Magnesium deficiency inhibits primary tumor growth but favors metastasis in mice

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Received 18 May 2004; received in revised form 12 July 2004; accepted 10 August 2004
Available online 21 August 2004

Abstract

The results of several experimental and epidemiological studies have shown an inverse correlation between Mg status and the risk of some cancers. However, relationship between magnesium and cancer is complex. The aim of our work was to examine the precise effect of Mg deficiency on transplantable mouse tumor growth and metastasis.

The results obtained indicate a significant retardation of primary tumor growth (up to 70%) in mice receiving Mg-deficient diet. However, Mg repletion caused in these mice significant increase of primary tumor burden. Analysis of cell cycle distribution showed a reduced percentage of cells in the S phase and an increase of cells in the G0/G1 phase of the cell cycle in LLC tumors caused by Mg deficiency. This is in agreement with the effect of low Mg level on cell growth observed in vitro. Interestingly, in mice inoculated with LLC cells and receiving low-magnesium diet, a higher metastatic potential was observed as compared to control mice.

In conclusion, our results demonstrate a direct role of magnesium in tumor growth and also point at deleterious effect of low magnesium status on tumor metastasis.

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Keywords: Magnesium deficiency; Tumor growth; Metastasis; Cell cycle

1. Introduction

Mg is the fourth most abundant cation in the body and the second most common cation in the intracellular fluid. The abundance of Mg within mammalian cells is consistent with its relevant role in regulating tissue and cell functions [1,2]. Several data strongly suggest that regulation of intracellular Mg availability is parallel to the molecular control of cell proliferation, and probably also cell differentiation and death [3,4]. Several lines of evidence on a
large variety of cell models support the role of Mg in the control of cell proliferation. The picture is clearest for normal cells whereas it is still controversial in tumor cells, because they appear to be more independent of extracellular Mg growth sustaining function than normal cells [4].

From the clinical point of view, considerable debate remains regarding the role of Mg in tumor growth, and the developing solid cancers was considered as a contraindication for Mg therapy [5]. The results of clinical assays of Mg deficiency in the patients with malignant tumors suggest a potential benefit; however, they are not conclusive enough and pose considerable practical problems [6]. However, serum level of MgII is frequently decreased in patients with solid tumors and this decrease is related to the advancement of malignancy [7,8]. In addition, several courses of chemotherapy in cancer patients may also lead to low Mg status [9]. Thus, it appears important to better understand the role of Mg in tumor development and to assess the Mg requirements and implications in healthy and cancer subjects.

There is also a growing evidence that inflammatory process may modulate cancer growth and metastasis [10,11]. Recent studies also indicate that low Mg level favors the inflammatory response and activates vascular endothelial cells [12,13]. Thus, the aim of the present work was to establish the relationship between Mg status and experimental mouse tumor growth and metastasis. For this purpose, the effect of Mg status regulated by variation of Mg in the diet was studied in murine models of subcutaneously growing solid tumors.

2. Materials and methods

2.1. Time course of Mg deficiency and blood leukocytes in mice fed Mg-deficient diet

The time course of Mg deficiency was evaluated in healthy, 10–12-week-old C57BL/6/JiW female mice fed with control or Mg-deficient diet. Mice were supplied by Animal Breeding Center of the Institute of Immunology and Experimental Therapy (IIET), Wroclaw, Poland, and maintained in standard laboratory conditions with demineralized water and food ad libitum. All experiments were performed according to “Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing and Education” issued by the New York Academy of Sciences’ Ad Hoc Committee on Animal Research and were approved by the 1st Local Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland.

The semi-purified diets contained (in g/kg): casein 200, wheat starch 650, corn oil 50, alphacel 50, DL-methionine 3, choline bitartrate 2, modified AIN-76 vitamin mix 10 (ICN Biomedicals, Orsay, France). MgO was omitted from the mineral mix in Mg-deficient diet. The Mg concentrations of diets, determined by flame atomic absorption spectrometric analysis (Perkin Elmer 400, Norwalk, CT), were 30 and 1000 mg/kg for deficient and control diet, respectively.

