Effect of Mg-deficiency on endothelial cell allogeneic activation in a model of isolated perfused mouse lung

F. Sabbagh¹, F. Lecerf¹, P. Maurois², P. Bac¹,2, M. German-Fattal¹,2

¹ CNRS UMR 8078, IPSC, Université Paris-Sud 11, Centre Chirurgical Marie-Lannelongue, 133, avenue de la Résistance, 92350 Le Plessis Robinson, France; ² Faculté de Pharmacie, Université Paris-Sud 11, 92296 Châtenay-Malabry Cedex, France

Correspondence: M. German-Fattal, CNRS UMR 8078, Université Paris-Sud 11, Centre Chirurgical Marie-Lannelongue, 133, avenue de la Résistance, 92350 Le Plessis Robinson, France. Tel.: (+00 33) 1 46 83 57 33. <michele.german@ccml.u-psud.fr>

Abstract. Following vascularized organ allotransplantation, an early intragraft inflammatory process is initiated by adhesion molecule-ligand interaction between recipient blood leukocytes and graft endothelial cells (EC). We have previously shown that chronic hypomagnesemia did not induce any inflammatory process in the lung, hence neither EC activation, nor lung remodelling. In the present study we have investigated the effects of allogeneic blood perfusion on lungs from magnesium-deficient mice in our experimental model of isolated mouse lung. After 3h of isogeneic or allogeneic perfusion, no inflammatory process was detected by histochemical examination of lung tissue; the mRNA levels of the adhesion molecules E-selectin, ICAM-1 and VCAM-1, and of the pro-inflammatory cytokines TNF-α and IL-2 in lung tissue, determined by reverse transcriptase-polymerase chain reaction (RT-PCR), were similar, and the expression of E-selectin and I-Aβ antigen on EC by immunohistochemical staining was undetectable. All of these markers were shown to be dramatically increased after allogeneic perfusion of lung from magnesium-non deficient mice. Our results clearly show that allogeneic perfusion of lungs from magnesium-deficient mice cannot induce EC activation or lung inflammation, indicating that hypomagnesemia in donors does not constitute an additional risk for allograft outcome and might allow to lighten the recipient’s immunosuppressive treatment.

Keywords: hypomagnesemia, isolated mouse lung, adherence molecules

Following vascularized organ allotransplantation, for example lung allotransplantation, an early intragraft inflammatory process is initiated by adhesion molecule-ligand interaction between recipient blood leukocytes and graft endothelial cells (EC), which represent the first barrier between the allograft and the host immune cells. The numerous injuries and stresses resulting from the different steps of lung transplantation, among which ischemia-reperfusion (I/R) injury, induce lung remodelling through inflammation and apoptosis, and ultimately organ damage/failure [1, 2]. The allogeneic activation of EC is characterized by the early expression of major histocompatibility complex (MHC) class II antigens, adhesion molecules, chemokines, and inflammatory cytokines [1, 3-8]. However, ECs are differentially regulated in space and time, according to the vessel-type, the organ and the microenvironment [9-11]. During inflammation, leukocytes tether to and roll on the EC surface. The cells then are arrested, spread and finally emigrate between ECs to reach the underlying tissue [12]. The inflammatory cytokines tumor-necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and interferon-gamma (IFN-γ) stimulate the expres-
sion of a series of adhesion molecules. IL-2, which is produced by stimulated T helper type 1 (TH1) cells, plays a pivotal role in the initiation of an immune response leading to the induction of lymphocyte proliferation [13]. In a model of isolated mouse lung perfused with fresh blood, which mimics lung transplantation conditions, we have shown that the adhesion molecules E-selectin, VCAM-1, ICAM-1 and LFA-3, and the cytokines TNF-α and lymphotoxin-α were up-regulated in lung tissue after 3 hours of continuous allogeneic perfusion [14, 15]. EC allogeneic activation led to lung apoptosis, which involved Fas ligand (FasL) and an increase of phosphorylase phosphatase activity [16].

Several experimental and clinical studies have suggested that the lungs might be a specific target of magnesium (Mg)-deficiency [17-20]. However, we have previously shown that chronic hypomagnesemia did not induce any inflammatory process in the lung, hence neither EC activation, nor lung remodelling [21]. Therefore, we hypothesized that severe Mg-deficiency might be responsible for a latent inflammatory status of lungs, which might be revealed following stresses, particularly allogeneic activation as seen in lung allotransplantation.

To test this hypothesis, in the present study we have investigated the effects of allogeneic blood perfusion on lungs from Mg-deficient mice in our experimental model of isolated mouse lung without ischemia. Lung alloactivation and inflammation was assessed by the expression of the adhesion molecules E-selectin, ICAM-1 and VCAM-1, and of the cytokines TNF-α and IL-2 in lung tissue at the end of the three-hour perfusion period. We also explored lung remodelling by apoptosis detection.

