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ARTICLES

Magnesium Restriction Induces Granulocytic Differentiation and Expression of P27^{KIP1} in Human Leukemic HL-60 Cells

Valeria Covacci, Nicodemo Bruzzese, Alessandro Sgambato, Arianna Di Francesco, Matteo A. Russo, Federica I. Wolf,* and Achille Cittadini

Institute of General Pathology, ''Giovanni XXIII Cancer Center'', School of Medicine, Catholic University, Rome, Italy

Abstract When cultured in Mg restricted medium, human leukemic HL-60 cells develop morphological and functional granulocytic differentiation. In 0.03 mM Mg, cells display the distinctive features of differentiation, without appreciable inhibition of proliferation. In 0.01 mM Mg, cells show terminal differentiation, accompanied by clear inhibition of proliferation. Such cells accumulate in the G0/G1 phase and subsequently die via apoptosis, similar to HL-60 cells that have been induced to differentiate by DMSO. These phenotypic changes are associated with a marked increase in the expression level of the cyclin dependent kinase inhibitor p27^{Kip1}. Cyclin E expression is also slightly increased in Mg restricted cells, whereas no changes are observed in the expression level of cyclin D1. We also show that during differentiation cell total Mg decreases, whereas $[Mg^{2+}]_i$ increases in both Mg-depleted and DMSO-treated cells. These data suggest that the maturation process is paralleled by a redistribution of intracellular Mg, leading to a shift from the bound to the free form. These changes could modulate the kinetics of Mg-dependent enzyme(s) that are involved in the control of the differentiation pathway. We propose that this model may represent an useful tool for the study of the mechanisms of cell differentiation and related events, such as aging and death. J. Cell. Biochem. 70:313–322, 1998.

Key words: proliferation; cell cycle; apoptosis; cyclins; p27^{Kip1}; cell magnesium; CD11b; myeloid differentiation; HL-60 cells

Mg, the second most abundant cation in mammalian cells, is involved in the regulation of a variety of biochemical reactions and biological activities, making it essential for life [Cowan, 1995; Hickie et al., 1983]. While the requirement for this cation has been fully investigated in vitro, little is known on the mechanisms whereby it regulates complex processes in cells. This is due to the lack of simple and direct methods for the study of Mg in living cells [Romani et al., 1992].

There is some evidence to suggest that Mg has a pivotal role in the processes of cell proliferation, differentiation, and aging. When normal cells become transformed they escape the regulation by Mg [McKeehan et al., 1980]. While tumor cells are independent from extracellular Mg, a variety of normal cell types requires elevated Mg concentration to maintain high rates of proliferation [Maguire et al., 1988; Ribeiro et al., 1984]. As compared to their normal counterpart, several neoplastic cells may nevertheless exhibit higher levels of intracellular Mg [Rubin, 1982a]. Furthermore, it has been described that spontaneously or virus transformed fibroblasts and other cells grown under Mg restriction may rapidly assume mor-

Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; $[Ca^{2+}]_i$, intracellular ionized calcium; BSA, bovine serum albumin; CDK, cyclin dependent kinase; DMSO, dimethylsulphoxyde; EDTA, ethylendiaminetetraacetic acid; FCS, fetal calf serum; HPLC, high performance liquid chromatography; IFNγ, interferon gamma; Mg, magnesium; $[Mg^{2+}]_i$, intracellular ionized magnesium; PBS, phosphate buffer saline; PMA, phorbol myristate acetate; ROS, reactive oxygen species; SDS-PAGE, polyacrilamide gel electrophoresis in the presence of sodium dodecyl sulphate.

Matteo A. Russo's permanent address is Department of Experimental Medicine and Pathology, Università "La Sapienza," Rome, Italy.

*Correspondence to: Federica Wolf, Institute of General Pathology, School of Medicine, Catholic University, L.go F. Vito,1, 00168 Rome, Italy. E-mail: ibipg@rm.unicatt.it

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phological and functional characteristics of differentiated cells [Rubin, 1981, 1982b; McKeehan et al., 1978; Hoftiezer et al., 1985]. Dietary restriction of Mg and cell Mg deficiency have also been shown to accelerate aging both in vivo and in vitro [Günther, 1991; Costello et al., 1992; Calviello et al., 1993].

While the molecular mechanisms of cell differentiation remain largely unknown, this process associates with several morphological and functional events varying from a loss of proliferation to remarkable changes in several proto-oncogenes mRNA levels and cell surface antigens [Collins, 1987].

Cell differentiation is usually associated with exit of the cells from the cell cycle. Cell cycle progression in mammalian cells is regulated by a family of enzymes known as cyclin-dependent kinases (CDKs) whose activity is dependent on the binding to specific regulatory subunits called cyclins. The activity of the cyclin/CDK complexes is negatively regulated by specific CDK inhibitors like $p16^{Ink4}$, $p21^{Waf1}$, and $p27^{Kip1}$ [Sherr et al., 1995b]. The mechanisms regulating cell cycle have a fundamental role in the control of cell proliferation and may also be involved in differentiation.