To evaluate the effect of magnesium deficiency on plasma Mg concentration and number of blood leukocytes, mice were fed either control or Mg-deficient diet for 3 days, 1, 2 and 3 weeks. After this time, mice were anesthetized with isoflurane (Forane) and blood was collected from retroorbital sinus into heparinized tubes. Plasma was obtained by low-speed centrifugation (2000×g). Concentration of Mg in plasma was determined by flame atomic absorption spectrometry analysis (Perkin Elmer 420) after dilution in lanthanum chloride solution containing 1 g La/l.

2.2. The effect of Mg deficiency on primary tumor growth

The experiments were performed on 10–12-week-old C57BL/6/JiW and C3H/JiW female mice (supplied by Animal Breeding Center of the IIET, Wroclaw, Poland), maintained in standard laboratory conditions with demineralized water and food ad libitum. They were fed control or Mg-deficient diet, starting from the day of tumor cell implantation until the end of experiment.

Lewis lung carcinoma (LLC) cells were received as a gift from National Cancer Institute (Bethesda, USA), 16/C mouse mammary adenocarcinoma cells were from Southern Research Institute (Birmingham, Alabama, USA), and C38 colon adenocarcinoma cells were purchased from National Institute of Radiobiology TNO (Rijswijk, The Netherlands). All lines were maintained in vitro and/or in vivo in syngeneic mice and stored at the liquid nitrogen at the Cell Culture Collection of IIET. Mice were inoculated subcutaneously (s.c.) into right flank region with 20% (v/w) (for LLC model) or 33% (for C38 model) suspension of tumor cells, taken from in vivo passage, suspended in 0.2 ml of Hanks medium. In 16/C model, mice were inoculated into the second mammary fat pad with 20% (v/w) suspension of tumor cells, taken from in vivo passage, suspended in 0.05-ml Hanks medium. When tumors became palpable, their maximum length and width diameters were measured and the tumor weights, calculated as $a^2 \times b/2$ ($a$=shorter diameter, $b$=longer diameter), were determined until completion of the study. Tumor growth inhibition (TGI) was calculated taking values of Mg-sufficient animals as 100%. The experiments were terminated 21 days after tumor cells inoculation.

2.3. Cell cycle distribution

2.3.1. In vivo studies

Samples of about 1 g from tumors grown in Mg-sufficient or Mg-deficient mice were collected 21 days after transplantation. Frozen samples were minced with scissors to disaggregate the tissue and obtain a tumor cell suspen-
sion. Samples of approximately $1 \times 10^9$ cells were washed in PBS, lysed by ddH$_2$O and centrifuged. Pellets were stained with propidium iodide-containing DNA-Prep and cell cycle distribution was studied by cytofluorimetric analysis of cell DNA content on a Coulter Epics 753 (Beckman Coulter, Inc. Fullerton, CA, USA).

2.3.2. In vitro studies

LLC cells were cultured in standard conditions, in EMEM medium, supplemented with 4 mM L-glutamine, 4.5 g/l glucose, 1.5 g/l NaHCO$_3$, antibiotics and 10% FBS. The only source of Mg in Mg-deficient medium (0.1 mM Mg) was FBS, whereas control medium was supplemented with MgSO$_4$ up to 1 mM Mg. Cells were grown in media of different Mg content for 7 or 12 days. Then, they were trypsinized, washed with PBS (without Mg$^{2+}$ and Ca$^{2+}$ ions) and suspended in cold 70% ethanol. Cells were washed and DNA was extracted with extraction buffer (0.2 mM Na$_2$HPO$_4$, 0.1 mM citric acid, pH 7.8). Then, cells were incubated for 30 min in darkness with fresh PBS buffer containing RNase A and propidium iodide. Fluorescence was measured using a FACs Calibur flow cytometer (Becton Dickinson, San Jose, CA). Data analysis was performed using a Becton Dickinson Cell Quest software.

2.4. Determination of Mg levels in plasma and tumors

Plasma and tumor Mg concentrations were evaluated in LLC model. The animals were fed either control or Mg-deficient diet for 21 days after s.c. inoculation of tumor cells. The Mg concentrations in plasma and dry-ashed tumors were determined by flame atomic absorption spectrometric analysis as described above.

