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Adenosine and Other Low Molecular Weight Factors Released by Muscle Cells Inhibit Tumor Cell Growth[1]

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ABSTRACT

In this study, we investigated the basis of the resistance of muscles to tumor metastases. We found that a low molecular weight fraction (M_r <3000) of skeletal muscle cell-conditioned medium (MCM) markedly inhibits the proliferation of carcinoma, sarcoma, or melanoma cell lines *in vitro*. The MCM exerts a cytostatic effect on tumor cell growth and arrests the cells in G_0/G_1 of the cell cycle. However, normal cell proliferation of cells such as bone marrow cells or fibroblasts was found to be refractory to the influence of the MCM. A reduction in melanoma growth was observed in mice treated with the MCM.

Adenosine was identified as one of the active components in the MCM by using high-performance liquid chromatography separations, mass spectra, and nuclear magnetic resonance analyses. At a concentration of 4 μM, equal to that found in the MCM, adenosine inhibits the proliferation of tumor cell lines (Nb2 lymphoma, K-562 leukemia, and LNCaP prostate carcinoma cells) while stimulating the proliferation of normal murine bone marrow cells. By similar methods, additional inhibitory components were detected in the MCM in a molecular mass range of 600–800 Da.

The ability of adenosine and other low molecular weight components to specifically inhibit tumor cell growth *in vitro* and *in vivo* may account for the resistance of muscle to tumor metastases.

INTRODUCTION

The inverse relation between cancer development and physical activity has been demonstrated both in animal and human studies. Animal model studies have shown that tumor growth is inhibited to a greater extent in exercised rats, as compared to a control that did not perform physical activity (1, 2). Giovannucci *et al.* (3) presented the results of a prospective study in 47,723 men, which showed that moderate level of physical activity is correlated to a substantially lower risk for colon cancer and adenoma. Thune *et al.* (4), in a prospective study carried out in 25,624 women, showed that greater leisure time activity is associated with a reduced risk of breast cancer. These studies indicate a potential link between muscle activity and increased resistance to development of cancer. Indeed, the rarity of tumor metastases in striated muscle is clinically established, and over the last 120 years, only a few reports of metastases in the muscle tissue have appeared in the literature (5–7).

Recently, we have shown that muscle cells derived from primary cultures of newborn rats are capable of producing a low molecular weight factor, designated MF,[3] which inhibits the proliferation of various tumor cell lines, including melanoma, sarcoma, and carcinoma, both *in vitro* and *in vivo* (8).

The MF is produced spontaneously by differentiated muscle cells. Although it is of rat origin, it affects both human and murine tumor cell proliferation. We have also shown that the MF does not inhibit growth of normal cells, such as proliferating bone marrow cells, fetal liver erythroblasts, or fibroblasts, from primary cultures. Cell cycle analysis reveals that the MF exerts a cytostatic rather than cytotoxic effect on proliferating tumor cells by arresting them in the G_0/G_1 phase of the cell cycle.

Here, we have further purified the active tumor-inhibitory components in the MCM, and we show that one of them is adenosine. Other low molecular weight antitumor components in the MCM were also detected.

MATERIALS AND METHODS

Preparation of MCM

MCM was obtained from the L-8 cell line (consisting of proliferating nontumorigenic myoblasts), which was purchased from the American Type Tissue Culture Collection (Manassas, VA). The cells were routinely maintained in DMEM containing 4.5 g% glucose and 15% FCS (Biological Industries, Beit Haemek, Israel).

For preparation of conditioned medium, the cultures were grown until confluence, medium was discarded, and the cells were washed twice with PBS and incubated for an additional 20 h in PBS. At the end of the incubation period, the supernatant was collected, centrifuged, filtered through 0.22-μm filter, and subjected to ultrafiltration through an Amicon membrane with a molecular weight cutoff of M_r 3000, because our previous work (8) showed that the inhibitory activity is detected in a molecular weight fraction lower than M_r 3000. MCM refers to this ultrafiltrated medium throughout.

Tumor and Normal Cells

Tumor Cells. tumor cell lines from murine, rat, or human origin were used. The murine cell lines included B-16-F10 melanoma, DA-3 breast carcinoma, and the LNCaP human prostate adenocarcinoma. All were purchased from the American Type Culture Collection, excluding the DA-3, which was kindly supplied by Dr. Y. Keidar from the Tel-Aviv University (Tel-Aviv, Israel). The rat cell lines included the Nb2-11C growth hormone-dependent lymphoma cell line and the Nb2-SP hormone-independent lymphoma cell line (9).

The human cell lines included SK-28 melanoma, MCF-7 breast carcinoma, and K-562 chronic myelogenous leukemia, all purchased from the American Type Culture Collection.

The cells were routinely maintained in RPMI 1640 containing 10% FCS and were transferred twice weekly to a freshly prepared medium.

Normal Cells. As a control for tumor proliferating cells, four normal proliferating cell types were used: bone marrow cells derived from the femur of C57BL/6J mice; fibroblasts derived from primary cultures of newborn rat skeletal muscle cells (prepared as described by us previously; Ref. 8); IM-9 human nontumorigenic lymphocyte cell line (American Type Culture Collection), which served as a normal control for the lymphoma cell lines described above; and L-8 myoblast rat cell line, which also served as the source for the MCM.

