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Low magnesium promotes endothelial cell dysfunction: implications for atherosclerosis, inflammation and thrombosis

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Abstract

Because (i) endothelial cells are important players in cardiovascular diseases and (ii) Mg deficiency promotes atherosclerosis, thrombosis and hypertension, we evaluated whether low concentrations of Mg could directly affect endothelial behavior. We found that low Mg concentrations reversibly inhibit endothelial proliferation, and this event correlates with a marked down-regulation of the levels of CDC25B. The inhibition of endothelial proliferation is due to an up-regulation of interleukin-1 (IL-1), since an antisense oligonucleotide against IL-1 could prevent the growth inhibition observed in cells exposed to low concentrations of the cation. We also report the up-regulation of Vascular Cell Adhesion Molecule-1 (VCAM) and Plasminogen Activator Inhibitor (PAI)-1 after Mg deficiency. VCAM is responsible, at least in part, of the increased adhesion of monocytoid U937 cells to the endothelial cells grown in low magnesium. In addition, endothelial migratory response is severely impaired. By cDNA array, we identified several transcripts modulated by exposure to low Mg, some of which—c-src, ezrin, CD9, cytohesin and zyxin—contribute to endothelial adhesion to substrates and migration.

In conclusion, our results demonstrate a direct role of low magnesium in promoting endothelial dysfunction by generating a pro-inflammatory, pro-thrombotic and pro-atherogenic environment that could play a role in the pathogenesis cardiovascular disease.
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Keywords: Endothelial cell; Magnesium; Atherosclerosis; Gene expression; Inflammation

1. Introduction

Endothelial cells maintain the functional integrity of the vascular wall. Beyond their role in controlling permeability, they are involved in the maintenance of a non-thrombogenic blood–tissue interface, in the modulation of blood flow and vascular resistance, in the regulation of immune and inflammatory reactions [1]. It is well accepted that endothelial dysfunction plays an important pathogenic role in several diseases, among which atherosclerosis [2], hypertension [3], diabetes [1] and thrombosis [1]. In particular, a huge amount of experimental evidence supports the paradigm of endothelial dysfunction as the common link between risk factors and atherosclerotic burden [2]. Indeed, endothelial dysfunction actively participates in the process of lesion formation by promoting leukocyte adherence, increasing chemokine secretion and cell permeability to lipids, enhancing LDL oxidation, stimulating vascular smooth muscle cell proliferation and migration and platelet activation [2].

In addition, endothelial cells are protagonists in angiogenesis, i.e. the branching and sprouting of capillaries from preexisting blood vessels, which is a tightly controlled event crucial in development, in reproduction and wound healing [4].

Because of their strategical location at the interface between blood and vessels, endothelial cells are readily exposed to various signals (cytokines, metabolites, ions, free radicals, shear stress), some of which may promote maladaptative functional changes. Among others, low magnesium (Mg) status has been reported to be important in the pathogenesis of cardiovascular diseases [5]. Interestingly, low magnesium status, which is frequent in western countries, is a common denominator in hypertension [6], coronary artery disease [7], thrombosis [8] and diabetes [9]. During experimental Mg deficiency hyperlipemia, inflammation and early atherosclerotic lesions have been observed

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[10]. Indeed, Mg fortification of drinking water succeeded in the inhibition of atherogenesis in APO-E-deficient mice fed with a high cholesterol diet [11]. Oral Mg therapy has been associated with significant improvement of endothelial function in patients with coronary artery disease [12] and with a decrease of plasma concentrations of triglycerides, VLDL and apo-B [13]. Mg deprivation also reduces tumor growth through unclear mechanisms [14]. Moreover, Mg is important in wound healing, since elevation in the concentration of Mg has been detected in early wound fluid, when cell migration into the wound is initiated [15].

Since Mg deficiency promotes atherosclerosis, thrombosis and hypertension, and inhibits tumor growth, all processes which recognize the endothelial cell as a crucial player, we have evaluated the effects of low concentrations of Mg on cultured endothelial cells.

2. Materials and methods

2.1. Cell culture, proliferation and migration

HUVEC were cultured in M199 containing 10% fetal calf serum, Endothelial Cell Growth Supplement (ECGS) (150 μg/ml) and heparin (5 U/ml) on 2% gelatin-coated dishes. The cells were routinely evaluated for the expression

