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Signaling Transduction Pathway of Angiotensin II in Human Mesangial Cells: Mediation of Focal Adhesion and GTPase Activating Proteins

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Human mesangial cells (HMCs) respond to angiotensin II stimulation, which modulates their physiological activities, i.e., contraction and proliferation. It has been revealed that focal adhesion kinase (FAK) and paxillin participate in the angiotensin II-mediated signaling and cytoskeletal rearrangements at focal adhesion. We investigated the influences of cell adhesion upon angiotensin II effects in HMCs. In adherent cells, both FAK and paxillin were tyrosine phosphorylated by angiotensin II, while the cell detachment completely inhibited the tyrosine phosphorylation of paxillin. Activation of p44/42 mitogen-activated protein (MAP) kinase by angiotensin II was accentuated in suspended cells. Moreover, p190, a member of Rho GTPase activating protein (GAP), and RasGAP were coprecipitated with paxillin in adherent cells and angiotensin II stimulation reduced the formation of paxillin–p190 and paxillin–RasGAP complexes. These results suggest that the formation of focal adhesion complexes accelerated by accumulation of mesangial matrices may inhibit the proliferation of HMCs by modulating MAP kinase activity and be related to mesangial cell depletion. © 1999 Academic Press

In various glomerular diseases and experimental glomerulonephritis, the accumulation of extracellular matrices (ECMs) in glomeruli is frequently accompanied with depletion of cells. For example, diabetic nephropathy can display the accumulation of ECMs in mesangium without any proliferative activity of mesangial cell. The pathological changes finally lead to glomeruloscrelosis. On the other hand, Thy. 1,1 nephritis demonstrates the mesangiolysis preceding to the massive cell proliferation in mesangium, which is followed by the accumulation of ECMs and depletion of cells [1]. These histopathological observations lead us to hypothesize that ECMs may play a role in the mediation of growth factor signalings.

A possible candidate growth factor which may be related to ECM suppression on mesangial cell proliferation is angiotensin II (A II). It is known that A II is a multifunctional peptide which promotes both constriction and proliferation of target cells. It plays a pivotal role in the vascular cell growth under both normal and pathological conditions [2,3,4,5,6] by its binding to A II receptors [6]. Receptors for A II are mainly type 1 (AT1) which are abundantly expressed on mesangial cell surface. A II is considered to promote the proliferation and constriction of human mesangial cells (HMCs), and is involved in the pathogenesis and progression of various glomerular disorders [7].

Evidence has been provided that A II induces tyrosine phosphorylation of paxillin and FAK prior to cell proliferation [8] accompanied by the activation of Ras, Raf-1 and MAP kinases [9]. In addition, this signaling event also provokes the cytoskeletal rearrangement resulting in cellular constriction [8]. Paxillin is a major component of focal adhesion [10], which contains binding sites for various signaling molecules [10,11,12], and cytoskeletal proteins [13,14,15]. It has also been revealed that the formation of focal adhesion preceded by cell attachment to ECMs results in assembly of actin-containing stress fibers, and activation of signaling pathway including MAP kinase cascade [16,17].

In brief, current evidences suggest that the accumulation of ECMs may alter the mesangial cell response to A II-stimulation through MAP kinase activation and protein tyrosine phosphorylation. Based on this hypothesis, we have now investigated the effects of A II on tyrosine phosphorylation of FAK and paxillin and MAP kinase activity in both suspended and adherent serum-deprived HMCs. To examine the influence of ECMs on A II-stimulated signaling, HMCs were plated

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on dishes coated with fibronectin or type I collagen. The mediation of GAPs was also investigated.