In the light of our interest in Mg homoeostasis and related functions, we have attempted to characterize the role of Mg in cell proliferation and differentiation. To this aim we have used the human leukemic HL-60 cell line since this is one of the most versatile model for the study of cell differentiation [Mishak et al., 1993; Collins et al., 1978]. Several compounds can in fact be used to promote differentiation of this cell line including anti-cancer agents, natural effectors such as retinoic acid and vitamin D_3, corticosteroids, prostaglandins, phorbol esters, Na-butyrate, and some polar solvents such as DMSO [Sawyres et al., 1991; Bloch, 1984].

Our data demonstrate that restriction of extracellular Mg induces granulocytic differentiation of HL-60 cells. When cultured in $\leq$ 0.03 mM Mg these cells exhibit the typical morphological and functional properties of differentiated cells as those observed after treatment with DMSO. Severe Mg restriction is accompained by terminal differentiation and cell death via apoptosis and these processes resemble those elicited by DMSO. Under Mg restriction, phenotypic changes are associated with a marked increase in the expression level of the CDK inhibitor $p27^{Kip1}$, suggesting a possible relationship between cell cycle control mechanisms and the differentiation process. Interestingly, HL-60 cells differentiated either by Mg restriction or DMSO show a decrease of total cell Mg, corresponding to a similar modification of Mg intracellular distribution. This indicates that changes in Mg content and distribution account for a common pathway of differentiation obtained under diverse experimental conditions.

MATERIALS AND METHODS

Cell Culture

Human promyelocytic leukemia HL-60 cell line, obtained from the Istituto di Ricerche Farmacologiche "Mario Negri," was maintained in log phase by seeding twice a week at density of 3×10^5 cells/ml. Cells were routinely cultured in RPMI 1640 medium (Bio Whittaker, Verviers, Belgium) supplemented with 10% heat-inactivated FCS (Biological Industries, Kibbutz Beit Haemek, Israel) and 2 mM glutamine in 95% air/5% CO_2 atmosphere at 37°C. As to Mg deficiency, cells were cultured in a Mg-free RPMI 1640 (Gibco, Grand Island, NY), supplemented with known amounts of $MgSO_4$. Mg-free FCS was prepared by dialization, with subsequent addition of 1.8 mM $CaCl_2$, according to Maguire [1988]. HL-60 cells were cultured in 0.03 mM Mg for 7 days and used thereafter. To achieve severe Mg restriction, HL-60 cultured in 0.03 mM Mg were transferred to 0.01 mM Mg and utilized on the 4th day.

Cell Proliferation, Differentiation, and Apoptosis

Cell number and viability were determined by cell counting in a Burker hemocytometer and by the trypan blue exclusion test, respectively. HL-60 granulocytic differentiation was also induced by 1.3% DMSO and cells were used on the 7th day according to Collins et al. [1978]. Light microscopy was performed on cytospin slides stained with May-Grunwald/Giemsa. For electron microscopy 4×10^6 HL-60 cells were fixed in 2.5% glutaraldehyde in PBS and post-fixed in osmium tetroxide 1.33% in PBS. A standard schedule was followed for further processing until the embedding in an Epoxy resin. Ultra thin sections were stained with uranil acetate and lead hydroxide and studied in a Philips CM-12 electron microscope.

Cell cycle was analyzed by flow cytometry. Aliquots of 10^6 cells were harvested by centrifu-

gation, washed in PBS, fixed with ice cold 70% ethanol and treated with 1 mg/ml RNase for 30 min. Propidium iodide was added to a final concentration of 50 mg/ml. Data were collected, stored and analyzed using the Multicycle software.

HL-60 cells oxidative burst was measured as ROS production by luminol amplified luminescence under basal, PMA, and Zymosan-stimulated conditions [Scatena et al., 1996].

Cell antigenic profile was characterized by FACScan flow cytometry. 10^6 viable cells were incubated for 30 min at 4°C with CD13, CD33, CD11b, CD16, and CD14 monoclonal antibodies specific for the monocyte and granulocyte lineage (Coultronics, Margency, France and Becton Dickinson, San Jose, CA), washed twice to remove excess unbound antibodies and analyzed by a Epics Profile II.

Apoptosis was quantified by scoring the morphological features of nuclear picnosis and chromatin condensation on acridine orange stained cells. At least 300 cells for each condition were counted. Double blind examination was routinely performed.