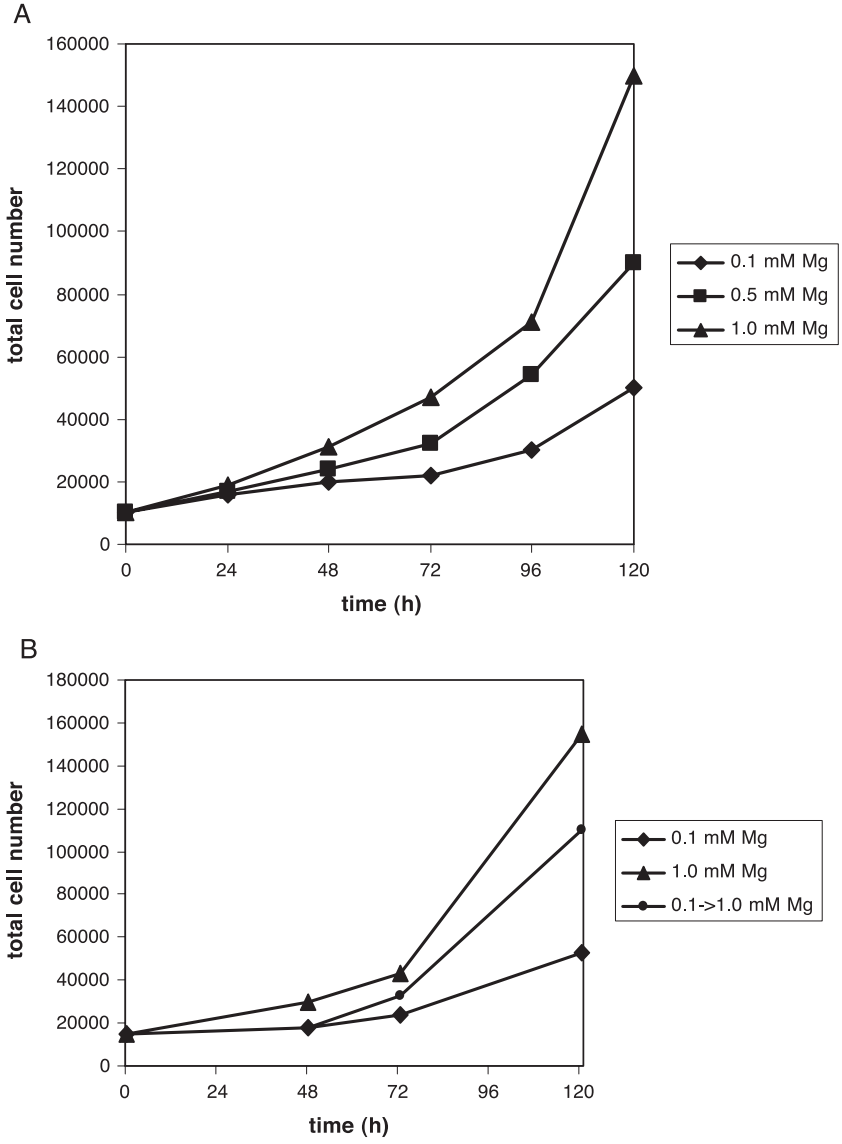

Fig. 1. Inhibition of endothelial proliferation by low magnesium. (A) HUVEC were cultured for different times in media containing 0.1, 0.5 or 1.0 mM Mg. Every 24 h, the cells were harvested by digestion with trypsin and viable cells counted using a Burker chamber. The figure shows a representative experiment in triplicate (out of five performed). Standard deviation never exceeded ± 4500 cells. (B) HUVEC were cultured in medium containing 0.1 mM Mg. Forty-eight hours later, the medium was supplemented with Mg to 1.0 mM concentration. Cells were counted as described after 72 and 120 h from the beginning of the experiments, which were performed in triplicate. Standard deviation never exceeded ± 3700 cells. The figure shows a representative experiment (out of three performed).

of endothelial markers, i.e. endothelial nitric oxide synthase, VE-cadherin and CD34, and utilized for five to six passages. All culture reagents were from Gibco. A magnesium-free medium was purchased by Bio Media (Boussens, France) and utilized to vary the concentrations of magnesium by the addition of $MgSO_4$. No significant difference was observed when we used $MgSO_4$ or $MgCl_2$ to add magnesium in the culture media (not shown). In all the experiments the cells were seeded in growth medium; after 24 h, the medium was changed to expose the cells to various concentrations of Mg. Proliferation assays were performed on HUVEC at low density (7500/cm^2), cultured in growth medium containing

