Clinical & Experimental Metastasis **17:** 531–535, 1999.

Oral administration of muscle derived small molecules inhibits tumor spread while promoting normal cell growth in mice

Sara Bar-Yehuda, Tamar Farbstein, Faina Barer, Gil Ohana & Pnina Fishman
Laboratory of Clinical and Tumor Immunology, The Felsenstein Medical Research Center, Tel-Aviv University, Rabin Medical Center, Petach-Tikva, 49100, Israel

Received 17 May 1999; accepted in revised form 8 November 1999

Key words: bone marrow, chemoprotection, muscle-derived small molecules, oral administration, tumor growth inhibition

Abstract

Tumor metastases are extremely rare in striated muscles. This is surprising given the fact that this tissue constitutes 60% of body weight. The present study focuses on small molecules produced and secreted by muscle cells which possess anti-cancer activity *in vivo*. Recently we have shown that a low molecular weight fraction (<1000 Dalton) of skeletal muscle cell conditioned medium (muscle factor-MF), markedly inhibits the proliferation of carcinoma, sarcoma or melanoma cell lines *in vitro*. The MF exerts a cytostatic effect on tumor cell growth and arrests the cells in the G0/G1 of the cell cycle. However, normal cell proliferation, such as bone marrow and fibroblasts, was stimulated following incubation with MF. In this study, the effect of orally administered MF on melanoma and sarcoma growth was examined in mice. The administration of MF to mice inoculated intravenously with melanoma (B16–F10) or sarcoma (MCA-105) cells, resulted in a statistically significant inhibition of metastatic lung foci. In a different model, melanoma was induced in the foot pad and after development of a local lesion, the leg was amputated. A prolonged survival time was observed in the MF treated groups. Since the MF stimulated bone marrow cell proliferation *in vitro*, we decided to test its efficacy as an inhibitor of the myelotoxic effect exerted by chemotherapy, *in vivo*. MF, administered after chemotherapy, restored the number of white blood cells and yielded an increased percentage of neutrophils compared with the decline in these parameters after administration of chemotherapy alone. Thus, it is indicated that MF exerted a systemic anti tumor and chemoprotective effect when given orally. It can be concluded that it is bioavailable and is not biodegradable in the digestive system. MF may be considered as a potential therapy for the prevention of tumor spread.

Introduction

The resistance of muscle to the development of tumor metastases is a well established clinical phenomenon [1–3]. Several mediators released by muscle cells may account for the rarity of metastases in this tissue: cytokines with anti cancer activity, such as TNFα , TGFβ, lymphocyte infiltrating factor and interferon γ [4–8]; lactic acid which inhibits tumor cell growth [9, 1]; proteolytic enzymes such as plasminogen activator inhibitor which prevent metastasis [10]. Moreover, the muscle sarcolemma serves as a physical barrier against tumor cell invasion [1].

Recently, we demonstrated that muscle cells are capable of releasing adenosine and other small molecules (designated MF) that inhibit the proliferation of melanoma, sarcoma, and carcinoma cell lines *in vitro* [11]. MF is water soluble, heat stable and resistant to the action of proteolytic enzymes. It has a cytostatic effect on tumor cell growth and arrests the cells in the G0/G1 of the cell cycle. However, normal cells such as murine bone marrow are stimulated following incubation with MF. *In vivo* studies showed that MF inhibited the development of melanoma lung foci when administered intraperitoneally to C57Bl/6J mice [12]. The stability of MF and its low molecular weight prompted us to test its efficacy, when administered orally, as an inhibitor of tumor spread. In addition, its differential effect on tumor and normal cells, led us to examine its capability to prevent, in bone marrow cells, the myelotoxic effect caused by chemotherapy. Indeed, we found that orally administered MF, induced anti cancer activity and acted as a chemoprotective agent.

Correspondence to: Pnina Fishman, Laboratory of Tumor and Clinical Immunology, The Felsenstein Medical Research Institute, Rabin Medical Center, Petach-Tikva, 49100, Israel. Tel: +972-3-9233227; Fax: +972-3-9233228; E-mail: pnina@mor-research.com

Materials and methods

Preparation of MF

MF was prepared from the muscle L-8 cell line (American Tissue Culture Collection). The cells were maintained in DMEM containing 4.5 g glucose and 15% fetal bovine serum (FBS) (Biological Industries, Beit Haemek, Israel).