Protein Extraction and Immunoblotting

Proteins were extracted from exponentially growing cells and were subjected to Western blot analysis as described previously [Sgambato et al., 1996]. Total cell lysates (50 mg) were electrophoresed by SDS-PAGE and then transferred to immobilon-P membranes (Millipore, Bedford, MA). Different dilutions were used for different primary antibodies as suggested by the suppliers. The polyclonal antibodies to cyclin D1 and E were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The polyclonal antibody to $p27^{Kip1}$ was from Santa Cruz Biotechnology (Santa Cruz, CA). Immunodetection was performed using the enhanced chemiluminescence kit for western blotting (Amersham, Arlington Heights, IL). Identical results were obtained when distinct protein extracts were tested with the same antibodies.

Cation and Adenine Nucleotide Determinations

HL-60 cell total Mg content was determined by atomic absorption spectrometry on acid extract (0.1 N HNO_3) of cell pellets, according to Wolf et al. [1997]. On similar samples H_2O was measured by gravimetric determinations. $[Mg^{2+}]_i$ and $[Ca^{2+}]_i$ were measured by differential spectrometry on Mag-fura 2 AM and Fura AM loaded cells, respectively [Wolf et al., 1996]. Adenine nucleotides were analyzed by HPLC [Stocchi et al., 1985].

Chemicals and Data Analysis

Proteins were measured by the Bio-Rad assay system using BSA as standard. All chemicals and reagents were of analytical grade. Deionized, double distilled water (<18 MΩ) was used throughout.

Data are presented as mean ± SE (number of experiments). Where vertical bars are not visible, SE falls within the line. Statistical analysis has been calculated by Student's *t*-test of unpaired data with significance being considered when $P < 0.05$.

RESULTS

Cell Proliferation and Differentiation

Figure 1 reports the growth rate of HL-60 cells cultured in control (0.5 mM Mg) and Mg deficient media. It can be observed that between 0.5 and 0.03 mM Mg there is no significant difference in cell number after 72 and 96 h incubation. On the contrary, 0.01 mM Mg causes marked reduction of cell number corresponding to a growth inhibition of about 70% at 96 hours. Cell viability remains higher than 95% in all conditions tested. Noteworthy, cells could be cultured in 0.03 mM Mg indefinitely, whereas in 0.01 mM Mg cell cultures exhaust after 6–7 days.

Cell cycle analysis of control, Mg-deprived, and DMSO differentiated HL-60 cells is reported in Figure 2. Cells cultured in 0.03 mM Mg display a distribution similar to that of control cells as much as S phase is about 27% and 25%, respectively. In HL-60 cultured in 0.01 mM Mg and in 1.3% DMSO, S phase significantly decreases to about 6% and cells accumulate in G0/G1, indicating a drastic reduction of cell proliferation.

HL-60 cells from Mg deficient media, observed by phase contrast microscopy, show evident morphologic changes. In contrast to the control, which has mostly a round shape, these cells appear reduced in volume with irregular cell boundaries. Figure 3a,b shows that HL-60 cells cultured in 0.03 mM Mg display a decrease of N/C ratio and clear nuclear segmentation that is typical of cell maturation along the granulocytic pathway. Electron microscopy (Fig. 3; 1–3) evidentiates the presence of cytoplasmic

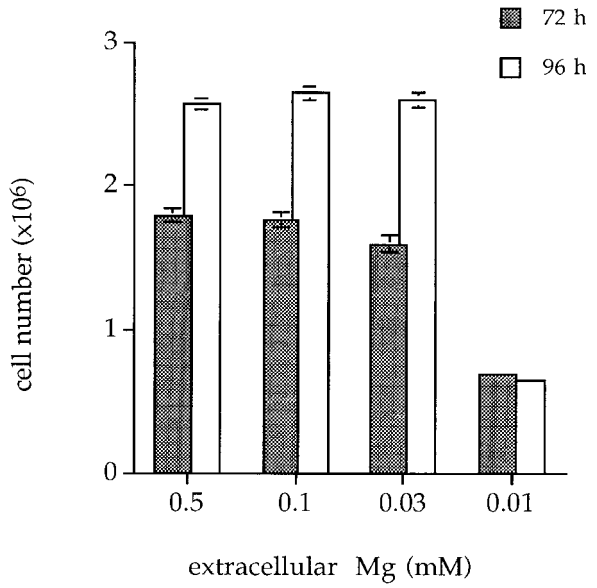

Fig. 1. Growth rate of HL-60 cells cultured under Mg restricted conditions. Cells were counted on 72 and 96 h after seeding. Viability was >95% in all conditions. At concentration from 0.5 to 0.03 mM Mg cell number is similar, while it sharply decreases at 0.01 mM Mg. Results are means ± SE of six different experiments.