2.5. The effect of Mg deficiency on metastasis

This experiment was performed in LLC model in C57BL/6/IiW female mice, inoculated with tumor cells according to the protocol described above. The animals were fed either control or Mg-deficient diet for 21 days after s.c. inoculation of tumor cells. Samples of lungs were fixed in 4% formaldehyde and embedded in paraffin, cut into 1689 (2004) 13–24.1689 (2004) 13–24.5-μm slices and stained with haematoxylin and eosin. Metastatic potential was expressed as a number of lung colonies counted under 50× magnification.

2.6. The effect of Mg deficiency followed by repletion on primary tumor growth

This experiment was performed on C57BL/6/IiW female mice, inoculated with LLC cells according to the protocol described above. Mice were fed either control or Mg-deficient diet for 13 days, starting from the day of tumor cells inoculation. After this time, mice were switched from Mg-deficient diet to Mg-sufficient (control) one. From the moment when tumors became palpable until the end of experiment, their weights were determined and the percentage of TGI was calculated. Mice were sacrificed 24 days after tumor cell implantation.

2.7. Statistical analysis

Results of all experiments are expressed as mean values±S.E. The statistical significance of differences between the groups was assessed using Student’s unpaired t-test and significance of mortality incidence was evaluated by $\chi^2$ test (Instat, GraphPad, San Diego, CA, USA). Results were considered as significant at $P<0.05$.

3. Results

3.1. Time course of Mg deficiency and blood leukocytes in mice fed Mg-deficient diet

In healthy C57/BL6/IiW mice fed Mg-deficient diet, we observed a rapid fall in the plasma Mg concentration during the first week of Mg deficiency. The concentration of Mg reached the level of about half of the control value. Then, in the course of the study, the Mg concentration remained stable at about one third of the control plasma level.
(Fig. 1A). No mortality in both studied groups was observed.

The total circulating leukocyte number increased progressively in the serum of mice receiving Mg-deficient diet during the first 2 weeks, reaching a twofold increase over the control, and remained stably elevated in the third week (Fig. 1B).

3.2. Effect of Mg deficiency on primary tumor growth

A significant retardation of primary tumor growth in mice fed Mg-deficient diet was observed in all three tumor models applied. The values of TGI in LLC, C38, and 16/C tumors, calculated at day 21 of experiment, were respectively 60%, 57%, and 75% (Fig. 2). In all models we observed a slight (5–15%) decrease in body weight in Mg-deficient mice. This is at least partly related to the lower tumor weight in mice receiving Mg-deficient diet.

No mortality was observed in any group of mice fed control diet and inoculated with tumor cells during the 21 days of the experiment; however, Mg deficiency led to the following mortality: 7% in mice inoculated with LLC cells, 27% in C38 model and 46% in C3H mice inoculated with 16/C cells. The latter result reached a statistically significant value (Fig. 3).

3.3. The effect of Mg deficiency on cell cycle

Cytofluorimetric analysis of cell cycle distribution in tumor samples of LLC after 21 days of transplantation showed that 13% and 16% of cells were apoptotic in tumors from Mg-sufficient and Mg-deficient mice, respectively (Fig. 4). The remaining cells, distributed in the different phases of cell cycle, showed that tumor grown in mice fed with Mg-deficient diet compared to the tumor grown in Mg-sufficient animals showed an increase of cells in the G0/G1 phase (57.5% vs. 51.1% in control cells) which corresponded to a reduction of cell in the S phase, (35.3% vs. 46.9%). A threefold increase of cells in G2/M phase was also observed in tumors grown in Mg-deficient mice (7.2% vs. 2.0%).

These observations are consistent with in vitro studies showing that Mg removal from culture medium leads to the inhibition of the proliferation rate of LLC cells (15–24% after 1 week of culturing in Mg-deficient medium). This is accompanied by a modification in cell cycle distribution of LLC cells in a time-dependent manner. The percentage of cells in G0/G1 phase increases by 26% after 7 days of Mg deficiency and by 37% after 12 days, whereas the percentage of cells in S phase decreases by 27% and 36%, respectively.

