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Muscle cells produce a low molecular weight factor with anti-cancer activity

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The present study describes a new low molecular weight factor released by muscle cells, which inhibits proliferation of tumor cells *in vitro* and *in vivo*, is highly specific towards tumor cells, and has no observable effect on normal cells' proliferation. What first prompted us to investigate this factor was the observation that tumor metastases are extremely rare in striated muscles. Co-culturing of striated muscle cells with malignant cells led to marked morphological alterations in the latter, in contrast to the same cells when incubated without muscle cells. A conditioned medium of striated muscle cells was prepared and its effect tested on a variety of cells. This conditioned medium (CM) inhibited proliferation of tumor cell lines of murine (B16 melanoma, Madison 109 lung carcinoma, MCA-105 sarcoma, ESB lymphoma), or of human origin (HTB-38 adenocarcinoma, T47D breast carcinoma, CX1 colon carcinoma). The proliferation of normal cells (bone marrow cells, fetal liver erythroid cells) was not affected by the CM. Flow cytometric analysis of B16 melanoma cells following incubation with the CM revealed that 63% ± 12 of the cells were in the G_0/G_1 phase of the cell cycle, compared to 47.8% ± 8 of cells incubated with a medium (not conditioned) only. The activity of the CM and of certain fractions thereof was also demonstrated *in vivo*: they prevented tumor growth in mice inoculated intraperitoneally with MCA-105 sarcoma cells. Partial purification of the CM revealed that the active component was a non-proteinaceous compound with a molecular weight of about 500 D. The results clearly suggest that muscle cells produce a low molecular weight factor which can selectively inhibit the proliferation of tumor cells *in vitro* and *in vivo*. This factor is neither species nor tumor specific.

Keywords: inhibition, muscle factor, proliferation, tumor cells

Introduction

It is a widely acknowledged fact that malignant tumors spread and metastasize in almost all of the body's organs, yet are very rare in voluntary striated muscles [1, 2]. Although primary tumors in these muscles have been reported, it would seem that striated muscle tissue is extremely resistant to metastatic cancer [1]. The current literature shows that in the last 120 years, very few cases of tumor metastases in striated muscles have been described [1, 2]. In a series of 500 autopsies of cancer patients, Willis found only four cases of carcinoma that metastasized in the muscles (0.8%) [3]. Several theories have attempted to account for the low frequency of isolated muscle metastases [1–3]. In principle there are two possible explanations: either the striated muscle directly affects the circulating malignant cells, or it affects them indirectly, by

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secreting a factor(s) which in turn inhibits their proliferation. To further investigate this hypothesis, we set up an experimental system to test the effect of conditioned medium (CM) from newborn rat muscle cells on the proliferation of malignant cells. Our observations suggest that muscle cells secrete a low molecular weight factor which can inhibit tumor growth both *in vitro* and *in vivo*.

Materials and methods

Primary cultures of newborn rat muscle cells
Primary cultures of newborn rat muscles were prepared according to Shainberg *et al.* [4]. The muscles of the hind legs of 24- to 48-h-old newborn rats (Wistar rats obtained from Anilab, Rehovot, Israel) were separated and minced into small pieces. Following trypsinization with 0.24% trypsin–versan solution, the cells were preplated (in order to remove fibroblasts) in tissue culture dishes for 30 min. The cells were then counted and seeded in enriched Dulbecco modified Eagle medium (DMEM) (Gibco, USA).

Morphological examination by scanning electron microscope (SEM)
A co-culture of ESB cells or HTB-38 cells with 5-day-old muscle cells was maintained for 24 h in a humidified atmosphere containing 5% CO_2 at 37°C. Thereafter the ESB cells were separated and prepared for SEM examination. Since HTB-38 cells are adherent cells, their co-cultures were fixed and prepared for ultrastructural examination without separation. Co-cultures of fibroblasts and malignant cells served as controls. Muscle cells and fibroblast cultures were examined before and after 24 h of co-culture with ESB cells. SEM was performed according to standard procedures [5], except for using plastic supports when adherent cells were examined.

Preparation of CM from muscle cells
Muscle CM was obtained from newborn rat muscle cultures which contained contracting muscle cells (detailed above). On day 5, the contracting muscle cells were washed three times with PBS. Fresh RPMI was added to the cultures for a further 24 h. At the end of the incubation period the supernatant was collected, centrifuged and kept at −70°C till use. This CM was designated as MF CM.

