Extracellular magnesium and \textit{in vitro} cell differentiation: different behaviour of different cells

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Abstract. The contribution of magnesium to cell differentiation is not clear. Some studies indicate that low extracellular magnesium promotes cell differentiation, while others reach opposite conclusions. We evaluated the effects of different concentrations of extracellular magnesium on the differentiation of three \textit{in vitro} experimental models: human endothelial cells seeded onto Matrigel; phorbol ester-treated myeloid leukemia U937 cells; and 3T3-L1 pre-adipocytes exposed to a hormonal cocktail containing dexamethasone and insulin. The differentiation of endothelial cells and pre-adipocytes seems to be independent of extracellular Mg concentration. Conversely, magnesium deficiency retards, while high extracellular magnesium accelerates phorbol ester-induced U937 cell differentiation, probably by interfering with calcium homeostasis or with the activity of kinases. We conclude that the extracellular magnesium concentration affects the differentiation of various cell types.

Key words: magnesium, cell differentiation, endothelial cells, pre-adipocytes, U937 cells

While clear evidence has been provided about the relevance of magnesium (Mg) in driving cell proliferation, little is known about its participation to the modulation of cell differentiation [1]. Differentiation, crucial during all stages of life, from embryo to adult, is triggered by soluble factors, such as growth factors, cytokines and hormones, or by signaling from components of the extracellular matrix, all activating the transcription of genes that control the acquisition of a differentiated phenotype. The genetic program activated in response to pro-differentiating signals is cell-specific.

For instance, the differentiation of pre-adipocytes into adipocytes is regulated by a complex network of transcription factors that control the expression of hundreds of genes responsible for establishing the mature adipocyte phenotype [2]. The main protein orchestrating the differentiation and function of adipocytes is peroxisome proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\)) [3]. PPAR\(\gamma\) induces the expression of adipocyte-specific genes through the binding of PPAR\(\gamma\)-retinoid X receptor heterodimers to a PPAR-response element, resulting in the promotion of intracellular fat storage.

Endothelial differentiation is a fundamental process for the development of the vasculature and for angiogenesis [4]. A convenient model for studying endothelial cell differentiation \textit{in vitro} is the tube formation assay on Matrigel, a reconstituted basement membrane extracellular matrix [5]. When plated onto Matrigel, endothelial cells rapidly attach, align, and form capillary-like tubules which contain a lumen and tight cell-cell contacts. Both transcription-dependent and -independent mechanisms are involved in endothelial cell tube formation on Matrigel. Recently, gene array experiments have shown that during Matrigel-induced tube formation, human
endothelial cells from the umbilical vein (HUVEC) up-regulate the expression of 86 genes and down-regulate the expression of 65, mainly associated with cell proliferation and metabolism [6]. By subtractive cDNA cloning, the cytoskeletal protein thymosin β4 has been found to be up-regulated early after seeding onto Matrigel [5]. Interestingly, in HUVEC transfected with thymosin β4, the rate of tube formation on Matrigel was accelerated, while an antisense oligomer against thymosin β4 inhibited tube formation on Matrigel [5].

Non-adherent, autonomously proliferating myeloid leukemia cells, including the human U937 cell line can be stimulated to differentiate along the monocyte/macrophage pathway by treatment with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) [7]. Indeed, in response to TPA, U937 cells become adherent, are growth-arrested and undergo several morphological and functional changes typical of normal monocytes and macrophages [7].

We asked whether different extracellular concentrations of Mg might affect cell differentiation. To this end, we selected the three aforementioned experimental models: HUVEC seeded onto Matrigel; TPA-treated myeloid leukemia U937 cells; and 3T3-L1 pre-adipocytes induced to differentiate into adipocytes.

