Influence of Extracellular Magnesium on Capillary Endothelial Cell Proliferation and Migration

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We investigated the role of extracellular magnesium on capillary endothelial cell migration and proliferation, components of endothelial cell function that play an important role in angiogenesis and wound healing. Cell migration and proliferation were tested in six different MgSO4 concentrations and in various culture conditions. The Boyden chamber procedure was used to evaluate migration of bovine adrenal cortex capillary endothelial cells. We found that low magnesium concentration inhibited cell migration, but a dose-dependent increase in migration was observed when magnesium level was increased beyond the normal serum concentration (up to 2.4 mM magnesium; \( p<0.0001 \)). Cell proliferation was also inhibited by very low magnesium concentration, an effect observed under all conditions studied. When cell proliferation was stimulated by acidic or basic fibroblast growth factors, it appeared that a ceiling was reached, as increasing magnesium concentration had no additional stimulatory effect. However, a dose-dependent increase in proliferation (\( p<0.005 \)) was observed when magnesium concentration was increased above the normal serum level (0.8 mM) in culture conditions that did not cause marked cell proliferation. Thus, magnesium has an important role in endothelial cell migration and proliferation: very low extracellular magnesium concentrations inhibit and supranormal levels enhance both migration and proliferation. These results suggest that magnesium deficiency might adversely influence the healing and reendothelialization of vascular injuries and the healing of myocardial infarction and might also result in delayed or inadequate angiogenesis, effects potentially leading to infarct expansion and inadequate collateral development. (Circulation Research 1990;67:645–650)

For several years our laboratory has been interested in the mechanisms responsible for the angiogenic response to myocardial ischemia. Because endothelial cell migration and proliferation are essential steps in angiogenesis,1 we have focused our efforts on the role of endothelial cells in this process. Endothelial cells also have been implicated in the control of both systemic and coronary vascular resistance,2–5 in the development of intravascular thrombii,6 and in atherogenesis.7,8 In our search for the underlying mechanisms that mediate the angiogenic response to myocardial ischemia, we were struck by the analogous role postulated for magnesium in the control of systemic and coronary vascular resistance,9,10 thrombosis,11 and atherogenesis.12,13 Therefore, as a first step to determine whether magnesium might influence endothelial cell function, and in particular those functional components that are believed to play an important role in angiogenesis, we have investigated the effect of varying extracellular magnesium concentrations on capillary endothelial cell migration and proliferation.

Materials and Methods

Cells

Capillary endothelial cells were isolated from bovine adrenal cortex by the method described by Folkman et al.14 The minced cortical tissue was incubated with 0.75% collagenase type II (Worthington Biochemical Corp., Freehold, N.J.) in phosphate-buffered saline containing 0.5% bovine serum albumin (BSA) at 37°C for 20 minutes. The mixture was filtered through a 112-μm Nitex nylon filter (TETKO Inc., Elmsford, N.Y.). The filtrate was centrifuged at 900 rpm (200g) for 8 minutes at 5–10°C. The pellet was resuspended in low glucose Dulbecco’s minimum essential medium (DMEM) and 10% calf serum (HyClone Laboratories, Inc., Logan, Utah) and washed three times. The final pellet was resuspended in low glucose DMEM supplemented with 10% calf
serum, 10 μl/ml retina-derived growth factor (RDGF),\textsuperscript{15} and 100 μg/ml heparin. The cells were then plated in gelatin-coated dishes and incubated at 37°C in 5% CO\textsubscript{2}. Colonies of capillary endothelial cells were identified and weeded free of contaminating cells. After reaching a diameter of 3–5 mm, cell colonies were transferred (by use of cloning rings) to new gelatin-coated dishes. Cells were maintained in low glucose DMEM containing 10% calf serum and 5 μl/ml RDGF. The media was changed every other day, and cells were passaged weekly at a split ratio of 1:30. The purity of the cultured cells was evaluated by phase-contrast microscopy and staining with fluorescent-labeled acetylated low density lipoproteins (Biomedical Technologies, Inc., Stoughton, Mass.) and anti-factor VIII (Atlantic Antibodies, An Incstar Co., Scarborough, Me.).

