Magnesium and the control of cell proliferation: looking for a needle in a haystack

Federica I. Wolf, Valentina Trapani, Achille Cittadini

Istituto di Patologia generale e Centro di Ricerche Oncologiche Giovanni XXIII, Università Cattolica del Sacro Cuore, Facoltà di Medicina A. Gemelli, 00168 Roma, Italy

Correspondence: F. Wolf, Istituto di Patologia generale, Università Cattolica del Sacro Cuore, Largo Francesco Vito 1, 00168 Roma, Italy
<fwolf@rm.unicatt.it>

Abstract. Experimental evidence supports the important role of magnesium in the process of cellular proliferation, even though cell magnesium homeostasis is not completely clarified. We were the first to describe some molecular characteristics of the magnesium-dependent regulation of the cell cycle, and others proposed a mechanism for the magnesium-dependent regulation of protein synthesis occurring in the early phases of cell proliferation. We will attempt to relate such mechanisms with pathologic conditions whereby cell proliferation is severely disregulated, as in the case of tumors. It is interesting to note that recently some efforts have been made to correlate magnesium transport systems with its functions within the cells. Few, but stimulating new data are emerging which relate molecularly defined ion channels with magnesium homeostasis and its functions. Old and new data are now being merged and corroborated by diverse experimental approaches including molecular genetics, proteomics, electrophysiology and biochemistry. This, together with the development of new techniques to measure cell magnesium content and distribution, will hopefully pave the way to unravel the intimate mechanisms of such an essential though undefined metabolic regulator. New and deeper appreciation of magnesium homeostasis will help in delineating the key role of this cation in the regulation of normal or pathologic cell proliferation.

Key words: cell cycle, p53, oxidative stress, oxidative DNA damage, 8-OHdG, tumor cells, Mg fluxes, TRPM7

Cell proliferation is a complex phenomenon, which is initiated by receptor-mediated mitotic signals triggered by hormones or growth factors, followed by phosphorylation-based signal transduction, which leads to activation of transcription factors. The consequent transcription of regulatory molecules affects cell cycle progression and eventually leads to mitosis. Theoretically, magnesium (Mg, referring to both ionized and bound forms) might reasonably be involved in every single step of this intricate though well-organized process: from receptor-mediated mitotic signals and transphosphorylation reactions to gene transcription and protein synthesis occurring prior to cell division [1]. Subsequently, Mg is implicated in DNA duplication, as polymerases and ligases require Mg-ATP2. Finally, Mg plays a role in the cytoskeleton re-arrangement leading to the formation of the mitotic spindle and cytokinesis.

Due to this plethora of functions, it is very complicated to define “how” and “when” Mg availability regulates cell proliferation. Experimental evidence clearly indicates the following:
– growing cells contain more Mg than resting ones [2];
– cell Mg required to sustain proliferation can be retrieved, irrespective of extracellular Mg availability.

Magnesium and cell proliferation: data from cell cultures

In the 1980s several investigators described the essential role of Mg in the proliferation of yeast and mammalian cells [5, 6]. Harry Rubin postulated the theory of “the coordinated control of cell proliferation”, and proposed that Mg was the key factor regulating the different steps of this complex process [6]. Rubin described the relationship between some steps of cell proliferation (growth rate, thymidine and uridine incorporation etc.) and the availability of Mg. These observations provided the conceptual foundations for probing the involvement of Mg in the different phases of the cell cycle. The first pieces of evidence linking Mg availability to cell cycle were described in yeast cells. It was shown that incubation of yeast cells in low Mg concentrations causes growth arrest, resulting in an increased percentage of cells in the G0/G1 phase, and a decreased percentage of cells in the S phase [5].

When dealing with cell proliferation in vitro, one should first consider the proliferative behaviour of the cell population under study. Primary cultures of diploid cells are characterized by a limited capacity of cell division depending on cell type, e.g. few passages in lymphocytes, dozens of passages in fibroblasts and even more in endothelial cells. However, most laboratory studies, are carried out on stabilized cell strains. In this case, cell populations are usually immortalized by transfection with SV40 or other retroviruses; immortalization allows use of the same cell population for a theoretically unlimited time. Alternative to immortalized strains are transformed cells, which are per se immortalized as well as carcinogenic if transplanted in nude mice. In this case the control of cell proliferation is impaired due to the expression of several oncogenes and the loss of function of tumor suppressor genes, such as p53, the leading modulator of cell cycle progression [7].

