Cytoskeletal Reorganization by Mycophenolic Acid Alters Mesangial Cell Migration and Contractility

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Abstract—Cytoskeleton alterations are a hallmark of mesangial cell activation during glomerulosclerosis. The aim of this study was to investigate whether mycophenolic acid (MPA) affects cytoskeletal organization and motility of human mesangial cells. Using the IP15 cell line, we found that treatment with 1 μmol/L MPA inhibited both receptor-dependent (angiotensin II) and receptor-independent (KCl) contractile responses, as well as serum-induced migration activity, suggesting alterations in the intracellular mechanisms that control mesangial cell motility. Immunofluorescence studies of MPA-treated cells provided evidence for decreased membrane disassembly/reassembly of α-smooth muscle actin and F-actin fibers, which was correlated with sustained quantitative and qualitative modifications of actin-associated proteins: calponin was overexpressed and became associated with actin fibers, whereas phosphorylation levels of cofilin and myosin light chain increased, suggesting both an activation of the mechanisms responsible for act polymerization and an inhibition of actin-depolymerizing processes. These observations support a stabilizing effect of MPA on the mesangial actin cytoskeleton, which constitutes an additive action by which MPA, beyond its anti-inflammatory, antiproliferative and antifibrotic properties, might protect against excessive mesangial activation in the context of various glomerulopathies and kidney transplantation. (Hypertension. 2003;42:956-961.)

Key Words: mycophenolic acid  mesangium  kidney  glomerulosclerosis  cytoskeleton

Several forms of renal disease, including hypertension-related injury, might lead to progressive sclerosis of glomeruli, which all result from the activation of mesangial cells. Mesangial cells are contractile pericytes of the renal glomerulus that undergo phenotypic changes in response to various stimuli. Upregulation of numerous cytoskeletal proteins, particularly α-smooth muscle actin (SMA), has been identified as a marker of mesangial activation that occurs during repopulation of the glomerulus after glomerular injury. Isolation and culture of human mesangial cells cause profound changes in cellular morphology similar to that seen during in vivo activation: increased cell flattening and adhesion, with elaboration of filopodia and lamellipodia structures that are sites of cytoplasmic growth and movement.

Mycophenolic acid (MPA) is an immunosuppressive drug that acts as a reversible inhibitor of inosine 5-monophosphate dehydrogenase. Because this enzyme is the key factor that controls the synthesis of guanidylic nucleotides, MPA decreases the intracellular content of GTP and thus, exerts a reversible antiproliferative activity on macrophages and lymphocytes. In this context, it is used for prevention of chronic heart and kidney rejection in triple-therapy regimens that associate cyclosporine, steroids, and mycophenolate mofetil. This allows cyclosporine dosage reduction and thus, limits chronic cyclosporine nephrotoxicity and hypertension, which constitute important risk factors for graft failure.

In addition, MPA was recently proposed to prevent glomerulosclerosis in various models of experimental or clinical glomerulopathies. Its renoprotective action was related to reduced infiltration of inflammatory cells, but direct effects on resident cells, mainly interstitial myofibroblasts and mesangial cells, have also been suggested. We and others have demonstrated that MPA inhibits mesangial proliferation and secretion of extracellular matrix proteins, all effects that might contribute to the favorable effects of mycophenolate mofetil in chronic fibrotic kidney diseases. However, inhibition of mesangial activation caused by MPA treatment was accompanied by a strong and sustained increase in SMA expression, which is usually associated with mesangial proliferation.