**Methods and materials**

**Cell culture and differentiation**

For all of the cells studied, an Mg-free medium (Life Technologies Ltd, Paisley, United Kingdom) was used to vary the concentrations of Mg by adding MgSO4. On the basis of previous reports [8, 9], we cultured cells in medium containing 0.1, 1.0 or 5.0 mM Mg; 1.0 mM Mg served as the control physiological concentration. Murine 3T3-L1 pre-adipocytes were maintained in DMEM containing 10% FBS, 1 mM glutamine, endothelial cell growth factor (150 μg/mL), 1 mM sodium pyruvate and heparin (5 units/mL) on 2% gelatin-coated plates. Tube formation on Matrigel was evaluated as a model to study endothelial differentiation [5]. Culture plates were coated with cold Matrigel (BD Bioscience, Buccinasco, Italy) and incubated for 30 minutes at 37°C. HUVEC were cultured for 36 h in media containing 0.1, 1.0 and 5.0 mM Mg. The cells were then seeded in their corresponding medium onto Matrigel and photographed using a phase-contrast microscope after different time intervals.

Human myeloid leukemia cell line U937 cells were grown in RPMI 1640 medium containing 10% FBS and 2 mM glutamine. After 72 h of culture in medium containing different concentrations of Mg, the cells were treated with 25 nM TPA (Sigma, St. Louis, MO, USA) for 12, 24, 48 and 72 h. To measure plastic adherence, the supernatant was decanted, adherent cells were harvested with a rubber policeman, and the total number of attached cells was determined using a hemocytometer.

**Western blot**

Western blot was performed on cell lysates. Proteins were separated on 12% SDS-PAGE, transferred onto nitrocellulose sheets at 200 mA overnight, and probed with anti-PPARγ 1-2, anti-p21 and anti-actin antibodies (from Santa Cruz Tebu-bio, Magenta, Italy). The SuperSignal chemiluminescence kit (Pierce, Rockford, IL, USA) was used to detect immunoreactive proteins. All of the experiments were repeated at least three times with comparable results.

**RT-PCR**

HUVECs were lysed in 1 mL Trizol and RNA was purified, cDNA was synthesized from 1 μg of total FBS. The medium was changed every two days. Oil Red O (Sigma Aldrich, Milan, Italy) staining was performed to detect intracellular lipids [10]. The cells were photographed using a phase-contrast microscope. For a quantitative assay, Oil Red O dye was extracted with isopropanol 100% and absorbance was measured at 500 nm by spectrophotometry.
RNA using oligo(dN)₆ primers using the Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics, Basel, Switzerland). PCR amplification was performed as follows: 45 sec at 94°C, 45 sec at 60°C, and 1 min at 72°C. The reaction was stopped after 35 cycles. The primers for thymosin β4 were: 5'-CAGACC AGACTTCGCTCGTA-3' and 5'-GCTTCTCCTGTTCAATCGT -3'. RT-PCR with specific primers for GAPDH was performed to normalize (sense 5'- CCACC-CATGGCAAATTCC ATGGGA-3'; antisense 5'-TCTAGACGGCAGGTCAGGTCCACC-3').

**Statistical analysis**

Statistical significance was determined using the *t* test and set at *p* values at 0.01.

**Results**

**HUVEC differentiation on Matrigel**

Under normal culture conditions, HUVECs seeded onto Matrigel attach within one h. Then, in four-six hours, the cells align and form lateral associations which progress to the formation of tubes over the next 8-10 hours [11].

After 36 h of culture in media containing 0.1, 1.0 or 5.0 mM Mg, HUVEC were seeded onto Matrigel and maintained in their corresponding medium. Figure 1A shows no difference in cell organization in HUVEC cultured in 0.1, 1.0 and 5.0 mM Mg at any of the time points evaluated. We also studied the expression of thymosin β4, which is known to be rapidly upregulated in HUVEC on Matrigel [5]. As shown in figure 1B, we detected thymosin β4 only in HUVEC seeded onto Matrigel for 4 h with no differences related to the various concentrations of Mg used to culture the cells.

