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The Role of Magnesium as Immunomodulator and Mediator between Immune and Colon Cancer Cells

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Abstract

***Background:** Magnesium is one of the most abundant ions in the cells and plays a significant role in maintaining the adequate function of various systems including the immune one. Studies have revealed association between magnesium and tumorigenesis. We sought to examine the effect of magnesium on the capacity of human peripheral blood mononuclear cells (PBMC) for cytokine production and its impact on the interplay between immune- and human colon cancer cells from two lines.*

***Methods:** PBMC were incubated with various concentrations of $MgSO_4$ as a source for magnesium and the secretion of TNFα, IL-1β, IL-1ra, IL-6, IL-2, IL-10, and IFNγ was evaluated. In another set of experiments various concentrations of magnesium were added to co-cultures of PBMC and HT-29 or with RKO colon carcinoma cells and cytokine secretion was examined.*

***Results:** Magnesium sulfate added to non-stimulated or mitogen-stimulated human PBMC at various concentrations exerted no effect on the secretion of the cytokines examined, except for reduced IL-6 and stimulated IL-1ra production at high magnesium concentrations. Inhibited secretion of TNFα, IL-1β, IL-6 and IFNγ and enhanced production of IL-1ra by HT-29 stimulated PBMC was observed upon incubation with magnesium sulfate, whereas that induced by RKO cells was not affected except for increased IL-2 production.*

***Conclusions:** In addition to its multiple physiological activities magnesium is an immunomodulator able to control inflammatory processes by participating in immune cells' activity. The results insinuate a supplementary role of magnesium in the immune dialogue between immune and cancer cells from certain human colon carcinoma lines.*

Keywords: *Magnesium sulfate; cytokines; mononuclear cells; colon cancer; immunity; cross-talk.*

Introduction

Magnesium is one of the leading ions involved in the maintenance of human health. It participates in a great number of enzymatic reactions and is therefore essential for the normal functions of vital organs such as the brain, heart and muscles [1]. Although maintaining a normal diet that contains vegetables and fruits provides enough amounts of magnesium for sustaining adequate plasma concentration, disturbances in its metabolism and particularly its deficiency may lead to serious morbid conditions, such as coronary artery diseases, diabetes, preeclampsia, hypertension and others [1-3]. Magnesium deficiency may develop due to inadequate diet, malabsorption,

chronic intestinal diseases,as well as to prolonged intake of antacids, proton pump inhibitors and certain antibiotics [4]. A review of the causes of magnesium deficiency stresses point that it may result not only from incorrect intake, but also due to modern methods applied in intensive agriculture that exhaust soil's magnesium content [5]. Particularly awareness has been paid to the connection between magnesium status and the immune responses in athletes. It has been reported that strenuous exercise may deplete body magnesium stores with subsequent immunological alterations, stimulation of inflammatory responses and secretion of pro-inflammatory cytokines, predominantly IL-6 [6,7]. Magnesium supplementation in patients with type 2 diabetes and hypomagnesemia caused not only improvement of HbA1C level and CRP, but also an inhibition in TNFα production [8,9]. Since its presence is imperative for the normal activity of the immune system magnesium deficiency may alter its functions. Magnesium participates in immune responses in numerous ways reviewed by Galland [10], one of them being activation of mononuclear cells for cytokine production. Studies have revealed that magnesium plays a consistent role in colorectal cancer development. Lower magnesium level in mice has been linked with promoted tumorigenesis [11]. Chemopreventive effect of magnesium has been observed in rats by Mori et al. [12]. Rats fed with high magnesium diet showed lower number of colon tumors, as well as a lower tumor multiplicity. A survey as for the risk of colon cancer in a large number of Swedish women has indicated that keeping a magnesium supplemented diet reduces significantly the occurrence of malignancy [13]. The protective role of higher dietary magnesium in cases of colorectal cancer has been reported by others [14-16]. On the other hand, since the number of epidemiological studies as for the beneficial role of magnesium as a colon cancer preventer is rather occasional and the findings are inconsistent, scientists call for additional research in order to elucidate the relationship between magnesium and the incidence of colon tumors [17]. Chen et al. [18] have carried out a meta-analysis on prospective studies to elucidate the association of magnesium with lower colorectal cancer incidence and came to conclusion that the reduction of this type of malignancy due to magnesium is rather restrained. Similar results were reported by others [19]. Considering the anti-inflammatory properties of magnesium on one hand and the well-established linkage between chronic inflammation and cancer development on the other hand [20], we aimed to examine the effect of magnesium on the capacity of human peripheral blood mononuclear cells (PBMC) to produce cytokines and its effect on the interplay between immune- and colon cancer cells from two human lines, i.e. HT-29 and RKO.

