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MAGNESIUM IN CELL PROLIFERATION AND DIFFERENTITION

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1. ABSTRACT

Compelling evidence shows that magnesium (Mg) content is directly correlated to proliferation in normal cells as Mg stimulates DNA and protein synthesis. Some data have demonstrated that upon mitogenic stimuli normal cell are able to increase their intracellular Mg content, likely by activating Mg influx. Mg deprivation, in turn, induces inhibition of DNA and protein synthesis thus promoting growth arrest. From a mechanistic viewpoint, Mg deprivation may influence cell cycle control by upregulating the cyclin inhibitor $p27^{Kip1}$ thus influencing cyclin E-dependent kinases. In many neoplastic cells, Mg is higher than in normal counterparts and this high Mg is maintained also against concentration gradient. Moderate *vs.* severe and acute *vs* chronic effect of Mg deprivation must be distinguished: severe Mg deprivation causes growth arrest also in tumor cells, while chronic Mg deprivation leads to an "adaptation" of tumor cells both to growth rate and Mg content. In tumor cells deranged Mg content and distribution is likely due to an inhibition of Mg efflux via the *Na-Mg antiport*. When differentiation process is induced by receptor mediated stimuli such as IFN-*alpha* and ATP, decrease of cell Mg content accompanies with activation of Mg efflux. Transformed cells may thus display high growth rate *also* because they retain a large amount of Mg. On their whole, these data strongly suggest that regulation of intracellular Mg availability *parallels* the molecular control of cell proliferation, and maybe also cell differentiation and death.

2. INTRODUCTION

Due to its abundance in the intracellular environment, magnesium (Mg, referring to element in general or total elemental content) is considered the divalent cation *"par excellence"*. The abundance of Mg probably reflects its involvement in a large variety of biological functions and enzymatic reactions (kinases, phosphorylases, dehydrogenases, etc.) (1). In fact, several *in vitro* studies have provided convincing evidence for interactions of Mg with phospholipids, proteins and nucleic acids, thus providing a rationale to understand how Mg can stabilize cell structure and influence enzyme activation or inhibition (2). By interacting with negatively charged moieties like $(RPO_4^-)_n$, $(RCO_2^-)_n$, (NTP^-), or polyamines, Mg can function as allosteric modulator of several enzymes. Alternatively, Mg can influence catalytic activity by bridging structurally distinct molecules. This is best exemplified by the ability of Mg to promote transphosphorylation reactions through the formation of ATP-Mg complexes which anchor substrates to the active sites of enzymes.

The role of Mg as a regulator of cell function and structure is more difficult to prove *in vivo*. Pioneering work by Rubin has implicated Mg as a key factor of the so-called "coordinated response of growth and metabolism", i.e., the upregulation of energy metabolism, and protein and DNA synthesis which precedes cell division (3). Maguire and co-workers have proposed Mg as a "chronic regulator" of cell functions, and have opposed such function to the "acute regulation" elicited by calcium (Ca) (4). Unfortunately, these intriguing theories have had little follow-up, mostly because of some technical limitations inherent to Mg analysis in complex biologic systems. These limitations include the lack of selective and sensitive techniques to measure intracellular Mg as well as the existence of very high background levels of Mg in both intra- and extra-cellular milieus, precluding accurate determination of those small variations which presumably enable Mg to influence cell functions. Mechanism(s) for Mg transport, regulation and distribution within the cell have therefore remained controversial or speculative.

Our group has had a long-time interest in the putative role of Mg in cell proliferation and differentiation. Mg might play such role at several distinct levels. Mg stabilizes DNA structure, promotes DNA replication and transcription, and influences RNA translation (5), presumably by acting as allosteric or catalytic modulator of critical enzymes such as topoisomerases, endonucleases or polymerases. Furthermore, Mg (*i*) induces ribosome assembly, thus influencing protein synthesis at a post-translational level; (*ii*) regulates the opening-closure of ion channels, which may account for pH changes preceding cell division; and (*iii*) couples energy metabolism to sustained protein synthesis and DNA duplication, as suggested by the aforementioned model of "coordinated response of growth and metabolism".

In this article, we will review our studies on the possible role of Mg in the regulation of growth and differentiation of several cell types. Our results will be compared to other prevailing hypotheses with the aim to reconcile different opinions and obtain a unifying picture of an otherwise controversial topic.

3. Mg CONTENT IN PROLIFERATING AND NON-PROLIFERATING CELLS

There is a large variability in cell Mg content between different species. Cells contain millimolar amounts of Mg, distributed in bound and free/ionized forms ($[Mg^{2+}]_i$) (90 and 10%, respectively). The vast majority of available data refer to total cell Mg. Several reports concur in the conclusion that proliferating cells have more Mg than non-proliferating cells. In principle, such difference would be in keeping with the promoting effects of Mg on protein and DNA synthesis (6,7), provided that one is comparing cell types having the same histologic origin. To establish a correlation between DNA synthesis and Mg content within the same cell type Cameron *et al* have examined mouse heart ventricle myocytes at different times after birth (7). These studies have shown that myocyte proliferation increases shortly after birth and then decreases at longer times. These time-related changes in DNA synthesis and proliferation were accompanied by consistent increases and decreases of *total* Mg. To understand whether Mg changes correlated with normal or neoplastic growth, these authors also studied normal dividing cells (*e.g.*, mouse and rat regenerating hepatocytes or mammary lactating epithelia) in comparison with hepato- or breast carcinoma cells. Such comparative analyses revealed that neoplastic transformation was not accompanied by significant increase of total Mg, lending support to the concept that Mg changes are confined to proliferation induced by physiologic stimuli. Such conclusion has not been confirmed in several other studies, showing an increase of Mg in transformed cells as compared to normal parent cells (8,9,10). Therefore, it has remained unclear whether Mg increase is a common feature of any dividing cells or a unique characteristic of either physiologically-induced or transformation-related events.

