Severe Mg-deficiency is not associated with endothelial cell activation in mouse lung

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Abstract. Several experimental and clinical studies suggest that the lungs are a specific target of Mg-hypomagnesemia, which is a common side effect of cyclosporin A therapy. Due to the possible effect of hypomagnesemia on lung allograft function, the aim of this study was to evaluate endothelial cell (EC) activation and tissue remodelling (apoptosis) in the lungs from mice fed Mg-deficient diets. Immunocytochemical examinations did not reveal any inflammatory process in Mg-deficient mice, infiltration of leukocytes (CD45+ cells), expression of I-Ab class II molecules, E-selectin or ICAM-1 on ECs, and apoptotic cells. Quantification of mRNAs for E-selectin, ICAM-1 and VCAM-1, which are the most pertinent adhesins expressed by ECs, and for the cytokines TNFα and IL-2, demonstrated that severe Mg-deficiency does not result in EC activation. The balance between the up-regulation of G-CSF-R and CCL4 genes, and the down-regulation of the OPN gene shown by the cDNA microarray technique might be responsible for the absence of development of an inflammatory response, lung EC activation, and lung remodelling. However, we can hypothesize that severe Mg deficiency results in a latent inflammatory status of the lungs, which might be expressed following immune stresses, like transplantation conditions.

Key words: hypomagnesemia, lung, gene expression, adherence molecules

Magnesium (Mg) is the second most abundant intracellular cation. It plays an essential role in numerous enzymatic reactions involving energy metabolism and fundamental cellular reactions [1-3]. It acts as an ionic membrane regulator and modulator of neuronal and muscle activity of the ion transfer through membrane channels [4] and stabilizes cellular membranes, organelles like ribosomes, and nucleic acids [3, 5]. Therefore, it is not surprising that alterations of Mg homeostasis would lead to metabolic, biological, or organic dysfunctions, and cause various clinical disorders. Experimental hypomagnesemia in weaning or adult rats or mice has been associated with an early inflammatory process, a decrease of immune responses, oxidative damage to which neuropeptides like substance P (SP) and calcitonin gene-related peptide (CGRP) may contribute, and endothelial cell dysfunction [6-15]. In vitro, culture of human umbilical vein endothelial cells (HUVEC) in low Mg concentrations was shown to inhibit cell growth and migration, to stimulate monocyte/endothelial cell interactions, and to up-regulate plasma plasminogen activator inhibitor (PAI)-1 expression [16]. Endothelial cells (EC) play a crucial role in maintaining the functional integrity of the vascular wall. They express a panel of markers, including factor VIII/von Willebrand antigen, platelet/endothelial cell adhesion molecule-1 (PECAM-1 or CD31), vascular endothelial cadherin (VE-cadherin or cad-5), and endothelial cell nitric oxide synthase (eNOS). The endothelium is a highly metabolically active organ...
that is involved in many physiological processes, including the control of vasomotor tone, barrier function, leukocyte adhesion and trafficking, inflammation, and hemostasis [17]. However, profound structural and functional heterogeneity exists along the vascular tree, between different organs and between two adjacent ECs of a single blood vessel [18, 19]. EC diversity is also reflected at the molecular and transcriptional levels [18]. Blood vessel type-specific and tissue-specific characteristics of ECs are differentially regulated in space and time, and are under the control of their microenvironment [20]. ECs in the lung express a unique repertoire of genes and gene products, although heterogeneity also exists between different segments of the vascular loop within the lung: microvessels, veins, arteries, bronchial or pulmonary circulation [19, 21-23]. Several experimental and clinical studies have shown a relationship between Mg status and pulmonary allergies or sudden infant death syndrome (SIDS), suggesting that the lungs might be a specific target of Mg-deficiency [24, 25]. From epidemic data, magnesium may play a role in the control of asthma or in the development of bronchospasm [26, 27], although magnesium supplementation does not appear to provide important clinical benefits in chronic stable asthma [28, 29]. Indeed, acute asthma was associated with lower erythrocyte Mg content, while plasma Mg concentrations remained unchanged [27]. Finally, Mg deficiency may be considered as an adjuvant nutritional disorder in asthma, but asthma per se does not depend only on Mg deficiency [30]. Hypomagnesemia is a common side effect of cyclosporin A (CsA) therapy [31-33]. It is associated with a faster rate of decline in kidney allograft function and increased graft loss in renal transplant recipients with chronic CsA nephropathy [34, 35]. Moreover, magnesium deficiency enhances the susceptibility of rat hearts to ischemia-reperfusion injury [36]. Conversely, it has been reported that the addition of ATP and MgCl2 to the preservation solution attenuated reperfusion injury following cold ischemia in rat lungs [37].

