Insights Into the Mechanisms Involved in Magnesium-Dependent Inhibition of Primary Tumor Growth

Jeanette A. M. Maier, Anna Nasulewicz-Goldeman, Matteo Simonacci, Alma Boninsegna, Andrzej Mazur, and Federica I. Wolf

Abstract: We have previously shown that a low Magnesium (Mg)-containing diet reversibly inhibits the growth of primary tumors that develop after the injection of Lewis lung carcinoma (LLC) cells in mice. Here we investigate some of the mechanisms responsible for the Mg-dependent regulation of tumor development by studying cell cycle regulation, tumor angiogenesis, and gene expression under Mg deficiency. The inhibition of LLC tumor growth in Mg-deficient mice is due to a direct effect of low Mg on LLC cell proliferation and to an impairment of the angiogenic switch. We also observed an increase of nitric oxide synthesis and oxidative DNA damage. Complementary DNA arrays reveal that Mg deficiency modulates tumor expression of genes involved in the control of cell cycle, stress response, proteolysis, and adhesion. Our results suggest that Mg has multiple and complex roles in tumor development.

Introduction

Magnesium (Mg) is involved in the regulation of a large number of biochemical reactions that are crucial to cell proliferation, differentiation, apoptosis, and angiogenesis (1). Because it functions as an allosteric modulator of several enzymes or bridges structurally distinct molecules, Mg stabilizes DNA, promotes DNA replication and transcription, influences RNA translation, and induces ribosome assembly (2). Compelling evidence shows that Mg is required for proliferation in normal diploid and transformed cells (3–5). Recently, the role of Mg in regulating cell proliferation was underscored by studies based on the deletion of the transient receptor potential melastatin (TRPM) 7, which is critical to Mg entry in eukaryotic cells (6). Interestingly, cells in which TRPM 7 was genetically deleted are Mg depleted and growth arrested (6).

The occidental diet is relatively deficient in Mg because of its low content in water and soils and because of the processing of some foods (7). Apart from being associated with a decreased dietary intake, Mg deficiency occurs in diabetes, metabolic syndrome, nephropathies, chronic alcoholism, and other age-associated diseases. In addition, diuretics and some anticancer drugs promote Mg waste, thus leading to hypomagnesemia (8).

Epidemiological studies about the relation between Mg content in drinking water and cancer provided a vast array of results: An inverse relationship was found for breast, prostate, and ovarian cancers; a protective trend for esophageal cancer, but no correlation for other tumors (8). A recent large epidemiological prospective study on Swedish women demonstrated that the incidence of rectal cancer was inversely related to the levels of Mg in the diet (9). An observational study comparing control subjects to patients affected by different kind of tumors (lung, breast, ovary, oropharyngeal, and hypopharyngeal cancers) showed that in cancer patients, serum Mg was lower \((P < 0.001)\) than in controls, and this correlated with the stage of malignancy (10).

The contribution of Mg availability to tumor growth is still debated, and both experimental and epidemiological evidences are fragmentary and sometimes contradictory. Although low serum Mg is detected in tumor-bearing organisms, including oncologic patients, Mg content is increased in tumors compared to their normal counterpart both in vivo and in vitro (3, 8, 11). This observation supports the hypothesis that growing tissues require more Mg than resting ones to sustain their proliferation rate. Because tumors are able to maintain high intracellular Mg in spite of decreased extracellular availability, they have been addressed as a very powerful “Mg trap” (3). The capability of tumor cells to sequester and keep up Mg is confirmed by experiments in vitro showing that the proliferation of tumor cells is less

J. A. M. Maier is affiliated with the Dipartimento di Scienze Precliniche LITA Vialba, Università di Milano, 20157 Milano, Italy. A. Nasulewicz-Goldeman is affiliated with the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53–114 Wroclaw, Poland. M. Simonacci, A. Boninsegna, and F. I. Wolf are affiliated with the Istituto di Patologia Generale, e Centro di Ricerche Oncologiche Giovanni XXIII, Facoltà di Medicina, Università Cattolica del Sacro Cuore, Largo F. Vito, 100168 Roma, Italy. A. Mazur is affiliated with the INRA, Clermont Ferrand/Theix, Centre de Recherche en Nutrition Humaine d’Auvergne, Unité de Nutrition Humaine, Equipe Stress Mètabolique et Micronutriments, 63122 Saint-Genès-Champanelle, France.
dependent from extracellular Mg than the normal counterpart (12). Thus, because Mg content and uptake are higher in neoplastic than in normal tissues, it is feasible that tumors are very efficient in balancing intracellular Mg. It remains to be elucidated whether and how Mg availability affects tumor growth in vivo. To address this issue, we have developed an experimental model of Mg deficiency in mice that are subcutaneously injected with Lewis lung carcinoma (LLC) cells. We followed tumor development from Day 12 to 21 after the injection, showing that the low-Mg-containing diet inhibits the growth of primary tumors while enhancing the number of lung metastatic foci. We also showed that growth inhibition is reverted by reintroducing Mg in the diet (13).