To explore the relationship between Mg content and cell proliferation we have used HL-60 human promyelocytic leukemia cells. This model has several distinctive advantages. First, cells in suspension do not need manipulations that could damage the permeability of plasma membrane and consequently interfere with measurement of intracellular Mg. Second, these cells can be induced to differentiate into phenotypically normal non-proliferating granulocytes or monocytes that are directly comparable to the parent proliferating cells. Our results show that inhibition of cell proliferation by differentiating agents such as dimethysulphoxyde (DMSO) or retinoic acid (RA) is accompanied by approx. 20% decrease in Mg content. Such decrease likely reflects important modifications of the bound pool of Mg; in fact, measurements in mag-fura2 loaded cells have shown that $[Mg^{2+}]_i$ does not decrease but actually increases during inhibition of cell proliferation (11). Under comparable experimental conditions electron probe microanalysis of the subcellular distribution of Mg indicates that decrease of Mg occurs in cytosol and mitochondria but not in nuclei in relation to differentiation- and growth arrest (12). This suggests that both quantitative and qualitative modifications of intracellular Mg may occur. Collectively, these findings confirm that proliferating or resting cells may differ with respect to Mg content and compartmentalization; however, it remains to be established whether such differences are confined to physiologic or neoplastic patterns of proliferation.

4. THE EFFECT OF EXTRACELLULAR Mg ON CELL PROLIFERATION

The observation that *intracellular* Mg content and distribution may vary with physiologic or neoplastic proliferation has led many investigators to explore the possible influence of *extracellular* Mg on these processes. Early in the 70s Rubin and co-workers studied the proliferation of chick embryo fibroblast and found that 2-deoxyglucose transport, glycolysis, and both DNA, RNA and protein synthesis, required the presence of Mg in the medium (6,13). A requirement for Ca was similarly observed, but the authors concluded that the metabolic effects of varying Ca in the medium were ultimately mediated by increases of $[Mg^{2+}]_i$ (14). According to these studies, high *extracellular* Mg stimulates protein synthesis and energy metabolism, thus allowing initiation of cell division. Two additional lines of evidence support such a role for *extracellular* Mg in cell proliferation. First, moderate decreases of *extracellular* Mg (from 0.8 to 0.1 mM) inhibited the proliferation of chick embryo fibroblasts the same way as did serum deprivation (15-17). Second, cell proliferation could be resumed by including Mg back in the culture medium (18).

The role of *extracellular* Mg as a positive modulator of cell proliferation has been confirmed in several other studies. For example, elevation of *extracellular* Mg, but not of Ca, has been found to stimulate the proliferation of newborn mouse keratinocytes and to extend the confluent phase of epidermal cell growth beyond 10 days; moreover, adult keratinocytes required high phosphate but also high Mg to increase DNA synthesis and DNA content in culture (19).

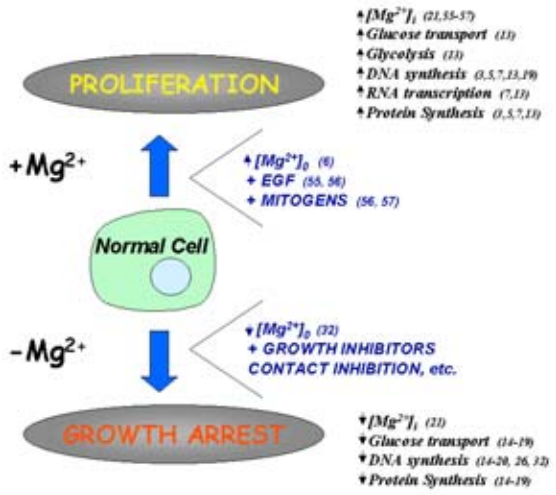


Figure 1: Effect of *extracellular* Mg on proliferation and biologic functions of normal cells. The most representative references in support of this interpretation are given in parenthesis.

In other studies, elevation of *extracellular* Mg above the physiologic concentration could stimulate the proliferation and migration of capillary endothelial cells. Opposite effects were consistently observed by decreasing extracellular Mg below physiologic levels, thus allowing to establish an important correlation between serum Mg and cellular mechanisms of angiogenesis (20). All these effects of *extracellular* Mg are most probably mediated by corresponding changes of $[Mg^{2+}]_i$. In fact, recent studies in human aortic endothelial cells have shown that elevation of *extracellular* Mg is accompanied by consistent increase of $[Mg^{2+}]_i$ within 2-10 minutes (21).

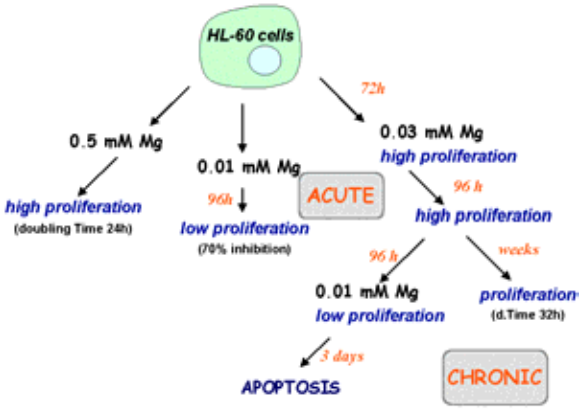
Whereas studies in normal cells have established a positive correlation between Mg availability and degree of proliferation (see figure 1 for a schematic review), similar experiments on *tumor cells* have yielded conflicting or inconclusive results. In the mid 70s it was found that removing Mg from the diet induces striking regression of the highly malignant Walker carcinosarcoma in the rat. Such effect of Mg deprivation was indeed specific, as switching back to Mg-supplemented diet restored tumor growth. In the same studies dietary restriction of Mg was found to potentiate the anticancer activity of a variety of chemotherapeutic agents (22). Whereas these findings highlighted Mg as a key factor of tumor growth and perhaps drug-sensitivity, other studies in SV 40-transformed human lung fibroblasts revealed that these cells could grow in the face of Mg removal from the culture medium (23-25*)*.