Because of a possible effect of hypomagnesemia on lung allograft function, the aim of this study was to evaluate EC activation and tissue remodelling (apoptosis) in lungs from mice fed a Mg-deficient diet. We analyzed the mRNA expression of a number of endothelial adhesion molecules (ICAM-1 or CD54, VCAM-1 or CD106, E-selectin or CD62E), which are expressed in the lung in inflammatory conditions and are of eminent importance for the migration of leukocytes to sites of inflammation. We quantified the major inflammatory cytokine tumor necrosis factor alpha (TNFα) and also IL-2, which plays a pivotal role in the initiation of an immune response leading to the induction of lymphocyte proliferation. These two cytokines influence manifold cells involved in inflammatory processes and graft rejection.

Materials and methods

Animals and diet
Female C57BL/6N slo 6 week-old mice (Harlan, Gannat, France) were given ad libitum for 26 days, either standard Mg-containing diet (control diet, 1400 ppm Mg2+, Harlan Teklad 2016 batch n° M013) and tap water for drinking (< 4 ppm Mg2+), or synthetic Mg-deficient diets (150 or 50 ppm Mg2+) prepared in the laboratory and deionized water to avoid consumption of Mg from normal drinking water. Mice were maintained at +22°C. All experiments were performed according to the recommendations of the INRA Ethics Committee (decree no. 87-848). At days 5, 14 and 26, control and Mg-deficient mice (n = 6 per group) were anesthetized with avertin (250 mg/kg, intraperitoneally) and injected with heparin (25,000 IU/kg intraperitoneally) as anticoagulant protocol. Blood was collected by heart-puncture. Lungs were excised: one part was immediately frozen in liquid nitrogen then stored at -80°C for histological and immunocytochemical studies, while total RNA was extracted from a second part and total proteins from a third part.

Blood determinations
Mg concentration in plasma was determined by a xytridyl blue complexometric method by routine procedures in an automated clinical biochemistry facility (AU400, Olympus, Rungis, France).

White cell count and leukocyte formula were performed in an automaton (ABX Pentra Vega Retic/ABX Hemato, Montpellier, France).

TNF-α quantification in lung extracts
Fresh lungs were sonicated 3 times for 10 sec on ice in a lysis solution containing 50 mM Tris/HCl, 150 mM NaCl, 1 mM diithiothreitol, 0.5 mM EDTA, 0.25 mM Na3VO4 (a protein tyrosine phosphatase inhibitor), 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 μg/mL aprotinin (a serine protease inhibitor) and 1 μg/mL leupeptin (an inhibitor of serine and cysteine proteases). These extracts were kept frozen at -80°C until use.

Total proteins were quantified by the Biuret colorimetric method (BC Assay Kit, Interchim, Montluçon, 226
identify apoptotic endothelial cells, double staining according to the manufacturer's instructions. To CD45 were analyzed on 7 Fluorescein labeling) method (available TUNEL (TdT-mediated dUTP-X nick end controls for the specific mAbs.

anti-rat (Serotec, Cergy Saint-Christophe, France). cytes, then FITC-conjugated rabbit polyclonal IgG Systems, Lille, France), which reacts with leukocyte Texas Red; rat IgG2b anti-mouse CD45 mAb (R&D (R&D Systems, Lille, France), then streptavidin-biotinylated polyclonal goat ICAM-1 streptavidin (Molecular Probes, Montluçon, France); biotinylated mouse E-selectin IgG2a mAb (BD Biosciences, Le Pont de Claix, France), then Alexa594-conjugated mouse E-selectin IgG2a mAb (BD Biosciences, Le Pont de Claix, France), then streptavidin-Texas Red (Amersham Life Science, Orsay, France), which reacts with leukocyte common antigen expressed on all types of leukocytes, then FITC-conjugated rabbit polyclonal IgG anti-rat (Serotec, Cergy Saint-Christophe, France).