For preparation of condition medium (CM), the cultures were grown until confluence, the medium discarded,

cells washed 3 times with PBS and incubated for additional 24 h in PBS. At the end of incubation, the supernatant was collected, filtered through 0.22 μ filter and subjected to ultrafiltration through an amicon membrane with a molecular weight cut off of 3 kD. This sample was designated as MF.

Partial purification of MF

The MF was loaded on a preparative RP-HPLC column. The diameter of the column was 2 inches and the length, 200 mm. Merck Lichrosphere C18 particles (12 μm in diameter) with a porosity of 60A, were loaded onto the column. During each run, 800 ml of the 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%. The rate of elution was 100 ml/min. Two-hundred ml fractions were collected and each was tested for its ability to inhibit B-16 melanoma cell growth (see below). A rotary evaporator was used to dry the active fractions.

Tumor and normal cells

MCA-105, a methylcholantrene induced sarcoma of C57BL/6J (kindly provided by Dr E. Kedar, Hadassa, University Hospital, Jerusalem, Israel) and B16–F10 melanoma cells were used. The cells were maintained in RPMI medium containing 10% FBS. Twice weekly cells were transferred to a freshly prepared medium. Normal bone marrow cells were obtained fromthe C57BL/6J mice femur. Cells were disaggregated by passing through a 25 G needle.

Cell proliferation assays

[^{3}H]-thymidine incorporation assay was used to evaluate cell growth. Tumor (3×10^3/well) or bone marrow cells (3×10^5/well) were incubated with 50% or 25% MF, in 96-well microtiter plates for 48 h. PBS served as a control, since MF was prepared in PBS. During the last 18 h of incubation, each well was pulsed with 1 μCi [^{3}H]-thymidine. Cells were harvested and the [^{3}H]-thymidine uptake was determined in an LKB liquid scintillation counter (LKB, Piscataway, New Jersey).

One activity unit (AU) was defined as the amount of MF or purified MF which exerted 50% proliferation inhibition of the B16–F10 melanoma cells *in vitro*.

In vivo *studies*

Male C57BL/6J mice (Harlan Laboratories, Jerusalem, Israel) aged 2 months, weighing an average of 25 g were used. Standardized pelleted diet and tap water were supplied.

Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the Rabin Medical Center, Petah Tikva, Israel.

To examine the capability of MF to inhibit tumor growth *in vivo*, two types of tumor models were used. Each group contained 20 mice and experiments were repeated at least 3 times. The first was the artificial lung metastasis model in which 2.5×10^5 B16–F10 melanoma or MCA-105 sarcoma cells were inoculated to mice intravenously (i.v.). Mice inoculated with the B16–F10 cells were divided into 5 groups. Two were treated orally twice daily with 4 AU or 8 AU purified MF (AU were calculated as was detailed above and diluted as needed with PBS before administered orally). The third group was treated orally with adenosine (0.5 ml from an adenosine solution of 30 μM) and the forth served as a control group and was treated with the vehicle which was PBS. The activity of MF in these studies was compared with the recognized cyclophosphamide regimen of 50 mg/kg body weight, one day after tumor inoculation (intraperitoneal injection).

The MCA-105 inoculated mice were treated with 4 AU/day of MF. Mice treated with PBS, served as controls.

In the B16–F10 model, mice were sacrificed after fifteen days, lungs removed and the black metastatic foci were counted using a Dissecting Microscope. In the MCA-105 model, mice were sacrificed after 30 days, lung weight of control and MF treated groups was evaluated. As an additional parameter, we monitored the number of mice that were free of lung metastases in each group.

In another model, 2.5×10^5 B16–F10 melanoma cells were injected to the footpad, where the tumor cells developed local lesions. When the tumor mass measured 1 cm^3, the leg was amputated. MF (4 AU) was administered orally twice daily and survival time monitored. The control group was treated with PBS.

To test the myeloprotective effect of MF, mice were orally treated one day after chemotherapy with 4 AU of MF. Cyclophosphamide (50 mg/kg) was administered i.p. Each group contained 10 mice and each experiment was repeated three times. 120 h after the chemotherapy, the mice were sacrified, blood samples were taken and bone marrow cells were harvested from the femur. The blood and bone marrow samples were pooled from each group and analysed as one sample. Blood cell counts were carried out in a Coulter counter and differential cell counts were performed on smear preparations stained with May–Grunvald–Giemsa solution. Bone marrow cell proliferation was tested *ex vivo* using [^{3}H]-thymidine incorporation as was specified above. The two control groups were treated with PBS or cyclophosphamide.