Different cellular types vary in their dependence on the extracellular Mg availability as to their proliferation rate. In general, diploid cells proved to be highly sensitive to changes in Mg availability: endothelial cells and fibroblasts decreased their growth rate to a considerable extent when the cells were maintained in 0.1 mM extracellular Mg [8-10]. Immortalized cells, such as HC11 mammary epithelial cells, usually grown in 0.8 mM extracellular Mg, were less sensitive and showed a 50% growth inhibition only upon exposure to 0.05 mM Mg [11]. Tumor cells proved to be the most resistant ones; for example, HL-60 leukaemia cells, MCF7 mammary carcinoma cells and Ehrlich ascites tumor cells showed an unaltered growth rate at 0.05 mM extracellular Mg [3, 11, 12]. However, most cells can adapt to non-physiological concentrations of extracellular Mg, as shown by the chronic adaptation to very high or very low Mg containing media [13].

High Mg availability, as obtained by increasing Mg concentration in the extracellular medium, accelerates cell proliferation, especially in diploid cells [14, 15]. When cells are Mg-depleted, the re-addition of Mg causes a rapid and substantial rise in the proliferation rate, suggesting that cell cycle arrest is reversible [16]. Nevertheless, a chronic adaptation to an increased extracellular Mg neither causes a substantial increase in the proliferation rate nor leads to a significant increase of cell Mg content [2]. The comparative study of the relationship between Mg availability and proliferation rate in cells characterized by different growth characteristics should help in delineating the mechanisms underlying Mg-dependent cell growth.

In the attempt to investigate how Mg could regulate cell proliferation at the molecular level, we showed that incubation in low Mg medium triggers the up-regulation of the cell cycle inhibitor p27kip1 both in immortalized and in transformed cells [11, 12]. Following our observations, other authors found Mg-triggered regulation of cell cycle inhibitory proteins in diploid cells, including p21cip1 in endothelial and smooth muscle cells [8, 14], and p21cip1 and p16ink4a in fibroblasts [9]. Further data supporting the capability of Mg to influence the cell cycle were obtained by DNA expression profiling. In mammalian epithelial cells, it was found that a low Mg content in the growth medium causes up-regulation of p53, as well as of junonji and numblike, two newly identified negative modulators of cell proliferation [18-20]. In parallel, cyclin D and F and the transcription factor E2F, which promote cell cycle progression by
activating cyclin-dependent kinases and transcription of S phase-specific genes, respectively, were down-modulated. Although no changes in proliferation rate and cell Mg content can be detected in mammary epithelial cells adapted to grow in high Mg-containing medium, RNA arrays uncovered the up-regulation of several cell cycle-related genes, specifically cyclin F, and ETS-related transcription factor.

Molecular mechanisms of Mg-dependent cell growth

Rubin proposed that mTOR, a phosphatidylinositol 3-kinase (PIK)-related kinase which initiates protein synthesis, might be the key activity modulating G1 protein synthesis [21]. This kinase is characterised by a $K_m$ for ATP of 1.0 mM, which is 50-100 times greater than that of most protein kinases. One millimolar would be close to the concentration of Mg within the cell, but one should also consider that Mg-ATP$^\text{2-}$ is the only active form of ATP. It follows that the limiting step of this specific kinase reaction would be the full availability of Mg to form Mg-ATP$^\text{2-}$ at a concentration close to 1 mM [21].

A signalling cascade linking proliferation to Mg has been described in some detail in smooth muscle cells grown in high Mg containing media. In this model a 2 mM Mg concentration was found to promote cell cycle activation by up-regulation of cyclin D1 and Cdk4 and down-regulation of p21$^{\text{cip1}}$ and p27$^{\text{kip1}}$. DNA and protein synthesis increased approximately threefold. It was shown that cell cycle stimulation occurs via the specific activation of an ERK1/2-dependent pathway [14].