Tumor cell lines
Tumor cell lines of murine or human origin were used. The murine cell lines included: Madison 109 (M109), a spontaneous, transplantable lung adenocarcinoma of BALB/c origin and MCA-105, a methylcholantrene-induced sarcoma of C57Bl/6J origin, kindly provided by Dr E. Kedar (Hadassah, University Hospital, Jerusalem, Israel), B-16-F10 melanoma cells (purchased from American Type Tissue Culture Collection, Rockville, MD, USA), and ESB cells derived from a mouse lymphoma [6]. The human cell lines included: HTB-38 cells derived from adenocarcinoma of human colon (purchased from American Type Tissue Culture Collection, Rockville, MD, USA), T47D breast carcinoma, CX1 colon carcinoma (kindly provided by Prof. M. Ravel from the Weizman Institute, Rehovot, Israel and Dr Traugort, Knoll. Ludvigshaffen, Germany, respectively), and M14 melanoma cells, kindly supplied by Prof. Ferrone (New York Medical College, Valhala, USA). The cells were routinely maintained in RPMI medium containing 10% fetal calf serum (FCS). Twice a week the cells were transferred to a freshly prepared medium. The cells were ready for experimental studies 2 days following splitting.

Normal control cells
As a control for the muscle cells and for the muscle CM, fibroblasts of the same origin, i.e. newborn rats, were used. Primary cultures of rat fibroblasts were obtained during the preplating process of the muscle cells as described above. The fibroblasts adhere to the plastic dishes and can be grown for several weeks in RPMI medium containing 10% FCS. Twice a week the cells were transferred to a freshly prepared medium. For preparation of CM, 3 days after splitting, the culture supernatant was discarded, replaced with RPMI medium and the cells were incubated for a further 24 h. Culture supernatants were then collected and when not immediately processed, were kept in the refrigerator at −70°C.

As a control for tumor cell proliferation, human bone marrow cells were obtained by sternal aspiration biopsy from patients without hematological disease during clinical work-up. Murine bone marrow cells were separated from the femur of C57Bl/6J mice. Embryonic mouse liver erythroblasts were obtained from mouse embryos at day 11 of gestation, according to previously reported methods [5].

Cell proliferation assays
Cells (1×10^4)/well of each murine tumor cell line, human tumor cell line or normal cells were incubated with two-fold dilutions of muscle CM in 96-well microtiter plates for 24 h. 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, USA).

Effect of the muscle CM on the cell cycle of B-16 melanoma cells

Flow cytometric analysis of the B-16-F10 melanoma cell cycle was carried out by propidium iodide staining according to Krishan [7]. Briefly, 1×10^6/ml B-16-F10 melanoma cells were cultured in RPMI medium containing 10% FCS and 50% muscle CM and were incubated for 24 h at 37°C in a CO_2 incubator. B-16-F10 melanoma cells cultured in RPMI and 10% FCS served as control. At the end of the incubation period, the cells were trypsinized, washed three times with PBS and resuspended in staining buffer containing 0.1% bovine serum albumin (BSA), 50 mg/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 & Co., Mountain View, CA, USA).

Characterization of the active component in the muscle CM

In order to determine whether the active component in the MF CM is a proteinaceous substance or not, the CM was subjected to a series of treatments including sensitivity to proteolytic enzymes, stability to lyophilization and effect of incubation at various temperatures. Following such treatments the CM was brought back to the original salt and protein concentration by dilution with the original medium, or if diluted, the dilution factor of the protein concentration was taken into account in evaluating the results. The [^{3}H]thymidine uptake assay with either HTB-38 or MCA-105 tumor cells was used as described above.