Cell Proliferation and Differentiation Assays

Cell Count Assay. The effect of MCM on the proliferation of rat Nb2-11C lymphoma cells was monitored by cell counting. The Nb2-11C cells were synchronized in the G_0/G_1 phase prior to cultivation with the MCM by transferring the cells to medium supplemented with 5% horse serum (Biological Industries) for overnight incubation. Cells (1.2×10^5 cells/ml) were cultured in 24-well plates in 1 ml of RPMI 1640 containing 5% horse serum, and 2-fold dilutions of MCM were added. Because the MCM consisted of PBS,

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[3] The abbreviations used are: MF, muscle factor; MCM, muscle cell-conditioned medium; HPLC, high-performance liquid chromatography; RP, reverse phase; LC, liquid chromatography; MS, mass spectra.

2-fold dilutions of PBS were added to synchronized Nb2-11C cells as controls. Cell proliferation was initiated by the addition of human growth hormone (Biotechnology General, Rehovot, Israel) to a final concentration of 2 ng/ml. The cultures were incubated at 37°C at 5% CO_2 and counted in Coulter Counter 48 h later. Inhibition of cell proliferation was calculated as follows:

$$\% \text{ inhibition} = \frac{A - B}{C - B} \times 100$$

where A = cell count of sample, B = control cell count without hormone, and C = control cell count with hormone.

This assay with the Nb2-11C cells was also used as the biological assay to detect inhibition of proliferation during the fractionation procedure.

The effect of the purified MCM on the proliferation of Nb2-SP and IM-9 was evaluated according to the same protocol described above for the Nb2 cells.

[^{3}H]Thymidine Incorporation Assay. Murine and human tumor and normal cells (1×10^4 cells/well of each) were incubated with RPMI 1640 containing 10% FCS and 50% MCM or purified MCM in 96-well microtiter plates for 48 h. Because the MCM was prepared in PBS, cultures containing tumor cells suspended in RPMI 1640 with 50% PBS and 10% FCS served as controls. During the last 6 h of incubation, each well was pulsed with 1 μCi of [^{3}H]thymidine. The cells were harvested, and the [^{3}H]thymidine uptake was determined in an LKB liquid scintillation counter (LKB, Piscataway, NJ).

Differentiation Assay. Cell differentiation was assessed by incubating 2×10^5 K-562 cells for 96 h in a culture system containing RPMI 1640, 10% FCS, and adenosine in a concentration of 4 μM. As a control, hemin, in a concentration of 5 μM, which is known to induce differentiation in the K-562 cells, was added. Following the incubation period, the cells were cytocentrifuged and stained with benzidine (10). Differentiated cells contain hemoglobin, which is clearly stained by the benzidine. Two hundred cells were analyzed in each sample, and results are expressed as percentage of stained cells.

Cell Cycle Analysis

Flow cytometric analysis of the cell cycle of K-562 myelogenous leukemia cells was carried out by propidium iodide staining according to Krishan (11). K-562 cells, at a concentration of 1×10^6/ml, were cultured in RPMI 1640, 10% FCS, and 50 or 25% MCM and were incubated for 24 h at 37°C in a CO_2 incubator. K-562 cells cultured in RPMI 1640 containing 50 or 25% PBS and 10% FCS served as a control.

At the end of the incubation period, the cells were washed three times with PBS and resuspended in staining buffer containing 0.1% BSA, 50 μg/ml propidium iodide, 0.1% Triton X-100, and 1 mg/ml RNase (boiled for 10 min). Samples were examined after 30 min of staining on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

To examine whether the effect of MCM on the cell cycle is reversible, in some cultures, following the 24-h incubation period with the MCM, the cells were centrifuged, the MCM was changed with RPMI 1640, and the cultures were incubated for additional 48 h. Cell cycle analysis was carried out as detailed above.

Fractionation and Partial Purification of the Inhibitory Activity Found in the MCM

HPLC Separations. The MCM was subjected to fractionation through four types of HPLC columns. During each purification step, fractions were collected and assayed for their ability to inhibit cell proliferation of Nb2-11C lymphoma cells. Each fraction was lyophilized prior to testing, suspended in RPMI 1640, and filtered through a 0.22-μm sterile filter.

The first column was a preparative RP-HPLC having a diameter of 2 inches and a length of 200 mm. The column was loaded with Merck Lichrosphere-C18 particles having a diameter of 12 μm and a porosity of 60 Å. During each run, 800 ml of the above filtrate were loaded, and the column was eluted with a gradient of acetonitrile at a rate of 2%/min, until it reached a level of 60% acetonitrile. The rate of elution was 100 ml/min, and 200-ml fractions were collected. A small portion (5 ml) from each fraction was dried and then used to test biological activity.