Efficacy of MF activity *in vitro* and *in vivo* was statistically evaluated using Student's *t*-test. For statistical analysis, comparison between the mean value of different experiments was carried out. The criterion for statistical significance was $P < 0.05$.

Results

Effect of MF on tumor and bone marrow cell proliferation in vitro

MF induced a dose dependent inhibitory effect on the growth of B16–F10 melanoma and MCA-105 sarcoma cells, while inducing a marked proliferative effect on bone marrow cells

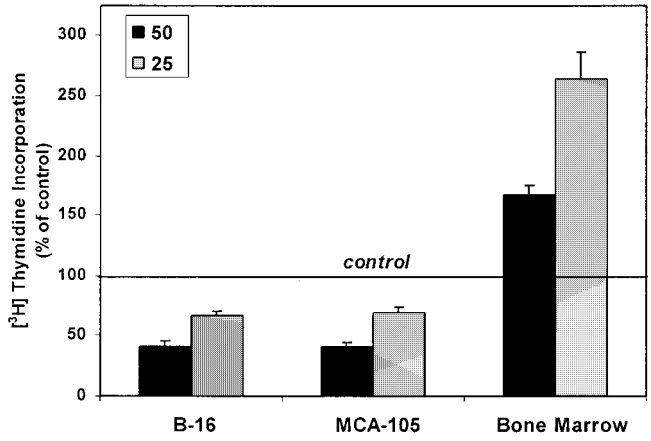

Figure 1. A differential effect of MF on tumor and normal cell proliferation. Inhibitory dose dependent effect on B16–F10 melanoma and MCA-105 sarcoma cells, while a stimulatory effect on bone marrow cell growth is noticed. Proliferation was evaluated by [^{3}H]-thymidine uptake.

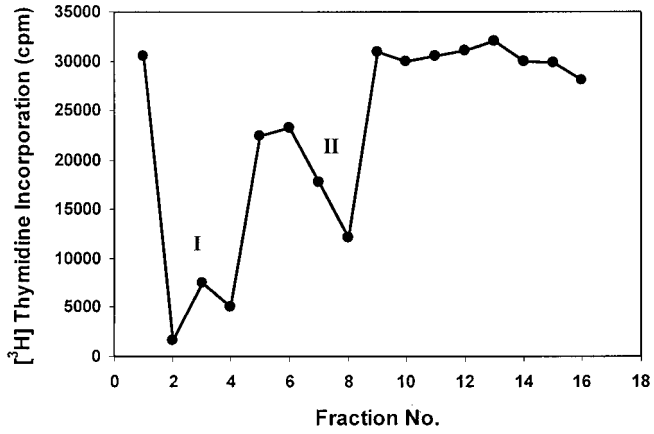

Figure 2. The biological activity of fractions eluted from the RP-HPLC regarding their capability to inhibit the proliferation of the B16-F10 melanoma cell line. The first inhibitory peak represents the salt, while the other one contains the specific inhibitory activity. Proliferation was evaluated by [^{3}H]-thymidine uptake.

(Figure 1). The inhibitory and stimulatory effects were statistically significant ($P < 0.001$, $P < 0.001$, respectively).

In an attempt to purify the active components of MF, it was loaded on a preparative RP-HPLC column. Figure 2 depicts the capability of various fractions, eluted from the RP-HPLC, to inhibit the proliferation of the B16–F10 melanoma cells. The first inhibitory peak, fractions 2–4, represents the salt that remained from the PBS vehicle (this has already been verified in our former study [12]). The second inhibitory activity was eluted at an acetonitrile concentration of 16–20% (fractions 7–9). The active fractions were pooled and tested for their efficacy to inhibit tumor growth *in vivo* (detailed below).

