DNA. Exercising animals demonstrated a 34.4% reduction in tumour volume ($P < 0.05$) but a 1.31-fold increase in energy charge in tumour tissue ($P < 0.05$). Labelling index (LI), DNA synthesis time (T_s) and potential doubling time (T_{pot}) were not significantly altered. These results suggest that the influence on tumour growth is closely related to the exercise period.

Key words: energy charge, BrdU, ATP, exercise
Eur J Cancer, Vol. 31A, Nos 13/14, pp. 2309–2312, 1995

INTRODUCTION

HYPERALIMENTATION IS known to stimulate experimental tumour growth [1, 2], while starvation has the opposite effect [3, 4]. Both starvation and refeeding in tumour-bearing animals significantly affect tumour DNA synthesis [4–7] and energy state [8]. Voluntarily exercising rats exhibit reduced tumour weights despite an early increase in food intake [9–11]. This paradoxical finding might be due to a substrate deviation (i.e. altered flow of nutrients from the tumour host to the tumour tissue) to skeletal muscle [10, 11]. Deviation of substrate has been proven when tumour-bearing animals are exposed to cold [12]. The aim of the present study was to evaluate the effects of exercise on tumour energy state, by analysing the contents of purine nucleotides by HPLC, and on tumour cell proliferation, measured by flow cytometry after incorporation of bromodeoxyuridine (BrdU) into tumour DNA.

MATERIALS AND METHODS

Animal and tumour model

Twenty female Wistar Furth rats (B&K Universal AB, Stockholm, Sweden) with initial body weights 170–180 g were allocated to the study groups matched for body weights: tumour-bearing exercising animals (TBE) and tumour-bearing sedentary animals (TBS). After adaptation to the cages, they were subcutaneously implanted with 1.5 mm^3 of viable Leydig cell sarcoma in each flank. This tumour does not metastasise when implanted in this location, is well defined [13] and induces well-described anorexia and cachexia [9–11].

The animals allocated to exercise were individually housed in cages open to the interior of a freely moving, wire-bottomed and non-motorised running wheel with a diameter of 33 cm (UNO Roestvaststaal BV, Arnhem, The Netherlands). These animals had free access to the running wheels throughout the experiment. The other group of animals were individually housed in standard cages. All animals had free access to a balanced diet (R34 Labfor, Lactamin, Stockholm, Sweden) and tap water. Room temperature was kept at 22 ± 1°C and the light : dark cycle was 12 : 12 h. Body weight, food intake and distance run were recorded throughout the experiment.

On day 13 after tumour implantation, the animals were anaesthetised with sodium pentobarbital (ACO, Solna, Sweden) intraperitoneally (35 mg/kg body weight). The tumours were measured with vernier calipers at the largest (a) and the smallest (b) superficial diameters and the tumour volumes were calculated [14], using the formula

$$V = a \times b^2/2.$$

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Revised 15 Jun. 1995; accepted 30 Jun. 1995.

Biopsies were rapidly dissected from non-necrotic parts of each tumour, and the animals were then killed by bleeding. One biopsy from each tumour was weighed and then dried for determination of water content.

The experimental model was approved by the Animal Ethical Committee of the University of Göteborg, Sweden.

Bromodeoxyuridine (BrdU) administration and tumour tissue preparation for bivariate flow cytometric analysis

In vivo uptake of BrdU (No. B-5002 Sigma St Louis, Missouri, U.S.A.) into tumour DNA was accomplished by intraperitoneal injection of BrdU 0.1 mg/g body weight 2 h after the exercise period and 4 h prior to termination. Solid pieces (about 100 mg) of tumour tissue were fixed directly in 70% ethanol and stored at 4° for at least 12 h. Nuclei extraction, BrdU and DNA staining were performed as described elsewhere [15, 16].

The specimens were analysed on a FACScan flow cytometer (Becton Dickinson, San José, California, U.S.A.) connected to a Hewlett Packard 310 computer (Hewlett Packard, San Diego, California, U.S.A.). The optic beam for simultaneous fluorescein and propidium iodide excitation was a 15 mW, 488 nm, air-cooled argon-ion laser. Wavelengths corresponding to the maximum emission were 525 nm (green fluorescence) and 610 nm (red fluorescence) for the respective fluorochromes.

