Erythrocyte magnesium influx and efflux in solid tumor bearing mice

C. Feillet-Coudray¹, A. Nasulewicz¹,², L. Jaffrelo¹, S. Thien¹, C. Coudray¹, M. Rambeau¹, E. Gueux¹, Y. Rayssiguier¹, A. Opolski², F.I. Wolf³, A. Mazur¹

¹ CRNH d’Auvergne, Unité Maladies Métaboliques et Micronutriments, INRA, Clermont-Ferrand, France; ² Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland; ³ Istituto di Patologia Generale e Centro di Ricerche Oncologiche Giovanni XXIII, Università Cattolica del Sacro Cuore, Facoltà di Medicina, Roma, Italy

Correspondence: Dr Christine Feillet-Coudray, Unité Maladies Métaboliques et Micronutriments, INRA, 63122 Saint Genès Champanelle, France. <feillet@clermont.inra.fr>

Abstract. Mg metabolism is modified in tumors and tumor-bearing organisms. In particular cancer patients often display elevated erythrocyte Mg levels. For a better understanding of the increased erythrocyte Mg content, we attempted to determine Mg fluxes in erythrocytes from tumor-bearing mice by Mg stable isotopes, using a method developed in our laboratory. To characterize the animal Mg status, blood and tissue Mg levels and hematological parameters were assayed. Results showed that in tumor-bearing mice total erythrocyte Mg was about 46% higher than in controls, whereas plasma and tissues Mg levels were not modified; red blood cells and hemoglobin as well as hematocrits were significantly decreased, while mean corpuscular volume and mean corpuscular hemoglobin were slightly but significantly increased in tumor-bearing mice compared to controls (by 3% and 4%, respectively), a picture corresponding to a normochromic, slightly macrocytic anemia. Erythrocyte Mg efflux was about 20% higher (404 + 59 vs 330 + 45 μmol/L, respectively, p < 0.05) in tumor-bearing mice compared to controls, whereas influx was not significantly modified (130 + 11 vs 122 + 19 μmol/L, respectively). Our data therefore exclude that the increased Mg content observed in erythrocytes of tumor-bearing mice could simply result from an increase of young Mg-enriched erythrocytes produced by the enhanced erythropoiesis which follows tumor-induced anemia.

Key words: magnesium, erythrocyte, influx, efflux, mice, tumor, erythropoiesis

The importance of magnesium (Mg), the second most abundant cation in the cell, has long been recognized. In fact, Mg is involved in many enzymatic reactions and in ion transport systems. For instance, Mg is essential for phosphorylation reactions, DNA transcription and protein synthesis, energy transfer, and lipid and carbohydrate metabolism [1]. Mg metabolism is extensively modified in cancer [2], an abundant Mg content could account for the higher metabolic rate of proliferating cancer cells. In fact, many tumors display higher Mg concentrations than corresponding normal tissues, and the increase in Mg content seems to correlate to tumor malignancy [2]. Interestingly, it has been observed that cancer patients have elevated erythrocyte Mg levels [3]. To explain this feature a decrease of Mg efflux from the red blood cell membrane has been hypothesized. The consequent accumulation of Mg inside the erythrocytes would allow the transport of this cation to the tumor where it could be released to sustain cell growth [2], as if erythrocytes could serve as vehicles not only of chemical species but also of cations. To
verify this hypothesis and improve our understanding of the significance of the distorted Mg distribution in cancer, we studied Mg fluxes in erythrocytes from tumor-bearing mice. Mg influx is basically driven by the electrochemical gradient via channels and carriers [4] while Mg efflux results mainly from a Na+ and ATP-dependent Mg2+ release via an Na+/Mg2+ antiport [5, 6]. Until recently, studies on Mg fluxes have been limited by the fact that simultaneous determination of Mg fluxes was not possible. In our laboratory, we have developed a new method using a stable isotope of Mg, which allows the simultaneous determination of Mg influx and efflux in erythrocytes [7].

For a better understanding of the regulation of cellular Mg, in particular during the neoplastic process, we used a mouse model in which we subcutaneously transplanted a solid tumor, namely Lewis lung cancer (LLC). We characterized the Mg status of these animals by measuring blood and tissue Mg levels and hematological parameters. Erythrocyte Mg influx and efflux were studied in vitro using Mg stable isotopes.