To get insights into the mechanisms involved in the inhibition of primary tumor growth in Mg deficient mice, we have evaluated the impact of low Mg on some pivotal events in tumor growth and development, namely, cell proliferation, angiogenesis, and oxidative stress. To better define the mechanisms involved in the inhibition of tumor growth under Mg deficiency, we also analyzed gene expression by complementary (c)DNA array and found the modulation of the expression of genes involved: 1) in cell cycle, 2) in oxidative stress response, and 3) in adhesion and proteolysis.

Materials and Methods

Animal Model

The experiments were performed in 10- to 12-week-old C57 BL/6/JW female mice. Mice were supplied from Animal Breeding Center of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland and maintained in standard laboratory conditions with demineralized water and food ad libitum. LLC cells were a gift from the National Cancer Institute (Bethesda, MD). Mice were inoculated subcutaneously into the right flank region with LLC cells (20% vol/wt) in 0.2 ml of Hanks medium (13). The Mg diet was initiated on the same day of the injection with LLC cells. 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 Local Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland.

Mice (10–15/group) were fed with either control or Mg-deficient diet (1.0 or 0.030 g/kg, respectively) for 21 days exactly as described in (13). At the end of the experiment, mice were sacrificed by cervical dislocation. Tumors were excised and immediately frozen in liquid nitrogen or fixed in formaline, embedded in paraffin, and sectioned (5–7 µm).

Cell Culture and Cytofluorimetry

LLC cells were grown in Eagle’s minimum essential medium containing 10% fetal bovine serum, glucose (4.5 g/l), NaHCO3 (1.5 g/l), penicillin (100 µg/ml), and streptomycin (100 U/ml) at 37°C in humid atmosphere saturated with 5% CO2. A Mg-free medium was purchased by Invitrogen (San Giuliano, Italy) and utilized to vary the concentrations of Mg by the addition of MgSO4. Control medium contained 1.0 mM Mg, whereas deficient medium contained 0.1 mM Mg. Cell cycle was analyzed by FACS after staining with propidium iodide by a standard protocol (14). For proliferation assays, the cells were cultured in 0.1 or 1.0 mM Mg for various times, trypsinized, stained with trypan blue solution (0.4%), and the viable cells were counted using a Burker chamber (5).

Western Blot

Tumors were lysed in 10 mM Tris-HCl (pH 7.4) containing 3 mM MgCl2, 10 mM NaCl, 0.1% sodium dodecylsulfate (SDS), 0.1% Triton X-100, 0.5 mM EDTA, and protein inhibitors, separated on SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose sheets. Western blot analysis was performed using antibodies against p21, p27, endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), or actin (Tebu Bio, Magenta, Italy). Secondary antibodies were labeled with horseradish peroxidase (Amersham Pharmacia Biotech, Milan, Italy), and immunoreactive proteins detected by the SuperSignal chemiluminescence kit (Pierce, Rockford, IL). The blots were quantified by densitometry.

Levels of Nitric Oxide (NO2/NO3)

To determine the levels of NO, total NO2/NO3 was measured by the Griess method, according to the manufacturer’s instructions (Oxis Research, Portland, OR) (15).

cDNA Array

RNA was extracted by the cesium chloride method from tumors derived from Mg-deficient mice and controls 21 days after the injection of LLC cells. Human (c)DNA expression array membranes consisting of 1176 known genes (AtlasTM Mouse 1.2 Array and Atlas Mouse Cancer 1.2 Array, Clontech, BD Biosciences, Clontech, Palo Alto, CA) were used according to the manufacturer’s instructions. The hybridization data were collected with Phospholmager (Molecular Dynamics, Sunnyvale, CA). The AtlasImage version 1.0 (Clontech) software was used to compare gene expression. Signal intensities between the compared arrays were normalized using the global mode (to develop a normalization coefficient) that uses an average value based on all the expressed genes (16).