Active fractions were dried on a rotary evaporator, dissolved in water, filtered, and injected into a Supelco-C18 5-μm column (4.6 × 250 mm) with porosity of 80 Å. The flow rate was 1 ml/min. The eluent was a water/acetonitrile gradient between 0 and 60% acetonitrile. The gradient rate was 3%/min; *i.e.*, it reached 60% after 20 min. One-ml fractions were collected and tested for biological activity, and the active fractions were dried in a concentrating centrifuge and then dissolved in 50 mM formic acid for the next chromatography step on a 5-μm Polyhydroxyethyl A column (9.4 × 200 mm; Poly LC, Columbia, MD), with the eluent being a 50 mM formic acid solution. Two hundred-μl samples obtained from the previous stage were injected into the Poly LC column and chromatographed at a flow rate of 0.5 ml/min, and 0.5-ml fractions were collected. The fractions were dried in a concentrating centrifuge, tested for activity, and further purified on a 5-μm Hypersil Cyanopropylsilane column (4.6 × 250; Shandon, Columbia, MD). Two hundred-μl samples were injected into the latter column with a water/acetonitrile gradient of 0–60% and a flow rate of 1 ml/min.

LC/MS Analysis. At the last purification step, two peaks of inhibitory activity, fractions A and B, were found and were subjected to LC/MS analysis.

Fraction A was analyzed using a method based on microprobe HPLC in combination with tandem mass spectrometry (LC/MS/MS) in the positive ionization mode. Chromatographic separations were performed on a Valupack C-8, 3-μm column (Keystone Scientific, Bellefonte, PA). The mobile phase, methanol:1% acetic acid (45:55, v/v), was delivered at a flow rate of 30 μl/min. The mass spectrometer (model TSQ-700; Finnigan Mat, San Jose, CA) was operated in the positive ion electrospray mode. A potential of 3900 V was applied to the electrospray needle to provide an ion spray of 0.2–0.8 μA during the run. The heated capillary was set at 200°C. Because the retention time at the specific HPLC conditions pointed toward a mass of 268 Da, which was presumed to be adenosine, the ion parent-to-daughter (m/z 268 to 136) reaction was monitored at 1 s for target ion m/z 136 (argon was used as the collision gas). The mass resolution was set at 1 atomic mass unit for both parent and daughter ions.

Fraction B was subjected to chromatographic analysis (liquid chromatograph model LC-10AD; Shimadzu Scientific, Tokyo, Japan) on a microprobe 100 × 1 mm column packed with 3 μm Valupack C8, using a gradient consisting of methanol and 0.2% formic acid. The mass spectrometer (model TSQ-700; Finnigan Mat) was operated in the positive ion electrospray MS mode.

***In Vivo* Studies.** The crude MCM (before ultrafiltration), the MCM, and a purified fraction of the MCM after the first step of HPLC (preparative column, adjusted through recovery calculations to the original concentration of the MCM) were all tested for their ability to inhibit the development of B-16-F10 melanoma in C57BL/6J mice. Each group included 20 mice that were i.v. inoculated with 2×10^5 B-16-F10 melanoma cells. The mice were treated by i.p. injection, twice daily by 0.5 ml of the test material, and a control group was similarly treated with PBS. After 18 days, the mice were sacrificed, and melanoma tumor foci were counted in the lung. In addition, hematological parameters, spleen, and body weight were measured in the treated and control groups. Statistical analysis was carried out using the Student's t test.

RESULTS

Effect of the MCM on the Proliferation of Tumor and Normal Cells. The growth of the Nb2-11c rat lymphoma cells was markedly inhibited following incubation with the MCM. Dose-dependent inhibition data are shown in Fig. 1*a*.

Murine or human cancer cells derived from solid or leukemic tumor cell lines showed a marked statistically significant ($P < 0.001$) inhibition of [^{3}H]thymidine uptake following incubation with 50% MCM (Fig. 1*b*).

The effect of purified MCM on the proliferation of normal cells was studied. Pooled fractions from a preparative RP-HPLC column, found to be active in inhibiting proliferation of tumor cells (fractions 9 and 10 from Fig. 4), were dried, resuspended in RPMI 1640, and tested to determine their ability to inhibit proliferation of bone marrow cells, fibroblasts, the L-8 muscle cells, and the nontumorigenic lymphoid cell line IM-9. The proliferation of the later was compared to that of the two lymphoid tumorigenic cell lines Nb2-11C and Nb2-SP. Fig.