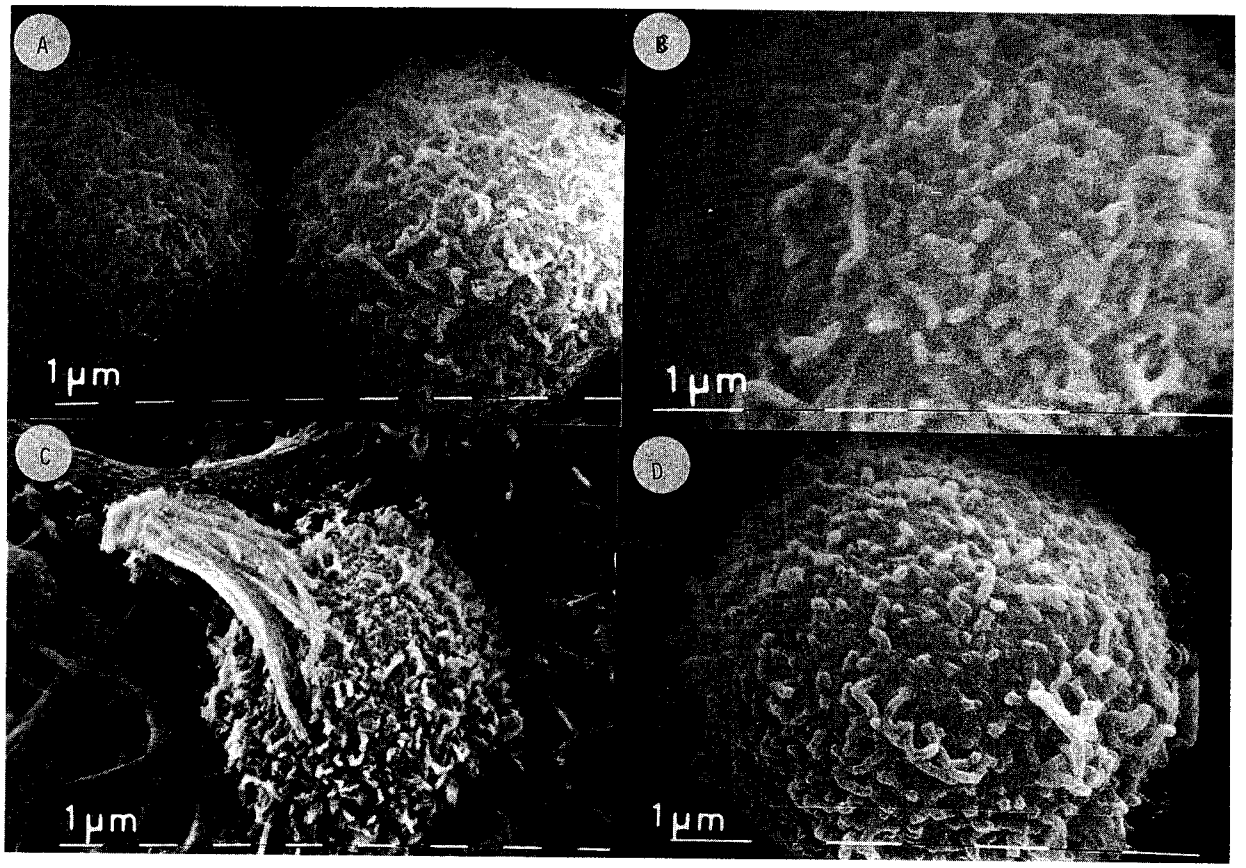

Figure 1. Scanning electron micrograph of ESB cells incubated with medium only (A, B), with myofibers (C) and with fibroblasts (D). The cells in (C) show marked surface damage.

Effect of proteolytic enzymes (trypsin and pronase). In order to determine the effect of trypsin, two procedures were used. In the first, MF was incubated with trypsin (0.5–2 mg/ml) for 4 h at 37°C, following which the trypsin activity was stopped by adding approximately 2-fold molar access of soybean trypsin inhibitor. In the second, the trypsin was removed on a column of P-aminobenzamidine-agarose. To test the effect of pronase, the MF was treated by bringing it into contact with pronase immobilized on a sepharose gel. In all experiments MF-free medium served as a control.

Lyophilization MF was lyophylized without prior dialysis and the lyophylizate was then redissolved in water and brought back to its original composition.

Heat treatment. MF was treated for various periods of time at temperature ranges of 4–100°C.

Molecular size of the active component in the MF. MF CM was fractionated by ultrafiltration on Amicon membranes with a molecular cut-off of 3 kDa. The effect of the filtrate and the retantate (which was recovered to the original volume) on the tumor cell growth was determined by the [^{3}H]thymidine uptake assay as described above.