Immunohistochemical Analyses

Detection of 8-hydroxy-deoxyguanine (8-OHdG) coupled with diaminobenzidine (DAB) (Vector, Burlingame, CA) was carried out as described (17). Semiquantitative evaluation
Figure 1. Cultured Lewis lung carcinoma (LLC) cell growth in control and low-Mg-containing medium. A: LLC cells were grown in control (1.0 mM) and low (0.1 mM) Mg medium. After 4, 6, and 8 days, the cells were trypsinized, stained with trypan blue solution (0.4%), and the viable cells were counted using a Burker chamber. B: Cell cycle distribution was analyzed by FACS on LLC cells after 4 and 8 days of culture in medium containing 1.0 or 0.1 mM Mg. In A and B, data are shown as the mean ± SD of 3 separate experiments. *, P < 0.05 vs. control.

Results

Impact of Low Mg on Cultured LLC Cells

To investigate a direct role of low Mg on cell proliferation, LLC cells were cultured in a medium containing 0.1 mM Mg or in control medium (1.0 mM Mg) and counted after 4, 6, and 8 days. A significant growth retardation (20%; P < 0.05) was observed in cells grown 8 days in low Mg vs. controls (Fig. 1A). We also evaluated the cell cycle distribution by FACS analysis and observed that low Mg promotes the accumulation of LLC cells into the G0/G1 phase of the cell cycle in parallel with a decrease of cells in the S phase (Fig. 1B).

Evaluation of Gene Expression by cDNA Array

A total of 21 days after the injection of LLC cells in mice under different dietetic regimens, RNA from the tumors was extracted. The pattern of gene expression was then examined by cDNA expression array. We found that Mg deficiency significantly modulated the expression of about 7% of the genes. The most prominent changes are listed in Table 1. By Western blot, we confirmed the upregulation of p27 and p21 in the tumors from mice on a Mg-deficient diet (Fig. 2). cDNA array also revealed the downregulation of cyclin B1 and D3. In addition, tumors from Mg-deficient mice overexpressed calpain

Table 1. Modulation of Gene Expression in LLC Tumors Grown in Mice on a Magnesium-Deficient Diet

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Induction of Repression</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upregulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione S-transferase</td>
<td>4.4</td>
<td>Scavenger, oxidative stress</td>
</tr>
<tr>
<td>DNA-inducible transcript 3</td>
<td>5.5</td>
<td>Cell cycle, apoptosis</td>
</tr>
<tr>
<td>Junonji</td>
<td>3.6</td>
<td>Transcription factor, cell proliferation</td>
</tr>
<tr>
<td>Calpain 1</td>
<td>4.5</td>
<td>Intracellular proteinase, adhesion</td>
</tr>
<tr>
<td>Deleted in colorectal carcinoma p21</td>
<td>3.4</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>p27</td>
<td>4.1</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>Downregulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasminogen activator, urokinase</td>
<td>4.4</td>
<td>Tissue structure, metastasis</td>
</tr>
<tr>
<td>Contactin 1</td>
<td>3.3</td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>Integrin P binding protein 1</td>
<td>5.4</td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>Gap junction membrane channel protein β3 and α5</td>
<td>3.2</td>
<td>Cell–cell and cell–ECM interactions</td>
</tr>
<tr>
<td>Presenilin 1</td>
<td>3.9</td>
<td>Cell–cell and cell–ECM interactions</td>
</tr>
<tr>
<td>Ajuba</td>
<td>4.0</td>
<td>Cell–cell interactions, signaling</td>
</tr>
<tr>
<td>CyclinB1</td>
<td>3.7</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>Cyclin D3</td>
<td>3.3</td>
<td>Cell cycle</td>
</tr>
</tbody>
</table>

a: Abbreviations are as follows: LLC, Lewis lung carcinoma; ECM, extracellular matrix. Membranes of 1176 known genes were used for hybridization (AtlasTM Mouse 1.2 Array and AtlasTM Mouse 1.2 Array Cancer, Clontech, Palo Alto, CA) as described in the Methods.
Evaluation of p27 and p21 in Lewis lung carcinoma tumors grown in control (1.0 g/kg) and Mg-deficient (0.03 g/Kg) mice. Western analysis was performed as described using antibodies against p21 and p27. After stripping, the blot was incubated with an antiactin antibody to show that comparable amounts of proteins were loaded per lane.

I and glutathione S-transferase (GST), whereas they downregulated urokinase and molecules involved in cell adhesion.