The calculations of labelling index (LI), DNA synthesis time (T_s), potential doubling time (T_{pot}) and DNA distribution were performed as described previously [5, 15, 17] using the Lysus II program (Becton Dickinson, San José, California, U.S.A.), assuming a uniform distribution and a linear progression of cells throughout S-phase. At least 10 000 events were collected in list mode for these calculations. Briefly, T_s and T_{pot} are derived from quantification of the relative movement (RM), which at time zero is assumed to be in mid-S and accordingly the value of 0.5. The RM will eventually reach a value of 1.0 when only the BrdU-labelled cells, which have not yet divided, now reside in G2. Thus, the increase in RM from 0.5 in the direction to 1.0 during the time from BrdU injection to tumour sampling represents the T_s T_{pot}, the cell division time not taking into account cell loss, can be calculated from:

$$T_{pot} = l \times T_s/\mathrm{LI}$$

where l is a correction factor for the age distribution of the tumour population and a value of 0.8 has been assumed [15].

Purine nucleotides

Solid pieces of tumour tissue were rapidly dissected and frozen in liquid nitrogen and then freeze-dried for 8 h (LYOVAC GT2, Leybold-Heraeus GMBH, Köln, Germany). Tumour tissue was then minced to powder. To 50 mg of dry powder, 0.950 ml 1.5 mM perchloric acid solution (PCA) containing 1 M EDTA was added and the extraction was performemd by gentle agitation for 20 min on ice. The precipitate was separated by centrifugation and neutralised before injection into the HPLC-system (Gynkotek, Germering, Germany) [18]. The column used was a prepacked reversed phase C18 column, SynChropak 100 RP C18 (5 mm; 3.0 × 250 mm) (VDS Optilab, Berlin, Germany) and the elution medium mobile phase consisted of 0.1 M ammonium dihydrogen phosphate buffer ($NH_4H_2PO_4$) with pH adjusted to 6.0 with 25% ammonium hydroxide (NH_4OH). The purine nucleotides were determined in a single run as described in detail elsewhere, and the concentrations of ATP, ADP, AMP, IMP, GMP, hypoxanthine, xanthine, uric acid and also NAD were calculated from the computer-integrated areas of the sample, relative to the areas obtained for the standard solutions.

The energy charge EC, was calculated according to the method of Atkinson [19]:

$$\mathrm{EC} = [\mathrm{ATP}] + 1/2[\mathrm{ADP}] / [\mathrm{ATP}] + [\mathrm{ADP}] + [\mathrm{AMP}].$$

Statistics

All values are presented as mean ± S.E.M. Statistical significance was evaluated with ANOVA and Student's t-test. A P-value less than 0.05 was regarded as significant. Correlation coefficients were evaluated according to Pearson.

RESULTS

Body weight, food intake and physical activity

The data shown in Table 1 are in accordance with results in our previous studies [9–11].

Tumour volume and water content

The total tumour burdens are presented in Table 1 with a significant difference between the groups. The water content did not differ between the groups.

Purine nucleotides and energy charge in tumour tissue

The results are based on averages of both tumours from each animal where applicable or otherwise on data from one tumour (Table 2). TBE animals had increased tumour content of uric acid and an increased energy charge while the content of AMP was decreased ($P < 0.05$). The same differences were encountered when results were based on data from each tumour separately, except for a non-significant difference in AMP content (results not shown).

When each tumour was evaluated separately, energy charge in tumours from TBE animals correlated inversely to distance run in the last 24 h of the experiment ($r = 0.606$; $P < 0.01$).

Tumour cell kinetics

The results are based on averages of both tumours from each animal where applicable or otherwise on data from one tumour (Table 3). Tumours with a labelling index of less than 0.10 were excluded. No differences were found when the results were based on data from each tumour separately (results not shown).