In vivo *studies*

C57BL/6J mice were injected intraperitoneally (i.p.) with 2.5×10^5 MCA-105 cells. The mice were treated twice daily by i.p. injections of 0.5 ml of either muscle CM or the low molecular weight fraction of the muscle CM. Mice treated with RPMI medium only served as a control group. Each group included between 7 and 10 mice. The mice were sacrificed on day 33 and in each animal the size of tumor foci was classified according to >2 cm, between 0.5–2 cm and <0.5 cm.

Statistical analysis in all studies was carried out using the Student's *t*-test.

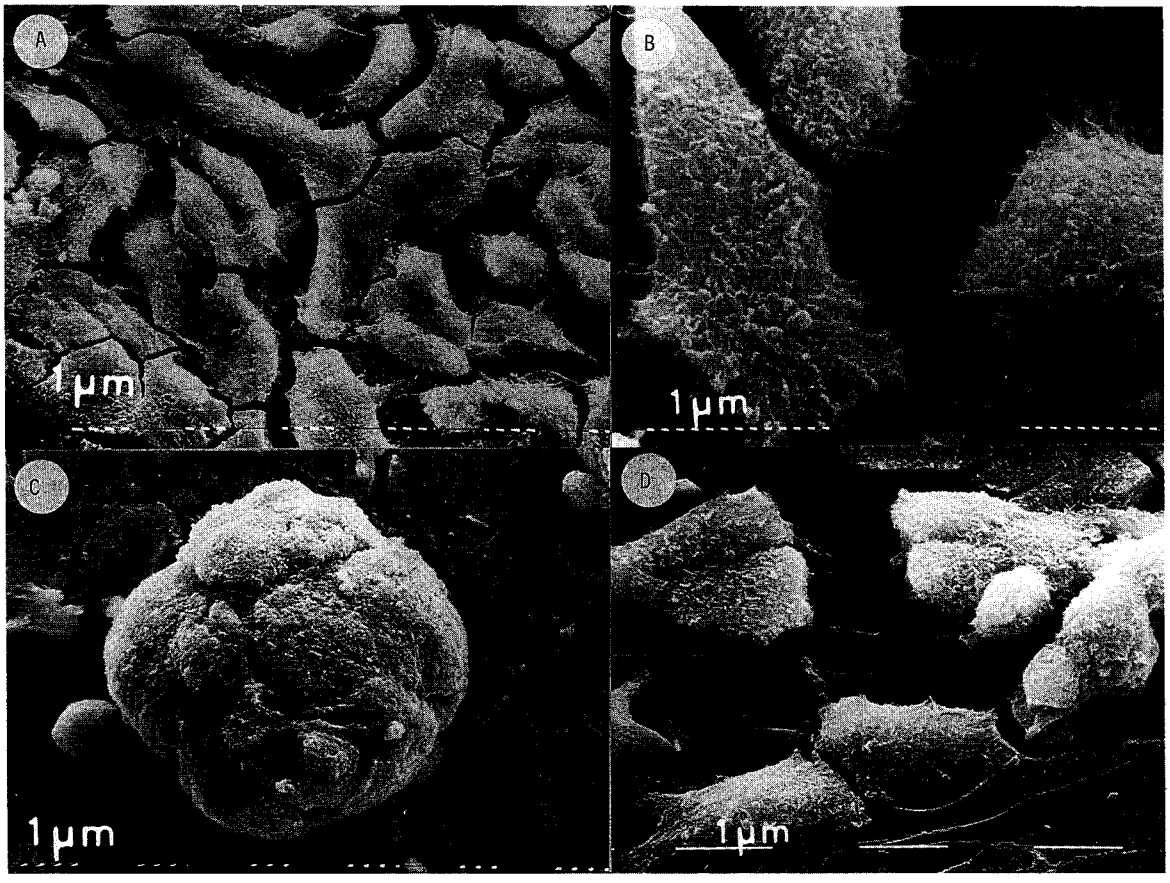

Figure 2. Scanning electron micrograph of HTB-38 cells incubated with medium only at lower (A) and higher magnification (B). Cells incubated with myofibers (C) show tendency to clumping and loss of the microvilli, whereas incubation with fibroblasts (D) did not cause any surface alterations.