Irrelevant immunoglobulins were used as negative controls for the specific mAbs.

Detection of apoptotic cell death was conducted on 7 μm cryostat sections using a commercially available TUNEL (TdT-mediated dUTP-X nick end labeling) method (In Situ Cell Death Detection kit, Fluorescein, Roche Diagnostics, Meylan, France), according to the manufacturer’s instructions. To identify apoptotic endothelial cells, double staining was performed using polyclonal rabbit anti-vWF immunoglobulin fraction, then TRITC-conjugated goat (Fab’)2 fragment anti-rabbit IgG2a monoclonal antibody.

mRNA quantification in lung tissue by SYBR Green real-time RT-PCR

Total RNA extraction from fresh lung, reverse transcription and quantitative PCR using the LightCycler™ method (Roche Diagnostics, Meylan France) were carried out as previously described [38]. The primers for E-selectin, VCAM-1, ICAM-1, TNFα, and IL-2 cDNA amplification were selected by using Oligo-l software (Med Probe, Oslo, Norway) to limit self-complementarity and to optimize sequence specificity [38, 39]. They were synthesized by Eurobio (Les Ulis, France). Results were expressed as arbitrary units (A.U.) using a single external standard curve (log concentration versus number of cycles) obtained from dilutions of a specific standard cDNA amplification product, and after standardization for GAPDH housekeeping gene. For length and specificity verification, amplimers were separated by electrophoresis onto a 1.5% agarose gel.

Gene expression analysis of lung tissue by the cDNA microarray technique

Gene expression was analyzed in lung tissue after 26 days of Mg-deficient diet (50 ppm Mg) using topic-defined PIQOR™ Cytokines & Receptors (274 genes) Mouse Microarray (Memorec Biotec GmbH, Köln, Germany). PIQOR™ Microarray experiments allow the identification of genes that are differentially expressed in different samples and the comparison of gene expression profiles in lung tissues from normal and Mg-deficient mice. The resulting data enable to compare amounts of specific RNAs between RNA samples. A minimum of 50 μg total RNA per sample is required; otherwise cDNA amplification is needed.

In a first step, quality of total RNA was controlled. Total RNA quality was checked by the Agilent 2100 Bioanalyzer system (Agilent Technologies, Massy, France), which gives electrophoregram with a RNA ladder reference in kb and elution diagrams (fluorescence versus elution time). The two prominent peaks within the elution diagrams represent ribosomal 28S and 18S RNAs. The peak areas of 28S and 18S RNAs were determined and the ratio of 28S/18S was calculated. A value greater than 1.5 indicates qualitative integrity of the RNAs.

The second step was hybridization of PIQOR™ Microarray. Cy3- or Cy5-probe labelling by reverse transcription was performed according to the
PIQOR\textsuperscript{TM} Instruction Manual. The fluorescent labelled probes were subsequently hybridized on topic-defined PIQOR\textsuperscript{TM} Cytokines & Receptors Mouse Sense Microarray and subjected to overnight hybridization using a hybridization station.

In a third step, the signal generated by the hybridization of a labelled, complementary nucleic acid sequence to an arrayed cDNA was detected and quantified using a scanner and appropriate software. Scanned Cy3 and Cy5 images were overlaid. Red colour indicates that the Cy5 signal intensity is higher than the Cy3 signal intensity. The respective gene is therefore overexpressed in the respective sample. Green spots indicate that the fluorescence intensity in the control sample is stronger induced than in the experimental sample. Yellow spots indicate that the signal intensities are equal for both probes. Each gene was spotted on 4 different positions on the array. Results for each gene are expressed as Cy5/Cy3 ratio and the respective coefficient of variation (CV in %, which refers to the average of the expression ratios of 4 spot replicates). A $<0.58$-fold down-regulation or a $>1.7$-fold induction of a gene in comparison to the control sample was considered as relevant.