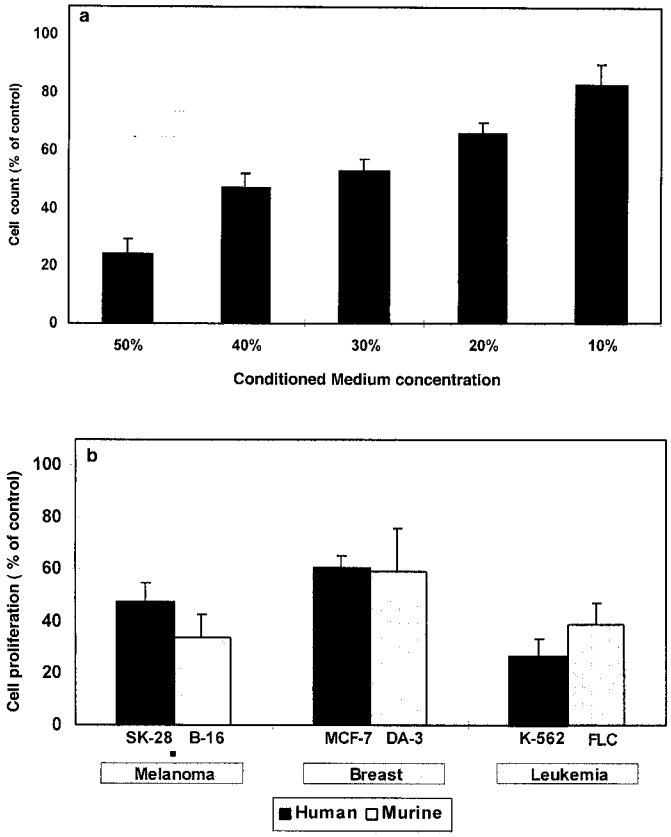

Fig. 1. *a*, inhibitory dose-dependent effect of the MCM, at a concentration of 50%, on the proliferation of synchronized Nb2-11c rat lymphoma cells. Proliferation was evaluated by determination of cell count. Maximal effect is seen in a concentration of 50%. *b*, inhibitory effect of the MCM on the proliferation of human and murine cell lines. Proliferation was evaluated by [^{3}H]thymidine uptake.

2*a* shows a stimulatory effect of the purified MCM on the proliferation of bone marrow cells, fibroblasts, and the L-8 muscle cells. However, the MCM inhibited the proliferation of the hormone-dependent Nb2-11C and the hormone-independent Nb2-SP tumor cell lines but had essentially no effect on the nontumorous human lymphocyte IM-9 cell line (Fig. 2*b*).

Effect of the MCM on the Cell Cycle of K-562 Leukemic Cells. Flow cytometric analysis of K-562 leukemic cells, incubated with or without MCM and stained with propidium iodide after RNase digestion, was carried out. The distribution of cells at different stages of the cell cycle was assessed. An increase in the number of cells in the G_0/G_1 phase and a decrease in the number of cells in the S phase was recorded in the samples incubated with 50% of MCM, as compared to the control (Fig. 3). A dose-dependent effect was seen when 25% MCM was added to K-562 cells (G_0/G_1: control, 54%; MCM, 64.7%; S: control, 40.7%; MCM, 30.6%). These results indicate the capability of the MCM to arrest the tumor cells in the G_0/G_1 phase of the cell cycle. The effect of the MCM was reversible when, after 24 h, the medium was changed to RPMI 1640, and cell cycle analysis revealed similar results to those of the control cultures.

Fractionation and Purification of the Active Component in the MCM. A method was developed for purification of the MCM consisting of several steps that entailed ultrafiltration through a M_r 3000 Amicon membrane, followed by four HPLC columns and LC/MS analysis, as described in "Materials and Methods."

Fig. 4 depicts the capability of various fractions eluted from different stages of HPLC purification to inhibit the proliferation of the Nb2-11C lymphoma cells. The first two columns, preparative RP-HPLC and Supelco C-18 RP-HPLC, resulted in an inhibitory activity that eluted as a single peak of activity at an acetonitrile concentration of 16–20%. In the Polyhydroxyethyl A column, two peaks with activity were detected, one sharp peak at 17–22 min (peak A) and the second, which was clear and broad, at 25–32 min (peak B). The inhibitory fractions of each peak were pooled and were rechromatographed using the Hypersil Cyanopropylsilane HPLC column. The active fraction of peak A eluted at 20–24% acetonitrile, whereas that of peak B eluted at 40–60% acetonitrile. Percentages of recovery in each purification step are specified on the graphs.

The active inhibitory components in the MCM are soluble in water. In addition, these components retained their biological activity following freezing/thawing, as well as after drying and redissolving in an aqua medium. In previous studies, we have demonstrated (8) that these inhibitory components were stable after heating and lyophilization. All of the above findings demonstrate the stability of the tumor-inhibitory components of the MCM.

Peak A was subjected to LC/MS analysis. The combination of retention time at the specific HPLC conditions (Fig. 5*a*), the mass of the parent ion (m/z = 268), and the mass of the daughter ion (m/z = 136; Fig. 5, *c* and *d*), as a chromatogram of commercial adenosine was analyzed in the same conditions and was found in the same retention time (Fig. 5*b*). These results proved that adenosine is present in fraction A. Quantitative analysis of fraction A revealed that the concentration of adenosine was 4 μM.