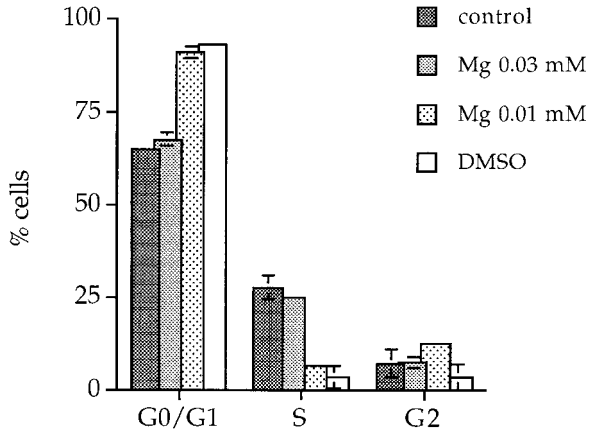

Fig. 2. Cell cycle distribution of HL-60 cultured in Mg restricted media or in DMSO. The cells grown in control and in 0.03 mM Mg display similar cell cycle distribution with S phase of about 25%. In 0.01 mM Mg and in 1.3% DMSO, HL-60 cells show cell cycle arrest in G0/G1. Results are mean ± SE of three separate experiments. Further technical details are under Materials and Methods.

granules exclusively in cells grown under Mg restriction. Most of these resemble the specific (secondary) granules, while a few appear rounded or football shaped, similar to the azurofilic (primary) granules. An immunochemical characterization will be necessary to better characterize these granules. The incubation under Mg restriction induces numerous changes also in the nuclear and cytoplasmic compartments. In particular, chromatin is more aggregated and nucleolus is single and well organized. The cytoplasm appears decreased, but richer in organelles, as mitochondria, Golgi apparatus and segments of rough endoplasmic reticulum.

In order to detect a specific functional differentiation we analyzed HL-60 cell superoxide-anion production by chemiluminescence assay. Figure 4 shows that HL-60 cells cultured in 0.03 and 0.01 mM Mg significantly increase their production of ROS after PMA and Zymosan treatment. The stimulation is of about 50% with PMA and 100% with Zymosan in the cells cultured in 0.03 mM Mg and it is even higher in the cells cultured in 0.01 mM Mg. Hence, both morphological and functional evidences suggest that Mg restriction leads to HL-60 granulocytic differentiation.

Table I reports the expression of CD11b, the cellular surface antigen specific for granulocytes, in HL-60 cells cultured in 0.5, 0.03, and 0.01 mM Mg as well as in 1.3% DMSO. While control cells show a CD11b reactivity of 3.6%, cells cultured in 0.03 and 0.01 mM Mg increase CD11b expression up to the 62.5% and 75%, respectively, similar to cells treated with DMSO (76.7%). It is interesting to note that Mg-depleted cells lose the acquired CD11b antigen upon addition of 0.5 mM Mg for 4 days (from 62.5% to 6.8%), suggesting that differentiation obtained by Mg restriction is reversible. CD14, which is specific for the monocyte/macrophage lineage, is expressed by about 1% in all the conditions tested, confirming that Mg restriction induces HL-60 cell differentiation toward granulocytes.

The major regulatory events leading to mammalian cell proliferation and differentiation occur in the G1 phase when cells decide whether to proceed through the cell cycle or undergo differentiation. To obtain insights into the molecular events associated with the phenotypic changes observed in Mg restricted HL-60 cells, we evaluated the expression levels of the major G1 cyclins, i.e. cyclins D1 and E, and of the CDK inhibitor $p27^{Kip1}$ in both Mg deprived and control cells. Figure 5 shows that the expression level of cyclin D1 is not affected by extracellular Mg while cyclin E expression increases at low Mg concentration. Under this latter condition, a marked increase in the level of expression of $p27^{Kip1}$ is also observed. As shown in Figure 5, this increase is directly correlated