Data analysis
The results of plasma Mg\textsuperscript{2+}, white blood cell count, mRNAs and TNF\textsubscript{a} quantification were recorded for each test group as mean values with SEM. The non-parametric Mann-Whitney $t$ test was used. A $p$ value of $p < 0.05$ was considered significant.

Results and discussion
Mice fed a Mg-deficient diet exhibited normal growth curve, since body weights at days 5, 14 and 26 were similar in mice fed normal or deficient diet. Mice fed a 50 ppm Mg diet exhibit a dramatic decrease in Mg concentration in plasma starting from day 5 ($0.92 \pm 0.11$ mmol/l \textit{versus} $1.31 \pm 0.04$ in control mice, $p < 0.01$ at day 5; $0.58 \pm 0.09$ mmol/l \textit{versus} $1.18 \pm 0.15$, $p < 0.01$, at day 14; $0.53 \pm 0.07$ mmol/l \textit{versus} $1.23 \pm 0.09$, $p < 0.01$, at day 26) (\textit{figure 1}). However only a transient increase in the number of blood neutrophils ($1.3$ fold, $p < 0.05$) was observed at day 5, without any changes in total leukocytes or lymphocyte populations. By contrast, in mice fed a 150 ppm Mg diet, Mg concentrations were transiently decreased (\textit{figure 1}) only at day 5 ($1.13 \pm 0.14$ mmol/l \textit{versus} $12.37 \pm 0.13$ in control mice, $p < 0.05$) and accompanied by a transient increase ($1.6$ fold, $p < 0.01$) in the number of blood leukocytes, both neutrophils ($1.86$ fold, $p < 0.05$) and lymphocytes ($1.46$ fold, $p < 0.05$). These data are in contradiction to previous studies in rats, which demonstrated that Mg deficiency is responsible for a non-specific inflammatory response characterized by an increase in the number and activation of blood neutrophils [7, 40]. The return of plasma Mg to a normal level following the moderate decrease observed as early as following 5 days of 150 ppm Mg diet, may be related to a compensatory phenomenon with either the activation of intestine Mg-carriers, which leads to an increased intake of Mg from the diet, or the mobilization of the intracellular Mg pool.

At the level of lungs, our histological studies did not reveal any inflammatory process in Mg-deficient mice. No infiltration of leukocytes (CD45\textsuperscript{+} cells) was observed by immunofluorescence examination.

![Figure 1. Concentration of Mg\textsuperscript{2+} (mmol/l) in plasma from mice fed either the control or a Mg-deficient diet (50 or 150 ppm Mg) for 5, 14 and 26 days. Data are mean ± SEM of 6 mice. *$p < 0.05$ and **$p < 0.01$ versus control diet.](image-url)
Data on the increase in proinflammatory cytokine production in blood from Mg-deficient rats are conflicting. Elevated levels of TNFα, IL-1β and IL-6 within 3 weeks of Mg deprivation were reported in rats by Weglicki et al. [41]. By contrast, only elevated levels of IL-6 were reported by Malpuech-Brugere et al. in weanling rats without a concomitant increase in TNFα, which was undetectable [7]. Again, data on the effect of endotoxin challenge on plasma TNFα levels in Mg-deficient rats are unclear. Nakagawa et al. [42] demonstrated that serum TNFα was detected neither in control nor in Mg-deficient rats aged 7-9 weeks, but increased after endotoxin challenge without significantly larger amounts in Mg-deficient rats than in controls, which was in contradiction to Malpuech-Brugere et al., who showed a significantly higher TNFα plasma value in weaning rats fed a Mg-deficient diet for 7 days than control rats following endotoxin challenge [43].

However, the effect of Mg-deficiency on TNFα expression in lungs has not been reported. In our studies, the levels of TNFα in lung extracts were not altered in Mg-deficient mice compared to control mice throughout the experiment, and were around 3 pg/mg of proteins.