**Materials and methods**

**Animals and diet**

Female C57BL/6NHsd (H-2b, Ms<sup>+</sup>) mice, 6 week-old (Harlan, Gannat, France), were given either standard Mg-containing diet (control diet, 1400 ppm Mg<sup>2+</sup>, Harlan Teklad 2016 batch n° M013) and tap water for drinking (≤ 4 ppm Mg<sup>2+</sup>), or synthetic Mg-deficient diet (50 ppm Mg<sup>2+</sup>) prepared in the laboratory and deionized water to avoid consumption of Mg from normal drinking water, ad libitum for 26 days. Mice were maintained at +22°C. All experiments were performed according to the recommendations of the INRA Ethics Committee (decree no. 87-848).

Female C3H/He (H-2k, Ms<sup>+</sup>) 6 week-old mice (Harlan, Gannat, France), fed standard Mg-containing food and tap water for drinking, were used as the H-2 allogeneic strain.

**Experimental model of isolated, ventilated and perfused mouse lung**

The experimental model of isolated, ventilated mouse lung perfused in a recirculatory manner with fresh mouse blood at a constant flow of 1 mL/min and at a steady pressure of 10 mmHg was previously described and validated [14]. The heart-lung block was taken out from C57BL/6 mice fed Mg-deficient diet for 26 days, after anaesthesia with averitin (250 mg/kg, intraperitoneally). The isolated mouse lung can be stably perfused with fresh mouse blood for periods of over 3 hours without clinical or histological signs of edema.

This model was used under the two following conditions: 1) isogeneic, i.e., lung from C57BL/6 mouse perfused with fresh whole blood from C57BL/6 mouse as a control for the experimental model, and 2) allogeneic, i.e., lung from C57BL/6 mouse perfused with fresh whole blood from C3H/He mouse (6 experiments for each condition).

In the two conditions, blood for perfusion was taken from mice fed a normal diet after injection of heparin (25,000 IU/kg) as anticoagulant protocol.

At the end of the 3 hours of perfusion, the lungs were washed with 3 mL of phosphate-buffered saline at a rate of 5 mL/min, then divided into two parts: the first part was snap frozen in liquid nitrogen then stored at -80°C until use for histo- and immunohistochemical examinations on tissue sections; the second part was placed into a denaturing solution for immediate total RNA extraction.

**Blood determinations**

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

**Histological and immunocytochemical studies**

All histological and immunocytochemical observations were carried out on 7 μm cryostat sections fixed with acetone for 10 min.

Sections were stained with hematoxylin-eosin (Harris haematoxylin, 10 sec; eosin 225 for histology, 10 sec) to assess histologic changes.

The expression of I-A<sup>+</sup>, E-selectin, and ICAM-1 was analyzed on endothelial cells to assess their activation. Moreover, CD45-positive cells infiltrating lung tissue were explored to assess an inflammatory process. Endothelial cells were identified by the expres-
mRNA quantification in lung tissue by real time RT-PCR

Total RNA extraction from fresh lung, reverse transcription and quantitative PCR using the SYBR Green LightCycler™ method (Roche Diagnostics, Meylan, France) were performed as previously described [14]. The primers for E-selectin, VCAM-1, ICAM-1, TNF-α and IL-2 cDNA amplification were selected by using Oligo-4 software (Med Probe, Oslo, Norway) to limit self-complementarity and to optimize sequence specificity [14, 15]. 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 the GAPDH housekeeping gene. For length and specificity verification, amplimers were separated by electrophoresis onto a 1.5% agarose gel.

Statistical analysis

In our experimental model of isolated ventilated lung, EC is the interface between perfusion blood and lung tissue. This physiologic mouse model without ischemia mimics the clinical heart-lung transplantation setting, since the isolated lung and the blood for lung perfusion represent the graft and the recipient’s blood, respectively. The perfusion pressure was steady during 3 to 5 hours. Hence, to ensure the reproducibility of the model, all determinations were performed at 3 hours of perfusion. In this model, the isogenic perfusion of lung reflects the consequences of both surgical and perfusion stress, and defines the basal status to which the effects of allogeneic perfusion were compared.

All the C57BL/6 and C3H/HE mice fed Mg-containing diet exhibited normal magnesiumemia. Mice fed Mg-deficient diet for 26 days exhibited a dramatic decrease in Mg concentration in plasma compared to control-diet mice (0.35 ± 0.04 mM versus 1.09 ± 0.04 mM, p < 0.001). These determinations confirmed that isogenic or allogeneic blood for lung perfusion contained a normal concentration of Mg, whereas perfused lungs were really taken from mice with hypomagnesemia.