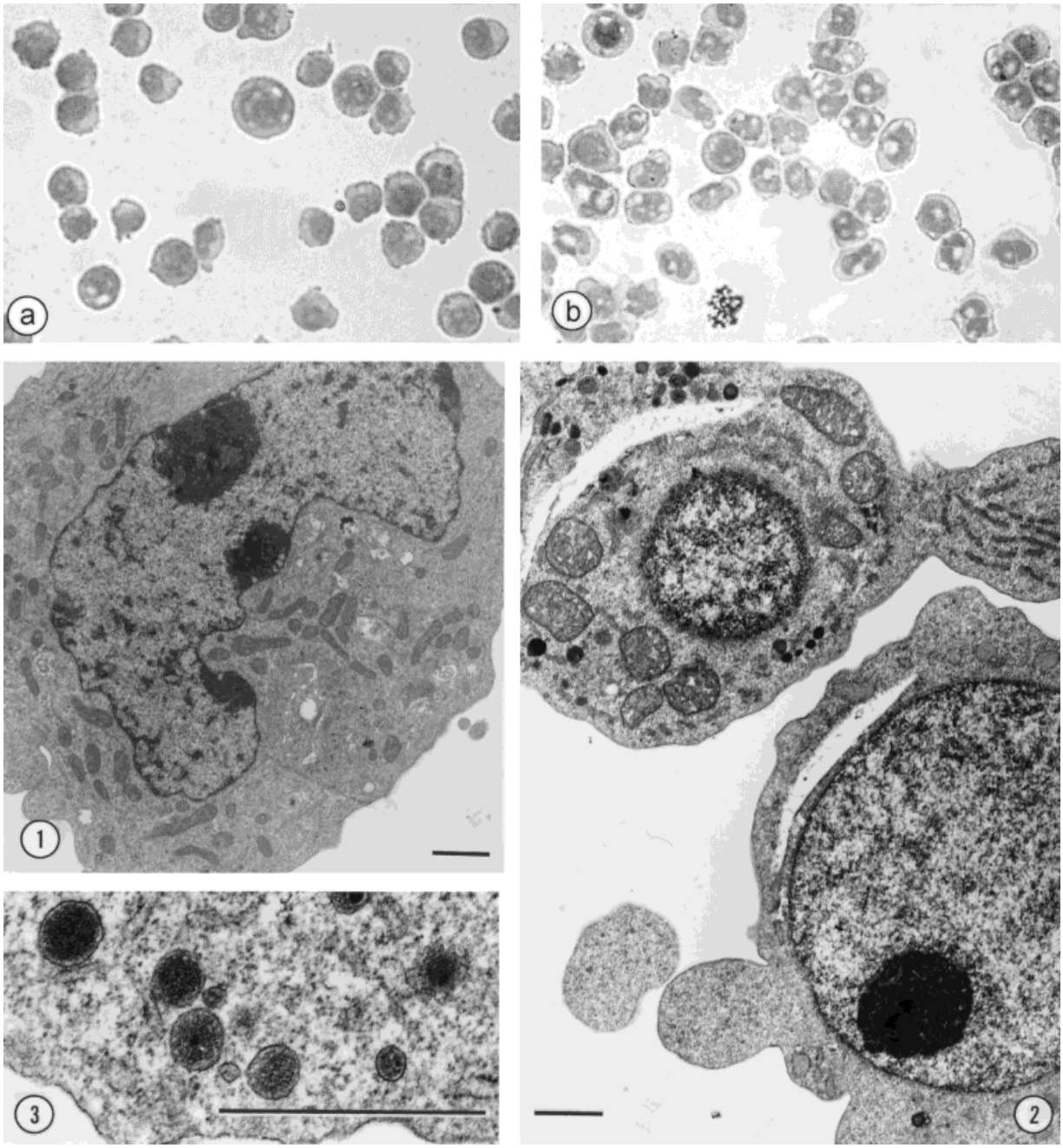

Fig. 3. Morphological analysis of control and Mg deprived HL-60 cells. Upper panels: light microscopy of cytospin slides. **a**: Control. **b**: cells cultured in 0.03 mM Mg. Mg restricted cells display reduction in size and clear signs of nuclear invagination (400x). Lower panels: HL-60 electron microscopy of (**1**) control and (**2**,**3**) HL-60 cells cultured in 0.01 mM Mg. Electron-dense granules are present exclusively in the cytosol of Mg-deprived cells (2,3). For other details see Results section. Scale bar = 1 μm.

with Mg restriction since it is evident at 0.03 mM and further increased at 0.01 mM Mg.

Apoptosis

Terminal differentiation is usually followed by cell death via apoptosis [Martin et al., 1990; Wyllie, 1992]. As shown in Table II, spontaneous apoptosis accounts for 8.2% of cells in control condition. When HL-60 cells are cultured in 0.03 mM Mg, apoptosis is 4.1%. After 4 days of culture in 0.01 mM Mg the value is 3.2%, but increases to about 30% on the 6th day. Similarly, DMSO treated cells display a value of 3.6% after 7 days of treatment, but it increases to about 50% on the 10th day. These data clearly show that, as DMSO treated cells, HL-60 cul-