Materials and Methods

Cell Preparation

The study was approved by the Ethics Committee of the Rabin Medical Center. Blood Bank donors gave written informed consent with an agreement to use components of their blood for medical research. Peripheral blood mononuclear cells (PBMC) were separated from venous blood by Lymphoprep-1077 (Axis-Shield PoC AS, Oslo, Norway) gradient centrifugation. The cells were washed twice in phosphate buffered saline (PBS) and suspended in RPMI-1640 medium (Biological Industries, Beith Haemek, Israel) containing 1% penicillin, streptomycin and nystatin, 10% fetal calf serum (FCS), and was designated as complete medium(CM).

Colon Cancer Cell Lines

HT-29 and RKO human colon cancer cell lines were obtained from the American Type Cultural Collection, Rockville, MD. The cells were maintained in CM containing Mc-COY'S 5A medium and modified eagle medium (MEM- Biological Industries Co, Beth-Haemek, Israel) respectively, supplemented with 10% foetal bovine serum (FBS), 2mM L-glutamine and antibiotics (penicillin, streptomycin and nystatin-Biological Industries Co, Beth-Haemek, Israel). The cells were grown in T-75 culture flasks at 37°C in a humidified atmosphere containing 5% CO_2.

$MgSO_4$ Preparation

$MgSO_4$ (Sigma Israel) was dissolved in CM at 250 mM and further dilutions were made in CM. $MgSO_4$ was added at the onset of cell cultures at concentrations of 0.4 mM, 1 mM and 2.5 mM.

Effect of MgSO4 on Cell Proliferation

The effect of $MgSO_4$ on the proliferation of PBMC, HT-29 and RKO was examined applying XTT proliferation assay kit (Biological Industries, Beith Haemek, Israel). In short: 0.1 ml aliquots of PBMC or HT-29 and RKO cells obtained after trypsinization and suspended at 10^5/ml in appropriate CM were added to each one of 96 well plates and incubated for 24 hrs in the absence or presence of $MgSO_4$ added at the onset of cultures at concentrations as indicated. At the end of the incubation period the cells were stained according to the manufacturer's instructions. The plates were incubated for 3 hrs at 37°C in a humidified incubator containing 5% CO_2 and the absorbance was measured at 450 nm using an ELISA reader.

Effect of $MgSO_4$ on Cytokine Production

1.0 ml of PBMC ($2x10^6$/ml suspended in CM) was incubated without (non-stimulated) or with LPS (50ng/ml) for TNFα, IL-1β, IL-6, IL-10, and IL-1ra production, or with PMA 1μg/ml and ionomycin 0.5μg/ml for IL-2 and IFNγ secretion. In another set of experiments, 0.5 ml of PBMC ($4x10^6$/ml of CM) was incubated with 0.5 ml of CM or with 0.5 ml of one of the colon cancer cell lines i.e. HT-29 or RKO ($4x10^5$/ml) suspended in appropriate CM. $MgSO_4$ was added at the onset of cultures at concentrations as described. Cultures without $MgSO_4$ served as controls. The cultures were maintained for 24 hrsat 37^0C in a humidified atmosphere containing 5% CO_2. At the end of the incubation period the cells were removed by centrifugation at 250 g for 10 min., the supernatants were collected and kept at -70°C until assayed for cytokines content.

Cytokine Content in the Supernatants

The concentration of TNFα, IL-1β, IL-6, IFNγ, IL-10, IL-1ra and IL-2 in the supernatants was tested using ELISA kits specific for these cytokines (Biosource International, Camarillo, CA) as detailed in the guide-line provided by the manufacturer. The detection levels of these kits were: 15 pg/ml for IL-6, and 30 pg/ml for the remaining ones.

Statistics

A linear mixed model with repeated measures and the assumption of compound symmetry (CS) was used to assess the effect of different concentrations of $MgSO_4$ on cytokine secretion by PBMC induced by colon cancer cells. SAS vs 9.4 was used for this analysis. Paired t-test was applied to compare between the level of cytokines produced with various concentrations of $MgSO_4$ and that found in control cultures. Probability values of $p<0.05$ were considered as significant. The results are expressed as mean ± SEM of 6 different blood donors.

Results

Effect of $MgSO_4$ on Cell Proliferation

24 hrs of incubation of PBMC, HT-29 or RKO cells with $MgSO_4$ at concentrations between 0.4mM and 2.5mM had no effect on the proliferation rate measured by XTT test ($p=0.58$, $p=0.68$ and $p=0.46$, respectively, data not shown)

Effect of $MgSO_4$ on Cytokine Production

No detectable level of any of the cytokines tested could be obtained in supernatants from 24 hrs cultures of either HT-29 or RKO cells incubated at the above mentioned conditions without or with $MgSO_4$ added at concentrations between 0.4mM and 2.5mM.