Migration

The Boyden chamber procedure was used to evaluate cell migration. The chamber consists of two compartments separated by a filter, and migration is measured by the number of cells crossing the membrane through pores of defined size. Blind well chambers (lower well, 200 μl; upper well, 800 μl) and 8-μm (pore size) polycarbonate polycrylonitrile-free filters (Neuro Probe, Cabin John, Md.) were used. Filters were coated with 5 μg collagen IV (Collaborative Research Inc., Bedford, Mass.). Collagen IV was used, despite its inhibitory effect on migration relative to collagen I, because it is the predominant type of basement membrane. Recently, confluent cells were dissociated with trypsin. The trypsin was inactivated with low glucose DMEM containing 10% calf serum. The cell suspension was divided into six aliquots, centrifuged (200g for 8 minutes), washed once, and resuspended in low glucose DMEM containing 0.1% BSA and 0, 0.4, 0.8, 1.6, 2.4, or 4.0 mM MgSO\textsubscript{4}. DMEM with 0.1% BSA or DMEM with 0.5% calf serum was added to the lower well of the chamber, and the collagen-coated filter was placed over it. The upper well was fixed in place, and the cell suspension (200,000 cells/well) was added to the upper well. The chambers were incubated at 37°C in 5% CO\textsubscript{2} for 6 hours. The filters were then removed, fixed, stained (Diff-Quick stain, American Scientific Products, McGaw Park, Ill.), and placed on glass slides. Nonmigrated cells on the top surface of the filter were wiped off. The number of cells that had migrated to the lower surface of the filter was determined by counting 10 fields for each filter at a magnification of ×400. Each sample was assayed in triplicate, and data were expressed as the mean±SD of the replicates. The actual magnesium concentration in the wells containing magnesium-free DMEM (Figure 1, column 1) was measured by atomic absorption spectrophotometry and found to be 0.004 mM. The presence of magnesium was probably due to the fact that the magnesium-free DMEM was supplemented with 0.5% calf serum and due to the endothelial cells, which may also contribute magnesium.

![FIGURE 1. Bar graph showing number of endothelial cells in the Boyden chamber in the six different magnesium concentrations that migrated toward 0.5% calf serum and toward bovine serum albumin (BSA). The bars represent the mean±SD of three counts of the migrated cells.](image)

Proliferation

Initial incubation period. The subconfluent capillary endothelial cells were trypsinized and harvested from the culture surface. All cells were then plated under the same standard culture conditions in gelatin-coated, 2-cm
\textsuperscript{2} wells in 24-well plates and incubated in low glucose DMEM (1.8 mM CaCl\textsubscript{2}) containing 7% calf serum and 5 μl/ml RDGF at 37°C in 5% CO\textsubscript{2}, for initial incubation periods as specified in Table 1.

Second incubation period. After the initial incubation period in which the culture conditions were similar in all the wells, the medium was aspirated, and media with six different MgSO\textsubscript{4} concentrations were added to the wells. Thus, the media consisted of DMEM with no MgSO\textsubscript{4}, 0.4 mM MgSO\textsubscript{4}, 0.8 mM MgSO\textsubscript{4} (normal [Mg\textsuperscript{2+}]), 1.6 mM MgSO\textsubscript{4}, 2.4 mM MgSO\textsubscript{4}, or 4.0 mM MgSO\textsubscript{4}. Seven percent calf serum, 1% glutamine, and 1% penicillin/streptomycin were added to all six media.

The measurement by atomic absorption spectrophotometry (MetPath, Rockville, Md.) of the magnesium concentration in the wells containing magnesium-free DMEM (supplemented with 7% calf serum and RDGF) was 0.04 mM after 14 hours of incubation with 5,000 freshly plated cells in 0.5 ml complete medium. The cells were counted at the end of the second incubation period using the Electro-Zone Cellscope counter (Particle Data, Inc., Elmhurst, Ill.). The same experiment was repeated three times with different protocols (Table 1): 1) Plating density for protocol 1 was 5,000 cells/well; the initial incubation period was 2 hours. The second incubation period was 72 hours, during which no basic fibroblast growth factor (bFGF) was added to the six different media (protocol 1a) or 1.5 ng/ml bFGF was added to all wells (protocol 1b). 2) Plating density for protocol 2 was 5,000 cells/well; the initial incubation period was 12 hours. The second incubation period was 72 hours, during which no bFGF was added...
(protocol 2a) or 1.5 ng/ml bFGF was added to the six different media (protocol 2b). 3) Low plating density for protocol 3 was 500 cells/well; the initial incubation period was 12 hours (protocol 3a) or 2 hours (protocol 3b). The second incubation period for protocols 3a and 3b lasted for 9 days, during which the cells were fed 1.5 ng/ml bFGF every other day. The purpose of the last experiment (protocols 3a and 3b) was an attempt to cause maximal replications by using low primary plating density of cells and continuously stimulating the cells by adding bFGF until the cells in any one of the wells reached 80% confluence. With maximal replications, we thought the cells might utilize their intracellular magnesium, so that the effect of low extracellular magnesium levels on cell proliferation would become more apparent.