Evaluation of DNA Oxidation

To evaluate whether the Mg-deficient diet could influence LLC growth by enhancing DNA oxidative damage, we measured the levels of 8-OHdG, the most indicative guanine oxidation adduct (17). Figure 3A reports indicative samples of tumors developed 21 days after the injection of LLC cells in normal and Mg-deficient mice. LLC tumor cells appeared dispersed, heterogeneous, pleomorphic, and dysplastic (Fig. 3A and Fig. 3B). Fig. 3B reports a semiquantitative evaluation of the nuclear staining for 8-OHdG of the tumor samples 12 and 21 days after the injection. The nuclear staining was less pronounced in tumors from control mice (1 g/kg Mg) than in Mg-deficient animals (0.03 mg/kg Mg). This difference became significant in tumors 21 days after the injection of LLC cells (b) ($P < 0.05$).

Apart from reactive oxygen species (ROS), a possible source of oxidative species could be nitric oxide and its derivatives. Interestingly, we observed an increase of NO production as detected by the Griess method (Fig. 4A), which correlated with the upregulation of endothelial NOS in tumors from Mg-deficient animals as shown by Western blot (Fig. 4B).

Evaluation of Tumor Angiogenesis

Because angiogenesis is one of the hallmarks of cancer (19), we quantified microvessel number in the tumors from mice on a normal or Mg-deficient diet after staining the sections with antibodies against CD31. We found a decrease of vessel density in mice fed with low Mg (Table 2), whereas the caliber of the vessels was not affected. Angioinvasivity was not observed (not shown). We also evaluated angiogenic factors, which are often upregulated in tumors (18,19). In our experimental model, no modulation of VEGF and bFGF was detected by Western blot in the tumors from Mg-deficient mice vs. controls (Fig. 5).

Figure 2.

Figure 3.

DNA oxidative damage of tumors from control and Mg-deficient mice. A: Immunohistochemical staining for 8-hydroxy-deoxyguanine (8-OHdG) in lung Lewis lung carcinoma (LLC) tumors 21 days after injection in mice fed normal a) or Mg-deficient diet b). The asterisk (b) indicates a vessel. Magnification ×20. B: Semiquantitative evaluation of nuclear staining of 8-OHdG diaminobenzidine by optical density (OD) was performed on samples from mice under normal or Mg-deficient diet 12 and 21 days after LLC injection. Data are the mean ± SD from 4 different samples; *$P < 0.05$ vs. 21 days LLC tumors from mice under normal diet.
Low Mg retards the growth of cultured LLC cell by a typical G0/G1 arrest, as demonstrated in other cell types (11,12,14). The latency of this inhibition is probably due to the higher resistance to extracellular Mg availability of tumor vs. normal cells (3). However, our results demonstrate that also the growth of LLC cells is ultimately dependent from extracellular Mg. These in vitro results are in agreement with our previous findings in vivo. Indeed, we described an inhibition of LLC tumor growth associated with a reduction of the number of the cells engaged in the S phase in mice on a low-Mg diet (13). Gene expression studies by cDNA arrays have shown that the growth inhibition of the tumors in Mg-deficient mice correlates with the downregulation of cyclin B1 and D3, crucial for the progression through the cell cycle, and the upregulation of p21 and p27, 2 cyclin-dependent kinase inhibitors, which block primarily the transition from the G1 to the S phase. The overexpression of p27 and p21 in the tumors from Mg-deficient mice was confirmed by Western blot. Interestingly, increased expression of p27 was also observed in murine breast HC11 cells and in HL60 leukemia cells under Mg deprivation (11,12), whereas p21 overexpression is associated with low-Mg-dependent growth inhibition of endothelial cells (15). The direct relationship between extracellular Mg availability and p27 expression was explored in detail in HC11 cells, where we described the Mg concentration- and time-dependent expression of p27 (12).