Materials and methods

Animals

Fifty C57BL/6 female mice, aged 12 weeks, were divided into two groups, a control group of 18 mice and an experimental group of 32 mice. Mice were housed under conditions of constant temperature (20-22°C) and humidity (45-50%) with a standard dark cycle (20.00-08.00 hours). Our institutional guidelines for the care and use of laboratory animals were applied. Mice received a control diet and demineralized water, ad libitum. The diet contained the following constituents (g/kg): casein 200, wheat starch 650, maize oil 50, alphacel (cellulose) 50, DL-methionine 3, choline bitartrate 2, modified AIN-76 mineral mix 35, AIN-76A vitamin mix 10 (ICN Biomedicals, Orsay, France). The Mg concentration in the diet was 1000 mg/kg. As one mouse consumed about 4g/d of control diet, each mouse received about 4 mg Mg/d. The diet started on the day of tumor cell implantation and was maintained until the end of the experiment.

LLC (Lewis lung carcinoma) cells were donated by the National Cancer Institute (Bethesda, USA). Thirty-two mice were inoculated subcutaneously (s.c.) in right flank region with a 20% (v/w) suspension of tumor cells taken from in vivo passage and suspended in 0.2 mL of Hanks medium (CaCl2 0.185g/L, MgSO4 0.09767g/L, KCl 0.4g/L, KH2PO4 0.06g/L, NaHCO3 0.35g/L, NaCl 8.0g/L, Na2HPO4 0.04788g/L, D-glucose 1g/L). The experiments were terminated 21 days after tumor cell inoculation.

Evaluation of Mg status

Blood was obtained from anesthetized mice (0.3µg pentobarbital/g body weight) by biopsy from the abdominal vein using a heparinized syringe. A blood counter (Animal Blood Counter, Strasbourg, France) was used to determine hematological parameters. Erythrocyte Mg influx and efflux were studied in vitro using Mg stable isotopes.

Abbreviations

RBC = red blood cells
MCV = mean corpuscular volume
Hb = hemoglobin
MCH = mean corpuscular hemoglobin
Ht = hematocrits

C. FEILLET-COUDRAY, ET AL.
France) after appropriate dilution with 0.14 M-HNO₃ using beryllium (Be) as the internal standard, and external calibration of the ICP-MS [8]. Mg²⁺ efflux and influx were calculated according to the following equations:

\[
\text{Mg}^{2+} \text{ efflux in } \mu\text{mol/L cells} = [\text{²⁴Mg}]_T - [\text{²⁴Mg}]_{T_0} \times \text{erythrocyte dilution in the cell suspension},
\]

\[
\text{Mg}^{2+} \text{ influx in } \mu\text{mol/L cells} = [\text{²⁵Mg}]_{T_0} - 0.1 \times [\text{²⁵Mg}]_{T}.
\]

where 0.1 is the natural abundance of ²⁵Mg.

Given the calibration process, the ICP-MS machine gives the quantitative results of each measured isotope as if the natural abundance for each isotope was 100%, while the natural abundance of Mg isotope is:

\[
\begin{align*}
\text{²⁴Mg} & = 0.789; \\
\text{²⁵Mg} & = 0.100; \quad \text{and} \quad \text{²⁶Mg} = 0.111 [9].
\end{align*}
\]

Therefore, it was necessary to subtract the amount of ²⁶Mg from the amount of ²⁵Mg in the erythrocyte. Indeed, erythrocyte ²⁵Mg represents the natural erythrocyte Mg but erythrocyte ²⁶Mg represents both the natural erythrocyte Mg plus ²⁵Mg derived from the incubation medium.

To exclude Mg²⁺ efflux caused by cell damage, erythrocyte hemolysis was systematically measured by hemoglobin (Hb) determination in the supernatants (cyanmethemoglobin, at 546 nm). A hemolysis in supernatants lower than 1.5% of total hemolysis, which did not increase significantly during the experiment, was considered to be within normal ranges and was therefore not taken into account.

**Statistical analysis**

Results were expressed as means ± SD. The statistical significance of differences between means was assessed using the Student’s t-test or Mann-Whitney test. The limit of statistical significance was set at p<0.05. Statistical analyses were performed using the GraphPad program (V3.00, GraphPad Software, San Diego, CA).

**Results**

**Body and organ weight**

Mean body weight was significantly higher in tumor-bearing mice than in control animals (table 1). However, when tumor weight was subtracted from the body weight of tumor-bearing mice, the mean values did not differ between these two groups. It is noteworthy that the weight of the spleen and liver of tumor-bearing mice was significantly higher than that of controls (200% and 20% respectively) (table 1).

**Hematological parameters**

Red blood cell (RBC) counts, Hb and hematocrit (Ht) levels were significantly decreased in tumor-bearing mice compared to controls, indicating severe anemia (table 2). Mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) were slightly but significantly increased in tumor-bearing mice (3% and 4%, respectively), suggesting a normochromic, slightly macrocytic anemia (table 2).