Elegant work by Rubin consistently showed low Mg media affected the growth potential of 3T3 fibroblasts but not of spontaneously or SV-40 transformed subclones (26). Other studies similarly showed that transformed fibroblasts and various types of tumor cells can grow in 0.02 mM Mg (7,27). Spontaneously transforming Swiss 3T3 fibroblasts also grow in low Mg media, but only after displaying anchorage-independent growth (28).

This picture becomes even more complicated if one appreciates that the effects of *partial* Mg deprivation are quite different from the effects of *total* Mg deprivation. Decreasing Mg concentration down to 0.03 mM does cause a gradual reduction of tumor cell growth; however, growth arrest and death only occur when Mg levels are decreased below this threshold. Moreover, the effects of Mg on tumor cells do not always exhibit reversibility upon Mg re-supplementation. This is the case for experiments in S49 lymphoma cell cultures. In such a case, low Mg medium committed a number of cells to death and the surviving clones did show the expected decrease of growth potential. The doubling time of cultures stabilized in 0.03 mM Mg was 3 times longer than it was for cells grown in normal Mg media. However, introducing Mg back into the culture medium did not resume growth rate, suggesting that Mg deprivation had imposed permanent modification(s) and selection of clones resistant to the absence of this cation (29). On balance, there is a large body of evidence to support the concept that tumor growth may not depend on *extracellular* Mg availability. Under particular conditions, Mg depletion can paradoxically elicit tumorigenic effects, as is in the case of T cell lymphoma-leukemia occurring in as many as 25% of Sprague-Dawley rats surviving after Mg-deficient diet (30). Cultures of some lymphomas consistently displayed increased growth rate when maintained in Mg-deficient medium (31).

In an attempt to reconcile the aforementioned inconsistencies, we have studied the *acute* and *chronic* effects of Mg deprivation on the growth of HL-60 cells. As to the *acute* effects, our results indicate that the growth rate of HL-60 cells is not affected by reduction of *extracellular* Mg from 0.5 to 0.03 mM, when assessed 96 hours after Mg removal; however, growth rate is inhibited by approx. 70% when similarly assessed 96 hours after seeding in 0.01 mM Mg (11). As regards the *chronic* effects, we have found that HL-60 cells can be grown indefinitely in 0.03 mM Mg, although the growth rate tends to decrease as compared with control cells (32 *vs.* 24 hours, unpublished observations); in contrast, cells grown in 0.01 mM Mg die within 6-7 days (figure 2). Similar experiments have been carried out in cultures of either normal (HC11) or tumor (MCF-7) mammary epithelial cells. In this model *extracellular* Mg deprivation from 0.5 to 0.05 mM did not influence the growth rate and DNA synthesis of tumor MCF-7 cells, when assessed at 72 hours; at the same checkpoint, HC11 cells exhibited 50% reduction of both growth rate and DNA synthesis (32).

Taken as their whole, our results seem to confirm that tumor cells are more resistant to Mg deprivation than are normal cells. Perhaps more importantly, our results also indicate that (*i*) Mg deficiency affects tumor growth only below a threshold concentration of 0.03 mM; and (*ii*) the ability of tumor cells to grow in low Mg media upon Figure 2: Effect of *extracellular* Mg deprivation in HL-60 cells. Different results depend on *acute* or *chronic* treatments.



HL-60 cells adapt to prolonged Mg-deprivation slightly modifying their doubling time.

prolonged treatment may reflect the selection of clones which require little or no Mg or the development of biochemical adaptations which compensate for low Mg availability. At the present time we would privilege the latter hypothesis, as the experiments were performed under conditions not specifically designed to select specific subclones.

5. THE EFFECT OF EXTRACELLULAR Mg ON THE CELL CYCLE

To better understand the mechanism(s) whereby divalent cations can influence cell proliferation, some authors have studied the effects of Mg and Ca on the distinct phases of cell cycle. Most of the data obtained in proliferating *normal* cells clearly showed that the transition from G_1 to S phases was strictly dependent on Ca availability (33). Whereas such conclusion points to a direct effect of Ca, others (3,7) have suggested that Ca acts indirectly, presumably by displacing Mg from intracellular binding sites. Hence, Mg, but not Ca, should be considered the ultimate regulator of cell cycle. Three lines of evidence supported the role of Mg in cell cycle regulation. *First*, WI-38 human fibroblasts progression into the S phase was delayed by Mg deprivation and such delay was proportional to the duration of Mg deprivation (34). *Second*, Mg deprivation was found to permit WI-38 human fibroblast to complete one cycle but cells then arrested in the pre-replicative G_1 phase of a subsequent cycle (34). Finally, studies in epidermal cells from newborn and adult mice have shown that increasing Mg from 1 to 5 mM in the culture medium decreased the percentage of cells in G_1 phase and increased the percentage of cells in S and G_2+M phases (19). Inasmuch as changes in Mg availability was also accompanied by increased thymidine incorporation, the passage of cells through the S phase probably reflected the ability of Mg to stimulate DNA synthesis.