Table 2. Tumor Vasculature From Control and Magnesium (Mg)-Deficient Micea

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Blood Vessels</th>
<th>Diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg-deficient mice</td>
<td>17 ± 6</td>
<td>978.1 ± 606.5</td>
</tr>
<tr>
<td>Control mice</td>
<td>32 ± 8*</td>
<td>997.8 ± 914.3</td>
</tr>
</tbody>
</table>

a: Specimens were stained with antibodies against CD31. Data are the mean ± SD of 4 separate experiments. * , P < 0.05.
ROS have a huge range of potential actions on cells, some of which can be envisaged as anticancer (cell cycle stasis, senescence, apoptosis), whereas others can be considered procarcin (promoting proliferation and invasiveness) (24). The different biological responses to oxidative species depend on the ROS and on the tissue-specific sensitivity to oxidative damage (24). Most attention has been paid to direct oxidative damage to DNA. Indeed, DNA oxidative damage, specifically 8-OHdG, has been associated to different pathologic conditions, among which are aging and proliferative disorders (25). Interestingly, we detected higher levels of 8-OHdG in tumors developed in Mg-deficient mice than in controls (>30%; P < 0.05). In the same conditions, we found an increase of NO in tumors from Mg-deficient mice, which correlated with the upregulation of eNOS as detected in several tumors (26). It is therefore feasible to propose that NO-derived reactive nitrogen species may contribute to DNA oxidative damage. Indeed, high concentrations of NO participate in mediating the inhibition of cancer (26). All together, these data suggest that during tumor growth, Mg deficiency enhances the levels of oxidative species that could affect the growth rate of LLC tumors.

To this purpose, it is noteworthy that by cDNA array, we found an upregulation of GST in tumors from Mg-deficient mice. Because the tissue levels of GST are a good marker of oxidative stress (27,28), this result suggests the activation of an adaptive response to counteract the increased production of free radicals consistently described under Mg deficiency in vivo. Indeed, increased mRNA for GST was found also in normal tissues (lung and thymocytes) of rodents under Mg-deficient diet, suggesting that the expression of this enzyme is modulated by the levels of Mg (16,29).

Apart from being a mediator of inflammation and a possible source of reactive species, NO has a role in tumor angiogenesis through the upregulation of VEGF, a potent angiogenic molecule often implicated in tumor neovascularization (18,19). In our experimental model, Mg does not affect the levels of VEGF and bFGF. We have recently shown in vitro that low Mg inhibits endothelial migration and proliferation, events absolutely required for the formation of new vessels, by desensitizing endothelial cells to the effects of angiogenic factors (15). Consequently, the fact that tumors from Mg-deficient mice develop fewer vessels than controls could be due to a direct effect of low Mg on endothelial cells and to impairment of the angiogenic switch in the tumor cells.

The influence of Mg on tumor development is even more complex because mice on a low-Mg diet develop more lung metastases than controls in spite of the smaller size of the primary tumor (13). Results from cDNA array suggest that Mg also influences the remodeling of the extracellular matrix by modulating the expression of proteases. Calpain I, an ubiquitous cytoplasmic nonlysosomal cysteine endopeptidase necessary for tumor invasion and overexpressed in a variety of tumors (30), was upregulated in tumors grown in Mg-deficient mice. On the contrary, urokinase and contactin-1, molecules with a role in invasion and metastasis (31,32), were downregulated by Mg deficiency. The altered expression of these molecules together with the lack of angioinvasivity suggests that primary tumors from Mg-deficient mice might be less locally invasive. On the other hand, the increase of lung metastases in Mg-deficient mice could be due to the marked inflammatory response observed in the lungs that promotes the upregulation of adhesion molecules (15,16), which facilitate lung colonization by circulating tumor cells.

In conclusion, we propose that Mg deficiency exerts a dual role on primary tumors in vivo: It inhibits LLC tumor growth directly by interfering with the cell cycle progression of the tumor cells and indirectly by inhibiting angiogenic switch and promoting oxidative stress. In this scenario, the immunoinflammatory response evoked by Mg deprivation, by producing cytokines and free radicals, may affect primary tumor growth and metastasis in opposite ways. Unfortunately, our in vivo model does not allow us to examine step by step the contribution of each of these events because the tumor lesions become detectable only 12 to 13 days after injecting LLC (13). The overall process and in particular, the contribution of immunoinflammatory response to tumor dissemination, deserve further investigations.

Acknowledgments and Notes

This work was supported by Collaborative Linkage NATO grant to AM, JAM, and FW (2002–04); COFINS, 2001064293 and 2003067599; MIUR 60% and line D1 2004–2006 to FW. Address correspondence to Jeanette A. M. Maier, Dipartimento di Scienze Precliniche LITA Vialba, Università di Milano, Via GB Grassi 74, 20157 Milano, Italy. Phone: 39-02-50319648. Fax: 39-02-50319659. E-mail: jeannette.maier@unimi.it.

Submitted 21 February 2007; accepted in final form 7 April 2007.

References