TNFα, IL-1β and IL-6 (Table 1)

24 hrs of incubation of PBMC with $MgSO_4$ at concentrations between 0.4 and 2.5 mM caused reduced production of TNFα, IL-1β and IL-6 by HT-29-stimulated PBMC ($F_{3,24}=12.96$, $p=0.0002$; $F_{3,24}=8.17$, $p=0.0019$, and $F_{3,24}=23.0$, $p<0.0001$, respectively). The secretion of TNFα was lowered by 22% and 20% ($p<0.001$) at 0.4 and 1 mM of $MgSO_4$ respectively, that of IL-1β was reduced by 15% ($p<0.001$), 12% ($p<0.005$) and 8% ($p<0.05$) at 0.4 mM, 1 mM and 2.5mM of $MgSO_4$, respectively, whereas the secretion of IL-6 was reduced by 5% (not significant), and 16% and 15% ($p<0.001$), respectively. At the same culture conditions, there was no effect of $MgSO_4$ on the secretion of the three cytokines by non-stimulated PBMC ($F_{3,24}=1.31$, $p=0.307$; $F_{3,24}=1.46$, $p=0.26$, and $F_{3,24}=1.8$, $p=0.19$, respectively), or by cells stimulated with LPS ($F_{3,24}=0.44$, $p=0.72$; $F_{3,24}=0.62$, $p=0.61$, and $F_{3,24}=1.08$, $p=0.38$, respectively). In addition, the secretion of the three cytokines by PBMC stimulated with RKO cells was also not affected ($F_{3,24}=1.36$, $p=0.293$; $F_{3,24}=1.98$, $p=0.16$, and $F_{3,24}=3.81$, $p=0.03$, respectively).

Table 1. *Effect of $MgSO_4$ on pro-inflammatory cytokine production by PBMC*

$MgSO_4$	0	0.4mM	1mM	2.5mM
TNFα, ng/ml				
Non-stimulated	0.76±0.04	0.84±0.04	0.78±0.04	0.76±0.02
LPS-stimulated	1.88±0.24	2.20±0.26	2.08±0.42	1.96±0.22
HT-29-stimulated	2.60±0.36	2.32±0.36***	2.36±0.34**	2.60±0.38
RKO-stimulated	2.44±0.34	2.70±0.38	2.74±0.44	2.70±±0.4
IL-1β, ng/ml				
Non-stimulated	1.08±0.10	1.12±0.08	1.00±0.04	0.94±0.02
LPS-stimulated	8.66±1.16	8.88±0.74	8.30±0.92	8.36±0.90
HT-29-stimulated	7.32±0.59	6.19±0.77***	6.47±0.63**	6.76±0.53**
RKO-stimulated	4.10±0.52	4.57±0.50	4.72±0.48	4.42±0.54
IL-6, ng/ml				
Non-stimulated	4.42±0.24	3.96±0.4	3.5±0.30	3.42±0.28
LPS-stimulated	17.10±1.36	16.36±1.14	17.06±1.56	14.32±1.10
HT-29-stimulated	18.85±0.39	18.37±0.49	15.85±0.69***	15.79±0.35***
RKO-stimulated	15.23±0.68	16.10±0.95	15.51±0.83	14.01±0.87

*Non-stimulated PBMC or cells stimulated with LPS, or with one of the colon cancer cell lines HT-29 or RKO were incubated for 24 hrs without (0) or with $MgSO_4$ at concentrations as indicated. The level of cytokines in the supernatants was tested by ELISA. The results are expressed as Mean ± SEM of 6 experiments (6 different blood donors). Asterisks represent statistically significant difference from cells incubated without $MgSO_4$ (**p<0.01, ***p<0.001).*

IL-2 and IFNγ (Table 2)

Supernatants collected from non-stimulated PBMC did not contain detectable amounts of IL-2 incubated without or with $MgSO_4$. The spontaneous production of IFNγ and the secretion of IL-2 or IFNγ by PMA/ionomycin stimulated PBMC were not affected following incubation with $MgSO_4$ at 0.4-2.5 mM (p=0.23, p=0.146 and p=0.72, respectively). However, at the same culture conditions, IL-2 or IFNγ secretion by HT-29-stimulated PBMC was reduced ($F_{3,24}$=15.4, p<0.0001 and $F_{3,24}$=3.76, p=0.03, respectively) and that induced by RKO cells was enhanced ($F_{3,24}$=83, p<0.0001, $F_{3,24}$=4.0, p=0.028, respectively). At 0.4 mM, 1.0 mM and 2.5 mM of $MgSO_4$ the production of IL-2 induced by RKO cells was enhanced by 25% (p<0.001), 19.5% (p<0.001) and 4.5% (p<0.01), respectively, and that of IFNγ was higher by 13% p<0.05) at 0.4 mM and 1.0 mM. At the same $MgSO_4$ concentrations, IFNγ synthesis induced by HT-29 cells was reduced by 11% (p<0.01).