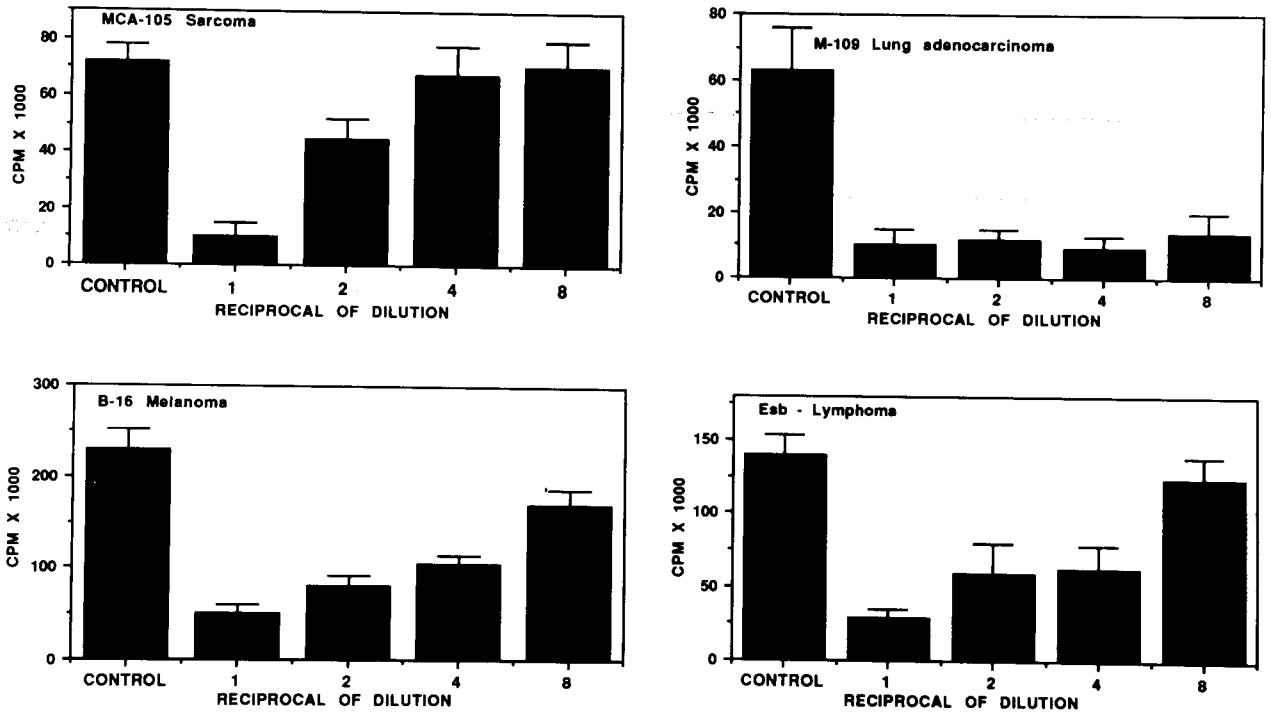

Figure 3. Murine tumor cell lines incubated with muscle CM show a dose-dependent inhibition or persistent inhibition of [^{3}H]thymidine uptake. [^{3}H]Thymidine incorporation is expressed as counts per minute (cpm) × 1000.