The requirement for Mg described above may well be in keeping with its general role in macromolecular synthesis and transphosphorylation reactions, that is biochemical events occurring throughout the cell cycle (34). More specific and time-confined functions of Mg have nonetheless been identified in yeast. In this cell type Mg might serve as a '*sensor*' of cell size and hence as a '*timer*' for cell cycle progression. In fact, the Mg content of yeast decreases as the volume of these cells increases, until spindle formation and chromosomes separation eventually occur. Once chromosomes have separated completely, an activation of Mg influx restores intracellular Mg levels and such processes appear to trigger spindle breakdown and consequent cell division (35,36). To reconcile the role of both Ca and Mg in cell cycle regulation, a model of mitosis has been developed based on the concept that cation transport and reciprocal fluctuations of $[Ca]_i$ and $[Mg]_i$ influence the SH/SS status and conformation of proteins, with ultimate effects on spindle protein polymerization (37). In principle, similar processes might also influence chromosome condensation; accordingly, a differential requirement for Mg has been observed in the prophase and telophase of fused Chinese hamster lung cells (38).

As far as *tumor* cells are concerned, studies on epithelial-like rat liver cells has shown that Mg-deficiency delays transit through the G_1 and S phases of normal cells but not of transformed cells (39). This is consistent with the observation that growth of transformed cells is relatively less sensitive to Mg availability than is in the case of normal cells (*cf. Section 3*). We have performed experiments which confirm this general trend when the effect of low Mg (0.05 mM) is studied in normal HC11 mammary epithelial cells *vs.* transformed MCF-7 cells (32). At the same time, we have data to suggest that Mg deprivation should not be considered as invariably ineffective on tumor cell growth. Studies on HL-60 cells indicate that lowering Mg concentration to 0.03 mM does not affect cell cycle distribution in comparison with normal Mg-supplemented cells. However, reducing Mg to as low as 0.01 mM eventually modifies cell cycle distribution, increasing the percentage of cells in G_0/G_1 phase by approximately 25% while decreasing the percentage in S phase by greater than 50% (11). Thus, "severe" Mg deficiency *can* arrest tumor cell growth under defined experimental conditions.

5.1. The effect of extracellular Mg on the regulation of cell cycle

Having confirmed that *extracellular* Mg deprivation may affect cell cycle, we have sought to establish how such effect is mediated at a mechanistic level. Recent studies have shown that progression through the various phases of the cell cycle is driven by a series of proteins which are called cyclins and represent positive regulators of a family of kinases (cyclin-dependent kinases, CDKs) (40-42). The sequential activation/deactivation of cyclin-CDK complexes govern the progression of eukariotic cells through the cell cycle. At the present time, three categories of mammalian cyclins have been identified: G1 cyclins, which are expressed maximally in the corresponding phase of the cell cycle; cyclin A, expressed in late G_1 and S phases; and G_2 cyclins, expressed in the G_2/M phase. The activity of cyclin-CDK complexes is under the control of two major categories of inhibitory proteins. One family is called INK4 and includes $p16^{ink4a}$, $p15^{ink4b}$, $p18^{ink4c}$ and $p19^{ink4d}$ which inhibit G1-type cyclins like CD4 and CD6. The other family is called

Cip/Kip and includes $p21^{cip1}$ and $p27^{kip1}$ and $p57^{kip2}$ which inhibit CDKs responsible for G_1 progression and S phase entry. In particular, $p27^{kip1}$ has been implicated in the regulation of cell response to extracellular signals such as serum deprivation, TGF-*beta*, contact inhibition or growth in suspension (43,41). Thus $p27^{kip1}$ is a major negative regulator of cell cycle progression.

Based on these premises, we have studied the effect(s) of Mg on the expression of major cyclins and cyclin inhibitors. Our results show that in HL-60 grown in Mg-deficient media the expression of $p27^{kip1}$ increases proportionally with Mg deprivation and associates with a slight increase of cyclin E but not cyclin D (11). The same pattern of $p27^{kip1}$ overexpression has been observed in HC11 mammary epithelial cells in which $p27^{kip1}$ expression correlates with the length and the extent of Mg deprivation as well as with cyclin E-associated kinase activity (32). These results provide unprecedented evidence that Mg can regulate cell cycle by modulating cyclin expression. It remains to be established whether the effect of Mg deprivation on $p27^{kip1}$ levels reflects changes in the synthesis and/or degradation of this inhibitory protein.

6. THE EFFECT OF Mg ON APOPTOSIS

During their life span many cells can choose either to proliferate or to undergo programmed death named apoptosis (44). Inasmuch as Mg deprivation can negatively influence proliferation, it might also (in)directly force the cell to undergo apoptosis. Irrespective of the stimuli eliciting programmed cell death, the morphologic pattern of apoptosis includes DNA degradation into large and subsequently small fragments. The first step of DNA degradation seems to depend on topoisomeraseII activity; the second step involves endonuclease-catalyzed oligonucleosomal fragmentation. Both topoisomeraseII and endonuclease are activated by Mg; topoisomeraseII is activated through allosteric and catalytic mechanisms (45), whereas endonuclease activation can be either Mg or Ca dependent or sensitive to Zn inhibition (46). In the face of its role as a positive modulator of topoisomeraseII and endonucleases, there has been no specific investigation on the influence of Mg movements and availability on such process. In a limited study, Patel *et al*. have shown that Mg influx into hepatocytes is a key event of apoptosis induced by the bile salt glycodeoxycholate (GDC). These authors found that treatment of hepatocytes with GDC was accompanied by net Mg influx, increase of $[Mg^{2+}]_i$, and concomitant DNA fragmentation. Removal of Mg, but not Ca, from the extracellular medium prevented the increase of $[Mg^{2+}]_i$ and consistently reduced DNA fragmentation (47). Therefore, they concluded that an increase of $[Mg^{2+}]_i$ can trigger endonuclease activation in hepatocytes. Several other studies have nonetheless concluded Mg-dependent endonucleases are not essential for the apoptotic process to occur (48,49).