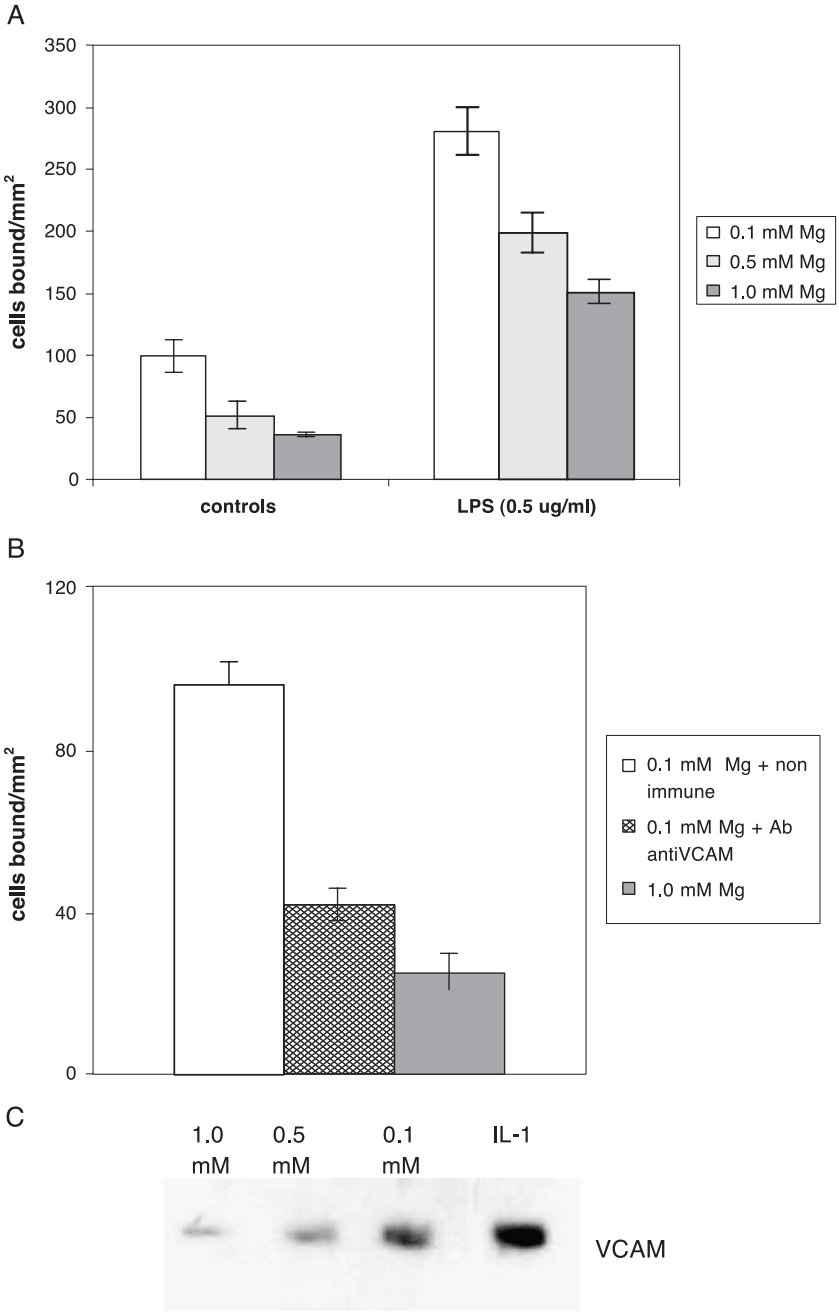

Fig. 2. Induction of HUVEC/U937 interactions by low magnesium concentrations. (A) Confluent HUVEC were cultured in 0.1, 0.5 or 1.0 mM Mg for 3 days and exposed or not to LPS (0.5 μg/ml). After 4 h, U937 cells were added. One hour later, the nonadherent U937 cells were rinsed off and the number of attached U937 cells was counted in 20 microscopic fields defined by an eyepiece. The figure shows a representative experiment in triplicate (out of six performed). (B) Adhesion assay as described above was performed in the presence of an anti-VCAM antibody (15 μg/ml). Control cells in 0.1 and 1.0 mM Mg were added with the same concentration of nonimmune rabbit IgG. The experiment was performed in triplicate. Data refer to means ± standard error. The figure shows a representative experiment (out of three performed). (C) Western blot was performed on HUVEC cultured in different concentrations of Mg for 3 days using an anti-VCAM antibody as described in Materials and methods. Densitometric analysis indicated that VCAM is threefold induced in cells cultured in 0.1 mM Mg for 3 days. Exposure to IL-1 (20 ng/ml) for 8 h was considered a positive control and determined a fivefold induction.

different concentrations of Mg. At various time points, the cells were trypsinized, stained with trypan blue solution (0.4%) and the viable cells were counted using a Burker chamber. In some experiments, HUVEC were incubated in the presence of an interleukin 1α (IL-1) antisense oligomer (50 μM) designed to recognize nine nucleotides upstream and nine downstream from the translation initiation codon [16]. Since this oligonucleotide proved to efficiently inhibit IL-1α translation [16], this is the only antisense oligomer we utilized in our study. The antisense oligomer does not modulate the levels of IL-1β [16]. As controls, we used the same concentrations of the corresponding sense oligomer. All the experiments have been repeated at least three times, always in triplicate.

Migration of HUVEC cultured in the presence of different concentrations of Mg was determined using an in vitro model of wound repair as previously described [17]. Briefly, confluent endothelial cells were wounded and treated with Hepatocyte Growth Factor (HGF) (20 ng/ml) for 18 h. The number of cells migrating from the wound origin was counted with a light microscope at 100× magnification using a grid. The values represent the mean ± standard deviation of five fields, each condition tested in triplicate.