Table 2. *Effect of $MgSO_4$ on IL-2 and IFNγ production by PBMC*

$MgSO_4$	0	0.4 mM	1.0 mM	2.5 mM
IL-2, ng/ml				
Non-stimulated	ND	ND	ND	ND
PMA/iono-stimulated	17.6±0.48	17.0±0.73	16.1±0.38	17.9±0.53
HT-29-stimulated	1.19±0.02	1.14±0.04	1.12±0.04	1.20±0.04
RKO-stimulated	1.13±0.04	1.40±0.02***	1.35±0.04***	1.18±0.04*
IFNγ, ng/ml				
Non-stimulated	1.28±0.06	1.30±0.06	1.30±0.04	1.14±0.04
PMA/iono-stimulated	31.40±2.65	29.81±3.7	32.8±3.83	30.81±3.25
HT-29-stimulated	2.64±0.18	2.34±0.2 **	2.36±0.18***	2.70±0.28
RKO-stimulated	2.86±0.50	3.22±0.46**	3.22±0.62*	3.04±0.62

*Non-stimulated PBMC (spontaneous) or cells stimulated with PMA or with one of the colon cancer cell lines HT-29 or RKO were incubated for 24 hrs without (0) or with $MgSO_4$ at concentrations as indicated. The level of cytokines in the supernatants was tested by ELISA. The results are expressed as Mean ± SEM of 6 experiments (6 different blood donors). ND-not detected level of IL-2 in the supernatant. Asterisks represent statistically significant difference from cells incubated without $MgSO_4$ (*p<0.05, **p<0.01, ***p<0.001).*

IL-10 and IL-1ra (Table 3)

The secretion of IL-10 or IL-1ra by non-stimulated PBMC or by cells stimulated with LPS was not affected following incubation with $MgSO_4$ at concentrations between 0.4 and 2.5 mM ($F_{3,24}$=1.66, p=0.21; $F_{3,24}$=0.23, p=0.87 for IL-10 and $F_{3,24}$=2.1, p=0.143; $F_{3,24}$=3.06, p=0.06 for IL-1ra, respectively). Moreover, $MgSO_4$ did not exert any effect on HT-induced IL-10 production ($F_{3,24}$=1.66, p=0.21) or on RKO-induced both cytokines secretion ($F_{3,24}$=1.46, p=0.26 for IL-10; $F_{3,24}$=1.92, p=0.17 for IL-1ra). However, the production of IL-1ra by HT-29 stimulated PBMC was significantly reduced following incubation with the above-mentioned concentrations of $MgSO_4$ ($F_{3,24}$=4.95, p=0.014) and was lowered by 10% (p=0.03) at 0.4mM of $MgSO_4$.

Table 3. *Effect of $MgSO_4$ on anti-inflammatory cytokine production by PBMC*

$MgSO_4$	0	0.4 mM	1.0 mM	2.5 mM
IL-10, ng/ml				
Non-stimulated	0.37±0.03	0.36±0.02	0.34±0.01	0.32±0.02
LPS-stimulated	2.14±0.26	2.20±0.34	2.26±0.36	2.07±0.30
HT-29-stimulated	1.95±0.18	2.35±0.08	2.24±0.15	2.53±0.22
RKO-stimulated	2.51±0.21	2.57±0.30	2.84±0.31	2.87±0.19
IL-1ra, ng/ml				
Non-stimulated	0.57±0.08	0.53±0.03	0.56±0.05	0.69±0.07
LPS-stimulated	0.53±0.02	0.60±0.05	0.71±0.06*	0.70±0.07*
HT-29-stimulated	1.77±0.06	1.59±0.09*	1.70±0.05	1.80±0.01
RKO-stimulated	1.50±0.06	1.67±0.03	1.64±0.09	1.54±0.09