MF inhibits the development of lung metastases in mice

In this set of experiments, we evaluated the capability of MF and purified MF to inhibit the growth of lung metastases. MF was tested in the MCA-105 sarcoma model, where it markedly decreased the lung weight in the treated mice

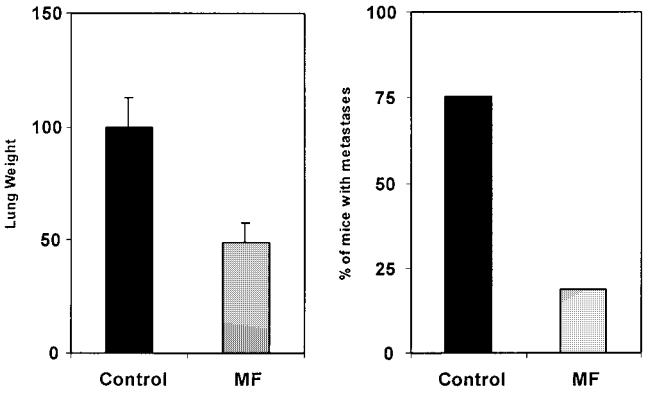

Figure 3. Effect of MF on the development of lung metastases in mice inoculated with MCA-105 sarcoma cells. MF markedly decreased the lung weight in the treated mice ($P < 0.001$) and increased the number of mice which were free of metastases.

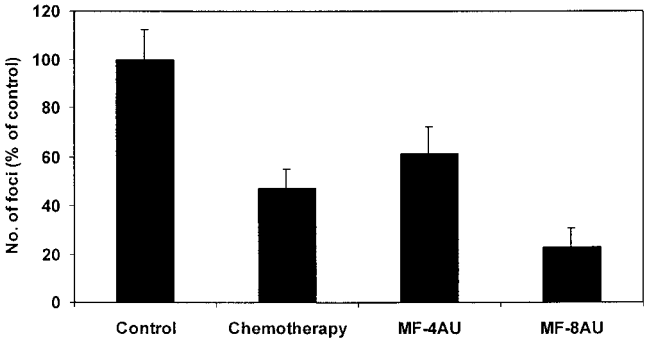

Figure 4. Effect of MF on the development of lung metastases in mice inoculated with B16–F10 melanoma cells. The number of melanoma foci was inhibited following treatment with 4 AU and 8 AU ($P < 0.01$, $P < 0.001$ respectively). The later being more effective than the chemotherapy.

($P < 0.001$), while increasing the number of mice free of metastases (Figure 3).

The efficacy of the purified MF was evaluated using the B16-F10 melanoma cells. A dose dependent, statistically significant inhibition of the number of melanoma lung foci was observed (4 AU: $P < 0.01$; 8 AU: $P < 0.001$). The high concentration of purified MF (8 AU) was even more effective than the chemotherapy in inhibiting melanoma cell growth (Figure 4). Adenosine was inactive and did not reduce the number of lung metastatic foci.

MF induces prolongation of survival time in amputated mice

On day 40 following the amputation, 90% of the control mice died, compared to 10% in the MF treated group (Figure 5).

MF protects mice from the myelotoxic effects of chemotherapy

Mice treated with cyclophosphamide exhibited a maximal decline in the number of peripheral blood leukocytes and neutrophils 120 h after chemotherapy. When MF was administered after chemotherapy, it restored the number of white blood cells ($P < 0.02$) and yielded an increased percentage of neutrophils ($P < 0.001$) (Figure 6).

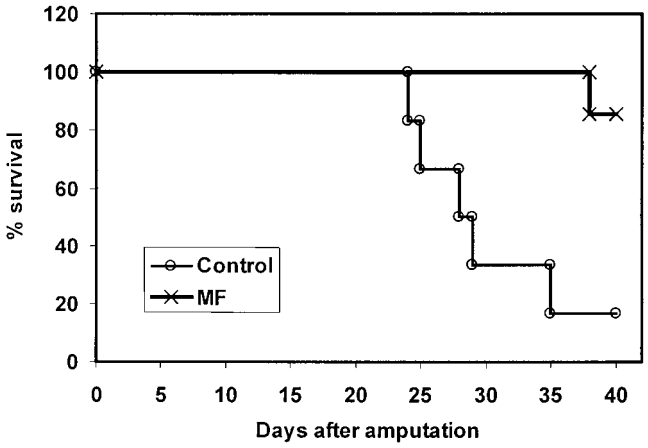

Figure 5. Prolongation of survival time of amputated mice treated daily with MF following the amputation.