The monocytoid cell line U937 was cultured in RPMI medium containing 10% FCS.

2.2. Adhesion assay

Confluent HUVEC were cultured in 24-well plates in medium containing different concentrations of Mg for different times. In some cases, HUVEC were incubated in the presence of IgGs against Vascular Cell Adhesion Molecule-1 (VCAM) or nonimmune IgGs (Santa Cruz). In other experiments, the cells were treated with lipopolysaccharide (LPS; 0.5 μg/ml). At the end of the incubation, the wells were rinsed three times and 2×10^5 U937 cells were added to each well. After 1 h, the nonadherent U937 cells were rinsed off and the wells were fixed with 1% glutaraldehyde. The number of attached U937 cells was counted in 20 microscopic fields defined by an eyepiece. The experiments were performed at least three times in triplicate [18].

2.3. Western blot

HUVEC were lysed in 10 mM Tris–HCl (pH 7.4) containing 3 mM $MgCl_2$, 10 mM NaCl, 0,1% SDS, 0,1% Triton X-100, 0,5 mM EDTA and protein inhibitors, separated on 10% SDS-PAGE and transferred to nitrocellulose sheets. Western blot analysis was performed using antibodies against Plasminogen Activator Inhibitor (PAI)-1, src, CDC25B or VCAM (Santa Cruz). Secondary antibodies were labeled with horseradish peroxidase (Amersham Pharmacia Biotech). The SuperSignal chemiluminescence kit (Pierce) was used to detect immunoreactive proteins according to the manufacturer's instruction. All the Western blots have been repeated at least three times on cell extracts from different experiments.

2.4. IL-1α synthesis

HUVEC were cultured in media containing different concentrations of Mg for different times. In another set of experiments, the cells were cultured in 0.1 and 1.0 mM Mg with an antisense oligomer against IL-1 and the corresponding sense oligonucleotide. IL-1 concentration in cell extracts was detected using Quantikine human IL-1 immunoassay according to the manufacturers' instructions (R&D Systems) [16]. The experiment has been repeated four times in triplicate.

2.5. cDNA array

The RNA utilized in these experiments was extracted by the cesium chloride method [18] from HUVEC cultured in 0.1 and 1.0 mM Mg for 3 days. Human cDNA expression array membranes consisting of 588 known genes (Atlas™ Human Cancer Array, Clontech, Palo Alto, CA, USA) were used as described by the manufacturer. The hybridization data were collected with PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA). The AtlasImage 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. Genes with a ratio of 2.0 or above were considered regulated.

3. Results

3.1. Inhibition of endothelial proliferation by low Mg

HUVEC were grown in the presence of different concentrations of extracellular Mg for various times. The considered physiological concentration of the cation is 1.0 mM Mg. In human, the lowest level of Mg in critically ill subjects was 0.5 mM [19] and concentrations ranging between 0.7 and 0.8 mM are frequent in apparently healthy

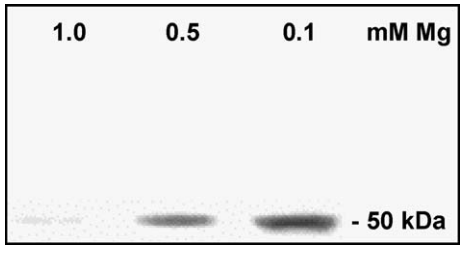

Fig. 3. Induction of PAI-1 expression by low magnesium concentrations. Western blot was performed on HUVEC cultured in different concentrations of Mg (1.0, 0.5 and 0.1 mM Mg) for 3 days as described in Materials and methods. By densitometric analysis, PAI-1 is increased by 2- and 3.8-fold in cells grown for 3 days in 0.5 and 0.1 mM Mg, respectively.

western people [20]. In rodents, after 8 days of experimental diet, a decrease in plasma Mg to a 0.14 mM can be detected [21,22]. Based on these findings, we utilized the following concentrations of Mg in the culture media: 0.1, 0.5 and 1.0 mM. In agreement with results obtained in other cell types [14], low Mg inhibited endothelial proliferation in a dose- and time-dependent manner (Fig. 1A). This inhibition was reversible: after 48 h in 0.1 mM Mg-containing medium, the concentration of Mg was restored to 1.0 mM and HUVEC rapidly began to grow as fast as the controls (Fig. 1B).