Table 1. Data on body weight difference (% and %/d), food intake (g/100 g body weight/d), running activity (m/d), tumour volume (mm^3) and tumour water content (%)

	TBE (n = 10)	TBS (n = 10)
Body weight difference during experiment (%)	−1.89 ± 1.05*	1.00 ± 0.63
Body weight difference in last 24 h (%/d)	−0.22 ± 0.36	−0.13 ± 0.19
Food intake in last 24 h (g/100 g body weight/d)	7.11 ± 00.25†	4.39 ± 0.24
Running distance in last 24 h (m/d)	7816 ± 649	—
Total tumour volume (mm^3)	5764 ± 622*	8787 ± 1162
Tumour water content (%)	81.500 ± 0.4	81.9 ± 0.3

*$P < 0.05$. †$P < 0.001$. TBE, tumour-bearing exercising animals. TBS, tumour-bearing sedentary animals.

Table 2. Contents of purine nucleotides in tumour tissue (mmol/g dry weight)

μmol/g dry weight	TBE (n = 10)	TBS (n = 10)
ATP	3.250 ± 0.351	2.789 ± 0.465
ADP	1.512 ± 0.166	1.588 ± 0.188
AMP	1.134 ± 0.237*	1.303 ± 0.091
IMP	0.121 ± 0.017	0.110 ± 0.007
GMP	0.841 ± 0.079	0.922 ± 0.048
Hypoxanthine	0.431 ± 0.032	0.483 ± 0.042
Xanthine	0.372 ± 0.075	0.512 ± 0.250
Uric acid	1.186 ± 0.072*	0.992 ± 0.044
NAD	0.731 ± 0.071	0.520 ± 0.091
Energy charge	0.683 ± 0.022*	0.604 ± 0.029

Results based on averages of both tumours where applicable or on data from one tumour.
*$P < 0.05$. Energy charge in tumour tissue according to Atkinson's formula: EC = [ATP] + 1/2[ADP] / [ATP] + [ADP] + [AMP].

Table 3. Flow cytometry after incorporation of BrdU into tumour DNA

	TBE (n = 10)	TBS (n = 8)
LI (%)	0.251 ± 0.035	0.238 ± 0.018
T_s (h)	7.660 ± 0.250	7.418 ± 0.413
T_{pot} (h)	29.380 ± 3.641	26.817 ± 3.471

Results based on averages of both tumours where applicable or on data from one tumour.
LI, Labelling index (%); T_s, DNA synthesis time (h); T_{pot}, potential doubling time (h). Tumours with a LI less than 0.10 were excluded.

DISCUSSION

We have previously demonstrated that voluntary physical exercise in the tumour-bearing rat can delay the onset of anorexia and cachexia [9]. Furthermore, protein metabolism, oxidative capacity and energy state in skeletal muscle are improved [9–11]. The peripheral insulin resistance seen in cancer is also normalised and the alterations in levels of anabolic and catabolic hormones are modified [11]. As a substrate deviation to the exercising host has been postulated, the aim of the present study was to evaluate tumour response as reflected by contents of purine nucleotides and energy charge, and cell division rate.

We have previously evaluated two experimental tumours, both subcutaneously transplanted in rats: an adenocarcinoma of the colon (NGW) and a Leydig cell sarcoma (LTW). Both induce the same type of anorexia and cachexia, but the time course with NGW is faster. Final weights of LTW tumours are most often significantly reduced in exercising animals, while this is not always true for those of NGW tumours. Both tumours are affected by exercise during the first week of experimentation as evidenced by smaller volumes (unpublished data). The reduction in tumour burden in exercising animals occurs despite an early and persistent increase in food intake. This paradoxical finding contrasts with the numerous reports on stimulating effects of hyperalimentation on tumour growth, and a substrate deviation in the tumour host has been hypothesised [9]. Indicators of such deviation have been shown in tumour-bearing animals exposed to cold [12].