This study was undertaken to define the specific effects of MPA on the mesangial cytoskeleton and their consequences on cell motility. Our results suggest that MPA prevents the cytoskeletal reorganization characteristic of the activated phenotype that is required for migration of mesangial cells during repopulation of the glomerulus. Therefore, MPA might modulate or inhibit key pathophysiologic responses of human mesangial cells in the course of chronic renal diseases.
Methods

All products used for cell culture were from Gibco BRL. The human mesangial cell line IP15 used in this study and culture conditions have been described previously. Cells were grown in RPMI-1640 medium supplemented with penicillin, streptomycin, sodium pyruvate, nonessential amino acids, 1 mmol/L HEPES buffer, and 10% fetal calf serum (FCS) (exceptions are mentioned). MPA and primary antibodies were from Sigma-Aldrich. The micromolar concentration of MPA used in most experiments was previously shown to inhibit mesangial cell proliferation and extracellular matrix synthesis without toxic effects and was within the range of plasma concentrations obtained in current clinical protocols. A 5-day treatment period was chosen to reach maximal GTP depletion.

Cell Contractility

Control or MPA-treated IP15 cells were sparsely seeded on 30-mm dishes and allowed to adhere overnight. Cell contractility was estimated by measuring the change in planar surface area (PSA) measured in Hanks’ balanced salt solution (HBSS) buffer at 37°C, as described in L’Azou et al. Phase-contrast images of isolated cells were numbered to determine the PSA before (time 0) and after (30 minutes) the addition of a contractile agonist (1 μmol/L angiotensin II or 50 mmol/L KCl). The PSA was measured with an image analyzer (Samba, Alcatel) to determine the kinetics of size changes, each individual cell being used as its own control.

Cell Migration Assay

Control or MPA-treated IP15 cells were seeded at confluence into 12-well culture plates (Falcon) and allowed to adhere overnight. Then the medium was changed to RPMI-1640 plus 0.2% FCS to inhibit cell proliferation. Two days later, wounds were made by scraping the cell layer with a sterile pipette tip. Images from 2 marked fields per well were digitized under phase-contrast view, immediately after wounding (day 0) and after 1 to 3 days in culture in RPMI-1640 plus 0.5% FCS, supplemented or not with 1 μmol/L MPA. Wound closure was quantified with use of NIH Image 1.62 (National Institutes of Health) software for the Macintosh computer as a percentage of the day-0 distance of the wound edges.

Western Blots

Total proteins were extracted from mesangial cells and subjected to Western blot analysis as described previously. Dilutions of primary antibodies were as follows: anti-myosin light chain (MLC), anticofilin, and anti-phosphocofilin, 1/1000; anti-tubulin and antivimentin, 1/2000; anti-SMA, 1/4000; and anti-basic calponin, 1/5000. After incubation with mouse or rabbit peroxidase-coupled secondary antibodies, immunoreactive bands were detected with enhanced chemiluminescence according to the manufacturer’s recommendations (ECL kit, Amersham).

MLC (20 kDa) Phosphorylation

The non-, mono- or diphosphorylated MLCs were separated by urea/glycerol polyacrylamide gel electrophoresis and subjected to Western blot analysis as described by Persechini et al. MLC bands were visualized after immunoblotting with an anti-MLC monoclonal antibody (1/1000). The phosphorylated forms of MLC presented a greater mobility because of the increase in negative charge. Quantitative analysis was performed with NIH Image 1.62 software for the Macintosh computer.

Immunolabeling was performed on IP15 cells cultured onto 2-well glass slides (Falcon, Becton Dickinson). After fixation and incubation with a specific primary antibody (anti-SMA and anti-basic calponin, 1/500; anti-tubulin, 1/200; and anti-vinculin: 1/4000), slides were incubated with the biotinylated secondary antibody (anti-mouse immunoglobulin, Amersham Life Science). F-actin was stained with rhodamine-labeled phalloidin (dilution, 1/450; Molecular Probes).