A common feature in the Mg-dependent control of cell growth is the modulation of cell cycle inhibitory proteins such as p27$^{\text{kip1}}$ and p21$^{\text{cip1}}$ [8, 11, 12]. We described in detail the time-dependent and concentration-dependent up-regulation of p27$^{\text{kip1}}$ in HC11 mammary epithelial cells in Mg-deficient media, as well as the inhibition of p27$^{\text{kip1}}$ expression under the opposite conditions (i.e. 45 mM extracellular Mg) [11]. These data led us to suggest that the extracellular Mg availability can “directly” affect the cell cycle by influencing the transcription of related genes. At present, though, we do not know the complete molecular pathway whereby low extracellular Mg can affect the expression of cell cycle inhibitory proteins.

The relationship between Mg and oxidative stress has been a matter of investigation in the last few decades. Since it is well accepted that low Mg triggers oxidative reactions, we reasoned that in diploid cells oxidative stress may mediate the p21$^{\text{cip1}}$- or p27$^{\text{kip1}}$-dependent inhibition of cell proliferation via up-regulation of p53. A possible pathway is that p53 is activated by oxidative DNA damage. Indeed, DNA damage, via a DNA-kinase, is able to induce phosphorylation of p53 and activation of its transcriptional activity. If so, we should be able to demonstrate that low Mg induces: 1) the formation of reactive oxygen species (ROS); 2) an increase in oxidative DNA damage; 3) an increase in p53 activity, all coupled to the increased expression of cell cycle inhibitory proteins (p21$^{\text{cip1}}$ or p27$^{\text{kip1}}$). In our hands, a direct correlation between low Mg availability and the formation of ROS was difficult to be demonstrated in vitro. Some correlation between ROS and low Mg was found only in endothelial cells stimulated with $\text{H}_2\text{O}_2$. In this specific circumstance, dichlorofluorescein (DCF)-detectable ROS slightly and transiently increased in the first hours of Mg depletion. Surprisingly, however, DCF-detectable ROS tend to decline after 24h of Mg depletion, remaining lower than in control conditions [22].

Indirect evidence of low Mg-induced oxidative stress may be extrapolated by other intracellular signals, such as the up-regulation of NFKB, a transcription factor that is activated by oxidative stress-related signals [23], found in several Mg-deprived conditions [24]. Conversely, the addition Mg to endothelial cells prior to a pro-inflammatory stimulus inhibits endothelial cell activation, via NFKB, supporting the hypothesis that Mg treatment may function as an anti-inflammatory agent during specific circumstances as preterm labor [25]. Other studies on thymocytes from rats grown under Mg deficiency suggest that Mg availability might affect the expression level of stress proteins, other potential, though indirect, triggers of the cell cycle inhibition [26].

Even though in mammary epithelial cells no correlation was found between intracellular DCF-detectable ROS and Mg availability, other pieces of evidence suggest that low Mg triggers a cellular response that may be ascribed to oxidative stress. In these cells, but interestingly also in other Mg-depleted conditions, gene profiling showed an up-regulation of the detoxifying enzyme glutathione S-transferase (GST) which also acts as a ROS scavenger. Indeed, we have recently demonstrated that GST activity correlates with Mg availability. We showed that in HC11 cells GST enzyme activity increased significantly 48 hours after Mg removal from the extracellular medium. Conversely, the addition of Mg to Low-Mg cells reduced GST activity to control
Magnesium fluxes and cell proliferation

Incubation of cells in low Mg-containing media should determine sizeable modifications of Mg pools inside the cells. Finding direct evidence of such a modulation of free cytosolic Mg by the use of specific fluorochromes has been very frustrating. Nevertheless, we know that many cell types respond to an incubation in Mg-free media by activating a measurable Na-dependent Mg efflux, as shown for example in [30 and see 31 for a review]. Mg efflux somehow should perturb intracellular Mg pools possibly affecting Mg-dependent enzymatic activities. There is limited experimental evidence to explain how the intracellular distribution of Mg is affected by removing extracellular Mg. Interestingly, some experimental data suggest that changes in bound pools might result more relevant than changes in free pools [11, 32]. This is due to the peculiarity of cell Mg homeostasis which is very dissimilar from that of calcium (Ca). Indeed, it should be underlined that Mg distribution between the intra- and extracellular milieu is driven by an electrochemical gradient which tends to take up Mg into the cell, in the absence of a large chemical gradient as in the case of Ca. Furthermore, it has been clearly shown that in cardiomyocytes, hepatocytes and vascular smooth muscle cells, Mg fluxes can be modulated by receptor-mediated stimuli such as hormones or growth factors [33, 34].