In the light of these conflicting reports, we have studied the effect of *extracellular* Mg on spontaneous and drug-induced apoptosis in HL-60 cells. We have evidence that both the degree of Mg deprivation, the experimental conditions *(acute vs. chronic* treatment), and the rate of proliferation of apoptosis-susceptible cells, may contribute to generate different results. When HL-60 cells are grown in very low Mg medium (0.01 mM), proliferation is low and apoptosis is minimal during the first few days of treatment; however, apoptosis becomes the prevailing mechanism of cell death after 6-7 days of treatment, that is the time of culture exhaustion. A very similar pattern of time-related apoptosis is observed in Mg-supplemented HL-60 cells that are induced to differentiate and to decrease proliferation by treatment with DMSO (11). Different results are obtained when HL-60 cells are grown in 0.03 mM Mg. Under this latter condition of moderate Mg deprivation, cell proliferation is high and spontaneous apoptosis is minimal; however, topoisomeraseII inhibitors, like etoposide, or intercalating agents, like cytosine-arabinoside (ARA-C), can be used to increase the population of cells committed to apoptosis. When these drugs are given to HL-60 cells, the apoptotic response is lower in Mg-deprived than in normal Mg medium, although proliferation is much similar in the two media. Reintroducing Mg during incubation with drugs restores apoptosis to the same extent as observed in control/normal Mg cells; however, this response depends on the drug being used, since Mg restores apoptosis by etoposide but not by ARA-C. Considering that neither drug is targeted to endonucleases, one first conclusion is that Mg can influence apoptosis upstream DNA fragmentation elicited by endonuclease (unpublished results). One other conclusion is that topoisomeraseII is very central to the modulation of apoptosis by Mg, as one would expect based on the aforementioned allosteric or catalytic effects of Mg on this enzyme (45).

As an alternative mechanism one can envisage a role for Mg in the regulation of mitochondrial functions. It has been described that mitochondria actively participate to the apoptotic process by various mechanisms such as: release of caspase activators, disruption of electron transport and energy metabolism and production of reactive oxygen species and cellular redox potential. In particular it would be interesting to investigate the role of Mg in the regulation of mitochondrial transition pore which control the inner transmembrane potential. Opening of this channel induces swelling and consequent rupture of outer membrane leading to the release of various substances which induce apoptosis, *i.e.* caspase activators (50). However, specific studies to investigate the role of Mg in the mitochondrial functions and apoptosis have not been performed at the present time.

7. THE EFFECT OF EXTRACELLULAR Mg ON CELL DIFFERENTIATION

Inhibition of cell proliferation is often considered a prerequisite to differentiative events. Having discussed that Mg deprivation can inhibit cell proliferation, one might wonder whether such condition might pave the road to differentiation, thus providing cells with one more option in addition to apoptosis. (figure 3). Clearly, such possibility must be considered within an extremely complex framework, as differentiation is governed by numerous and

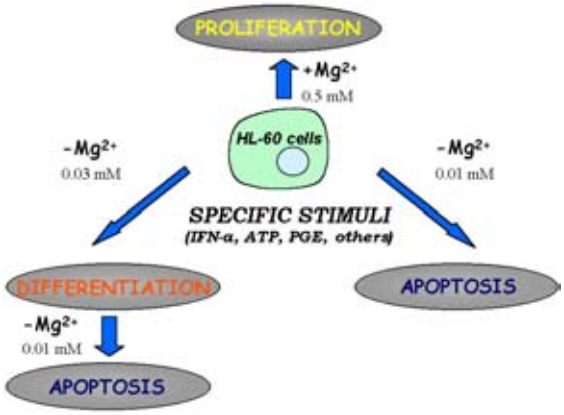


Figure 3: Effect of Mg on growth, differentiation and apoptosis of HL-60 cells. Unifying picture trying to summarize the possible role of Mg in these biologic functions.

different factors as receptor-mediated stimuli, growth inhibitors, contact inhibition, intercellular junctions, etc.

The differentiating effects of Mg deprivation have been described in very few reports. Rubin has shown that Mg-restricted media induce reverse differentiation of transformed fibroblasts and make these cells to change shape, to require more serum, and to become more sensitive to anchorage-dependent growth (51,52). Such effects are nonetheless transient; in fact, long-term Mg deprivation eventually induces differentiated fibroblasts to return back to the transformed phenotype and biological functions of the parental cells. Such reversibility presumably reflects biochemical adaptation(s) to low *extracellular* Mg and compensatory change(s) in *intracellular* Mg content, thus allowing differentiated cells to regain biologic functions of the transformed phenotype (52). The effects of *extracellular* Mg deprivation on *intracellular* Mg content and associated cell functions will be discussed in depth in the next chapter. In contrast to the above findings, other works have challenged the concept that Mg deprivation induces differentiation. Studies on keratinocytes or pancreatic islet RINm5F cells have shown that either high or low Ca levels in the medium would induce differentiation (19,53). In the case of RINm5F cells, low Mg media could only affect the synthesis of insulin otherwise associated with this tumor line, suggesting that the effects of Mg deprivation may be confined to the induction of functional rather than morphologic differentiation. Further evidence against the differentiating potential of Mg deprivation has been obtained in experiments with HL-60 cells grown in less than 0.01 mM Mg. Under these conditions, the addition of differentiating agents like DMSO, retinoic acid and others, failed to induce the canonical signs of cell maturation (reduction of nitrobluetetrazolium, non-specific esterase activity and monoclonal antibody expression), showing that there may be conditions when Mg deficiency can apparently antagonize, rather than stimulate, differentiation by chemical or biologic agents. Inasmuch as re-introduction of Mg in the culture medium enabled DMSO-treated cells to differentiate, the authors also concluded that Mg deprivation could compromise appearance of the differentiated phenotype but not of cell commitment to differentiation. Such conclusion can be tentatively reconciled with the well known inhibition of protein synthesis by Mg deprivation (54). Clearly, one might wonder whether such conditions are specific enough to probe the possible role of Mg in differentiation. Severe Mg deficiency and non specific inhibition of such general processes as protein synthesis might well have influenced factors other than differentiation, *e.g.*, viability and proliferation. Consistent with this, we have found that a combination of Mg deprivation with a differentiating agent causes massive death of HL-60 cells within few days (unpublished).