METHODS

Materials. 75-cm^2 tissue culture flasks were obtained from Costar (Cambridge, MA, USA). Culture dishes (100 mm in diameter) coated with Poly-L-lysine, type I collagen or fibronectin were from Iwaki Glass (Tokyo, Japan). Mouse monoclonal immunoglobulin G (IgG) 2a antibody to phosphotyrosine (PY20), protein A-Sepharose 4B, and protein-G Sepharose 4B were purchased from Zymed (San Francisco, CA, USA); rabbit polyclonal IgG antibody to FAK from Upstate Biotechnology (Lake Placid, NY, USA); mouse monoclonal IgG antibody to paxillin, rabbit polyclonal antibody to p190 and rabbit polyclonal antibody to RasGAP from Transduction laboratories (Lexington, KY, USA); and Elk-1 fusion protein, mouse monoclonal IgG phospho-specific antibody to p44/42 MAP kinase (Thr202/Tyr204), and rabbit polyclonal phospho-specific antibody to Elk-1 (Ser383) from New England Biolabs (Beverly, MA, USA). Human A II was obtained from Research Biochemicals (Natick, MA, USA); SDS-polyacrylamide gels and polyvinylidene difluoride (PVDF) membranes from Atto (Tokyo, Japan); and ECL immunoblot reagents from Amersham (Arlington Heights, IL, USA).

Cell culture and treatment. HMCs were isolated from the normal part of human kidneys resected from a patient with renal cell carcinoma as described previously [18]. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, rendered quiescent at 85~95% confluence by serum deprivation for 48 h, and then exposed to agonists at various concentrations for the indicated times. For stimulation of suspended cells, quiescent HMCs were harvested with 0.025% trypsin and 0.01% EDTA diluted in phosphate-buffered saline (PBS). Cells were treated with trypsin neutralizing solution (Clonetics, Walkersville, MD), washed three times with RPMI 1640, and resuspended in serum-free RPMI 1640 in the absence or presence of A II. To examine the influence of cell-ECM adhesion on A II effect, 1×10^7 harvested cells were plated on each coated dish and cultured in serum-free RPMI 1640 for indicated times and then followed with or without additional incubation in 5 μ MA II for 10 min.

Immunoprecipitation and immunoblot analysis. Immunoprecipitation and immunoblot analysis were performed essentially as described previously [19]. Cells were lysed in RIPAbuffer, containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 1% deoxycholate, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin (1 μg/ml), 0.5 mM vanadate and 50 mM NaF. Cell lysate was then cleared by centrifugation. For coimmunoprecipitation study, RIPAbuffer was substituted by TNE buffer, containing 50 mM Tris-HCl (pH 7.8), 10% NP-40, 20 mM EDTA, 2mM PMSF, and 0.5 mM vanadate. The protein concentration of the resulting supernatant was measured with DC protein assay kit (Bio-Rad, Hercules, CA, USA), and 500 μg of protein from each lysate were then incubated for 2 h at 4°C with 500 μl of the same buffer containing the appropriate antibody. For incubations with mouse monoclonal or rabbit polyclonal antibodies, 20 μl of protein G-Sepharose 4B or protein A-Sepharose 4B were added respectively to the appropriate tubes, which were then rotated for 2 h at 4°C. The sepharose beads were separated by centrifugation, washed three times with the same buffer, resuspended in SDS sample buffer, boiled for 3 min, and subjected to electrophoresis on 7.5% SDS-polyacrylamide gels [20]. The separated proteins were electrophoretically transferred to a PVDF membrane. They were then incubated overnight at 4°C with 1% bovine serum albumin in PBS, washed twice with PBS containing 0.1% Tween 20, and incubated for 1 h at room temperature with PY20 or other appropriate antibodies (1:1000 dilution). After washing three times with PBS containing 0.1% Tween 20, each membrane was incubated with horseradish peroxidase-linked secondary antibodies (1:1000 dilution) and immune complexes were detected with ECL reagents. For reproving study, membranes were incubated in reproving buffer, containing 62.5 mM Tris HCl (pH 6.8), 2% deoxycholate and 100 mM mercaptoethanol, for 30 min at 50°C, washed with PBS containing 0.1% Tween 20 and incubated with appropriate antibodies. The extent of protein tyrosine phosphorylation was quantified using ImageQuant analyzer (Molecular Dynamics, Sunnyvale, CA, USA). Each experiment was performed at least three times independently.