A direct proof of a causal relationship between EGF-induced cell proliferation and Mg fluxes is limited to a study on BGH1 myocytes where an increase in free cytosolic Mg, measured by the fluorochrome mag-fura2, was associated to EGF-stimulated thymidine uptake [35]. Another piece of evidence that intracellular free Mg may be modulated during cell proliferation is that described in smooth muscle cells incubated in high Mg medium [14]. The difficulties in obtaining relevant data associating cytosolic Mg with cell stimulation are probably due also to the lack of specific and sensitive methods to trace intracellular Mg movements.

The effect of decreased extracellular Mg availability on cell proliferation may be alternatively ascribed to a mechanism similar to a ion-sensing receptor or to a direct modulation of ion-specific channels [36]. The recently identified inward transporters of Mg, namely TRPM6 and TRPM7 ion channels, possess unique structural characteristics [37]. The cytosolic C-terminus of TRPM6 and TRPM7 shows an α-kinase activity that has inspired the definition of this class of channels as “Chanzymes” [38]. The possibility that these proteins may act both as channels and kinases suggested that they might play a role in the regulation of Mg homeostasis and related cell functions [39]. The activity of TRPM6/7 seems to be modulated by Mg, whether in the form of a Mg-ATP complex or in the form of a Mg²⁺ ion that becomes available inside or outside of the cell [38]. However, the most exciting aspect is the potential contribution of the channel-associated kinase activity to the transport of Mg and, in particular, to the regulation of cell signals and functions. Recent data suggest that the TRPM7 channel is regulated by changes in cyclic AMP and protein kinase A activity [40]. Such activation of Mg influx is quite surprising, since the Mg efflux through the Na/Mg antiporter was also found to be activated by cAMP and PKA signals [30, 31]; however, as long as the molecular structure of this outward transporter is not clarified, this apparent contradiction cannot be solved. Besides autophosphorylation, the TRPM7 kinase domain seems to be involved in the phosphorylation of other target proteins including annexin and the myosin IIA heavy chain [40]. An elegant recent work investigated the modulation of TRPM7 and the calcium release-activated calcium current (I_{Ca, rAC}), whose activity is important for refilling intracellular Ca stores, during the cell cycle progression in mast cells [41]. Results show that, while I_{Ca, rAC}-dependent Ca transport is tightly modulated during different phases of the cell cycle, TRPM7 seems to be up-regulated only during the G₁ phase. This observation is interesting from two points of view: 1) it underscores the substantial difference between intracellular regulation of Ca and Mg and their consequent role, as acute vs. chronic regulators of cell functions, respectively [42]; 2) the increase of Mg influx in G₁ is consistent with the modulation of cell cycle regulatory proteins (cyclin D1, Cdk4,
Mg availability during protein synthesis [6, 21].

consistent with Rubin's hypothesis on the need of high and mammary epithelial cells [16, 13] and also con-
in high or low Mg conditions, i.e. smooth muscle cells

animal studies

Magnesium and cell proliferation: data from animal studies

One of the major concerns of the effect of Mg on cell proliferation in vivo involves tumor development. The contribution of Mg availability to tumor growth in vivo has been much debated, but both experimental and epidemiological evidence is very fragmentary and sometimes contradictory [4 see for a review]. In the attempt to better define the effect of Mg availability on tumor growth we have developed an experimental model of Mg deficiency in mice that have been subcutaneously injected with cells from solid tumors. We have shown that a Mg-deficient diet reversibly inhibits the growth of primary tumors from Lewis lung carcinoma (LLC), 16/C mammary adenocarcinoma and C38 colon carcinoma cells. The re-addition of Mg to the diet led to rapid growth of tumor mass, which reached a larger size than that grown in control conditions. Interestingly, in the case of LLC cells, low Mg availability, while reducing primary tumor size of about 60%, increased the number of lung metastatic foci [16]. To get insights into the mechanisms involved in the inhibition of primary tumor growth in Mg deficient mice, we evaluated the impact of low Mg not only on tumor growth and development, but also on some pivotal events of tumor development, i.e angiogenesis, and metastatization, by taking advantage also of gene expression analysis by cDNA array. Most effects of Mg availability described in vitro were confirmed in tumors grown in vivo. In particular, 1) tumors from Mg-deficient mice were G0/G1 arrested; 2) p27kip1 and p21cip1 cell cycle inhibitory proteins were up-regulated in tumors from Mg deficient-animals; 3) the presence of 8-hydroxydeoxyguanosine (8-OHdG), the most relevant indicator of DNA oxidative damage, was higher in tumors from Mg-deficient mice compared to tumors from animals on a normal diet [46]. In principle, low extracellular Mg could induce oxidative DNA damage by two different mechanisms: first, by increasing intracellular ROS; second, by influencing DNA repair mechanisms [47, 48]. Indeed, the two effects are not mutually exclu-
sive. In mice, a low-Mg diet induced a relevant oxidative status, which can account for a sizable increase of oxidative DNA damage. In fact, hypomagnesiaemia in vivo induces inflammation, cytokine production and activation of phagocyte oxidative burst; these features may well account for a relevant oxidative damage [17, 49, 50, 51 for a review].