*Non-stimulated PBMC or cells stimulated with LPS or with one of the colon cancer cell lines HT-29 or RKO were incubated for 24 hrs without (0) or with $MgSO_4$ at concentrations as indicated. The level of cytokines in the supernatants was tested by ELISA. The results are expressed as Mean ± SEM of 6 experiments (6 different blood donors). Asterisks represent statistically significant difference from cells incubated without $MgSO_4$ (*p<0.05).*

Discussion

The protective role of magnesium in inflammatory responses of the organism is one of the multiple functions of this essential nutrient and merits particular attention. It has been reported that magnesium deficiency promotes macrophage capacity for pro-inflammatory cytokine production, particularly TNFα, IL-1 and IL-6 leading to low-grade inflammation [21]. Human umbilical vein endothelial cells cultured in low magnesium media expressed up-regulation of pro-inflammatory cytokines such as IL-2, IL-3, IL-8, and IL-15 [22]. Suzuki-Kakisaka et al. [23] have observed that magnesium inhibited TNFα and IL-6 production by LPS stimulated cord blood mononuclear cells. Related findings have been reported by Sugimoto et al. [24]. Magnesium at a concentration of 5mM caused a decrease in IL-1β and IL-6 production by mesenchymal stem cells, whereas that of the anti-inflammatory cytokine IL-10 was increased [25]. On the other hand, low extracellular magnesium promoted release of the pro-inflammatory cytokines IL-1, IL-1β, IL-6 and TNFα in rat alveolar macrophages [26-28]. Dietary supplementation with mineral complex containing magnesium, phosphorus, potassium selenium and copper to cows during lactation caused an increase in cytokine levels compared to controls [29]. In co-cultures of Th2 lymphocytes and antigen-presenting cells high magnesium concentration significantly inhibited IL-4 and IL-10 secretion by Th2 cells reflecting the effect of extracellular magnesium concentration on the immune responses [30]. In the present study magnesium inhibited the pro-inflammatory cytokine IL-6 production by both stimulated and non-stimulated PBMC when applied at 2.5 mM concentration only. Conversely, the release of the anti-inflammatory cytokine IL-1ra was elevated. The maximal concentration of 2.5mM magnesium sulfate used in the present study is a level known to be clinically effective in vivo. [23].The discrepancy in cytokine production detected in our experiments and those reported by other investigators can be attributed to disparity in the experimental procedures. In the present study we worked with PBMC isolated from adult donors, whereas Suzuki-Kakisaka et al. [23] used THP-1 and cord blood mononuclear cells. We have previously shown that the in vitro effect of vitamin A on Th1 cytokine production (IL-2 and IFNγ) by human cord blood mononuclear cells from preterm newborns differs from that of adult PBMC, but both cell types

responded similarly as for Th2 cytokine production (IL-10) [31]. These findings are supported by previous reports indicating that neonatal mononuclear cells differ from those of adults in cytokine profile and sensitivity to the in vitro effect of drugs on cytokine production [32-33]. The presently observed effect of magnesium on the immune interplay between PBMC and colon carcinoma cells is of interest. While the secretion of all pro-inflammatory cytokines by PBMC co-incubated with HT-29 cells was inhibited except for IL-2, that of PBMC incubated with RKO cells was not affected with the exception of IL-2, its release being increased. Notably, in the presence of magnesium the production of the anti-inflammatory IL-1ra by HT-29 cells stimulated PBMC was enhanced, this effect was not achieved with RKO cells. These findings indicate that magnesium can modulate the immune cross-talk between PBMC and cancer cells of a certain type only. Since in the course of the present study we used magnesium sulfate as a source of magnesium one may argue that the results are due not to the magnesium itself but to the sulfate moiety of the magnesium sulfate. As for that possibility we wish to draw the reader's attention to the work of Sugimoto et al. [24] who have examined the role of magnesium sulfate as innate immunomodulator and the results of their study clearly indicated that the magnesium itself has been the mediator of the immune properties and not the sulfate component. Similar conclusions have been reached by others [22-23]. In our hands, the effect of magnesium on IL-6 and IL-1ra production by PBMC did not differ between LPS stimulated or non-stimulated cells. Comparable findings have been reported by Nowacki et al. [34] who did not observe a significant effect of magnesium on TNFα and IL-6 production by LPS stimulated cells using whole human blood. Moreover, higher magnesium concentration reduced the cytokine production by un-stimulated cells. Based on these and previous observations the authors suggest that the effect of magnesium on cytokine production is closely associated with the magnesium status.

In summary, magnesium is involved in a vast number of physiologic reactions including alleviation of inflammatory processes by immunomodulation of cytokine producing cells. The present results enlighten the role of magnesium in the cross-talk between immune- and cancers cells obtained from certain type of human colon carcinoma cell lines.

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