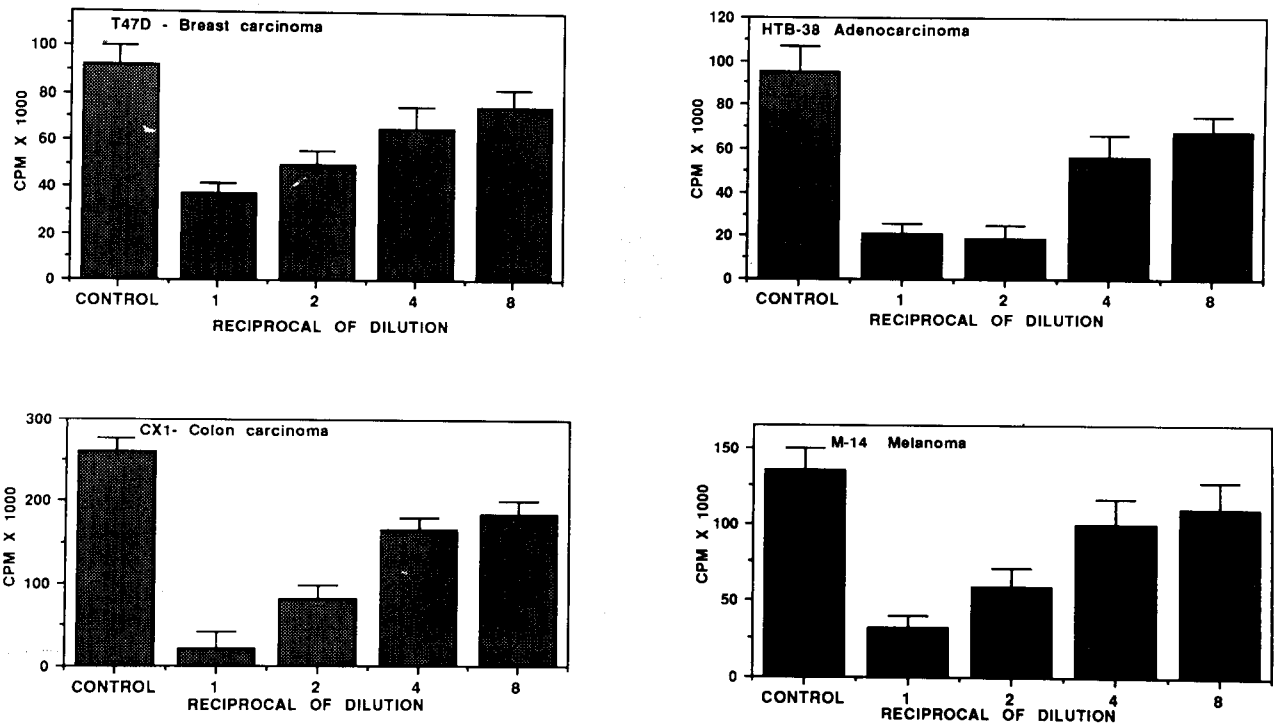

Figure 4. Human tumor cell lines incubated with muscle CM show a dose-dependent statistically significant inhibition of [^{3}H]thymidine uptake. [^{3}H]Thymidine incorporation is expressed as counts per minute (cpm) × 1000.

Results

Morphological examination of muscle/tumor co-cultures

Co-cultures of muscle and cancerous cells were analysed with a SEM. The morphology of ESB lymphoma cells is characterized by their numerous microvilli as shown in Figure 1A and B. On the other hand, when these cells were incubated with myofibers, the ESB cells showed marked surface alterations, such as disarray of the microvilli (Figure 1C). When incubated with fibroblasts the cells did not show any surface damage (Figure 1D).

Parallel experiments with HTB-38 cells revealed that cells incubated with medium only were characterized by their oval and polygonal shape and possessed numerous microvilli on their surface (Figure 2A, B). Incubation of these cells with muscle fibers caused clumping of the cells with loss of membranal borders and surface alterations (Figure 2C). Incubation with fibroblasts did not alter the morphology of the cells (Figure 2D).

Effect of muscle CM on the proliferation of tumor and normal cells

Murine or human tumor cells incubated with muscle CM showed a dose-dependent, statistically significant inhibition of [^{3}H]thymidine uptake ($P<0.001$) (Figures 3 and 4). The M-109 murine adenocarcinoma cells were invariably inhibited at all concentrations of muscle CM. There were no differences between the effect of the muscle CM on murine or human derived tumor cells.

Incubation of normal proliferating cells, i.e. human or murine bone marrow cells and embryonic liver erythroblasts with the muscle CM did not show any inhibitory effect on [^{3}H]thymidine uptake. In the case of rat fibroblasts, the cells were even stimulated following incubation with the muscle CM (Figure 5).

Fibroblast CM, which served as a control for the muscle CM, failed to inhibit the proliferation of both murine and human tumor cells.