Figure 1. Contractile activity of human mesangial cells is inhibited by MPA. A, Mesangial cell contractility after 30 minutes in the presence of angiotensin II (1 μmol/L) or KCl (50 mmol/L). Graph shows the decrease in PSA measured in IP15 cells under control conditions (white bars) or pretreated for 5 days with 1 μmol/L MPA (gray bars). Data are mean±SE of at least 50 cells from 3 different cultures. *P<0.0001 vs no agonist; §P<0.0001 vs untreated cells. B, Distribution of mesangial contractile responses to angiotensin II. Graph shows reparation of the IP15 cell population as a function of the variation in PSA measured after 30 minutes in the presence of angiotensin II (1 μmol/L). x² analysis yielded a significant difference (P<0.001) in the contractile response between control cells (white bars, n=50 cells) and cells pretreated for 5 days with 1 μmol/L MPA (gray bars, n=68 cells). Data are from 3 different cultures.

Statistical Analyses

Results are reported as mean±SEM. Statistical significance, defined as P<0.05, was evaluated with a 1-factor ANOVA followed by the Scheffé test (Stat-View v5.0, SAS Institute). Statistical analyses were not performed on Western blot gels because of the semiquantitative nature of these tests. The phosphorylation levels of MLC, which are expressed as percentages, form a binomial distribution that was subjected to angular transformation; the resulting data had a normal distribution and were analyzed with a 1-factor ANOVA.

Results

MPA Treatment Decreased Human Mesangial Cell Contractility and Migration Activity

Image analysis measurements showed that the mean baseline PSA of MPA-treated cells was not significantly different from that of untreated cells (5103±301 arbitrary units, n=196, vs 4938±249 arbitrary units, n=234, respectively). No significant spontaneous decrease in PSA was observed in either control or MPA-treated IP15 cells maintained for 30 minutes in HBSS alone (Figure 1A).
Addition of 1 μmol/L angiotensin II induced a rapid, time-dependent decrease in IP15 PSA that reached maximal effect at 30 minutes. The intensity of the contractile response was heterogeneous among the cell population (Figure 1B) but had a normal distribution and was independent of initial cell size. The mean PSA decrease in the IP15 population in response to 1 μmol/L angiotensin II was 10.59 ± 1.60% (P < 0.0001 vs nonstimulated cells; Figure 1A), a result that was similar to that measured by the same technique in primary cultures of mesangial cells.10 Similarly, addition of 50 mmol/L KCl induced a receptor-independent, significant contraction of IP15 cells (6.81 ± 1.14%, P < 0.0001 vs control; Figure 1A). However, 5-day treatment with MPA prevented significant changes in PSA in response to both agonists (angiotensin II, 1.56 ± 0.83%; KCl, 0.09 ± 0.77%; Figures 1A and 1B).

In a migration assay in wounded cultures of confluent, growth-arrested cells, a significant reduction in wound surface was observed as soon as day 1 and reached −40 ± 3% at day 3 (Figure 2). No significant change was observed in cultures that were pretreated with 1 μmol/L MPA, indicating a loss of migration activity. Removing MPA from the culture medium led to a recovery of mesangial migration capacity that became significant after 3 days (Figure 2). It is well known that cell migration and contractility processes require cytoskeletal proteins, mainly actin and tubulin. Because MPA treatment reduced in vitro mesangial cell motility, we then studied how it might affect cytoskeletal protein content and organization in IP15 cells.

Cytoskeletal Protein Expression and Reorganization in Response to MPA Treatment

MPA induced a dose-dependent increase in SMA expression and a concomitant decrease in tubulin, the maximal effect being reached at 0.5 μmol/L (Figure 3A). Calponin accumulation levels returned to control values when MPA was removed from the culture medium for 48 hours. The absence of an observable decrease in SMA accumulation after removal of MPA (Figure 3B) was caused by the long half-life of this protein (4 to 5 days). No variation was observed in the expression of vimentin, the main component of mesangial intermediate filaments (Figure 3A). Immunofluorescence and confocal microscopy of MPA-treated cells showed a less dense but still organized microtubule network when compared with untreated cells (Figures 4A and 4B). F-actin or SMA labeling showed fibers within control cells, together with actin accumulation at cell edges into the membranes ruffles (Figures 4C, 4E, and 4G). In MPA-treated cells, actin labeling showed a well-developed network of thick actin fibers, but protruding structures were never observed (Figures 4D, 4F, and 4H). Because membrane ruffling results from actin cytoskeleton turnover,13,14 the changes in actin expression observed in MPA-treated cells.
might be associated with a higher polymerization rate and/or a lower depolymerization rate of filaments. Therefore, we compared the expression pattern of 2 main actin-associated proteins: smooth muscle (basic) calponin and cofilin, in control and MPA-treated IP15 cells.