**3T3-L1 pre-adipocyte differentiation in vitro**

To explore whether different concentrations of Mg affect adipogenesis, 3T3-L1 cells were exposed to medium containing 0.1, 1.0 or 5.0 mM Mg and induced to differentiate with a hormonal cocktail up to day 8. At different time points, we stained the cells with Oil Red O to visualize the presence of lipid droplets in the cytosol. We did not observe any difference between 3T3-L1 cultured in different concentrations of Mg (figure 2A), and this was confirmed by spectrophotometry (figure 2B). We then investigated whether the expression of PPARγ is modulated during the differentiation of 3T3-L1 cells cultured in 0.1, 1.0 or 5.0 mM Mg. The total amounts of PPARγ were evaluated at the beginning of the experiment (day 0) and after exposure to the hormonal cocktail for 4 and 8 days. Western blot revealed that the levels of PPARγ markedly increased 4 days after the treatment with the differentiation cocktail, independently from the extracellular concentration of Mg (figure 2C). After 8 days, when the cells had reached terminal differentiation, PPARγ levels were decreased.

**Myeloid leukemia U937 cell differentiation in vitro**

To study the effect of different concentrations of extracellular Mg on macrophage differentiation, we used the differentiation system of human myelocytic U937 cells [12], which can be induced to differentiate into adherent macrophage-like cells by phorbol esters [13]. In parallel, cell cycle arrest occurs. We exposed the cells to TPA and counted the number of attached cells. TPA clearly induced U937 cell differentiation with a pronounced increase in adherent cells within the first 24 h. Our results show that high concentrations of extracellular Mg accelerated, while low Mg retarded TPA-induced U937 adhesion to plastic (figure 3A). We also investigated the levels of the cyclin-dependent kinase inhibitor p21, which is induced after 24 h exposure of U937 cells to TPA. Figure 3B shows that p21 is undetectable in untreated cells and is upregulated by TPA. Interestingly, the total quantity of p21 is less pronounced in TPA-treated U937 cells under Mg restriction than controls. No significant differences were observed in p21 levels between U937 cells in 1.0 and 5.0 mM Mg-containing medium.

**Discussion**

Differentiation is orchestrated by a variety of influences, such as growth factors, hormones, cell-cell contacts and cell-extracellular matrix...
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Interaction. Even though Mg is a versatile electrolyte that has been shown to be involved in many cellular processes [14], the relationship between Mg and cell differentiation remains unclear.

Mg-restricted media rapidly reversed the transformed phenotype of fibroblasts, thus suggesting that Mg promotes pro-differentiating events [15]. In C2C12 myogenic cells, Mg deficiency upregulated Myod, one of the transcription factors fundamental for myogenesis [16].

Conversely, in pancreatic islet RINm5F cells, both low and high extracellular Mg impaired insulin synthesis without altering cell morphology [17]. Furthermore, Mg deficiency reversibly antagonized the differentiation of promyelocytic leukemia HL60 cells exposed to differentiating agents such as DMSO and retinoic acid [18]. Our results on U937 cells are in agreement with this finding. U937 cells normally grow in suspension, but upon exposure to TPA they extend pseudopodia and became adherent to each other and to the surface of the culture dish. We observed that Mg restriction retards TPA-induced cell differentiation. To this end, since i) p21 is a primary