Effect of the muscle CM on the cell cycle of B-16 melanoma cells

Table 1 summarizes the flow cytometric analysis of B-16 melanoma cells, incubated with or without MF CM and stained with propidium iodide after RNAse digestion. The table presents the distribution of the cells in the different stages of the cell cycle. A statistically significant increase in the number of cells in the G_0/G_1 phase was recorded in the cells incubated with MF CM, as compared to those incubated with

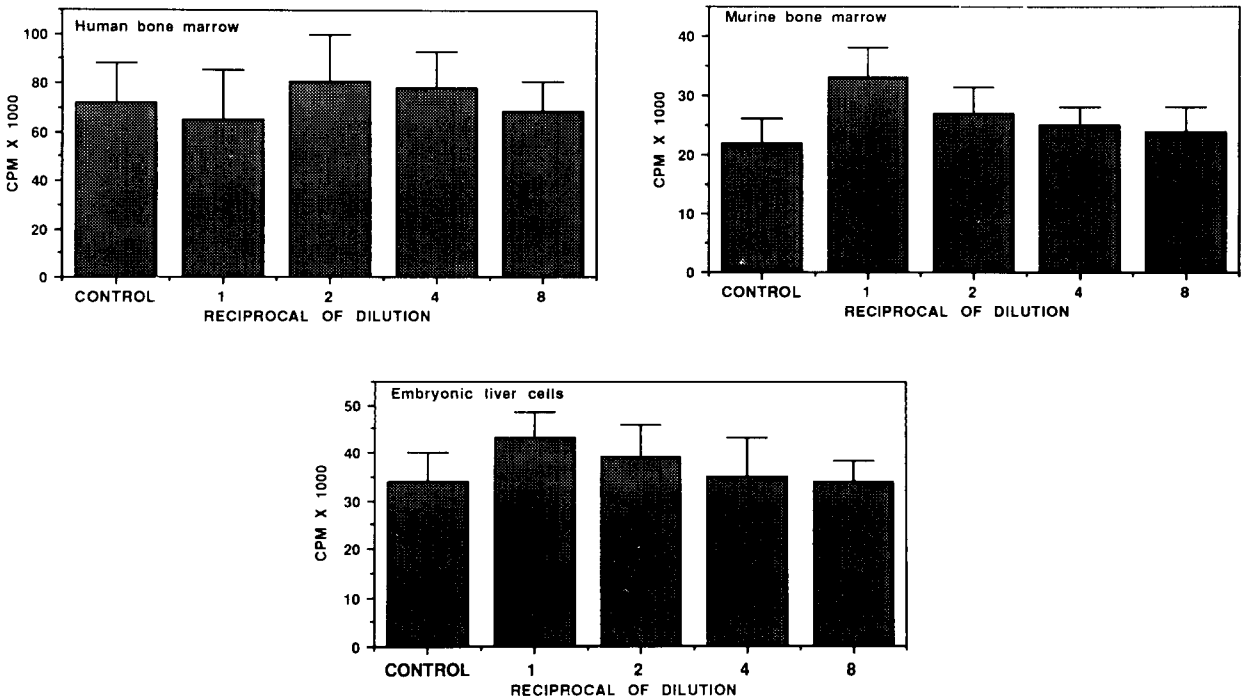

Figure 5. Normal murine or human cells incubated with muscle CM. There is no inhibitory effect on [^{3}H]thymidine uptake. [^{3}H]Thymidine incorporation is expressed as counts per minute (cpm) × 1000.

RPMI medium (47.8% ± 8 and 63% ± 12, respectively, $P < 0.01$).

Characterization of the active component in the muscle CM

Following treatment of the MF CM for various durations at temperatures ranging from 4 to 100°C, the samples were incubated with both HTB-38 and MCA-105 cells. There was no decrease in inhibitory potency at all tested temperatures including boiling at 100°C. The active component was found to be stable under both lyophilization treatment and the two proteolytic enzymes, i.e. trypsin and pronase.

To determine the size of the active component, the MF CM was fractionated by ultrafiltration on Amicon membranes with molecular cut-offs of 10, 3 and 0.5 kDa (the retantate was defiltered in each case with at least one additional PBS volume). In the case of the 10 and 3 kDa membranes, essentially all the inhibitory activity (above 90%) was found in the first two filtrates, and with the 0.5 kDa membrane, approximately 80% of the activity was found in the filtrates and some activity (20%) was retained in the second retantate. Dialysis of the MF containing CM through membranes with cut-offs of 12 and 3 kDa, showed that the active component escapes through both membranes. The above results show that the MF has a molecular weight roughly in the order of 500 D.