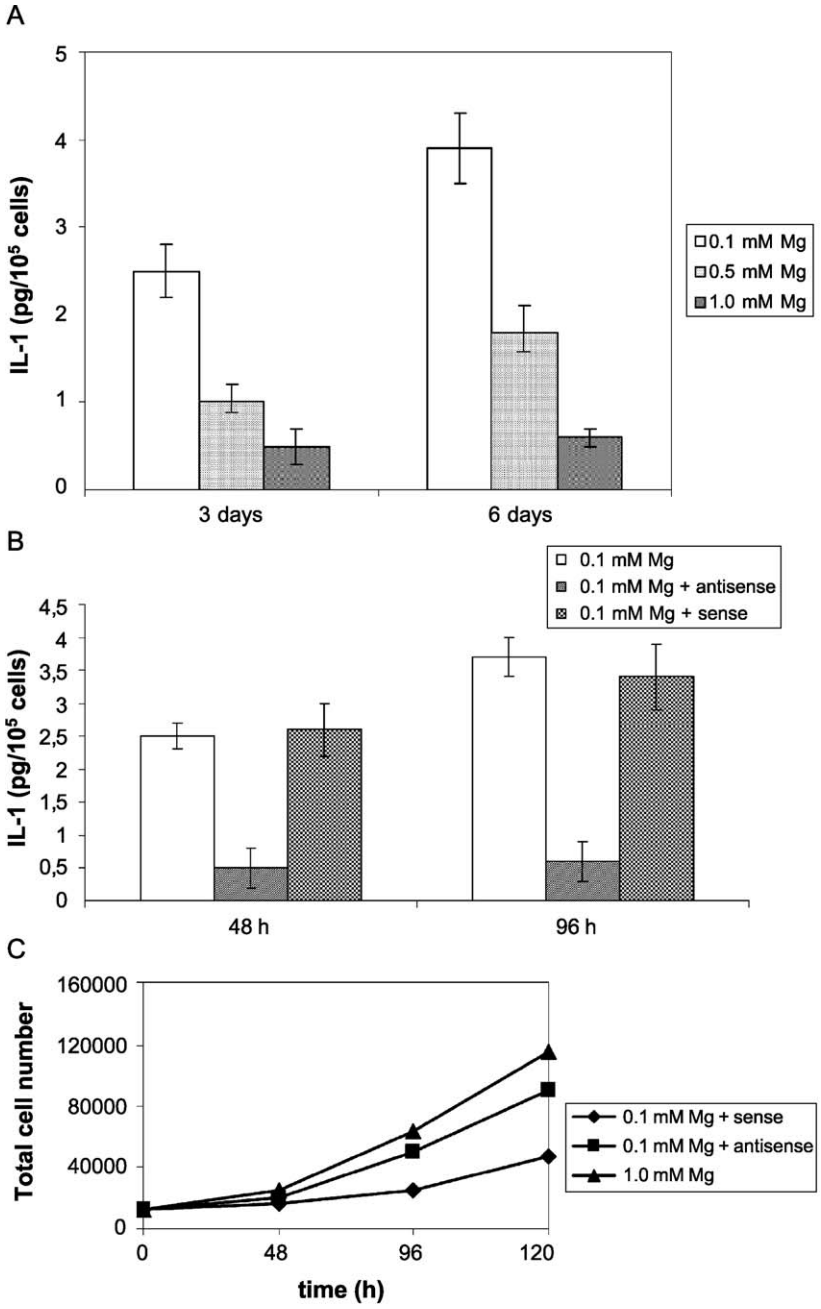

Fig. 4. IL-1 synthesis in low Mg and its role in modulating endothelial proliferation. (A) The cells were cultured in the presence of different concentrations of Mg. Cell extracts were utilized to measure the levels of IL-1 as described in Materials and methods. Data are expressed as the means ± standard error. The figure shows the results of a representative experiment (out of three performed). (B) HUVEC were cultured in 0.1 mM Mg in the presence of an antisense oligomer against IL-1 or its control sense oligonucleotide. Cell extracts were utilized to measure the levels of IL-1 as above. The experiment was repeated three times, with similar results. (C) HUVEC were grown in medium containing 0.1 mM Mg in the presence of an anti-IL-1 antisense (0.1 mM Mg + antisense) or its corresponding sense oligomer (0.1 mM Mg + sense). Every 48 h, cells were trypsinized and counted with a haemocytometer. Cells grown in 1.0 mM Mg were used as a control. The experiment was performed in triplicate. Standard deviation never exceeded ± 4500 cells. The figure shows a representative experiment (out of three performed).

3.2. Modulation of endothelial adhesiveness and thrombotic properties in HUVEC cultured in low Mg

Since one of the earliest events in the formation of the atherosclerotic plaque is the adhesion of monocytes to the endothelial lining, we evaluated the interaction of the monocytoid U937 cells to HUVEC cultured in low Mg for 3 days and found an increase in endothelial-U937 cell interactions (Fig. 2A). This increased adhesion is due, at least in part, to an up-regulation of VCAM. Indeed, when we incubated the cells in the presence of 0.1 mM Mg and an antibody against VCAM, we detected a significant reduction of the adhesion between U937 cells and HUVEC (Fig. 2B), whereas no effect was exerted by the antibody on cells cultured in 1.0 mM Mg (not shown). Accordingly, 2.9-fold increase of VCAM mRNA was detected by quantitative RT-PCR (not shown) and confirmed by Western blot (Fig. 2C). Densitometric analysis indicated that VCAM is threefold induced in cells cultured in 0.1 mM Mg for 3 days, while exposure to IL-1 (20 ng/ml) for 8 h determines a fivefold induction. No modulation of E-selectin and ICAM-1 was found both by RT-PCR and Western blot in cells grown in low Mg (not shown).