In vitro MAP kinase activity assay. MAP kinase activity was examined by *in vitro* MAP kinase assay. Briefly, cells were lysed in RIPAbuffer and resulting supernatants containing 300 μg protein were incubated with phospho-specific antibody to p44/42 MAP kinase for 12 h at 4°C. Twenty microliters of protein A sepharose 4B were added into the lysate and rotated for 3 h at 4°C. Sepharose beads were separated by centrifugation and washed twice with RIPAbuffer and twice with kinase buffer, containing 20 mM Tris (pH 7.5), 5 mM β-Glycerophosphate, 2 mM Dithiothreitol, 0.1 mM Vanadate and 10 mM $MgCl_2$. Sepharose beads in each tube were suspended in 50 μl Kinase buffer supplemented with 200 μM Adenosine 5′-Triphosphate (ATP) and 2 μM Elk 1 fusion protein for 30 min at 30°C and the reaction was terminated with 25 μM 3×SDS sample buffer. After boiled for 3 min, samples were subjected to electrophoresis on 12.5% SDS-polyacrylamide gels and electrophoretically transferred to PVDF membrane, which was then blocked and washed as described in immunoprecipitation and immunoblot analysis. After incubation with phospho-specific antibody to Elk1 (1:1000 dilution) for 12 h at 4°C, the membrane was washed three times with PBS containing 0.1% Tween 20 and phospho-Elk1(Ser383) was detected as described in immunoprecipitation and immunoblot analysis.

RESULTS

The lysates of adherent HMCs incubated with 0,1, or 5 μ MA II for 10 min were immunoprecipitated with antibodies to FAK or to paxillin and they were subjected to immunoblot analysis with PY20. A II induced the phosphorylation of both FAK (120 kDa) and paxillin (65~70 kDa) in a dose-dependent manner (data not shown).

We further investigated whether adhesion of HMCs to ECMs is essential for A II-induced protein tyrosine phosphorylation and MAP kinase activation. The results showed that both FAK and paxillin did not exhibit tyrosine phosphorylation in suspended cells. Nonadherent HMCs in suspension cultures did not alter the A II induced tyrosine phosphorylation of FAK, however, tyrosine phosphorylation of paxillin mediated by A II was not observed. In vitro kinase assay demonstrated that A II stimulation prominently provoked MAP kinase activation in suspended HMCs (Fig. 1).

To examine the influence of ECM-difference on A II stimulated signaling, HMCs were plated on coated dishes with fibronectin, type I collagen or poly-L-lysin and incubated in serum-free RPMI for 1 h or 6 h, followed by additional incubation with or without 5 μ MA II for 10 min. As shown in Fig. 2, MAP kinase was activated transiently without A II supplement 1 h after plating and resumed to the quiescent control level within 6 h in the absence of A II. In contrast, the addition of A II suppressed the ECM-mediated MAP kinase activation at 1 h, while it reproduced the MAP kinase activation in quiescent HMCs at 6 h of culture (Fig. 2). The difference of ECMs did not influence on the effect of A II on MAP kinase activity evidently.