Table 1. Percentage of B-16 melanoma cells in the different stages of the cell cycle following incubation with or without MF CM

Cell cycle	Control (medium)	MF CM
G_0/G_1	47.8 ± 8	63 ± 12
S	45.9 ± 9.1	30.9 ± 5.2
$G_2 + M$	6.3 ± 2.9	5.2 ± 1.7

In vivo *studies*

The crude CM and the low molecular weight fraction were tested for their ability to prevent tumor growth *in vivo* as compared to a control group (treated with non-conditioned RPMI medium). In each animal, the size of tumor foci was classified according to >2 cm, between 0.5–2 cm and <0.5 cm. Table 2 summarizes the results. Both the crude MF CM and the low molecular weight fraction were extremely effective in reducing tumor size.

Discussion

The observation that metastases in striated muscles are extremely rare served as the clinical basis of the present study. We demonstrated that co-culturing of embryonic rat striated muscle cells with tumor cell lines of various origins results in surface morphological alterations in the tumor cells. Furthermore, medium conditioned by such muscle cells significantly inhibited tumor cell growth. The effect of the CM was species and tumor non-specific; in contrast, normal non-malignant cells were refractory or even stimulated by this CM. Fractionation of the muscle CM revealed that the active component is a small molecule, the molecular weight of which is about 500 D.

The muscle factor (MF) is distinct from other known growth inhibiting factors which are all glycoproteins with a much higher molecular weight. These include, for example, IL-1, TGF-β, interferons or oncostatin which have anti-proliferative effects on malignant cells and whose molecular weight range is 15–25 kDa [8–11]. Another distinctive difference between these anti-growth factors and the MF is that it is not proteinaceous as shown, among other things, by its

Table 2. Effect of MF CM and the low molecular weight fraction on the development of MCA-105 sarcoma in the peritoneum of C57BL/6J mice. The mice were sacrificed following 33 days of treatment and in each animal the size of tumor foci was classified according to >2 cm, between 0.5–2 cm and <0.5 cm. Both the crude MF CM and the low molecular weight fraction were extremely effective in reducing tumor size

	No. of mice	Mice with no tumors	Mice with tumors		
			>2 cm	0.5–2 cm	<0.5 cm
Control	8	–	6	2	–
Crude MF	7	1	–	–	6
Low MW factor	10	3	1	3	3

resistance to proteolytic enzymes and high temperature. Moreover, it also differs from chemotherapeutic agents in clinical use in that it affects only tumor cells, and has no apparent effect on normal cells.

Several studies suggest that the rare occurrence of intramyofiber metastases in skeletal muscle is the outcome of cell to cell interactions. According to Zacks *et al.* [12, 13], it is possible that the sarcolema, which is very resistant to enzymatic, mechanical and chemical alterations, could serve as a barrier against tumor cell invasion. Therefore, the usual type of skeletal muscle involvement by metastatic cells, manifests itself by infiltration of tumor cells in the connective tissue between muscle fascicles [14]. The explanation that malignant cells are "recruited" by normal muscle nuclei undergoing malignant transformation [15] has not yet been proven. Ioachim [16] suggests a mechanism of tumor cell "engulfment" by the muscle cells, similar to that observed in emperipolesis. Carr *et al.* [17] have studied the ultrastructure of neoplastic invasions of skeletal muscles and other organs and concluded that the particular type of malignant cells which they used invaded the tissues by peripheral cell division and active cell migration. The present study points to a completely different mechanism of metastatic inhibition by striated muscle cells, namely, the secretion of a factor of low molecular weight which preferentially inhibits tumor cell proliferation. The results obtained from the cell cycle kinetics point to a cytostatic mode of action of the MF, by which it arrests the proliferating malignant cells in the G_0/G_1 phase of the cell cycle. This factor is constitutively released by muscle cells in the body and thus may have a physiological role in preventing tumor cell proliferation. The observed low molecular weight of the factor and its low concentration in the CM, together with its non-proteinaceous nature, may suggest that its mode of activity is similar to that of steroidal hormones. Biochemical studies to explore the nature of the MF are underway and include purification through different types of HPLC columns and subsequent analysis by mass spectra and NMR.

The *in vivo* results obtained with the original CM and the purified substance reinforce the view that the MF acts as an inhibitor of tumor cell growth, suggesting that the MF could be used clinically as a therapeutic agent complementing surgery or chemotherapy.

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