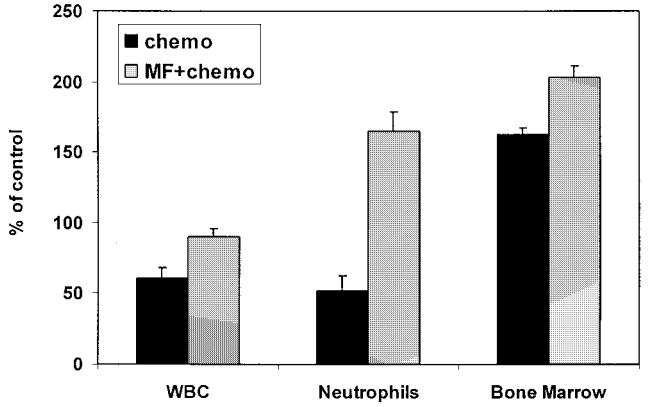

Figure 6. The *in vivo* protective effect of MF on the myeloid system in mice treated with chemotherapy. White blood cell counts,% of neutrophils counted in the blood smears and [^{3}H]-thymidine uptake by bone marrow cells are expressed each as % of control (naive untreated mice). Chemotherapy alone decreased the number of leukocytes and % of neutrophils. When MF was given prior to chemotherapy, it restored the number of white blood cells and yielded an increase in the percentage of neutrophils. A marked bone marrow cell regeneration was seen when MF was administered prior to the chemotherapy.

Ex vivo proliferative capability of bone marrow cells was used in this study as a parameter to test the response of bone marrow cells to external simuli. When MF was given in combination with chemotherapy, it induced a stimulatory effect on bone marrow cell growth ($P < 0.01$) (Figure 6).

There were no changes in the number of red blood cells, hemoglobin levels and platelets in the groups treated with cyclophosphamide or MF.

Discussion

The present study describes the efficacy of orally administered MF, as a systemic inhibitor of tumor spread and a chemoprotective agent. The anti-tumor effect was evidenced by the reduction in the number of lung metastases and prolongation of survival time.

The rationale which led us to examine the ability of muscle cells to release small molecules with anti-cancer activity, originated from two clinical observations: (i) the rarity of tumor metastases in skeletal muscles [1–3] and (ii) the inverse relation between cancer development and physical activity [13–16].

Indeed, in two earlier studies, we demonstrated the capability of muscle-derived small molecules to act as potent anti-tumor agents against a broad spectrum of tumor cells *in vitro*. These studies further showed the *in vivo* efficacy of MF, when injected i.p. to mice previously inoculated with melanoma cells [11, 12].

In the present study, we observed a differential effect of MF on tumor and normal bone marrow cells *in vitro*. The course of action on tumor and normal cells was similar regarding dosage and response time. These results served as the basis for calculating the number of MF activity units in samples that were later used for *in vivo* studies. Indeed, a dose dependent reduction in the number of lung metastatic foci was shown correlating to that of the *in vitro* results.

The efficacy of MF against melanoma growth was further confirmed by our studies where MF prolonged the survival time of mice. Our results show that MF activity is tumor non specific, since it also inhibited the development of sarcoma lung metastases. MF's stimulatory activity on bone marrow cells *in vitro*, prompted us to test this effect *in vivo*. Administration of MF prior to cyclophosphamide, prevented the decline in the number of leukocytes and neutrophils, thus avoiding the myelotoxic effects of chemotherapy. The increased bone marrow cell proliferation observed ex vivo following MF treatment, expresses a regeneration of myeloid progenitors. This could lead to the maturation of more granulocytes and their subsequent migration to the peripheral blood. While chemotherapy has been a leading therapy for many neoplasms, it damages the bone marrow. To counteract this phenomenon, colony stimulating factors (CSFs) that can accelerate recovery and shorten the severe nadir are currently used. However, some CSFs were found to stimulate the growth of tumor cells, which exhibit receptors for G-CSF and GM-CSF [17, 18]. In distinction, the capability of MF to inhibit tumor cell growth while simultaneously protecting the myeloid system against the toxicity of chemotherapy, renders MF a preferable agent in cancer treatment. The above results support the existence of a direct correlation between *in vitro* and *in vivo* studies. The three effects that were shown *in vivo*, i.e., dose dependent inhibition of tumor cell proliferation, tumor non specificity and the stimulatory activity on bone marrow cell proliferation, were also observed *in vivo*. Moreover, the results indicate that MF is very stable and bioavailable, thus exerting a systemic effect when administered orally.