The effect of each different medium was tested in eight different wells, and an aliquot of each well was counted three times without the investigator knowing from which protocol the cells were derived.

**Statistical Analysis**

All the counts are expressed as mean±SD of three blind counts of all eight wells. The differences among the six groups of cells within each protocol were compared by one-way analysis of variance with the Bonferroni adjustment. Repeated paired t tests were also used to control the comparisonwise error rate. Statistical significance was defined as p<0.05.

**Results**

**Cell Migration**

The effect of different magnesium concentrations on the migration of capillary endothelial cells was tested both with 0.5% calf serum and in serum-free BSA.

Figure 1 demonstrates the mean number of cells that migrated in the six different magnesium concentrations, both toward a serum-free medium (BSA only) and toward a medium with 0.5% calf serum. Although the number of cells that migrated was greater when calf serum was present in the lower chamber, there was a dose-dependent increase in migration to 2.4 mM when magnesium was increased beyond the normal concentration of 0.8 mM with (p<0.0001) or without (p<0.0001) serum. Low magnesium (0.4 mM) had the same effect as normal magnesium, but when magnesium was absent from the medium (0.004 mM magnesium in the well), migration was inhibited, especially in the absence of serum.

**Cell Proliferation**

The effect of six different magnesium concentrations on capillary endothelial cell proliferation was tested under various conditions (Table 1). In all, proliferation was lower in the wells containing very low magnesium concentration (0.04 mM), regardless of other factors like seeding density, length of initial or second incubation periods, and the presence or absence of growth factor in the medium. Figure 2 depicts the mean cell numbers in protocols 1a and 1b. In both, the initial plating density was 5,000 cells/well, the initial incubation period was 2 hours, and the second incubation period was 72 hours; in protocol 1a, no growth factor was added to the six different media during the second incubation period, whereas in protocol 1b, bFGF was added. Cells exposed to the lowest (0.04 mM) magnesium concentration proliferated

**TABLE 1. Culture Conditions Under Which the Effect of Various Magnesium Concentrations on Cell Proliferation Was Tested**

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Plating density (cells/well)</th>
<th>Initial incubation period</th>
<th>Second incubation period</th>
<th>bFGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>5,000</td>
<td>2 hr</td>
<td>72 hr</td>
<td>No</td>
</tr>
<tr>
<td>1b</td>
<td>5,000</td>
<td>2 hr</td>
<td>72 hr</td>
<td>Yes</td>
</tr>
<tr>
<td>2a</td>
<td>5,000</td>
<td>12 hr</td>
<td>72 hr</td>
<td>No</td>
</tr>
<tr>
<td>2b</td>
<td>5,000</td>
<td>12 hr</td>
<td>72 hr</td>
<td>Yes</td>
</tr>
<tr>
<td>3a</td>
<td>500</td>
<td>12 hr</td>
<td>9 days</td>
<td>Yes</td>
</tr>
<tr>
<td>3b</td>
<td>500</td>
<td>2 hr</td>
<td>9 days</td>
<td>Yes</td>
</tr>
</tbody>
</table>

bFGF, addition of basic fibroblast growth factor during second incubation period.
ated at the lowest rate whether or not growth factor was added. When no bFGF was added to the medium, a dose-dependent increase in proliferation was observed ($p<0.005$). When bFGF was present, the proliferation rate tended to increase as the magnesium concentration was increased from 0.4 to 2.4 mM; however, this trend did not achieve statistical significance. Further increment of the magnesium concentration to 4.0 mM caused a relative reduction in proliferation ($p<0.005$).