To shed more light on this issue we have studied the effect(s) of reducing *extracellular* Mg on the differentiation characteristics of HL-60 leukemia cells in comparison with sister cells induced to differentiate by DMSO treatment. HL-60 cells grown in 0.03 mM Mg displayed some signs of cell differentiation such as increased expression of the surface antigen CD11b; moderate increase of the neutrophyl-like oxidative burst; reduced nuclear/cytoplasm ratio, and changes of nuclear shape and cytoplasm staining. After a week in 0.03 mM Mg, cells were passed down to 0.01 mM Mg. Under these conditions, cells appeared to undergo terminal differentiation, as evidenced by the same growth arrest and programmed death patterns as observed in DMSO-differentiated cultures. Moreover, electron microscopy revealed the appearance of cytoplasmic granules resembling those specific to neutrophyls (11) (figure 3).

Whereas the above comparisons between low Mg- or DMSO- treated cells support a role for this ion in differentiation, other findings on the very same model system call for cautionary remarks. For example, we have growing evidence that HL-60 may become somehow "resistant" to differentiation induced by stepwise maintenance in 0.03 and 0.01 mM Mg, consistent with the Rubin's model of transient rather than permanent effects of Mg deprivation (51); or with the Maguire's hypothesis of a gradual selection of subclones resistant to Mg deprivation (29). The relationship between Mg and cell differentiation thus remains open to debate from both biologic and technical viewpoints.

8. Mg EFFLUX AND REGULATION FROM PROLIFERATING AND NON-PROLIFERATING CELLS

To understand whether an intracellular cation displays regulatory activity one must identify specific mechanisms for the regulation of its concentration within the intracellular compartments where it supposedly influences biologic functions. In eukaryotic cells concentration of *intracellular* Mg is remarkably high, yet it is still way below the electrochemical equilibrium. This simple observation hints that specific mechanisms maintain intracellular Mg within a physiologic range; such mechanisms can act by limiting Mg influx within the cell or by promoting Mg efflux into the extracellular milieu. Mg influx or efflux has been studied by different technical

approaches: by monitoring movements of radio-labeled Mg; by chelating Mg with selective fluorochromes; by electrochemical detection with specific microelectrodes; or by atomic absorption spectrometry (55,56). These technical approaches have contributed a great deal to the understanding of the regulation of *intracellular* Mg. Nevertheless, the picture has remained largely incomplete, especially in eukaryotic cells.

Over the last few years there has been convincing evidence that Mg fluxes, and hence $[Mg^{2+}]_i$, are influenced by receptor-mediated stimuli, some of which have *mitogenic* implications. For example, Grubbs has shown that stimulation of BC_3H1 myocytes with epidermal growth factor was accompanied by clear-cut increase of $[Mg^{2+}]_i$ and simultaneous onset of DNA synthesis (57). Such increase of cytosolic Mg occurred 5-7 min after EGF addition, lasted approximately 20 min, and was ascribed to the stimulation of Mg influx through plasma membrane as assessed by measurements of $^{28}Mg^{2+}$ uptake. However, similar increases of $[Mg^{2+}]_i$ could not be detected in all myocytes, suggesting heterogeneous responses to EGF. Erratic responses were similarly observed in single cell studies of $[Mg^{2+}]_i$ in Swiss 3T3 fibroblasts stimulated with bombesin or EGF *plus* insulin, presumably reflecting the differential expression of specific surface receptors (58). On the other hand, receptor-mediated mitogen activation of human lymphocytes is followed by increase of $[Mg^{2+}]_i$ only in cells having high $[Ca^{2+}]_i$, regardless of the presence or absence of extracellularly available $[Mg^{2+}]_i$. This suggests that $[Ca^{2+}]_i$ can increase $[Mg^{2+}]_i$ by releasing Mg from intracellular stores rather than by promoting Mg influx (59). Taken as their whole, these results demonstrate that a correlation exists between an increase of $[Mg^{2+}]_i$ and the induction of proliferation; however, it remains to understand why such processes can be so variable within the same cell population. In addition to the uncertainty regarding the influence of receptor expression on Mg movements, two more questions remain unanswered at the present time. The first question is: can $[Mg^{2+}]_i$ increase after Mg uptake from the extracellular space or does such increase reflect a release from intracellular stores, as demonstrated in the limited study on human lymphocytes? In principle, one might answer that these two mechanisms are not mutually exclusive, since increased Mg influx could be associated with increased Mg release from intracellular stores. Whereas mobilization of Mg from intracellular stores may undoubtedly account for an increase of $[Mg^{2+}]_i$, it would nonetheless be insufficient to explain the increase of *total* Mg which is observed in proliferating cells. The second question is: should $[Mg^{2+}]_i$ always be preceded by $[Ca^{2+}]_i$ transients, as is in the case of lymphocytes, or should these two processes be considered independent, as is in the case of the $[Ca^{2+}]_i$ independent increase of $[Mg^{2+}]_i$ in EGF-stimulated myocytes? Irrespective of the ultimate mechanism(s) leading to the increase of $[Mg^{2+}]_i$, the observation that mitogenic stimuli support such increase provides one more basis to conclude that *intracellular* Mg correlates positively with proliferation (*cf. Section 3*). It is then reasonable to conclude that changes in $[Mg^{2+}]_i$ might influence the activity of enzymes crucial to the proliferation process.