We then investigated the effects of Mg-deficiency upon lung activation by the quantification of mRNAs for the most pertinent adhesins expressed by lung ECs and for the cytokines TNFα and IL-2. In mice fed 50 ppm Mg-diet, the level of VCAM-1 mRNA was transiently significantly decreased at day 14 (0.30 A.U. ± 0.11 versus 0.45 A.U. ± 0.10, in Mg-deficient and control mice, respectively, p < 0.05). E-selectin mRNA was undetectable and the levels of ICAM-1, IL-2 and TNFα mRNAs were not significantly different between the two groups (figure 2). This last result is in keeping with the similar amounts of TNFα evidenced by ELISA in the lungs from both Mg-deficient and control mice. Surprisingly, in mice fed a 150 ppm Mg-diet, the level of TNFα mRNA was transiently significantly increased at day 5 (0.039 A.U. ± 0.016 versus 0.016 A.U. ± 0.010, in Mg-deficient and control mice, respectively, p < 0.05). then ICAM-1 at day 14 (1.12 A.U. ± 0.4 versus 0.64 A.U. ± 0.26, in Mg-deficient and control mice, respectively, p < 0.05), and ultimately VCAM-1 at day 26 (1.31 A.U. ± 0.26 versus 0.88 A.U. ± 0.19, in Mg-deficient and control mice, respectively, p < 0.05) (figure 3). These results suggest that the activation of the proinflammatory cytokine TNFα in the lung is an early event, which in turn leads to lung EC activation with the successive overexpression of the adhesins ICAM-1 then VCAM-1. Hence, the absence of activation of adhesion molecule expression observed in mice fed a 50 ppm Mg-diet could tie up with the fact that no TNFα up-regulation occurred. IL-2 mRNA levels were not significantly different between Mg-deficient and control mice, and E-selectin mRNA was undetectable. Since ICAM-1 and VCAM-1 are the most important members of the immunoglobulin superfamily for EC-T cell interactions [44], the absence of T-cell activation to produce IL-2 may be the consequence of poor EC activation. Cytokines strongly influence the expression of adhesion molecules on ECs and form a complex communication network causing results which are determined by the interaction of several cytokines [45]. Therefore, it has to be taken into consideration that, apart from TNFα and IL-2, other cytokines, including cytokines secreted by ECs themselves, may be involved in the different patterns of expression of the adhesion molecules observed in lungs from mice given a 50 or 150 ppm Mg-diet.

Double-staining and immunofluorescence examination obviated no expression of I-Aβ class II molecule, E-selectin or ICAM-1 on ECs identified by the expression of von Willebrand antigen, confirming the absence of EC activation. Consequently, the absence of EC or lung cell apoptosis demonstrated by the TUNEL method was not surprising. These results are in agreement with the observation that adhesion of monocyte-like U937 cells to ECs cultured in Mg-deficient medium was increased without any relevant induction of the well characterized adhesion molecules VCAM, ICAM, E-selectin and P-selectin expression [16].

All our findings demonstrate that in our model in mice, severe Mg-deficiency does not result in an inflammatory process and remodelling in lungs, which is in contradiction to Nasulewicz et al who have recently demonstrated that severe Mg-deficiency in mice was associated with significant changes in the expression of genes and resulted in inflammatory and oxidative processes and consecutive remodelling in lung tissue [46]. By contrast, our results suggest that moderate Mg-deficiency induce an early but transient inflammatory process and EC activation, which appear to be overcome with time. However, these mice were older, from a different breeding centre and of a different strain, which may explain our particular immunological pattern.

To further confirm our findings, which appeared to be in contradiction to the literature, we identified genes differently expressed in the lungs of Mg-deficient mice after 26 days of 50 ppm Mg2+-diet, using cDNA microarrays for mRNA expression
A total of 274 genes encoding for cytokines, chemokines, growth factors and receptors were analyzed. In conditions of severe Mg deficiency (26 days of 50 ppm Mg-diet), we found that a gene coding for G-CSF-R (granulocyte colony stimulating factor receptor precursor = CD114 antigen) was markedly up-regulated (2.08 fold versus control mice, with a coefficient of variation of 4%). G-CSF-Rs are high-affinity receptors for G-CSF, which have been reported on hematopoietic cells but also on nonhematopoietic cell types, including vascular EC and cells from the lung. G-CSF is the major regulator of neutrophil production, differentiation and activation, and induces human ECs to migrate and proliferate [47]. G-CSF-R has no intrinsic kinase activity but recruits cytoplasmic tyrosine kinases and ultimately activates signal transducer and activator of transcription (STAT) proteins [48]. STAT3 has been implicated in G-CSF-mediated growth arrest preceding differentiation, while activation of STAT5 has been linked to proliferation and survival signalling. Interestingly, G-CSF was shown to induce anti-apoptotic proteins and to inhibit apoptotic death [49]. Hence, the absence of apoptosis that we observed by the