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**Figure 1.** The effect of different concentrations of Mg on HUVEC differentiation. HUVEC were cultured for 36 h in 0.1, 1.0 or 5.0 mM Mg before being seeded onto Matrigel. **A)** Photographs were taken 1, 4 and 8 h after seeding onto Matrigel. **B)** RT-PCR using specific primers was performed after 4 h on Matrigel. GAPDH was used to show that comparable amounts of RNA were used per lane.
Figure 2. The effect of different concentrations of Mg on 3T3-L1 pre-adipocyte differentiation. 3T3-L1 cells were induced to differentiate in 0.1, 1.0 and 5.0 mM Mg for 4 and 8 days. A) The cells were stained with Oil Red O after 4 and 8 days of differentiation, and photographed (20× magnification). B) Oil Red O was extracted with isopropanol and absorbance measured at 500 nm. C) Cell lysates (70 µg) from 3T3-L1 cells, induced to differentiate, were separated by SDS-PAGE. Western blot was performed with antibodies against PPARγ. The blot was incubated with an anti-actin antibody to show that comparable amounts of protein were loaded per lane.
Figure 3. The effect of different concentrations of Mg on U937 cell differentiation. The cells were cultured in medium containing different concentrations of Mg for 72 h and then treated with TPA (25 nM) for different times. A) After 12, 24, 48 and 72 h in the presence of TPA, adherent cells were harvested with a rubber policeman and counted. Data are shown as the mean ± standard deviation (*p < 0.01). B) Cell lysates (70 μg) from U937 cells induced to differentiate for 24 h, were separated by SDS-PAGE. Western blot was performed with antibodies against p21. The blot was incubated with an anti-actin antibody to show that comparable amounts of protein were loaded per lane.

response gene strictly related to the early differentiation program of many hematopoietic cell lines including U937 cells [19] and ii) p21 plays a role in facilitating U937 differentiation [20], it is worth noting that the levels of p21 are lower in TPA-treated cells under Mg restriction than in cells cultured in 1.0 or 5.0 mM Mg. The induction of p21 in U937 cells by TPA is p53-independent and mediated through the activation of protein kinase C (PKC)/MAPK pathways [21]. Considering that the transphosphorylation from ATP is Mg-dependent, it is likely that Mg deficiency might impair the aforementioned pathways. Alternatively, since Mg acts as an intracellular second messenger, it is possible that Mg restriction impairs transient Mg influx necessary for several cell activities. While no differences were observed in p21 levels between cells cultured in 1.0 versus 5.0 mM Mg, our results indicate that high extracellular Mg accelerates U937 cell differentiation. This could be due to the well known antagonisms between Mg and calcium leading to an imbalance in the intracellular Mg/calcium ratio with consequent alterations of calcium-mediated signals, and/or to a potentiation of transphosphorylation reactions. All together, these data
concerning Mg and leukemia cell differentiation suggest that controlling and correcting Mg homeostasis in leukemic patients might be important for better outcomes.

Recently, we have shown that excessive extracellular Mg blocks pre-osteoblast differentiation [8], which is in agreement with a previous report showing that high extracellular Mg inhibited matrix mineralization in the prechondrogenic cell line ATDC5 [22]. The contrasting results about the effect of high Mg on the differentiation of U937 cells on one side and condrocytes and osteoblasts on the other are not surprising, since differentiation requires the activation of cell-specific genetic programs that can be differentially affected by Mg.

The differentiation of endothelial cells and pre-adipocytes seems to be independent from extracellular Mg. Indeed, we detected no effects of different concentrations of Mg in HUVEC induced to differentiate on Matrigel. Accordingly, we found no alteration in the expression of thymosin β4, an early gene induced by culture on Matrigel. Thymosin β4 is involved in the differentiation of many cell types, including HUVEC [5]. Since the reorganization of actin filaments into fiber bundles is essential for tube formation on Matrigel, it is interesting that thymosin β4 is a G-actin-sequestering protein directly implicated in the cytoskeletal remodeling required for tube formation [5].

We did not observe any change in the accumulation of lipid droplets or in the extent of PPARγ upregulation in pre-adipocytes cultured in different concentrations of Mg and induced to differentiate with a hormonal cocktail. Our results, however, are in disagreement with a previous report demonstrating depressed differentiation in primary porcine stromal-vascular cells induced to differentiate into adipocytes in Mg-deficient medium [23]. The contrasting results can be ascribed to the completely different experimental model used.

Together, these findings suggest that fundamental differences exist between various cell types in the contribution of Mg to cell differentiation.

**Disclosure**


**References**


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