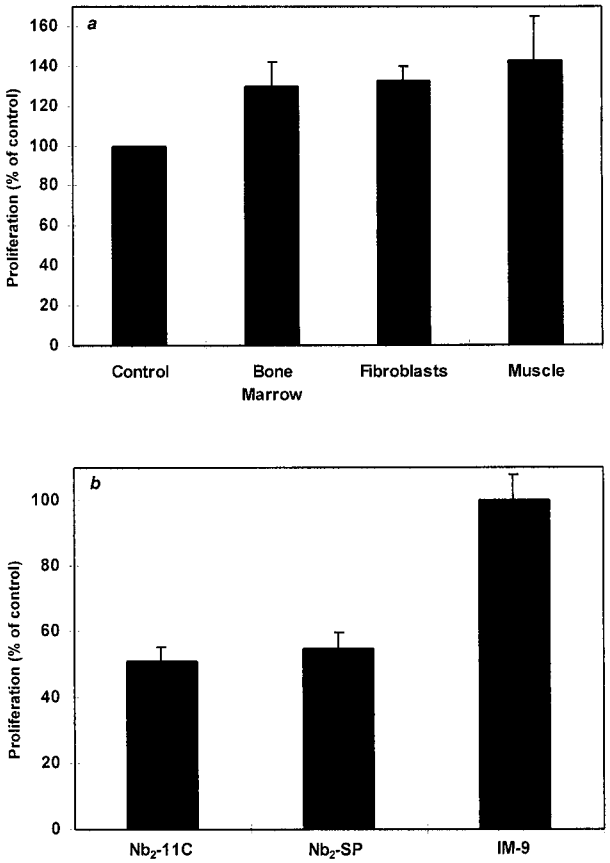

Fig. 2. *a*, effect of the MCM on the proliferation of normal murine bone marrow cells, rat fibroblasts, and L-8 muscle cells. *b*, the two lymphoma rat cell lines Nb2-11c and Nb2-SP in comparison to the nontumorigenic IM-9 lymphocyte cell line. The four types of normal cells, *i.e.*, bone marrow cells, fibroblasts muscle cells, and the IM-9 lymphocytes, were stimulated by the MCM. Cell proliferation was evaluated by [^{3}H]thymidine uptake (*a*) and by cell count (*b*).

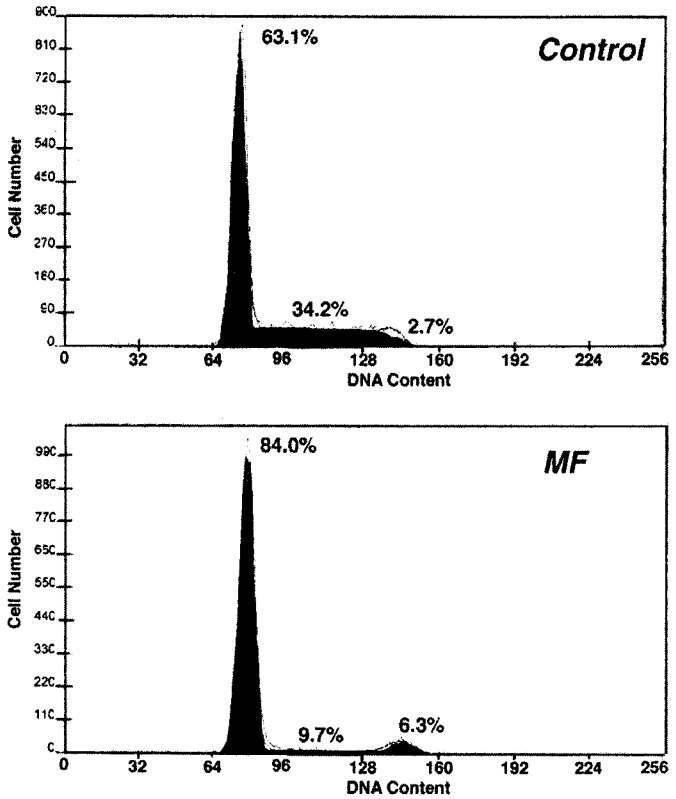

Fig. 3. Flow cytometric analysis of the cell cycle status of K-562 myelogenous leukemia cells following incubation with 50% MCM. An increase in the number of cells in the G_0/G_1 phase is seen. Distribution of the cells at the different stages of the cell cycle is specified.

Analysis of fraction B in the range of 600–1800 mass units revealed seven separate peaks, three of them (within the range of 600–800) having biological activity. Subsequent chromatographic analysis using three different HPLC columns (Valupack C18, 3 μm, 150 × 3 mm; Valupack C8, 3 μm, 150 × 3 mm; Hamilton, PRP1, 9 μm, 150 × 4 mm) confirmed the correlation between the same three masses in the range of 600–800 Da and the biological activity.

Effect of Adenosine and Fraction B on the Proliferation of Tumor and Normal Cells. Adenosine at a concentration of 4 μM was added to Nb2 lymphoma, K-562 leukemia, and LNCaP prostate adenocarcinoma cells and to normal bone marrow cells, fibroblasts, and the L-8 muscle cells. Cell proliferation using [^{3}H]thymidine uptake was determined. The proliferation of the three tumor cell lines was markedly inhibited ($P < 0.001$), whereas a statistically significant stimulation of bone marrow cells (121 ± 9%; $P < 0.01$)) and fibroblasts (137 ± 10%; $P < 0.01$) was recorded, whereas the L-8 muscle cells were refractory to the effect of adenosine (Fig. 6). Adenosine did not induce differentiation of K-562 cells, as was observed by counting percentage of cells stained for benzidine (control, 2%; hemin, 31%; adenosine, 2%).

Fraction B inhibited the proliferation of Nb2 lymphoma cells and B-16 melanoma cells (52 ± 7% and 45 ± 9%, respectively; $P < 0.001$), whereas bone marrow cells or fibroblasts were refractory to its effect.

Effect of Adenosine and Fraction B on the Proliferation of Tumor Cells. K-562 cells were incubated in the presence of 4 μM adenosine to assess its capability to induce cell differentiation. Hemin, which is known to induce differentiation in the K-562 cells, was added as a positive control. Cell counts showed 2% in the control group, 33% in the positive control group, and 3% in the adenosine-treated group.