In the present study, 3 hours of allogeneic lung perfusion induced no inflammatory process in lungs as demonstrated by the absence of leukocyte infiltrates, pneumonia, edema, or disseminated intravascular coagulation in histological examination. We have previously shown that lungs from Mg-deficient mice exhibited no inflammatory process after 26 days of 50 ppm-Mg diet, which was in contradiction to the recent report from Nasulewicz et al. [22]. Since in our experiments: 1) Mg-deficiency by itself does not result in lung inflammatory process and 2) allogeneic activation of lungs from Mg-deficient mice is not capable of inducing any inflammatory response, we suggest either that lungs from Mg-deficient mice are not in a latent inflammatory status, or that three hours of allogeneic activation is not a period of time sufficient for its expression.
To assess lung-EC activation induced by 3 hours of allogeneic perfusion, we first quantified the mRNAs for the adhesion molecules E-selectin, ICAM-1 and VCAM-1 in lung tissues. We have previously demonstrated that the expression of these molecules was unchanged in lungs from Mg-deficient mice [21], whereas they were early up-regulated on lung ECs from Mg-sufficient mice by allogeneic activation [14]. Surprisingly, the mRNA levels were not significantly modified after isogeneic or allogeneic perfusion for the three selectins (figure 1): 3.5 ± 0.87 versus 3.75 ± 0.85 A.U. (not significant, n.s.), in isogeneic and allogeneic conditions, respectively, for E-selectin; 14.7 ± 2.9 versus 9.2 ± 2.4 A.U. (n.s.), in isogeneic and allogeneic conditions, respectively, for ICAM-1; 2.1 ± 0.94 versus 1.96 ± 0.30 A.U. (n.s.), in isogeneic and allogeneic conditions, respectively, for VCAM-1. By contrast, the allogeneic perfusion of lungs from Mg-non deficient mice in the same conditions induced an early persistent dramatic increase in the mRNAs for these adhesion molecules in lung tissue: 18.3-fold, 2.2-fold and 2.2-fold increase compared to isogeneic perfusion, for E-selectin, ICAM-1 and VCAM-1, respectively [14].

We also quantified the mRNAs for the most important pro-inflammatory cytokine TNF-α and for IL-2, which results from T-cell activation, since these two cytokines have been demonstrated to be dramatically up-regulated in allograft [13, 23]. Neither TNF-α nor IL-2 mRNA levels were previously found to be altered in lung from Mg-deficient mice [21]. As expected from the absence of EC activation, the levels of mRNAs for these two cytokines were not altered after 3 hours of allogeneic perfusion: 0.69 ± 0.12 versus 0.65 ± 0.33 A.U. (n.s.), in isogeneic and allogeneic conditions, respectively, for TNF-α; 0.33 ± 0.15 versus 0.83 ± 0.47 A.U. (n.s.), in isogeneic and allogeneic conditions, respectively, for IL-2 (figure 1). By contrast, we have previously shown that the allogeneic perfusion of lungs from Mg-non deficient mice in the same conditions induced an early dramatic increase in the mRNAs for TNF-α in

Figure 1. Quantitative expression of E-selectin, ICAM-1, VCAM-1, IL-2 and TNF-α mRNAs by real-time RT-PCR in lungs from magnesium-deficient mice, after 3 hours of perfusion either with isogeneic or with allogeneic fresh whole blood. Results are expressed as arbitrary units (A.U.). Data are mean ± SEM of 6 mice.
lung tissue (4-fold increase compared to isogeneic perfusion), and a transient 1.5-fold increase of IL-2 mRNA level at 1 hour of perfusion [14].

We further confirmed this unexpected absence of lung EC allogeneic activation by immunohistochemical examination of lung slices after 3 hours of perfusion. The expression of I-A\(^d\) and E-selectin, which are the most pertinent markers for EC activation, was undetectable both after isogeneic and allogeneic perfusion. By contrast, we have previously reported that the expression of these two markers was dramatically increased on ECs from Mg-non deficient mice after 3 hours of allogeneic perfusion: 23-fold and 62.5 fold increase, for E-selectin and I-A\(^d\) antigen, respectively [16].

As a consequence of the lack of lung EC activation and inflammatory process, no apoptotic cell could be detected in lungs from Mg-deficient mice by the TUNEL method, before or after 3 hours of isogeneic or allogeneic perfusion, indicating the absence of tissue damage and remodelling. By contrast, EC activation and TNF-\(\alpha\) up-regulation after 3 hours of allogeneic perfusion of lungs from Mg-non deficient mice induced lung tissue apoptosis, since the number of lung apoptic cells was significantly increased: 3.2-fold increase compared to isogeneic perfusion [16].

These results clearly demonstrate that in our model of isolated, ventilated mouse lung perfused with fresh blood, allogeneic perfusion of lungs from Mg-deficient mice cannot induce EC activation or lung inflammation. In lungs from Mg-non deficient mice, interactions between allogeneic blood leukocytes and ECs induce an early dramatic up-regulation of the selectins, which initiate and mediate tethering and rolling of leukocytes on the EC surface, corresponding to reversible adhesion of leukocytes to ECs. Thereafter, the ligands ICAM-1, VCAM-1 and LFA-3 for leukocyte integrins on ECs are overexpressed [15]. Induction of adhesion molecules on EC is mediated by IL-1 and TNF-\(\alpha\) [4, 5, 24]. This sequence of events occurs within a few hours in the inflammatory process and, in the case of vascularized organ allotransplantation, leads to tissue damage in the graft and ultimately, in the absence of immunosuppressive regimen, to the graft loss [25, 26].

Since we have demonstrated that Mg-deficiency by itself does not induce adhesion molecules, TNF-\(\alpha\) or IL-2 up-regulation in the lung, we hypothesized that Mg-deficiency may lead to a latent inflammatory status of the lung, which would probably be revealed by a further stress. Therefore, we used lungs from Mg-deficient mice in our physiological model of iso-

References