In tumor tissues, oxidative DNA damage could therefore be responsible for the observed growth arrest. Were this the case, the mechanism could not be ascribed to a p53-related up-regulation of p27kip1 and p21cip1, as postulated previously for diploid or immortalized cells. In fact in LLC, a highly undiffer-
entiated neoplastic cell line, p53 tumor suppressor function is likely inactivated. One may hypothesize an alternative redox-mediated mechanism of growth arrest yet to be identified. On the other hand, low Mg-dependent growth arrest may account for the decreased vascularization of tumors grown in Mg-deficient animals. Impairment of angiogenesis under low Mg availability has been previously described [52].

In striking contradiction to low Mg-dependent growth arrest, we found that, in tumor-bearing mice under Mg-deficient diet, lung metastatic foci were more abundant than in control ones. We believe that in these specific circumstances the immuno-inflamatory response to low Mg, as suggested also by cDNA arrays of lung tissue from mice under a Mg-deficient diet, is primarily responsible for creating a condition favourable to extravasation and implantation of circulating tumor cells [46, 53].

Concluding remarks

Magnesium availability can affect cell proliferation to a variable extent: diploid cells are more dependent on extracellular Mg availability than immortalized or transformed ones. Irrespective of the proliferative behaviour of the cell studied, low Mg-induced growth arrest was found to be associated with up-regulation of p16ink4a, p21cip1 and p27kip1 inhibitors of cell cycle progression. Low Mg-induced oxidative stress is at present the best candidate for triggering
Figure 1. Possible mechanism of low Mg-dependent growth arrest in diploid cells. Oxidative stress-mediated up-regulation of cell cycle inhibitory proteins through a p53-dependent mechanism or through other pathways such as NFkB, stress signals or gene expression.

Figure 2. Possible mechanism of low Mg-dependent growth arrest in immortalized or transformed cells. The oxidative stress-mediated signals cannot be ascribed to p53 activation, but to other pathways where low Mg may affect directly or indirectly up-regulation cell cycle inhibitory proteins.
MAGNESIUM AND CELL PROLIFERATION

such an anti-proliferative signal; however, different pathways may be involved depending on whether the cells are diploid, immortalized or transformed. Figures 1 and 2 summarize the proposed mechanisms of low Mg-dependent regulation of cell proliferation in normal and immortalized/transformed cells. The effects of low Mg availability on cell proliferation in vivo are complicated by several associated mechanisms, first and foremost the low-Mg induced immuno-inflammatory response, which triggers cytokine production, oxidative stress, and endothelial dysfunction, which all participate to low Mg-related effects.

More experimental models and investigative tools are necessary to find the needle in the haystack of the mechanism whereby low Mg availability affects cell proliferation, a phenomenon that is particularly crucial in tumor development but may be also involved in other pathophysiologic conditions that are associated with altered Mg availability as, for example hypertension, diabetes and neuronal injury.

Acknowledgments

This work was supported by MIUR 60% and linea D1 2004-2007. The collaboration with Colleagues from INRA, Clermont-Ferrand and Università di Milano is greatly acknowledged (Collaborative Linkage NATO grant to AM, JAM and FIW 2002-04).

References


19. Toyoda M, Kojima M, Takeuchi T. Junonii is a nuclear protein that participates in the negative regulation of