***In Vivo* Studies.** B-16-F10 melanoma cells were inoculated by i.v. injection into four groups of mice. The mice were treated twice daily by an injection of crude MCM, MCM, or a purified MCM chromatographed by preparative RP-HPLC. Mice were sacrificed after 18 days, and the different parameters were monitored as depicted in Table 1. The number of melanoma foci in the lungs of the mice were reduced following the three different treatments (in all groups, $P < 0.001$; Table 1).

Statistically significant differences could be detected among the treated groups, suggesting that the same active components are re-

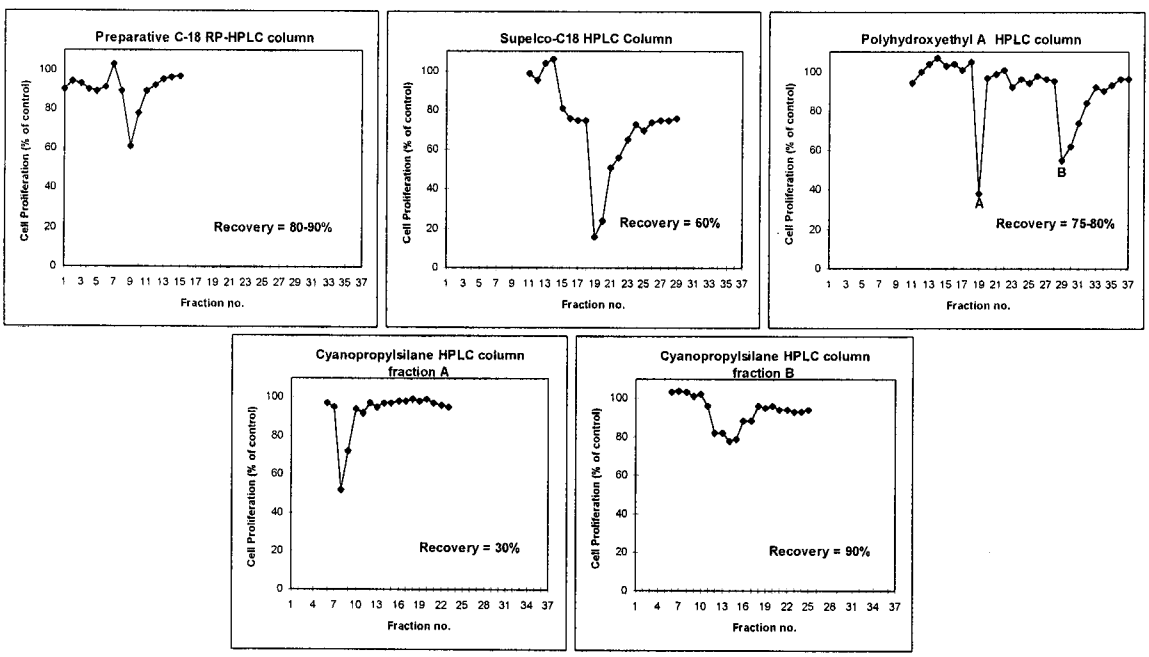

Fig. 4. The biological activity of fractions eluted from HPLC columns regarding their capability to inhibit the proliferation of the Nb2-11C lymphoma cells. Active inhibitory fractions from each column were pooled and passed through to the next column. Cell count assay was used.