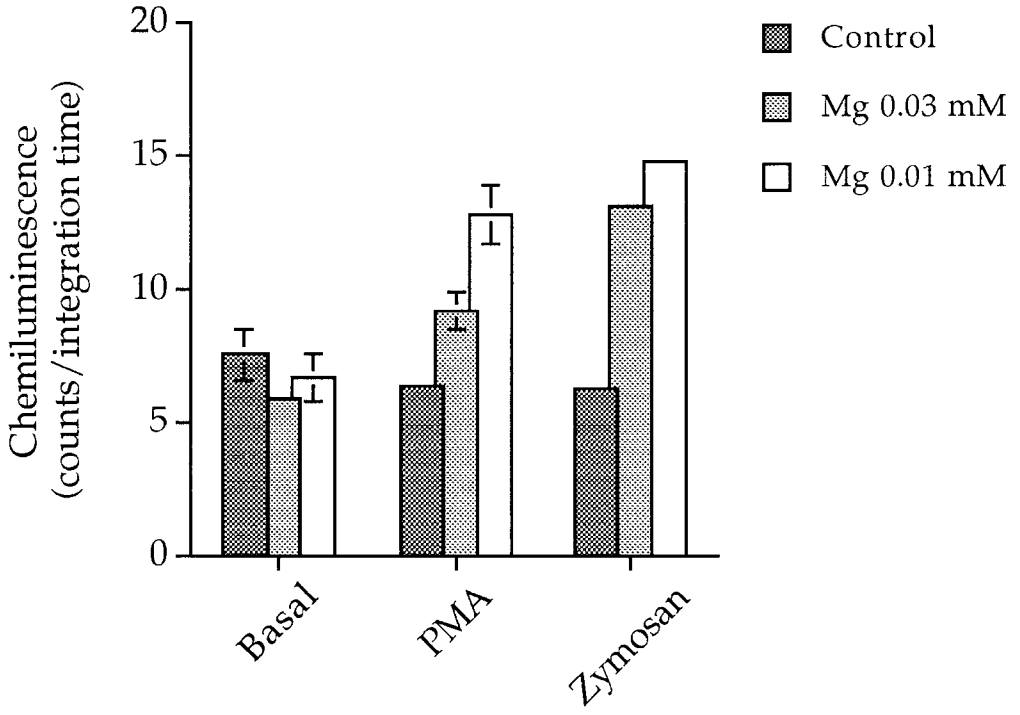

Fig. 4. Reactive oxygen species production in HL-60 cells induced to differentiate by Mg restriction. ROS were monitored by Luminol chemiluminescence analysis under basal and stimulated (PMA and Zymosan) conditions. Data are means ± SE of triplicates of a representative experiment.

TABLE I. CD 11b Expression of HL-60 Cells Cultured Under Mg-Restricted Conditions or in the Presence of DMSO

	CD 11b (%)
Control	3.6 ± 1.3 (4)
0.03 mM Mg^{2+}	62.5 ± 4.1 (4)*
0.01 mM Mg^{2+}	75.2 ± 2.8 (3)*
DMSO	76.7 ± 0.8 (4)*

*$P < 0.001$. Data are mean ± SE (number of separate experiments).

tured in 0.01 mM Mg behave as a terminally differentiated population.

Cell Adenine Nucleotides Content

Considering the deep involvement of Mg in cell energy metabolism and the relationship between ATP and Mg [Koss et al., 1993], we analyzed HL-60 cell adenine nucleotide content in order to compare cell energy state in the cells cultured under Mg restriction or in the presence of DMSO. The results reported in Table III indicate that the cells exhibit nearly the same ATP, ADP and AMP content. This suggests that the ability of Mg deficiency to induce cell differentiation, associated (0.01 mM) or not (0.03 mM Mg) with growth arrest, does not involve changes in cell energy metabolism.

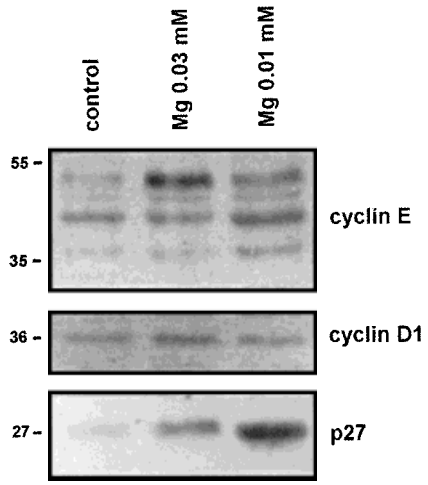

Fig. 5. Western blot analysis for the expression of cyclin E, cyclin D1, and $p27^{Kip1}$. Technical conditions described in Materials and Methods. Molecular weight markers (KDa) indicated on the left. Mg restriction is associated with a marked increase in the expression level of $p27^{Kip1}$. The expression level of cyclin E is also increased, while no changes are observed in cyclin D1 expression.

Intracellular Mg Content and Distribution

Table IV reports Mg content and distribution of control and HL-60 cells differentiated under

TABLE II. Effect of Mg Restriction on HL-60 Cells Spontaneous Apoptosis*

	Apoptotic nuclei (%)
Control	8.3 ± 0.7 (39)
0.03 mM Mg	4.1 ± 0.4 (25)
0.01 mM Mg (4th day)	3.2 ± 1.0 (6)
0.01 mM Mg (6th day)	32.7 ± 3.0 (3)
DMSO (7th day)	3.6 ± 1.2 (3)
DMSO (10th day)	52.0 ± 4.2 (3)

*Apoptosis determined by acridine orange staining. Data are mean ± SE (number of separate experiments).