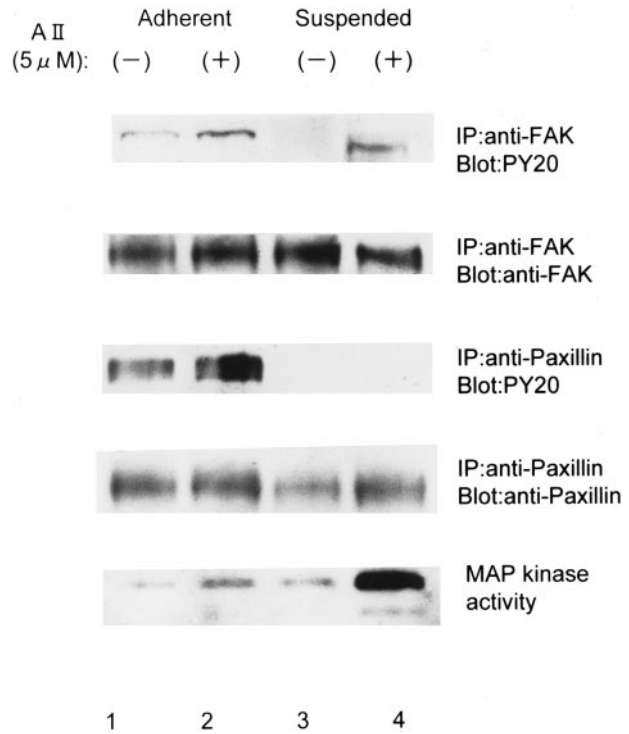

FIG. 1. Effects of A II on tyrosine phosphorylation of FAK and paxillin and MAP kinase activity in adherent and suspended HMCs. Adherent and suspended cells were incubated in the absence or presence of 5 μ MA II for 10 min, after which cell lysates containing equal amounts of protein were subjected to immunoprecipitation (IP) with antibodies to FAK and paxillin, followed by immunoblot analysis (Blot) with antibodies as indicated. MAP kinase activity was evaluated by *in vitro* MAP kinase assay. Extents in tyrosine phosphorylation of Elk-1 fusion protein are indicated.

Moreover, coprecipitation study revealed that p190 was associated with paxillin only in adhesive HMCs (Fig. 3A and 3B). A II stimulation diminished the formation of p190-paxillin complex in HMCs, which was observed in adhesive quiescent cells. RasGAP was also coprecipitated with paxillin in both adherent and suspended cells (Fig. 4A and 4B). A II stimulation reduced the formation of RasGAP-paxillin complex only in adherent HMCs.

DISCUSSION

Agonists of seven transmembrane domain G protein-coupled receptors, including AT1 receptor, stimulate tyrosine phosphorylation of FAK and paxillin in various cell types [10,24]. FAK has the binding site for paxillin and is involved in its tyrosine phosphorylation [10].

In this report, we have shown that the responsiveness of HMCs to A II stimulation differs between adherent and non-adherent cultures. In adherent HMCs, A II rapidly stimulated tyrosine phosphorylation of both FAK and paxillin. In suspended HMCs, A II-induced paxillin tyrosine phosphorylation was not observed, while FAK was tyrosine phosphorylated to the same extent as was observed in adherent cells. Miyamoto et al. demonstrated that paxillin accumulation to focal adhesion sites requires the ECM-integrin interaction and the concurrent intracellular protein tyrosine phosphorylation [16,25]. Thus, the loss of A II-induced tyrosine phosphorylation of paxillin in non-adherent HMCs (Fig. 1) may be attributable to the absence of ECM-integrin interaction.

The extent of A II-induced MAP kinase activation was also different between adherent and non-adherent HMCs. MAP kinase was further activated by A II stimulation in suspended cells compared with adherent cells (Fig. 1). This result suggests that the cell adhesion resulting in focal adhesion formation may inhibit A II-induced MAP kinase activation and, consequently, the growth signal via MAP kinase cascade in HMC culture system.

It has been reported that adhesion to fibronectin can induce transient MAP kinase activation via c-Src in fibroblasts within 1 h [26]. In the present study, this transient MAP kinase activation was reproduced in HMCs by plating cells on fibronectin, type I collagen and poly-L-lysine, though the difference in the extent of MAP kinase activation among ECMs was not clear (Fig. 2). Furthermore, the following A II stimulation suppressed this transient activation of MAP kinase (Fig. 2). These results imply thatA II can suppress MAP kinase activity in adherent HMCs and the prominent MAP kinase activation by A II stimulation in non-adherent HMCs (Fig. 1) may be due to the loss of this inhibitory effect.