To explore the relationship(s) between regulation of intracellular Mg content and cell proliferation we have looked at possible differences of Mg efflux in normally proliferating *vs.* DMSO-differentiated non-proliferating HL-60 cells. (60). The most established mechanism for the control of intracellular Mg involves the so-called *Na/Mg antiport*, that is a plasma membrane system coupling Mg efflux with Na influx. The driving force for this exchange is furnished by the inwardly directed concentration gradient of Na (see *ad hoc* reviews in this volume). To detect Mg efflux accurately, HL-60 cells have been incubated in Mg-free media; under these conditions, usually referred to as *0-trans*, the appearance and detection of Mg in the supernatants reflect Mg efflux. In HL-60 cells, Mg efflux is driven primarily by the aforementioned *Na-Mg antiport*, since Mg efflux decreases by more than 50% after replacement of extracellular Na with choline-Cl or treatment of cells with imipramine, the most potent inhibitor of *Na-Mg antiport* (61). Undifferentiated HL-60 cells usually exhibit higher basal rates of Mg efflux than differentiated neutrophyl-like HL-60 cells; however, both cell types respond to cAMP stimulation with a 50-70% increase of Mg efflux. These results indicate that Mg efflux is regulated by intracellular cAMP and, therefore, point to the possible involvement of PKA in the regulation of *Na-Mg antiport*. Besides acting on the antiport directly, cAMP could also stimulate the release of Mg from intracellular stores, thus providing the antiport with more exchangeable Mg (62). Once again, these two mechanisms should not be viewed as mutually exclusive and could very well contribute to modify intracellular Mg.

In earlier studies in Ehrlich ascites cells and rat spleen lymphocytes, we had found that Mg efflux was significatively increased by several receptor-mediated stimuli, such as IFN-*alpha*, ATP and PGE1 (63,64). The mechanism underlying such receptor-mediated stimuli involves phospholipase-dependent release of arachidonic acid and consequent formation of prostaglandins by cyclo-oxygenase. PGE formed through this pathway can activate adenyl-cyclase, thus increasing cAMP which eventually stimulates the *Na-Mg antiport* (65) (figure 4).

Comparative analysis of parental *vs.* neutrophyl-like HL-60 cells has revealed that IFN-*alpha*, ATP and PGE1 cannot stimulate Mg efflux from the former but can stimulate Mg efflux by greater than 50% in the case of the neutrophyl-like HL-60 cells. These results suggest that differentiation and consequent growth arrest are accompanied by modification of the mechanisms which regulate Mg efflux. As a result, proliferating HL-60 cells do release more Mg after stimulation with cAMP, showing that the *Na-Mg antiport* retains intrinsic sensitivity to regulation by this agonist; however, the same cells *do not* respond to receptor-mediated stimuli which would generate cAMP through adenyl-cyclase. It follows that HL-60 cells present

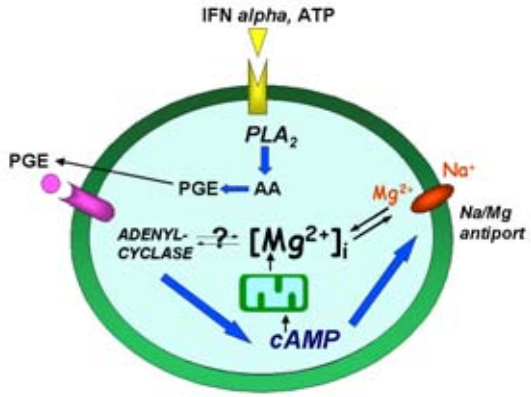


Figure 4: Intracellular regulation of Mg in cells that respond to receptor-mediated stimuli such as IFN-*alpha* and ATP. Receptor-mediated stimuli such as IFN-*alpha* and ATP elicit signal transduction which can involve elevation of $[Ca^{2+}]_i$, as in the case of ATP, or not, as in the case of IFN-*alpha*. Both signals activate phospholipaseA inducing arachidonic acid release from plasma membrane. Prostaglandins produced by arachidonic acid *via* cyclo-oxygenase, stimulate adenyl-cyclase which synthesize cAMP. *Na-Mg antiport* is stimulated by elevation of cAMP, likely by a PKA-dependent mechanism. Stimulation of Mg efflux influences $[Mg^{2+}]_i$, probably by releasing Mg from intracellular stores or binding site. It is possible, that $[Mg^{2+}]_i$ influences adenyl-cyclase thus down-regulating the same Mg efflux.

with high Mg content and resist to signals which otherwise would decrease *intracellular* Mg. Such anomalous behavior fits well into the concept that the uncontrolled growth of these malignant cells requires high Mg levels. In contrast, neutrophyl-like HL-60 cells contain less Mg, suggesting that Mg efflux may have been stimulated during the process of differentiation. As a matter of fact, these cells are sensitive to receptor-mediated stimuli which stimulate Mg efflux and consequently decrease *intracellular* Mg, as one would expect based on the assumption that low proliferation and low Mg usually coincide.