Bacterial LPS is a potent inducer of endothelial adhesiveness. When cultured in low concentrations of Mg for 3 days, endothelial response to LPS (0.5 μg/ml) is markedly increased in a dose-dependent fashion (Fig. 2A).

One of endothelial functions is the maintenance of non-thrombogenic blood–tissue interface [1]. We evaluated the expression of PAI-1, which is increased in a variety of clinical conditions such as coronary artery disease and venous thrombosis [23]. Fig. 3 shows higher amounts of PAI-1 (Mr 50 kDa) in HUVEC grown in low Mg-containing medium than in controls as detected by Western blot analysis. In particular, by densitometric analysis, PAI-1 is increased by 2- and 3.8-fold in cells grown for 3 days in 0.5 and 0.1 mM Mg, respectively.

3.3. Enhancement of IL-1 synthesis in endothelial cells cultured in low Mg

Elevated levels of circulating cytokines, among which IL-1, have been detected in rats fed with low Mg diet [10]. IL-1 profoundly affects endothelial behavior and is a potent inhibitor of endothelial cell growth [16,24]. We therefore measured the synthesis of IL-1 in HUVEC grown in media containing different concentrations of Mg for 3 and 6 days by ELISA. We found that cells cultured in low Mg produce higher amounts of the cytokine than controls and this event was dose-dependent (Fig. 4A).

Since IL-1 is an antagonist of endothelial proliferation, we anticipated that a link could exist between the increased levels of IL-1 and the growth inhibition in HUVEC cultured in low Mg. Therefore the cells were incubated in a medium containing 0.1 mM Mg in the presence of an antisense oligomer against IL-1, previously shown to be successful in repressing IL-1 translation [16]. As demonstrated in Fig. 4B, the antisense oligomer, but not the control sense oligonucleotide, markedly reduced IL-1 levels in HUVEC cultured in 0.1 mM Mg. Fig. 4C shows that the antisense oligomer prevented the growth inhibition of HUVEC grown in Mg-deficient medium, while the sense oligomer added to the medium containing 0.1 mM Mg did not exert any effect on cell proliferation.

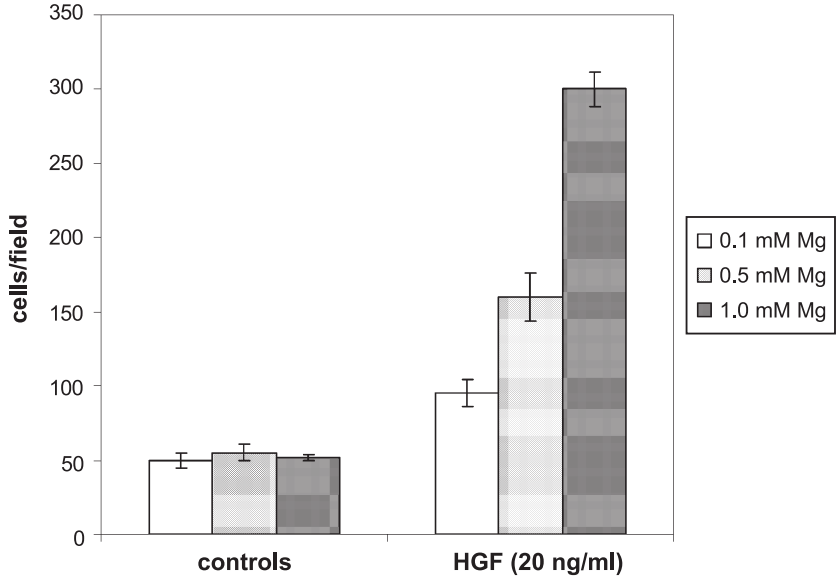

Fig. 5. Impairment of endothelial migration by low magnesium. After 3 days culture in low Mg containing medium, confluent HUVEC were wounded and treated with HGF (20 ng/ml) for 24 h. The number of cells migrating from the wound origin was counted with a light microscope at 100 × magnification using a grid. The values represent the mean ± standard deviation of five fields, each condition tested in triplicate. The figure shows a representative experiment (out of six performed).