**Magnesium status**

Plasma and tissue Mg levels were not modified in tumor-bearing mice in comparison to controls (table 3). On the other hand, erythrocyte Mg levels were significantly higher in tumor-bearing mice (46% higher than the controls) (table 3). It is worth noting that the tumor Mg content was almost similar to liver Mg content when expressed in total Mg content (Mg concentration in the organ x weight of the organ) instead of Mg concentration (388 ± 121 µg of Mg versus 521 + 49 µg, respectively), highlighting the abundance of Mg in the tumor in comparison with other organs.

| Table 1. Body mass and selected tissue mass of control and tumor-bearing mice. |
|----------------------------------|------------------|
|                                | Control mice     | LLC-bearing mice |
| **n**                          | 18               | 32               |
| **Mice (g)**                   | 20.3 ± 0.8       | 22.5 ± 1.0*      |
| **Tumor (g)**                  | -                | 1.95 ± 0.56      |
| **Liver (g)**                  | 0.87 ± 0.10      | 1.06 ± 0.10*     |
| **Spleen (g)**                 | 0.097 ± 0.011    | 0.293 ± 0.079*   |

Mean ± SD of n mice per group, statistical significance: *p < 0.001.

| Table 2. Hematological parameters of control and tumor-bearing mice. |
|----------------------------------|------------------|
| **RBC (10⁵/mm³)**               | Control mice     | LLC-bearing mice |
|                                 | 8.42 ± 0.35      | 5.26 ± 1.09***   |
| **MCV (µm³)**                   | 44.4 ± 0.6       | 45.7 ± 2.3*      |
| **Hb (g/dL)**                   | 13.25 ± 0.46     | 8.59 ± 1.57***   |
| **MCH (pg)**                    | 15.8 ± 0.4       | 16.4 ± 0.8**     |
| **Ht (%)**                      | 37.4 ± 1.5       | 23.9 ± 4.3***    |

Mean ± SD, statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001.
Erythrocyte Mg influx and Mg efflux

As shown in figure 1, erythrocyte Mg influx was not modified in tumor-bearing mice in comparison with controls (130 ± 11 versus 122 ± 19 µmol/l, respectively). However, the corresponding efflux was about 20% higher in tumor-bearing mice than in controls (404 ± 59 versus 330 ± 45 µmol/l, respectively, p < 0.05).

Discussion

In order to better understand the increased erythrocyte Mg content associated to cancer, we explored both global Mg status, red blood cell characteristics and Mg transport from erythrocytes in mice bearing lung cancer (LLC).

We observed no differences in plasma Mg levels between tumor-bearing mice and control animals. Decreased [3, 10] but also unmodified [11] or increased [12-14] plasma Mg levels have been reported in patients with various types of cancer. It is well known, however, that plasma Mg level (accounting for 1% of total body Mg) is not necessarily indicative of organism Mg status. While low plasma Mg values reflect a depletion, in some circumstances low intracellular Mg2+ levels may coexist with normal plasma levels. Mg levels in the tibia and muscles are the best markers of Mg status. In fact, more than 60% of total body Mg is present in bone tissue and 25% in muscles. Our results show that the neoplastic process does not influence Mg levels in these tissues. Moreover, no modification in Mg levels was observed in other tissues (kidney, lung, liver) of tumor-bearing mice. We can thus conclude that Mg content of tumor tissue does not derive from Mg depletion of other organs of cancer-bearing animals.

Erythrocyte Mg levels were significantly higher in tumor-bearing mice, data in accordance with clinical observations described in a large majority of cancer patients [3, 12, 15]. However, the mechanisms behind this phenomenon remain merely speculative. Durlach [2] has suggested that increased erythrocyte Mg levels may be due to a change in erythrocyte Mg fluxes to selectively provide the tumor tissue with a large amount of this cation essential to cell growth. If this was the case one could envisage an increased Mg influx which could serve to overload the erythrocytes and/or a decreased Mg efflux, which could be modulated according to the extracellular conditions, e.g. tumor tissue would selectively stimulate Mg efflux to provide abundant Mg for tumor cells.

By the use of stable isotope enriched media and ICP-MS detection, we were able to study Mg2+ influx and efflux simultaneously in non-loaded Mg cells [17]. We previously showed that in physiologic conditions, Mg fluxes from mice erythrocytes are proportional to extracellular Mg availability.