3.4. Determination of Mg levels in plasma and tumor tissue

Plasma and tumor Mg concentrations were evaluated in LLC model at the end of experimental period. Mg deficiency led to a marked decrease (84% as compared to the control group) in the concentration of Mg in plasma (Fig. 5A). Furthermore, we observed that Mg deficiency led
to a slight, but significant, decrease in the concentration of Mg in tumor tissue (5%) (Fig. 5B).

### 3.5. Effect of Mg deficiency on metastasis

Interestingly, in mice inoculated with LLC cells and receiving low-magnesium diet, a higher metastatic potential, expressed as the number of metastatic foci in lungs, was observed as compared to those fed Mg-sufficient diet. The mean number of metastatic colonies in the lungs of control mice was $3.7 \pm 0.9 \ (n=7)$, whereas in Mg-deficient mice, $7.6 \pm 2.9 \ (n=8, \ P<0.05)$. Moreover, we found an increased number of animals with lungs colonized by tumor metastatic cells in Mg-deficient mice in comparison to control (43% vs. 21%).

### 3.6. The effect of Mg deficiency followed by repletion on primary tumor growth

A significant inhibition of LLC tumor growth (44%), evaluated 13 days after tumor cells inoculation, was observed in mice fed Mg-deficient diet. However, switch to the control (Mg-sufficient) diet caused a significant increase of primary tumor growth (42%) as compared to the

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### Table 1

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Fig. 5. Plasma (A) and tumor (B) concentration of Mg in C57BL/6 mice bearing LLC tumors and fed either Mg-deficient or control diet. Values are mean±S.E., N=12–15 measurements per group. *P<0.05, **P<0.001.

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Fig. 6. Effect of Mg depletion followed by repletion on LLC primary tumor growth in C57BL/6 mice. Mice were fed Mg-deficient diet for 13 days starting from the day of tumor cell inoculation and then switched to Mg-sufficient diet for 11 days. The control group was continuously fed with Mg-sufficient diet. Tumor weights were determined at days 13 and 24 of experiment. Values are mean±S.E. *P<0.05, **P<0.01.
control group continuously receiving Mg-sufficient diet (Fig. 6).

4. Discussion

In 1974 Milles, in a short letter to Lancet [14], provided the first observation that in Mg-deficient rats, the growth of Walker carcinosarcoma 256 was inhibited and that the return to Mg-adequate diet induced a remarkable, almost explosive, increase in tumor growth. The inhibitory effect of low Mg diet on tumor growth was further confirmed in the other models: Yoshida ascite tumors in rats [15], human breast cancer cell line (HTB123/DU4475) growing in athymic nude mice, mammary rat male adenocarcinoma R3230AC [16,17]. On the other hand, no significant effect on tumor growth (Ehrlich carcinoma) was observed by other authors [18]. These discrepancies and the lack of precise evaluation of the tumor growth in the time course of deficiency pushed us to undergo a more careful assessment of the effect of low Mg status on tumor growth. To establish the time course and the severity of Mg deficiency, mice were fed with Mg-deficient diet for various periods of time. The results confirm that Mg deficiency could be easily induced in mice by a deficiency of Mg in diet, as shown by the rapid fall of Mg concentration in the plasma (Fig. 1A). To demonstrate the effect of Mg deficiency on tumor growth, we have selected three different models of transplantable mouse tumors. Our results clearly show that Mg deficiency markedly inhibits the growth of all tumors studied. Magnesium is required for almost every metabolic process taking place in a cell, including protein and DNA synthesis [19,20]. Magnesium ions seem to be especially important for rapidly growing and frequently dividing tumor cells. Thus, one of the mechanisms that might be responsible for these effects is the inhibition of protein and DNA synthesis, impairing intracellular metabolism and cell division, leading in consequence to the retardation of tumor growth. These macroscopic data are confirmed by analysis of cell cycle distribution which showed a reduced percentage of cell in the S phase of the cell cycle in LLC tumors grown in mice fed a Mg-deficient diet compared to tumors grown in animals fed sufficient Mg diet (Fig. 4). It is interesting to observe that in tumors grown in low-Mg fed animals the G2/M phase is 2.5-fold increased compared to tumors grown in control mice, suggesting that besides the G0/G1 arrest, also accumulation of cell in G2/M may be due to decreased Mg availability. Based on this observation, a tentative explanation of massive tumor regrowth following Mg reintroduction could be ascribed, on one hand, to G0/G1 arrest, as suggested by previous data showing that Mg availability can influence the expression level of cell cycle regulatory proteins [21] and, on the other hand, to cells in G2/M phase, as these cells could promptly complete their cell cycle once Mg will be available again through the diet.