The candidate molecules, which are involved in the inhibition of MAP kinase activity by A II, are p190 and RasGAP, the members of GAPs. p190 is the 190kDa protein which was characterized as a inhibitor of Rho family proteins containing two functional domains; GTPase domain and RhoGAP domain [27]. It has been shown that p190 forms a complex with RasGAP, the inhibitor of Ras, in cells stimulated with growth factors [28,29] and both p190 and RasGAP are tyrosine phos-

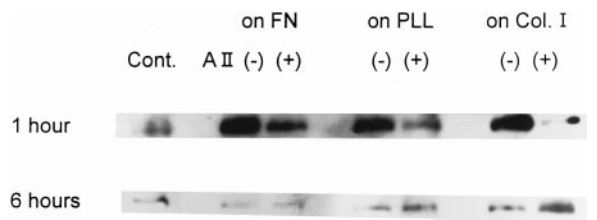

FIG. 2. MAP kinase activation by cell adhesion to ECMs and the effect of A II stimulation on the extent of induced MAP kinase activity. HMCs were harvested, plated on fibronectin (FN), poly-L-lysine (PLL), and type I collagen (Col. I) coated dishes as indicated and incubated in serum-free RPMI 1640 for indicated times (1 h or 6 h) at 37°C, which were followed by incubation in the presence (A II (+)) or absence (A II (−)) of 5 μ MA II for 10 min. Cells were then lysed in RIPA buffer and resulting supernatants containing 300 μ g of protein were subjected to *in vitro* MAP kinase activity assay. Cont. stands for the MAP kinase activity of quiescent adherent HMCs in serum deprived culture before harvested. Extents in tyrosine phosphorylation of Elk-1 fusion protein are indicated.

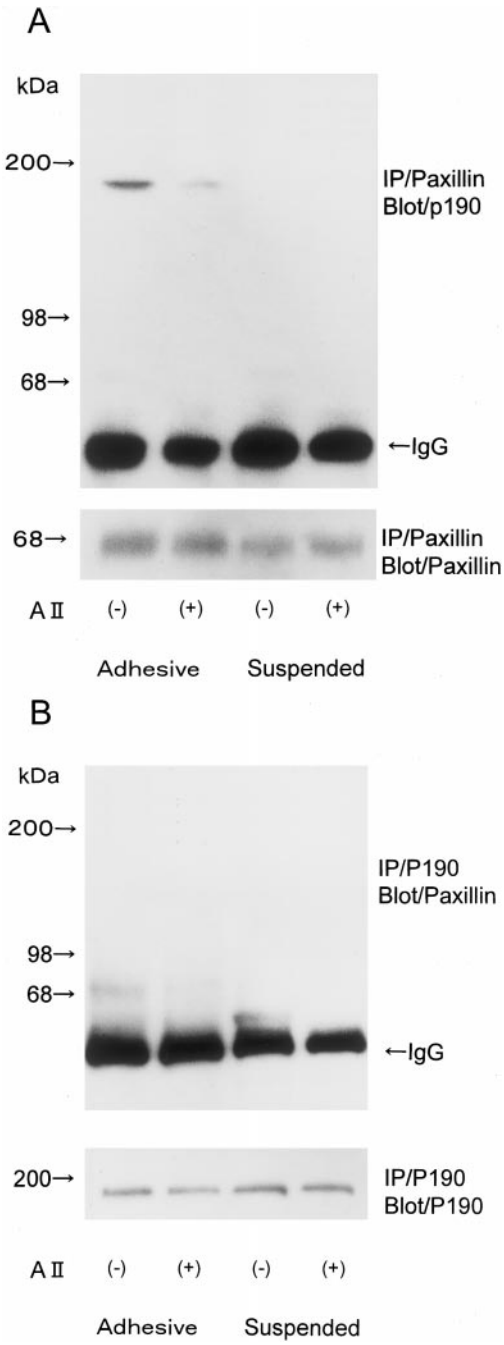

FIG. 3. Coprecipitation study on paxillin and p190. Adherent and suspended HMCs were incubated in the presence or absence of 5 μ MA II for 10 min, after which cell lysates containing the same amount of protein were subjected to immunoprecipitation (IP) with antibodies to paxillin (A) and p190 (B), followed by immunoblot analysis (Blot) as indicated.