When the initial incubation period was long (12 hours after seeding, protocols 2a and 2b, Table 1), cell proliferation during the second incubation period was high, especially in those wells to which bFGF was added (protocol 2b). The high proliferation rate was observed regardless of magnesium concentration (Figure 3). Cell number was lower in the wells containing 0.04 mM magnesium in protocol 2a ($p<0.005$) and in the wells containing 0.04 mM ($p<0.01$) and 4 mM ($p<0.001$) magnesium in protocol 2b.

In protocols 3a and 3b, the initial plating density was low (500 cells/well), and the second incubation period was long (9 days), during which bFGF was added to the medium every other day. The difference between protocols 3a and 3b was the length of the initial incubation period, which was long (12 hours) in 3a and short (2 hours) in 3b. Figure 4 summarizes the results of the two parts of protocol 3. The length of the initial incubation period had a marked effect on final cell number. When the initial incubation period was long, proliferation rate was high regardless of the magnesium concentration. In fact, the various magnesium concentrations did not significantly alter this high proliferation rate, with the exception of cells that grew in the very low magnesium (0.04 mM) medium. The proliferation rate of these cells was lower than that achieved with higher magnesium concentration ($p<0.01$). On the other hand, when the initial incubation period was short (protocol 3b), a much lower proliferation rate was observed, and cell growth was markedly reduced both when magnesium was very low and when magnesium concentration was high (4.0 mM, $p<0.001$).

In all the experiments, the length of the initial incubation period had a significant effect on cell proliferation. The final number of cells counted in each well was much higher when the initial incubation period was longer.

**Discussion**

Influences of magnesium on cell proliferation have been suggested by a number of reports over the past decade. Rubin et al. studied the magnesium requirements of nontransformed and spontaneously transformed Balb/c 3T3 cells. Decreasing the concentration of magnesium in the medium from 1.0 to 0.01 mM reduced the rate of proliferation of both groups of cells without a significant decrease in the total intracellular magnesium. In cultured normal human lung fibroblasts, McKeehan and McKeehan found that low concentrations of magnesium were required for attachment and maintenance of viability; more magnesium was required for proliferation, particularly in low serum.

The present investigation was undertaken to define more precisely the role of extracellular magnesium in endothelial cell migration and proliferation, components of endothelial function that are believed to play important roles in angiogenesis. We found that magnesium significantly altered both migration and proliferation, but the precise effects depended upon the specific culture conditions. In general, cell migration and proliferation were inhibited by very low magne-
sium concentrations and enhanced by higher than normal concentrations (>0.8 mM), when the cells did not come into contact with bFGF.

The inhibitory effect of very low magnesium concentrations was observed under all conditions we studied: short or long initial incubation periods and in the presence or absence of bFGF. In contrast, the stimulatory effect of higher than normal concentrations of magnesium on cell proliferation was observed only under certain incubation conditions. For example, the length of the initial incubation period determined the number of subsequent cell replications during the second incubation period, when the effects of magnesium were being assessed. When the initial incubation period was only 2 hours, cell proliferation during the second incubation period was relatively modest when no bFGF was added. Under these conditions, magnesium exerted a dose-related effect on proliferation (Figure 2, protocol 1a). When the initial incubation period was long (12 hours), cell proliferation during the second incubation was very rapid even in the absence of bFGF. Under these conditions of rapid cell proliferation, increasing the magnesium concentration above normal serum levels had no effect (Figure 3). Likewise, magnesium had no effect on proliferation when the initial incubation period was only 2 hours but a relatively rapid rate of cell proliferation was induced during a long second incubation period by the addition of bFGF (Figure 4).

These findings suggest that magnesium influences capillary endothelial cell proliferation under culture conditions that do not intrinsically lead to marked stimulation of cell proliferation. These results also suggest that when culture conditions favor the rapid proliferation of cells, a ceiling may be reached that obviates any further facilitatory effect of increasing magnesium levels above normal serum levels. Lowering magnesium concentration, however, still leads to inhibitory effects on cell proliferation.

The reason for the lack of a magnesium effect under culture conditions causing marked cell proliferation is uncertain. As suggested, it is possible that any culture conditions that markedly stimulate cell proliferation raise the proliferative rate to a ceiling that is intrinsic to those particular conditions, so that increasing the concentration of an agent with mild stimulatory actions such as magnesium will exert no additional effect. This might apply not only to the experiments in which bFGF was added during the second incubation but also to those experiments with a long preincubation period, since RDGF that was present in the initial incubation medium is a crude mixture that contains, among other constituents, both acidic fibroblast growth factor and bFGF. Alternatively, with the 12-hour seeding period, most of the wells had large confluent areas of cells that by contact inhibition caused the magnesium effect to be smaller or absent.