TABLE III. Adenine Nucleotide Content of HL-60 Cells Cultured Under Mg Restriction or in the Presence of DMSO*

	nmol/10^6 cells		
Treatment	ATP	ADP	AMP
Control	12.4 ± 4.3	1.7 ± 0.7	0.8 ± 0.1
0.03 mM Mg	14.9 ± 3.2	1.9 ± 0.5	0.6 ± 0.2
0.01 mM Mg	15.9 ± 0.4	1.2 ± 0.05	0.4 ± 0.1
DMSO	12.2 ± 0.5	1.1 ± 0.2	0.5 ± 0.2

*Data are mean ± SE of three separate experiments.

Mg restriction or with DMSO. Under 0.03 and 0.01 mM extracellular Mg, cell total Mg content decreases by about 17% and 40%, respectively. HL-60 cells induced to differentiate by DMSO similarly exhibit 24% decrease of total intracellular Mg. As to the free intracellular Mg, HL-60 cells grown under Mg restriction display a clear and significant increase from about 0.3 mM in the control to about 0.45 mM (+50%). Interestingly, DMSO-treated cells also show a similar increase of free intracellular Mg. On the basis of the data on total and ionized Mg it is possible to calculate the size of the free and bound Mg pools, by considering cell H_2O content and assuming it mostly confined into the cytosol. H_2O content was of 16.6 ± 0.5 (4), 13.1 ± 0.15 (4), and 10.4 ± 0.5 (4) mg/mg protein in control, Mg-depleted and DMSO-treated cells, respectively. The last two columns of Table IV report the size of the free and bound intracellular Mg pools calculated from the above mentioned data. It can be observed that the bound pool significantly decreases in Mg-depleted and DMSO-treated cells. Despite the increase of $[Mg^{2+}]i$, the size of the free pool remains almost unchanged in all conditions tested, due to the reduction of H_2O content of the differentiated cells.

Changes in cell Mg content often accompany with consistent increase or decrease of Ca. Keeping this in mind, we measured the cytosolic free Ca concentration during Mg-restricted culture. $[Ca^{2+}]_i$ is 146.3 ± 8.8 (3) nM in control and 138 ± 7.5 (3) nM in cells grown in 0.03 mM extracellular Mg, thus concluding that $[Ca^{2+}]_i$ is not affected by Mg restriction.

DISCUSSION

The results presented in this paper demonstrate, for the first time, the definite possibility to induce differentiation of HL-60 cells by manipulating a cell constitutive cation. Thus, lowering extracellular Mg to ≤ 0.03 mM leads the cells to develop along the granulocytic pathway, without affecting either cell viability or cell energy metabolism (Table III). The original HL-60 cell population, which consists of a majority of promyelocytes, changes to more mature cells, as judged by both morphological (Fig. 3) and functional criteria (Fig. 4 and Table I). At 0.01 mM Mg, a marked inhibition of cell proliferation also occurs, followed by cell death via apoptosis (cf. Figs. 1,2 and Table II).

Although cell maturation usually accompanies with the arrest of proliferation, the two processes may not be mutually exclusive. In fact, HL-60 treatment with IFNγ has been reported to induce monocytic differentiation without growth arrest [Smith et al., 1993]. Moreover, sodium butyrate leads to megacaryocytic differentiation without cytostasis in K562 erythroleukemia cells [Murray et al., 1993]. In our experimental model, restriction of Mg to 0.03 mM does not alter either cell growth or cell cycle distribution of HL-60 cells, whereas both growth inhibition and cell arrest in G0/G1, are observed in 0.01 mM Mg, similar to DMSO-treatment (cf. Figs. 1 and 2). Hence, Mg restriction to 0.03 mM induces HL-60 cell differentiation apparently before affecting cell growth, as shown in the case of IFNγ [Smith et al., 1993].

Mg restriction is also associated with a marked increase in the expression level of the CDK inhibitor $p27^{Kip1}$, a negative regulator of cell cycle progression in a variety of tissues and conditions [Polyak et al., 1994a,b]. The expression level of cyclin E is also increased, while no changes are observed in cyclin D1 expression (Fig. 5). Despite the observed increase in the expression level of cyclin E, the overexpression of $p27^{Kip1}$ is expected to inhibit the CDK activity associated with both cyclins D1 and E, which

TABLE IV. Intracellular Mg Pools in HL-60 Cells Cultured Under Mg Restriction or With DMSO

	Total Mg^{2+} (nmol/mg protein)	$[Mg^{2+}]_i$ (mM)	Free Mg[a] (nmol/mg protein)	Bound Mg[a] (nmol/mg protein)
Control	99.6 ± 1.1 (7)	.29 ± .04 (6)	4.8	94.8
0.03 mM Mg	82.7 ± 2.4 (7)*	.43 ± .04 (5)*	5.6	77.1
0.01 mM Mg	63.4 ± 1.7 (3)*	.46 ± .07 (9)*	6.0	57.4
DMSO	76.0 ± 1.6 (20)*	.47 ± .05 (5)*	4.8	71.2