3.4. Inhibition of endothelial migration in HUVEC cultured in low Mg

In several pathologic and pathophysiologic conditions as well as in development, angiogenic factors stimulate endothelial cells to migrate and then to proliferate [4]. The response to HGF, an angiogenic factor, was then evaluated. HUVEC were cultured in medium containing low concentrations of Mg for 3 days before the addition of HGF (20 ng/ml) for additional 24 h. Fig. 5 shows that low Mg concentrations impaired endothelial migration in a dose-dependent manner. Similar results were obtained with Fibroblast Growth Factor (FGF)-1 and -2 (not shown).

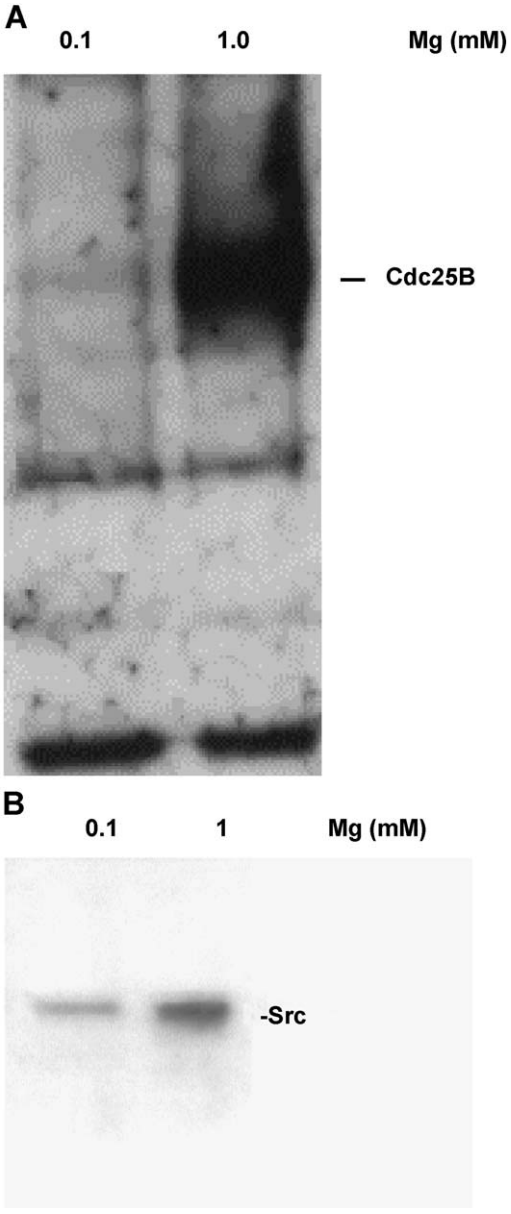

Fig. 6. Down-regulation of CDC25B and c-src by low magnesium. Cell extracts from HUVEC cultured in 0.1 or 1.0 mM Mg were separated on a 10% SDS-PAGE, transferred to nitrocellulose and Western blot was performed using (A) anti-CDC25B or (B) anti-c-src antibodies and visualized by chemiluminescence as described. By densitometric analysis, CDC25B is increased by ninefold in cells grown for 3 days in 0.1 mM Mg, while c-src is threefold induced.

3.5. Modulation of gene expression in HUVEC cultured in low Mg

In an effort to gain more insights into the response of endothelial cells to low concentrations of Mg, we used cDNA arrays to survey the transcriptional activity of a large number of genes in HUVEC cultured in normal or low Mg-containing medium for 3 days. Genes with a ratio of 2.0 or above were considered positively regulated. Using these criteria, we found six genes down-regulated: c-src (threefold reduction), ezrin (sixfold), cytohesin (fourfold), CD9 (fourfold), zyxin (fourfold) and CDC25B (14-fold). Two genes were found up-regulated: the DNA excision repair protein ERCC1 (twofold) and the AXL tyrosine kinase receptor (threefold).

Since low Mg impairs endothelial proliferation, a particular attention has been devoted to the modulation of CDC25B, which plays a role in promoting cell cycle progression. By Western blot analysis, we confirmed a marked reduction (ninefold) in the total amount of CDC25B (Fig. 6A). By the same technique, we also confirmed that the total amount of c-src is reduced in HUVEC cultured in low magnesium (threefold) (Fig. 6B).

4. Discussion

Mg is the second most abundant intracellular cation, after potassium, and is a cofactor in more than 300 enzymatic reactions involving energy metabolism, protein and nucleic acid synthesis. Indeed, it activates a large array of enzymes either by bridging distinct molecules or by functioning as an allosteric modulator through its interaction with negatively charged moieties [25]. Compelling evidence shows that Mg content correlates with normal cells proliferation since Mg stimulates DNA and protein synthesis [26]. In addition, a cellular calcium–Mg antagonism has been described and considered relevant in modulating cell functions [27].

Evidence has accumulated to suggest that extracellular Mg concentrations play a critical role in modulating endothelial activities [28]. Indeed, Mg levels influence the synthesis of nitric oxide [29], intracellular calcium release [28], the uptake and metabolism of low-density lipoproteins [30], the permeability to water and albumin [31], and the proliferation of endothelial cells [32]. In addition, Mg deficiency enhances free radical-induced cytotoxicity in endothelial cells [33].