In the present study, we showed that in erythrocytes from tumor bearing mice Mg efflux increased significantly. Thus, the increase of erythrocyte Mg content in tumor-bearing mice did not result from a retention of Mg inside the cell. The increase in erythrocyte Mg efflux was more probably associated with the increase of Mg in the cell (total cell Mg was 45% higher than in control cells), as we observed a positive correlation between Mg efflux and intracellular erythrocyte Mg levels in mice fed adequate, marginal

### Table 3. Magnesium status of control and tumor-bearing mice.

<table>
<thead>
<tr>
<th></th>
<th>Control mice</th>
<th>LLC-bearing mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma Mg</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mmol/L)**</td>
<td>0.975 ± 0.050</td>
<td>1.010 ± 0.090</td>
</tr>
<tr>
<td><strong>Erythrocyte Mg</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mmol/L)</td>
<td>2.21 ± 0.15</td>
<td>3.22 ± 0.44*</td>
</tr>
<tr>
<td><strong>Tibia Mg</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µg/kg fresh weight)</td>
<td>2400 ± 255</td>
<td>2309 ± 222</td>
</tr>
<tr>
<td><strong>Kidney Mg</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µg/kg fresh weight)</td>
<td>457 ± 48</td>
<td>443 ± 77</td>
</tr>
<tr>
<td><strong>Lung Mg</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µg/kg fresh weight)</td>
<td>71.1 ± 4.7</td>
<td>75.2 ± 7.5</td>
</tr>
<tr>
<td><strong>Liver Mg</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µg/kg fresh weight)</td>
<td>489 ± 36</td>
<td>492 ± 31</td>
</tr>
<tr>
<td><strong>Muscle Mg</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µg/kg fresh weight)</td>
<td>282 ± 37</td>
<td>294 ± 45</td>
</tr>
<tr>
<td><strong>Tumor Mg</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µg/kg fresh weight)</td>
<td>-</td>
<td>199 ± 31</td>
</tr>
</tbody>
</table>

Mean ± SD, statistical significance: *p < 0.001; **: n = 9 for controls and n = 8 for tumor-bearing mice.

### Figure 1. Mg influx and efflux in erythrocytes from control and from tumor-bearing mice. Mean ± SD, statistical significance: *p < 0.05 n = 9 for controls and n = 8 for tumor-bearing mice.
Mg-deficient or severe Mg-deficient diet and in mice selected for high and low erythrocyte Mg levels [16, 17]. If one could demonstrate that Mg efflux from erythrocytes of tumor-bearing mice could be modulated in different circumstances, these cells would be most suitable to provide the tumor tissue with the excess Mg required for its metabolism, confirming that these cells could be exploited not only as pharmacological but also as electrolyte carriers [18]. At present, however, our results do not conclusively prove this hypothesis since these cells seem non specifically more prone to lose Mg than those of controls.

In addition, our results showed that the increase in erythrocyte Mg of tumor-bearing mice does not result from an increased transport of Mg into the cell as Mg influx was not modified in erythrocytes from tumor-bearing mice. It is possible that the higher erythrocyte Mg levels in tumor-bearing mice may stem from an increase in the proportion of young erythrocytes, as proposed by Durlach [2]. It has been demonstrated that Mg levels decrease with increasing age of the erythrocytes, as total Mg is four-fold higher in reticulocytes than in mature erythrocytes [19, 20]. This interpretation is supported by the data showing that tumor-bearing mice had severe anemia and impressive splenomegaly. In fact, since hemolysis affects selectively older erythrocytes [21] and since young erythrocytes have a slightly larger mean volume than mature erythrocytes [22], the 40% higher erythrocyte Mg content in mice inoculated with LLC cells might reflect, at least in part, an increased erythropoiesis due to regenerative anemia.

Conclusion

This study demonstrates that the increased Mg content observed in erythrocytes of tumor-bearing mice is not due to decreased Mg efflux or increased Mg influx. On the contrary erythrocytes from tumor bearing mice displayed higher Mg efflux suggesting a tendency to lose excess Mg which could be utilized by the tumor tissue to sustain cell growth. It is also possible that the increased Mg content observed in erythrocytes of tumor-bearing mice is due, at least partly, to enhanced erythropoiesis induced by tumor associated anemia, leading to the presence of younger, Mg-enriched erythrocytes. Further studies are necessary to clarify the intimate significance of the increased erythrocytes Mg content associated with cancer.

Acknowledgements

We are grateful to D. Bayle, C. Lab and J.-C. Tressol for their technical assistance. This work was supported in part by a collaborative linkage NATO grant, a Polonium bi-national grant, the Prix de Recherche du Centre Évian pour l’Eau and the Ligue Nationale Contre le Cancer.

References