In our previous study [12], adenosine was identified as one of the molecules which is released by muscle cells and inhibits *in vitro* tumor cell growth, while promoting normal cell proliferation. We cannot attribute the *in vivo* activity found in the present work to adenosine, since when given orally, it was inactive. We hypothesize that adenosine metabolizes rapidly in the intestinal tract into inosine, which is an inactive metabolite. We have shown in a previous study [12] that the active components of MF are low molecular weight compounds (600–800 Dalton). In this study we have

proved that they exert an inhibitory effect on tumor cells and a stimulatory one on bone marrow cells *in vivo*. This may indicate that the active molecules may be other nucleosides which are analogues of adenosine but are more stable and thus may exert systemic effect.

In summary, muscle cells are a source from which small molecules with anti tumor, as well as bone marrow protective activity, may be purified.

The *in vivo* efficacy of MF when given orally and the marked difference in the susceptibility of neoplastic and bone marrow cells, suggests that MF is a promising substance which should be further evaluated for clinical applications.

Acknowledgement

This work was supported by a grant from Can-Fite Technologies Ltd., Tel Aviv, Israel.

References

1. Sarma DP, Weilbaccher TG, Love GL. Intramyofiber metastasis in skeletal muscle. J Surg Oncol 1985; 30: 103–05.
2. Pellegrini AE. Carcinoma of the lung occurring as a skeletal muscle mass. Arch Surg 1979; 114: 550–55.
3. Seely S. Possible reasons for the high resistance of muscle to cancer. Med Hypotheses 1980; 6: 133–37.
4. Husmann I, Soulet L, Gautron J et al. Growth Factors in skeletal muscle regeneration. Cytokine & Growth Factor Rev 1996; 7: 249–58.
5. Birnbaum M, Trink B, Shainberg A et al. Activation of the interferon system during myogenesis *in vitro*. Differentiation 1990; 45: 138–45.
6. Rosztoczy I, Megyeri K, Papos M. Interferon production by normal mouse tissues in organ cultures. Interferon Res 1989; 9: 509–15.
7. Kurek JB„ Nouri S, Kannourakis G et al. Leukemia inhibitory factor and interleukin-6 are produced by diseased and regenerating skeletal muscle. Muscle Nerve 1996; 19: 1291–301.
8. Lafyatis R, Lechleider R, Roberts AB et al. Secretion of transcriptional regulation of transforming growth factor-beta 3 during myogenesis. Mol Cell Biol 1991; 7: 3795–803.
9. Takada S, Nagato Y, Yamamura M. Effect of cyclic polylactates on tumor cells and tumor bearing mice. Biochem Mol Biol Int 1997; 43: 9–17.
10. Lee E, Vaughan DE, Parikh SH et al. Regulation of matrix metalloproteinases and plasminogen activator inhibitor-1 synthesis by plasminogen in cultured human vascular smooth muscle cells. Circ Res 1996; 78: 44–9.
11. Djaldetti M, Sredni B, Zigelman R et al. Muscle cells produce a low molecular weight factor with anti-cancer activity. Clin Exp Metastasis 1996; 14: 189–96.
12. Fishman P, Bar-Yehuda S, Vagman L. Adenosine and other low molecular weight factors released by muscle cells inhibit tumor cell growth. Cancer Res 1998; 58: 3181–87.
13. Hoffman SA, Paschkis KE, DeBias DA et al. The influence of exercise on the growth of transplanted rat tumors. Cancer Res 1991; 22: 597–99.
14. Baracos VE. Exercise inhibits progressive growth of the Morris hepatoma 7777 in male and female rats. Cancer J Physiol Pharmacol 1989; 67: 864–70.
15. Giovannucci E, Ascherio A, Rimm EB et al. Physical activity obesity and risk for colon cancer and adenoma in men. Ann Intern Med 1995; 122: 327–34.
16. Thune I, Brenn T, Lund E et al. Physical activity and the risk of breast cancer. N Engl J Med 1997; 336: 1269–75.
17. Berdel WE, Danhauser-Riedl S, Steinhauser G et al. Various human hematopoietic growth factor (interleukin-3, GM-CSF, G-CSF) stimulate clonal growth of nonhematopoietic tumor cells. Blood 1989; 19: 80–3.
18. Berdel WE, Danhauser-Riedl S, Oberberg D et al. Effects of hematopoietic growth factors on malignant nonhematopoietic cells. Semin Oncol 1992; 19: 41–5.