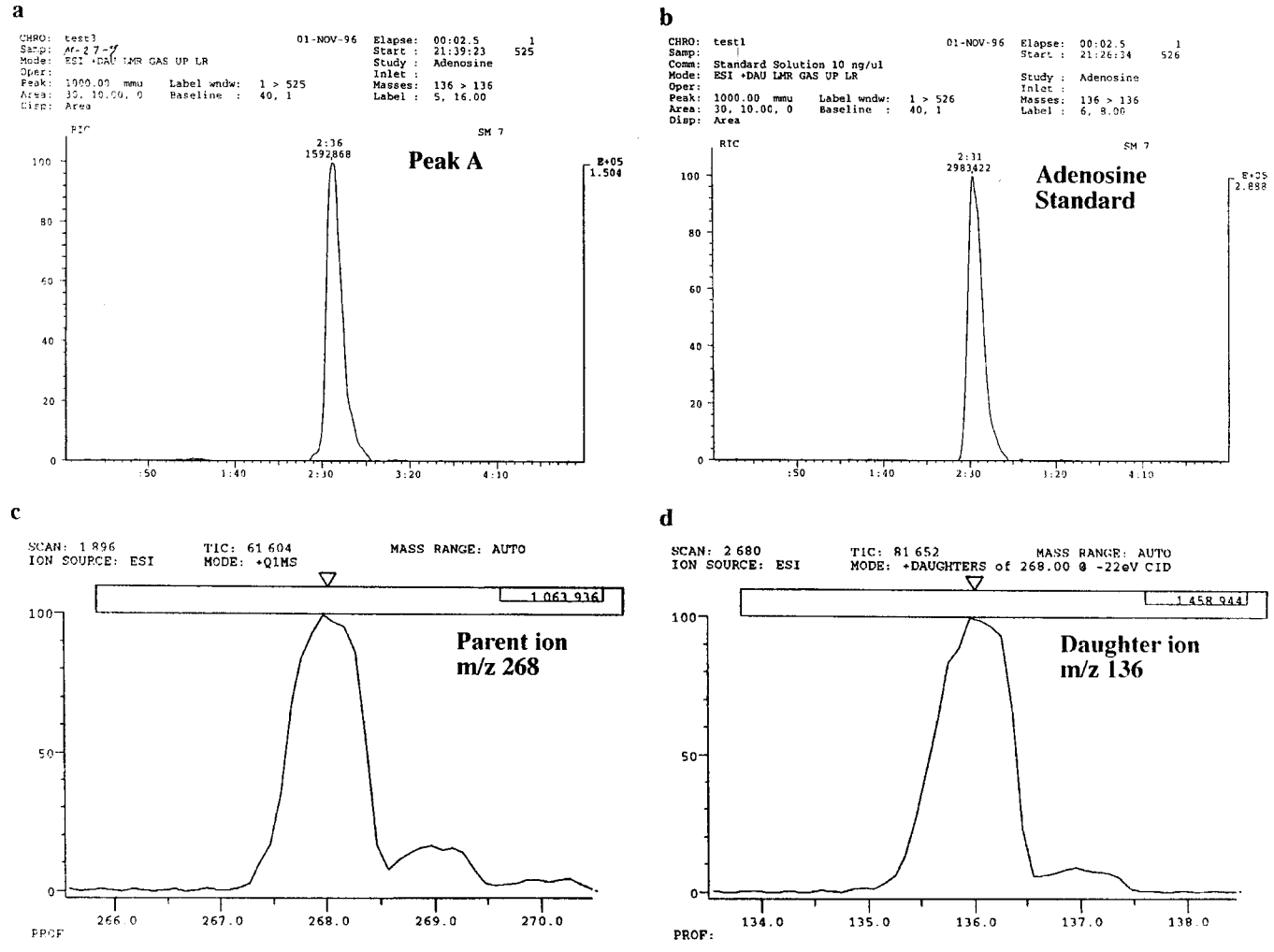

Fig. 5. LC/MS analysis of peak A. The combination of retention time of peak A (*a*), the mass of the parent ion (m/z = 268; *c*), and the mass of the daughter ion (m/z = 136; *d*) were found to be unique for adenosine. A chromatogram of synthetic adenosine (*b*) was analyzed in the same conditions and was found in the same retention time.

sponsible for the inhibitory activity. There were no differences in body or spleen weight or in WBC count or hemoglobin levels. In the differential blood cell count, a sharp tendency toward monocytes was observed following treatment with the three samples.

DISCUSSION

This work was undertaken to further study the phenomenon of muscle resistance to tumor metastases. The specific antiproliferative activity of MCM toward tumor cells was demonstrated by four different methods: decreased cell count, reduced [^{3}H]thymidine incorporation, cell cycle arrest in the G_0/G_1 phase, and inhibitory effect *in vivo* on tumor cell growth.

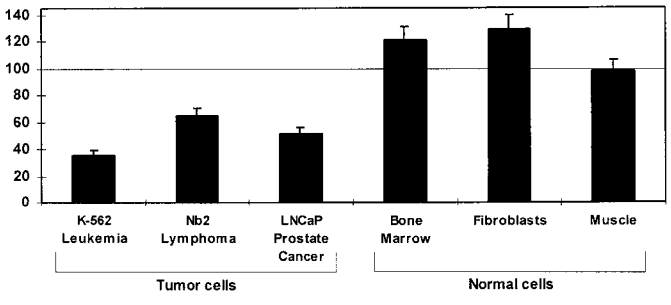

Fig. 6. Effect of adenosine at a concentration of 4 μM on the proliferation of normal and tumor cells, as measured by [^{3}H]thymidine incorporation assay.

Tumor cells originating from melanoma, carcinoma, leukemia, or lymphoma cell lines were markedly inhibited following incubation with the MCM, whereas normal cells such as bone marrow cells, fibroblasts, or muscle cells were refractory to its effect.

Although of a rat origin, the MCM affected both human and murine tumor cells. Thus, it may be concluded that the active components in the MCM are not tumor and species specific.

The capability of muscle cells to secrete these factors was independent of the differentiation state of the muscle cells. In this study, skeletal myoblast newborn rat cells were used, whereas in our previous work (8), well-differentiated myocytes derived from primary cultures of the same origin were used. However, based on the *in vitro* biological tests that we present here, it may be concluded that the well-differentiated contracting muscle cells release a higher level of inhibitors as compared to myoblasts. This finding also supports the previously postulated link between muscle activity and reduced risk to cancer development.