As observed with SMA, MPA induced a dose-dependent and reversible increase in basic calponin expression, the maximal effect being obtained at doses in the micromolar range (Figure 3B). By the same time, total cofilin expression was slightly enhanced by MPA, which most notably increased the amount of its phosphorylated form (Figure 3B). Both calponin and phosphocofilin stimulations were reversed by removing MPA from the culture medium. Immunolabeling showed nuclear localization of calponin in untreated IP15 cells (Figure 4E), but after MPA treatment, calponin was observed at the cytoplasmic level and was found associated with actin filaments (Figure 4F).

The MPA-induced inhibition of mesangial migration could be caused by either modifications in cytoskeleton dynamics or alterations in cell adhesion structures. Double immunolabeling showed that MPA treatment did not induce the loss of focal adhesions from the mesangial cells, as evidenced by the maintenance of punctate vinculin and its association with actin fibers (Figure 4H).

**MLC Phosphorylation Level Increased in Response to Angiotensin II in Control but Not MPA-Treated Mesangial Cells**

Cell contractility and actin organization are also controlled by MLC phosphorylation level, which was thus assessed in IP15 cells. Semiquantitative analysis indicated that mono- and diphosphorylated forms represented 18.3±3.0% and 9.1±2.2% of total MLC in untreated cells, respectively (Figure 5). In response to MPA, a significant and sustained increase in the accumulation level of both mono- and di-phosphorylated forms was observed, rising to 27.5±3.8% and 20.3±2.0% of total MLC, respectively, after 5 days of treatment (P<0.015 vs control). Similar levels were measured in cells cultured for longer periods (7 to 10 days) in the presence of MPA (data not shown).

Control cell activation by 1 μmol/L angiotensin II led to a rapid and transient increase in MLC phosphorylation. Preliminary experiments indicated that the maximal levels of mono- and di-phosphorylated MLCs were observed between 7 and 15 minutes after addition of the contractile agonist (mean levels measured after 10 minutes: monophosphorylated MLC, 28.5±10.7%; diphosphorylated MLC, 25.6±6.3%; P<0.01 vs nonstimulated cells) and then decreased to reach 24% and 17% at 30 minutes, respectively. However, 10 minutes of treatment with angiotensin II had no significant additional effect on MLC phosphorylation level in MPA-treated cells (monophosphorylated MLC, 44.7±18.6%; diphosphorylated MLC, 26.5±3.1%); no further increase was observed within 30 minutes of stimulation.