Figure 2. Quantitative expression of ICAM-1, VCAM-1, TNFα and IL-2 mRNAs by real-time RT-PCR in lungs from mice fed either the control or 50 ppm Mg diet for 5, 14 and 26 days. Results are expressed as arbitrary units (A.U.). Data are mean ± SEM of 6 mice. *p < 0.05 versus control diet.
TUNEL method may be related to G-CSF-R up-regulation.

A gene coding for the chemokine CCL4 (macrophage inflammatory protein 1-beta = MIP-1β) produced by the hematopoietic cells was also found to be up-regulated (2.26 fold) but with a coefficient of variation of 34%. CCL4, like CCL3 (macrophage inflammatory protein 1-alpha = MIP-1α) is a monocyte chemoattractant and coactivator. Additionally, the MIP-1 proteins have chemoattractant and adhesive effects on lymphocytes. They orchestrate acute and chronic inflammatory host response at sites of injury or infection mainly by recruiting proinflammatory cells. During allograft rejection, early CCL4 intragraft expression (1.5-48 h after cardiac transplant) was found to be T cell dependent and increased significantly with time after transplantation [50]. MIP-1 proteins mediate their biological effects by bonding to cell surface C'C chemokine receptors, namely CCR5 for CCL4, which belong to

Figure 3. Quantitative expression of ICAM-1, VCAM-1, TNFα and IL-2 mRNAs by RT-PCR in lungs from mice fed either the control or 150 ppm Mg diet for 5, 14 and 26 days. Results are expressed as arbitrary units (A.U.). Data are mean ± SEM of 6 mice. *p < 0.05 versus control diet.
the G-protein-coupled receptor superfamily. However, the exact downstream events induced by CCL3 and CCL4 remain to be characterized [51].

By contrast, a gene coding for the osteopontin precursor was found to be down-regulated by severe Mg-deficiency (0.45 fold with a coefficient of variation of 4%). Osteopontin (OPN) is one of the major extracellular proteins called matricellular proteins (MCP) involved in cell to matrix communication and in several inflammatory diseases by regulating inflammatory cell accumulation and function at sites of inflammation and repair, through inhibition of nitric oxide (NO) expression [52]. It is also a known chemoattractant for macrophages and neutrophils. OPN acts as a survival, cell-adhesive, and chemotactic factor for ECs, induces EC migration and up-regulates EC migration induced by vascular endothelial growth factor (VEGF), protects ECs from apoptosis via NF-κB activation [53, 54]. OPN acts as a cytokine that plays important roles in monocyte/macrophage functions. It was shown to be strongly expressed on alveolar macrophages in inflammatory lung situations like acute respiratory distress syndrome, where it might play a protective role against excessive production of NO [55]. It is interesting to point out that, like OPN in our model, IL-1β and TNFα following endotoxin challenge from rat alveolar macrophages cultured in low-Mg2+ medium [16].

Conclusions

In our model of severe hypomagnesemia in mice, the balance between the up-regulation of G-CSF-R and CCL4 genes, and the down-regulation of OPN gene might be responsible for the absence of development of an inflammatory response, lung EC activation, and lung remodelling, despite an enhanced mRNA expression of chemoattractant factors for neutrophils and macrophages. However, we can hypothesize that severe Mg-deficiency induces a latent inflammatory status of lungs, which might be revealed following stresses, particularly immune stresses. We are currently investigating this hypothesis in our experimental model of isolated mouse lung perfused in allogeneic conditions, which mimics lung transplantation conditions.

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