Muscle cells are known to secrete cytokines possessing antiproliferative activity toward tumor cells such as tumor necrosis factor, lymphocyte-infiltrating factor, IFN, or transforming growth factor β (12–16). The capability of the muscle cells to secrete these antigrowth factors cannot serve as the sole explanation for its resistance to

Table 1 *Effect of crude, M_r <3000 ultrafiltrated, and purified L-8-conditioned medium on the development of melanoma lung metastatic foci and physiological and hematological parameters in C57BL6/J mice*

	No. of foci	Mass (g)	Spleen mass (g)	WBC ($\times 10^3$)	Lymphocytes (%)	Granulocytes (%)	Monocytes (%)	HGB (g/dl)
Control								
Mean	61.1	21.07	143.27	7.01	91.46	3.03	5.53	11.09
SE	10	0.55	10.69	0.53	0.75	0.39	0.48	0.3
Crude								
Mean	22.27	22.22	146.27	7	83.97	4.01	12.02	10.01
SE	5.15	0.54	8.67	0.46	1.61	0.73	1.413	0.37
M_r <3000								
Mean	18.46	21.0	150.3	7.51	84.3	3.82	11.44	10.84
SE	5.31	0.96	10.82	0.95	2.29	0.62	1.92	0.34
Pure								
Mean	25.2	21.08	136.2	7.82	85.29	3.47	11.14	10.9
SE	5.08	0.57	7.36	0.81	0.83	0.4	0.62	0.22

metastases invasion because these cytokines are produced by other body organs that are susceptible to metastases. It is possible that low molecular weight proliferation inhibitors secreted by muscle cells with specific antitumor activity may account for low incidence of metastases in this organ.

To define the active components in the MCM, purification procedures involving different HPLC purification steps and LC/MS analysis were carried out. Following four HPLC steps two active peaks, peaks A and B, were found to have tumor-inhibitory activity. Mass spectra analysis of peak A revealed the presence of adenosine. We then determined that the MCM contains adenosine at a concentration of 4 μM. Introduction of adenosine at this concentration to cultures of tumor proliferating cells, *e.g.*, lymphoma, prostate carcinoma, or leukemia cells, markedly inhibits the proliferation of these cells but stimulates the proliferation of normal cells, such as bone marrow cells or fibroblasts. The L-8 myoblasts were not affected by the adenosine, a result that emphasizes the specific inhibitory effect of adenosine on tumor cell proliferation.

Adenosine is known as a regulator of several physiological processes. For example, it affects cardiac rhythm (17, 18), it acts as a vasoconstrictor in the kidney, and it acts as a vasodilator in other vascular beds (19). Adenosine is also known to exert effects on blood cells, such as inhibition of platelet aggregation (20) and stimulation of erythropoietin production (21), and it has an anti-inflammatory effect via the inhibition of inflammatory cytokine production (22, 23). A differential effect of adenosine on normal and cancer cells was previously reported by Bajaj *et al.* (24), who showed that proliferation of lymphocytes derived from patients with chronic lymphocytic leukemia was inhibited by adenosine, whereas proliferation of normal lymphocytes was inhibited to a lesser extent. Other *in vitro* studies have shown an inhibitory effect of adenosine on tumor cell growth (25, 26), and *in vivo* studies have shown an inhibitory effect on tumor development in mice (27, 28). Extracellular adenosine may bind or react with cell membranes through three different pathways: it can bind to specific cell surface receptors A1 and A2, which activate the G-protein/cyclic AMP pathway (29); it can enter the cells through nucleoside transporters (30); and it can be metabolized by membranal enzymes to inosine, an inactive metabolite (31, 32). The distribution of cell surface adenosine receptors, nucleoside transporters, and enzymes metabolizing adenosine is different between normal and tumor cells. This fact may account for the opposite effects of adenosine on cancerous and normal cells. Adenosine did not induce differentiation in K-562 cells, and the mechanism through which it inhibits tumor cell growth yet has to be explored. Peak B represents another factor with antiproliferative activity, in addition to adenosine, which may act additively or synergistically with adenosine in the inhibition of tumor cell growth.

LC/MS analysis of peak B revealed the presence of three molecular weight species in the range of M_r 600–800 having antiproliferative activity toward tumor cells.

The beneficial effect of exercise on cancer prevention has been demonstrated recently in two prospective studies. After several years of follow-up, it has been shown that there is a lower risk of developing breast cancer (in women) or colon cancer (in men) in physically active individuals (3, 4). Thune *et al.* (4) have suggested several mechanisms for the decreased incidence of breast cancer among women who engage in regular physical activity, related to hormonal balance alteration, such as interruption in the menstrual cycle, which subsequently leads to a lower cumulative exposure to estrogen, thereby inhibiting carcinogenesis in the breast. However, these explanations cannot account for the lower risk of cancer in males who engage in physical activity (3). The ability of muscle cells to release adenosine (a higher level is released following aerobic exercise) and other factors possessing the anticancer activity may account for the rarity of metastases in this organ and may be part of the explanation for the lower risk of developing cancer in physically active individuals. Exercise that activates muscle cells induces an increased production of adenosine (33, 34), which may subsequently act as a humoral physiological protective factor against tumor development.

The unique features of the factors described in this study include: (*a*) low molecular weight, (*b*) high thermal stability, and (*c*) water solubility may contribute to their bioavailability. Indeed, when administered to mice inoculated with melanoma cells, the MCM whether in a crude or purified form was capable of reducing the number of metastatic foci in the lungs. These unique characteristics may be important in the potential clinical use of these compounds in the development of novel anticancer therapies.

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