*$P < .05$ vs. control.
[a]Calculated on the basis of H_2O content reported in the text. Data are mean ± SE (number of separate experiments).

mainly regulate the G1 to S transition [Draetta et al., 1994; Sherr et al., 1995a]. Increase of $p27^{Kip1}$ is already evident at 0.03 mM Mg but it is probably not sufficient to induce a cell cycle arrest since there is no change in cell cycle distribution under these conditions (cf. Figs. 2,5). Increased expression of $p27^{Kip1}$ has been observed in response to a large number of antimitogenic signals, including TGF-β, rapamycin, growth factor deprivation, and contact inhibition [Sherr et al., 1995b]. $p27^{Kip1}$ also accumulates during oligodendrocyte differentiation [Duran et al., 1997]. In myelomonocytes, ectopic expression of $p27^{Kip1}$, as well as of $p21^{Cip1}$, leads to both cell cycle arrest and induction of surface macrophage-specific markers indicative of differentiation [Liu et al., 1996]. Thus, as already demonstrated for $p21^{Cip1}$ [Sherr et al., 1995b], an increased expression of $p27^{Kip1}$ may play an important role in the growth arrest associated with cell differentiation. Experiments are in course to further characterize the role of $p27^{Kip1}$ in differentiation of HL-60 and other cell lines. Maturation induced by Mg restriction looks, in fact, a very promising model to better understand the relationship between proliferation and differentiation.

To investigate the role of Mg in the regulation of HL-60 cells proliferation and differentiation, we studied Mg content and compartmentalization. Differentiation, induced by either Mg-restriction or DMSO, is accompanied by a significant decrease of cell total Mg content without any appreciable changes of the free intracellular pool (see Table III). Measurements of $[Ca^{2+}]_i$ in control and Mg restricted HL-60 cells confirm that the observed effect on cell proliferation is specific for Mg, since decrease of Mg in culture media does not interfere with cell $[Ca^{2+}]_i$.

The analogy between Mg restriction and DMSO treatment may help clarify the interpretation of our data. The mechanism of action of DMSO is still unclear. Among various possibilities, it has been proposed that this compound modifies cell plasma membrane surface potential [Arcangeli et al., 1993], eventually leading to ion flux regulation and second messenger production. From this point of view it seems reasonable to hypothesize that the effect of Mg restriction may be mediated by two different mechanisms. The first one could depend on the effect of Mg on plasma membrane surface potential that, in turn, may be followed by signal transduction and/or modification of intracellular Mg content and distribution. The second mechanism could involve the direct regulation of the transmembrane Mg flux by extracellular Mg. Although not fully characterized, Mg efflux can indeed be modulated by a variety of receptor-mediated stimuli [Wolf et al., 1994, 1996, 1997], which may well include extracellular Mg. To this regard, we have previously described a direct effect of external Mg on intracellular metabolic reactions in a mammalian tumor cell line [Bossi et al., 1989]. Moreover, recent studies in *Salmonella* have characterized a Mg receptor that influences bacterial virulence [Vescovi et al., 1996].

In any case, it is clear that both Mg restriction and DMSO treatment deeply modify Mg homoeostasis in HL-60 cells, as evidenced by major changes in cell total content and distribution of this cation (see Table IV). It is possible that the rearrangement of intracellular Mg, with a shift from the bound to the free pool, could alter the kinetic of Mg-dependent enzyme(s) involved in the control of the maturation pathway. For example, it has been recently demonstrated that Mg ions modulate the capabilities of particular nuclear proteins to recognize a consensus core nucleotide element for the transcription factor c-myc in murine brain [Ogita et al., 1996].

Altogether our data suggest that Mg may substantially contribute to the maturation of HL-60 cells. Modulation of extracellular Mg

allows to identify two different cell populations: a) cells which rapidly differentiate without undergoing growth arrest, in 0.03 mM Mg, and b) cells which differentiate into mature granulocytes, in 0.01 mM Mg. We also show that differentiation deeply involves intracellular Mg, with net loss of total Mg mostly regarding the intracellular bound pool. On this ground we believe that the data described in this paper indicate that HL-60 cells grown under Mg restriction, and particularly under severe Mg restriction (0.01 mM), exhibit all the characteristics of a terminally differentiated cell population, mimicking the physiological processes of maturation, and senescence. This experimental model, in which differentiation is obtained by simply manipulating a cation without any concurrent treatment with pharmacological agents, may prove useful to characterize the molecular mechanisms underlying cell differentiation and aging.

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