Magnesium depletion from culture medium also modifies LLC cell cycle distribution, increasing the percentage of cells in G0/G1 phase and decreasing the percentage of cells in S phase (Fig. 4). These changes are accompanied by the inhibition of the proliferation rate. The inhibitory effect of Mg deficiency on cell proliferation seems to be one of the basic mechanisms but not the only mechanism underlying the retardation of primary tumor growth in Mg-deficient mice. In our studies, we observed that in mice receiving low Mg diet, the number of tumor blood vessels was decreased by 50% as compared with control group (data not shown). These data are consistent with our observations made on microvascular endothelial cells (Maier et al. unpublished data). Microvascular endothelial cells cultured in low Mg medium reveal decreased proliferation and migration potential. Impairment of tumor angiogenesis process, a crucial step in tumor development and progression, may also explain the inhibitory effect of Mg deficiency on tumor growth [22,23].

In this study we have also shown, as it was previously reported by Milles [14], that the return to Mg-sufficient diet, after a period of Mg-deficient one, led to massive tumor regrowth. This could be somehow explained by the quite high resistance of tumor cells to mineral deficiencies. In the lack of Mg, tumor cells might enter a kind of “dormant” state, when they do not proliferate, but at the same time, they do not die. Re-supplementation of Mg enables these dormant cells to take up a rapid proliferation again.

We have also shown that the total tumor Mg level in Mg-deficient mice was only slightly reduced despite the fact that plasma Mg concentration was highly decreased. This supports the generally recognized belief that cellular Mg content is tightly regulated and varies only slightly even when the extracellular concentration is dramatically decreased [24]. This underlies also the crucial role of extracellular Mg in pathobiological phenomena that occur during Mg deficiency.

In our work, we also have observed the occurrence of mortality in Mg-deficient tumor-bearing mice. This may be explained by an aggravation of Mg deficiency by rapidly proliferating and growing tumors. However, a more precise evaluation of Mg status and Mg metabolism in tumor bearing mice is necessary to evaluate the modifications in Mg metabolism and the need for Mg in the process of tumor growth. In addition, we have previously shown that Mg-deficient animals are particularly sensitive to the septic shock [25]. Thus, it could be suggested that TNF (or other mediators) produced by tumor cells may have more deleterious effects in Mg-deficient animals than in controls.

Despite the inhibitory effect on primary tumor growth, Mg deficiency seems to favor metastasis. In the lungs of Mg-depleted mice, approximately twice more metastatic foci could be detected in comparison to control ones. Further studies, especially on the molecular level, may shed some light on the influence of Mg deficiency on cell–cell interactions and metastatic process. One of the phenomena...
that might have an important role in the increased metastatic potential of LLC tumors grown in Mg-deficient mice is an inflammatory response. The proinflammatory effect of Mg deficiency can contribute to the enhanced endothelial cell activation [26] and thus to the increased adhesion of tumor cells to endothelium.

In summary, it clearly appears that low Mg status affects tumor growth; however, the mechanism of such effect remains still obscure. The study on the differences in gene and protein expression in tumor cells submitted to low Mg may be very useful to clarify the molecular mechanisms explaining Mg$^{2+}$ effects at cellular and molecular levels.

From the clinical point of view, the evaluation of Mg status in cancer patients might be of particular practical value. In patients suffering from a various types of tumors, Mg deficit results from a disease progression itself and may be aggravated by some anti-tumor treatments. In such cases, Mg supplementation is a common clinical practice. Our studies show that we should be very careful concerning Mg repletion in patients—this may stimulate a process of growth.

Acknowledgements

This work was supported in part by collaborative linkage NATO grant (to AM, AO, JM, FW), Polonium bi-national grant (to AM and AO), Prix de Recherche du Centre Evian pour l’Eau (to AN) and La Ligue Nationale Contre le Cancer (to YR). The authors thank D. Bayle for skillful technical assistance.

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