Several other reports have shown that ATP and IFN-*alpha*, alone or in combination with other agents, inhibit proliferation and/or induce differentiation of various cell types, although not all these studies have attempted to establish a correlation between these phenomena and changes in Mg movements. Studies in HL-60 cells have shown that IFN-*alpha* enhances the differentiating effects of retinoic acid (66); in the same cells, extracellular ATP triggers the cytosolic synthesis of cAMP (67) and induces cAMP-mediated differentiation (68). Similar observations have been extended to other cells. For example, ATP inhibits Friend erythroleukemia cell growth (69) and displays cytostatic effects in mice bearing Ehrlich tumor cells (70). These findings tentatively support our hypothesis that growth arrest, phenotypic differentiation or even cell death can occur after extracellular stimuli which increase Mg efflux.

The experiments described above indicate that (*i*) the increase of *intracellular* Mg observed in proliferating cells should be ascribed primarily to a sort of down-regulation of Mg efflux; and (*ii*) the main reason for such apparent down-regulation might be identified in a reduced responsiveness to receptor-mediated stimuli. Studies by Maguire and colleagues support this hypothesis, showing that differences in hormone sensitivity largely account for the different content and distribution of Mg in actively growing or resting S49 lymphoma cells (71). As regards HL-60 cells, Mattera *et al.* have shown that undifferentiated or differentiated elements exhibit different sensitivity to activation of phospholipaseA_2 by extracellular ATP (72). Such differences might have obvious consequences on ATP-influenced Mg movements.

Recent studies from our laboratory have shown that receptor-mediated stimuli can influence not only Mg efflux but also Mg mobilization from intracellular stores. In fact, we have found that IFN-*alpha* increases [Mg2+]i in differentiated HL-60 cells (60); inasmuch as IFN-*alpha* also stimulates Mg efflux under the same conditions, one cannot escape the conclusion that Mg efflux is accompanied by mobilization of Mg from intracellular stores, preventing net decrease of [Mg2+]i (figure 4). Similar responses are lacking in tumor cells, which do not release Mg nor mobilize Mg from bound pools. These observations point to an-in-depth modification of Mg homeostasis and show that tumor cells are refractory to stimuli which otherwise would trigger movements of this ion.

9. CAN WE STILL CONSIDER Mg AS A PHYSIOLOGIC CELL REGULATOR ?

A major scope of this review has been to show that Mg is required for several cell functions; hence, several homeostatic process have been evolved with the aim to constantly adapt Mg levels to physiopathologic events. On balance, we propose that biochemical and biologic evidence for the involvement of Mg in the regulation of cell cycle, proliferation, apoptosis or differentiation largely outweighs the uncertainties derived from the lack of specific or highly sensitive techniques to study Mg in intact cells. Perhaps more intriguingly, there is some evidence to suggest that Mg can regulate its own homeostasis and hence its intervention in biologic processes, as one would expect from an authentic cell regulator. This is best exemplified by the case of the complex interactions between Mg and adenyl-cyclase. We have extensively described that activation of adenyl-cyclase by receptor-mediated stimuli can control Mg efflux by stimulating the *Na-Mg antiport*. We now would like to emphasize that $[Mg^{2+}]_i$ *per se* can, in turn, regulate adenyl-cyclase activity; in so doing, Mg would eventually regulate its own efflux and hence total levels within the cell environment. Early studies by Maguire and colleagues have shown that Mg binds to guanine nucleotides at the cytoplasmic side of adenyl-cyclase, influencing the V_{max}. These authors also proposed a role for Mg in the hormonal regulation of adenyl-cyclase, showing that Mg participates in the formation of hormone receptor-cyclase complexes (71,73). The influence of Mg on adenyl-cyclase has been subsequently confirmed by elegant studies in L6E9

myoblasts. These cells provided the investigators with the unique advantage to compare high *vs.* slow proliferation and early *vs.* late differentiation simply by studying adenyl-cyclase at specific time intervals (1,3,6 and 10 days after seeding). In this model it was possible to establish that adenyl-cyclase activity undergoes different regulation at a post-receptor level, depending on the presence of specific subsets of G proteins and on the availability of Mg as an allosteric modulator. Such possibility was attested by changes in the affinity of both G proteins and Mg for adenyl-cyclase in cell cultures of different age, and hence of different proliferation and differentiation states (74). Thus, modifications of $[Mg^{2+}]_i$ might control cAMP synthesis and stimulation of Mg efflux in such a manner that Mg levels remain constantly adequate to the cell requirements (figure 4).

10. PERSPECTIVES

Technical difficulties in the measurements of intracellular Mg content together with heterogeneity of cell models and response have biased the possibility to better understand the complex mechanisms underlying *intracellular* Mg regulation. Nevertheless, there are compelling evidence to confirm that high *intracellular* Mg is correlated with proliferation rate of normal cells. Some evidence are given to suggest that receptor-mediated stimuli which inhibit cell proliferation induce a decrease of cell Mg, by activating Mg release from the cell. The possibility that Mg could control its own $[Mg^{2+}]_i$, by regulating adenyl-cyclase should be investigated in depth by more specific experimental protocols. In conclusion, we would like to reiterate that modulation of cell Mg homeostasis *parallels* the molecular control of cell proliferation, differentiation and death. More work has to be performed to better understand the regulation of *intracellular* Mg, its relation to intracellular compartments and to Ca. Studies of Mg homeostasis at cellular level and molecular characterization of Mg exchange systems should be encouraged. A deeper understanding of these mechanisms will be of great help to better define the molecular basis of the role of Mg in regulation of such important cell functions.

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