The NGW tumour was found to be polyclonal in nature, and could thus not be evaluated in this study as uptake of BrdU in the different clones interfere (unpublished data). The LTW tumour was inconsistently polyclonal and thus somewhat heterogeneous, but the incorporation pattern of BrdU was sufficiently reproducible for measurements of tumour cell proliferation. In order to facilitate rapid dissection of non-necrotic parts of the LTW tumours, they had to be small and thus sacrifice was carried out on day 13 instead of days 30–32, as in the aforementioned studies. Furthermore, tumour burden had to be estimated by volume instead of weight since biopsies were taken.

In the present study, energy charge in tumour tissue from both groups was lower than previously shown for normal tissue [10] and thus in accordance with data on sedentary mice [8]. Unexpectedly, energy charge was increased in the tumours from exercising animals as compared to those from sedentary controls, despite reduced tumour burden.

Voluntary exercise affects most of the purine nucleotides and increases energy charge in fast-twitch glycolytic skeletal muscle, but not in the slow-twitch one [10]. The energy cost is higher in this tissue [20] and there is a lower degree of oxidation [21], and thus it is energetically more sensitive to ischaemia [22, 23]. As respiration and glycolysis have been demonstrated to be reduced in tumours from sedentary animals as compared to normal tissue, the present findings might thus reflect profound changes in tumour metabolism. Contributing factors behind the present improvement in tumour energy state due to exercise might be nutrient availability by increased food intake [8] and tumour perfusion by increased cardiac output. The inverse correlation between distance run and energy charge in tumour tissue indicates a direct effect on tumour metabolism proportional to the amount of exercise.

The disturbance in energy metabolism was further reflected by a decreased content of AMP ($P < 0.05$) without a further degradation to IMP. This indicates that different tissues have different degradation pathways of ATP at intermediate steps of a stress situation. The increase in uric acid in the TBS group indicates loss of phosphorylated metabolites, as hypoxanthine and xanthine are further degraded with the concomitant generation of $O_2^{\cdot-}$. DNA synthesis time (T_s) and potential doubling time (T_{pot}) were only numerically increased in the present study. Both values were expected to be increased as the final tumour burdens have been shown to be reduced by voluntary exercise in this and our previous studies. The pattern found was obviously due to postexercise effects as BrdU was injected 2 h after the exercise period, i.e. at 9 p.m., and the biopsies were taken 6 h after the exercise period. Most of the exercise and food intake takes place during the night in this experimental model. Our results suggest that the effects of exercise are closely correlated to the exercise periods and cease when the resting state takes place. However, cell death (apoptosis) to some extent cannot be excluded. The decrease in DNA synthesis following starvation has been shown to be preceded by a decrease in tumour ornithine decarboxylase activity (ODC) [4]. This enzyme, necessary for polyamine synthesis, has a relatively short turnover of 10–20 min, and alterations in ODC activity during exercise might thus not be detected in the postexercise period. Irreversible inhibition of ODC by difluoro-methyl-ornithine (DFMO) inhibits experimental tumour growth [24] with a maintained energy state in tumour tissue [8]. Whether ODC activity and/or availability of polyamines are limiting factors for tumour cell proliferation during voluntary exercise remains to be elucidated.

In conclusion, this study shows that energy charge in tumour

tissue is increased in voluntarily running rats. Concomitantly, tumour burden is reduced despite an increase in food intake. No significant reduction in tumour cell proliferation could be found in the postexercise period. The skeletal muscle might have a superior energy availability which is closely correlated to the exercising state *per se*.

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Acknowledgements—This work was supported by the Swedish Medical Research Council (project Nos B95-17X-00536-31A and B94-17X-07184-10C), the Göteborg Medical Society, the Sahlgrenska Hospital Foundation, the University of Göteborg, the Assar Gabrielsson Foundation, The King Gustav V Jubilee Clinic Cancer Research Foundation in Göteborg, the Gunnar, Arvid and Elisabeth Nilsson Foundation, and the Inga-Britt and Arne Lundberg Foundation. We thank Anette Arvidson, Marareta Jonsson, Lilian Karlsson and Lola Reinholdtsen for technical assistance.