**Discussion**

The central finding of this work is the inhibition of contractile and migratory responses in human mesangial cells treated with MPA, which was associated with alterations in cytoskeletal organization. Actin cytoskeleton alteration in the glomerulus is postulated to be important in the pathogenesis of glomerulosclerosis. The ability of activated mesangial cells to migrate or contract depends on cytoskeletal dynamics, which is primarily modulated by interactions between actin and associated proteins.
Among them, binding of basic calponin increases the rate of polymerization and prevents depolymerization of F-actin, leading to formation of tight bundles with actin filaments oriented parallel to each other, as we observed in MPA-treated cells (Figure 3D). Calponin also inhibits actin movement over myosin heads, thus affecting smooth muscle contraction. Conversely, cofilin binds and depolymerizes filamentous F-actin and inhibits the polymerization of monomeric G-actin; its activity is regulated by phosphorylation, because phosphorylated cofilin does not bind to either G-actin or F-actin and is thus unable to depolymerize actin filaments. Our findings that MPA promoted both basic calponin overexpression and association with actin filaments, along with an increase in cofilin phosphorylation level, indicate that MPA treatment of mesangial cells might result in decreased actin turnover, as also suggested by the loss of peripheral membrane ruffling, leading to fiber stabilization that affected the capacities of cell migration and contractility. Additionally, microtubule network integrity also controls actin dynamics. The decrease in tubulin content and microtubule density in response to MPA might thus contribute to reduced actin turnover in the lamellipodia and inhibit cell migration. In contrast to actin and associated proteins, the expression level or location of vimentin, the main component of mesangial intermediate filaments that are important to maintain cell integrity, were not affected by MPA treatment. This absence of change in the mesangial vimentin network has been already described in pathologic models that cause actin fiber disorganization, such as the diabetic glomerulus.

MLC phosphorylation is another critical step in the formation of actin stress fibers and the induction of cell migration and is the essential factor that determines the extent to which smooth muscle and nonmuscle cells contract, because it regulates the Mg-ATPase activity of myosin type II. Addition of angiotensin II to the culture medium induced a rapid (within 10 minutes) increase in the amount of both mono- and di-phosphorylated MLC. This preceded cell surface reduction, which was maximal at 30 minutes, whereas MLC phosphorylation level (especially the diphosphorylated form) began to decrease as soon as 20 minutes after addition of the agonist. Thus, cell contraction needs only transient phosphorylation of the regulatory MLC that is rapidly dephosphorylated by myosin phosphatase. In contrast, MPA treatment induced a sustained increase in the proportion of phosphorylated to total MLC, which could be observed up to 10 days after treatment. This might constitute an additional mechanism by which MPA stabilizes actin fibers, because hyperphosphorylation of MLC on Ser-19 and Thr-18 was shown to induce aggregation of thick actin bundles in HeLa cells. Another hypothesis is that mesangial cells were in a state of maximal contraction in the presence of MPA. However, the mean baseline cell surface of MPA-treated cells was similar to that of control cells, and volume measurements of trypsinized cells gave no evidence of significant differences between both cell populations. In MPA-treated cells, angiotensin II did not stimulate a sufficient additional increase in MLC phosphorylation to allow cell contraction, suggesting that components of the pathway leading to MLC phosphorylation were saturated or that...
mechanisms leading to MLC dephosphorylation were inactivated by MPA.

Perspectives
Our data are consistent with a model in which MPA inhibits mesangial cell migration and contractility by blocking actin fiber disassembly/reassembly, and at least a part of its effects is mediated through sustained cofilin and MLC phosphorylation and calponin overexpression. Different members of the small GTPases of the Rho family regulate the balance of actin cytoskeletal reorganization characteristic of the activated phenotype that is required for migration of mesangial cells after glomerular injury and which is considered a critical event in glomerulonephritides. Moreover, MPA altered mesangial cell contractility, which is involved in the modulation of mesangial capillary blood flow. It has been recently reported that mycophenolate mofetil, alone or in synergy with losartan, improves renal function (increased creatinine clearance) in a rat model of chronic cyclosporin nephropathy, without any effect on systolic blood pressure. In a rat model of subtotal renal ablation, mycophenolate mofetil prevented the reduction in single-nephron glomerular filtration rate, despite persistent systemic hypertension. MPA might also exert favorable effects in hypertensive glomerular diseases in which mesangial cell activation plays a key role, such as lupus nephritis. IgA nephropathy, and chronic allograft nephropathy, through reduced mesangial mobility and contraction. So far as the in vivo effects of MPA on mesangial motility are concerned, this might contribute to an improvement of local hemodynamics by mycophenolate in experimental nonimmune renal disease.

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