Because (i) endothelial cells are important players in atherogenesis and angiogenesis, and (ii) Mg deficiency promotes atherosclerosis, thrombosis, hypertension and inhibits tumor growth, we asked whether low concentrations of Mg could affect endothelial behavior. We focused our attention on cell proliferation and migration, critical steps in

angiogenesis, on endothelial/mononuclear cell interactions, early event in atherogenesis, and on the modulation of gene expression.

We here demonstrate that low Mg concentrations inhibit HUVEC proliferation, and this event correlates with a marked down-regulation of the levels of CDC25B, a phosphatase which plays a key role in controlling G2–M progression [34]. Indeed, the reduction in the levels of CDC25B could explain the negative regulation of the G2–M transition of vascular endothelial cells by the phorbol ester PMA and diacylglycerol [35].

The role of Mg in modulating eukaryotic cell growth is controversial. Whereas studies in normal cells have established a positive relation between the levels of Mg and the extent of proliferation, conflicting results have been obtained in tumor cells. For instance, low Mg medium affected the growth of 3T3 fibroblasts, but not of their transformed counterpart [26]. In accordance, it is noteworthy that Mg deficiency delays the transit through the G1 and S phase of normal but not transformed cells [36].

We also show that endothelial cell growth inhibition is reversible upon Mg re-supplementation, suggesting that no permanent modifications occur as a consequence of the deprivation of the cation. It is likely that the modulation of cell growth by Mg is mediated by corresponding changes in intracellular Mg, which would influence the activity of enzymes crucial to the proliferation process. Moreover, since Mg frequently acts as a natural Ca antagonist [37], it is conceivable to propose that an imbalance between Ca and Mg may play a role in modulating cell growth. However, it should be recalled that intracellular Mg is not significantly modulated in endothelial cells chronically exposed to low concentrations of the cation [38].

Since (i) Mg deficiency activates an inflammatory response and elevates the concentration of circulating cytokines in rodents and (ii) IL-1, a pro-inflammatory cytokine, is a potent inhibitor of endothelial cell growth, we anticipated that an up-regulation of IL-1 may be responsible for the growth inhibition observed in HUVEC cultured in low Mg. Indeed, IL-1 was up-regulated and an antisense oligonucleotide against IL-1 could prevent the growth inhibition observed in cells exposed to low concentrations of the cation. Apart from its effect on cell growth, IL-1 profoundly affects endothelial gene expression [1]. Indeed, it is conceivable that the up-regulation of VCAM and PAI-1 we detected after Mg deficiency might be ascribed to the high levels of IL-1 synthesized. However, it is unlikely that all the effects we observe are due to the up-regulation of IL-1. For instance, IL-1 induces the expression of E-selectin and ICAM-1 and we did not detect any modulation of these adhesion molecules in our system (unpublished results). We propose that VCAM is responsible, at least in part, of the increased adhesion of monocytoid U937 cells to the endothelial monolayer. The overexpression of VCAM and PAI-1 may contribute to the high incidence of atherosclerotic lesions and thrombotic events observed in Mg deficiency both in experimental models and in patients.

It is noteworthy that Mg deprivation has been linked to an exaggerated response to immune stresses in experimental models [10]. Analogously, we found that low Mg renders endothelial cells more susceptible to the detrimental effect of LPS. We propose that low Mg promotes an inflammatory response of endothelium which could be exacerbated by immune stress. This could be of particular importance in patients chronically affected by low Mg status, such as diabetics, alcoholics, intensive care unit and cancer patients.

By cDNA array, we isolated several transcripts modulated in HUVEC grown in media containing low Mg. We observed the up-regulation of AXL, the receptor for Growth-arrested-specific gene (Gas)-6, which has been shown to protect HUVEC from apoptosis [39]. It is noteworthy that AXL and Gas-6 expression are temporally correlated with neointima formation [40]. The down-regulation of several molecules has been detected: ezrin, CD9, cytohesin and zyxin, all contributing to endothelial adhesion to substrates and migration. By Western blot analysis, we confirmed the down-regulation of c-src, which is required for endothelial migration. This result can explain, at least in part, the reduced response of HUVEC to chemotactic factors in low Mg media. Accordingly, a recent report has proposed Mg as a newly recognized receptor-mediated chemoattractant for endothelial cells [41]. Together with proliferation, cell migration is pivotal in angiogenesis. These results may have implications in different situations, from tumor growth and metastatization to wound healing.

Previous works have linked low levels of Mg to dyslipidemia, which would be responsible for atherogenesis. Our data show that low magnesium directly affects endothelial cells, therefore indicating that the endothelial dysfunction caused by low Mg can contribute to generate a pro-inflammatory, pro-thrombotic and pro-atherogenic environment leading to cardiovascular disease.

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