The mechanisms responsible for the effects of magnesium on cell proliferation are probably complex. The inhibition of endothelial cell proliferation by very low extracellular magnesium concentration could have one or more mechanisms. A decrease in high-energy phosphates and overall decline in protein and carbohydrate synthesis could certainly be involved. Magnesium depletion to the point of affecting purine and pyrimidine synthesis and DNA and RNA polymerase activities would seem a less likely mechanism, based on work in other cells, but verification would require specific confirmation in capillary endothelial cells. Regulation by magnesium of cyclic AMP (cAMP) in some cells suggests that a cAMP-dependent mechanism could be involved. Additionally, excess [Ca2+] secondary to low extracellular [Mg2+] could conceivably inhibit cell growth by exhausting calcium-dependent ATPases.

The only information about the effects of magnesium on growth of endothelial cells of which we are aware is a recent report by Knedler and Ham. They found that increasing the concentration of Mg2+ (in MCD131 with 5% dialyzed fetal bovine serum) from 0.48 to 5 or 10 mM markedly increased the proliferation of sparse human umbilical microvascular endothelial cells. The mechanism of this effect was not elucidated. Curiously, the authors found almost no effect of the heparin binding growth factors (bFGF and acidic fibroblast growth factor) on these cells, an observation that is at odds with studies of other types of cultured endothelial cells.

Similar mechanisms may influence the effects of magnesium on endothelial cell migration that we found: cell migration was slow in very low magnesium (0.004 mM), intermediate in low (0.4 mM) and in normal (0.8 mM) extracellular magnesium, and increasingly rapid as the magnesium concentration was increased (1.6 and 2.4 mM). Interestingly, high concentrations of dibutyl cAMP inhibit fetal bovine aortic chemokinesis. As a cofactor for adenylate cyclase and cAMP-dependent phosphodiesterase, it is conceivable that elevated magnesium levels could alter cellular cAMP and thereby alter the rate of cell migration.

Alternative mechanisms are suggested by the fact that magnesium is required for the assembly of actin polymers and for myosin ATPase activity, two key components of the motor responsible for cell migration. In addition, endothelial cell migration is stimulated by fibronectin, a glycoprotein whose effects on the attachment of endothelial and other cells have been extensively documented. Interestingly, the affinity of the fibronectin receptor is influenced by magnesium concentration.

The complexity of the interactions of the many factors that may influence cell proliferation and migration is illustrated by the report that 50 μM verapamil or nifedipine inhibited keratinocyte migration, but not smooth muscle migration. If increasing extracellular magnesium lowered [Ca2+], an inhibition of endothelial migration might have been anticipated, but we found increased migration.
The effects of magnesium on endothelial cell migration and proliferation may have pathophysiological significance. Thus, deficiency of magnesium is common and can be caused, for example, by dietary deficiency and by diuretics. Given our results, it is possible that chronic magnesium depletion, by inhibiting endothelial cell migration and proliferation, could delay or prevent complete reendothelialization of vascular injuries. This could contribute to thrombosis and, therefore, to excessive subintimal proliferation of smooth muscle cells, changes that could lead to the development of atherosclerosis. The latter effect might be the result of mild but long-term magnesium deficiency, as has been suggested by both epidemiological and experimental studies. In the healing of myocardial infarction, magnesium depletion may result in delayed or inadequate angiogenesis potentially leading to infarct expansion and inadequate collateral development. In addition, hypomagnesemia has recently been reported to exert a vasorelaxing effect in coronary vessels with intact endothelium, whereas in vessels with injured endothelial cells, a similar lowering of magnesium may increase coronary vessel tone and result in vasoconstriction. Such actions may exert important effects on vascular tone that may play a role in modulating coronary and systemic vascular resistance. Whether attempts to increase extracellular magnesium concentration will influence cell proliferation and migration in vivo is unknown, but it is intriguing to speculate on the possible salutary role of such an intervention in facilitating the healing of vascular injury, in preventing atherosclerosis, hypertension, and coronary spasms, and in facilitating coronary collateral growth in the setting of chronic myocardial ischemia.

References


KEY WORDS • angiogenesis • fibroblastic growth factor • wound healing
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