phorylated by A II in adherent rat aortic smooth muscle cells [29]. Thus, a possibility exists that activated p190 suppresses A II-induced MAP kinase activation through cooperation with RasGAP which inactivates Ras located in the upper stream of MAP kinase cascade. It can also be speculated that p190 may suppress MAP kinase activity by inhibiting the activity of Rho and G proteins coupled with AT1 receptors.

In this study, we showed that p190 and RasGAP were coprecipitated with paxillin in both quiescent and A II stimulated adherent HMCs. It seems that A II stimulation reduces the amount of p190 and RasGAP coprecipitated with paxillin in adherent cells (Fig. 3 and 4). In suspended HMCs, the formation of p190-paxillin complex was not observed with no relation to A II stimulation (Fig. 3). The formation of RasGAP-

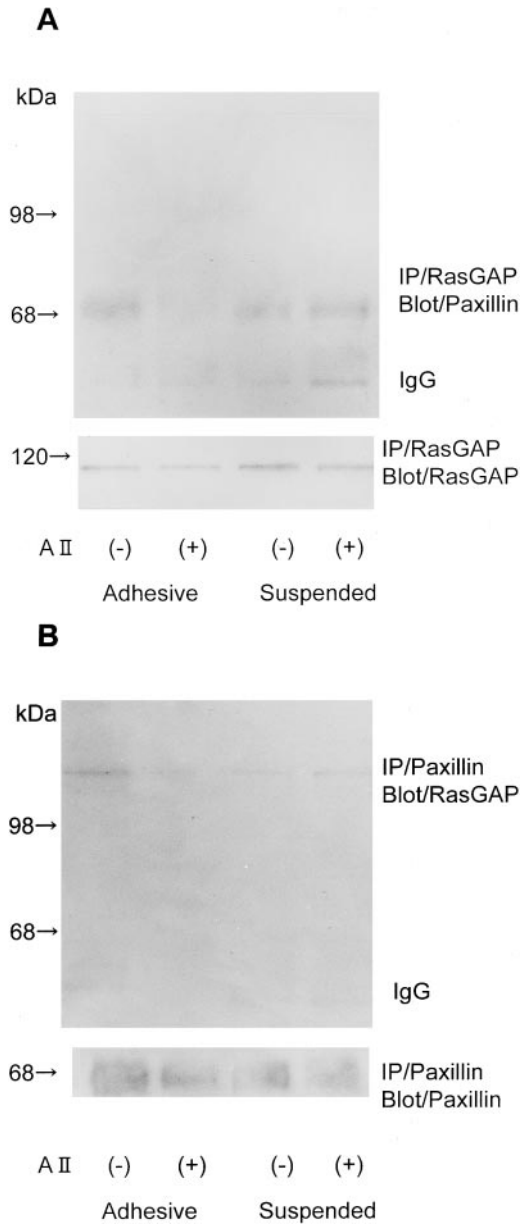

FIG. 4. Coprecipitation study on paxillin and RasGAP. Adherent and suspended HMCs were incubated in the presence or absence of 5 μ MA II for 10 min, after which cell lysates containing the same amount of protein were subjected to immunoprecipitation (IP) with antibodies to RasGAP (A) and paxillin (B), followed by immunoblot analysis (Blot) as indicated.

paxillin complex was detected in suspended cells, though the amount of RasGAP coprecipitated with paxillin was not influenced by A II stimulation (Fig. 4).

Miyamoto et al. reported that concurrent ECM-integrin interaction and intracellular protein tyrosine phosphorylation are essential for the focal accumulation of signaling proteins, including p190 and RasGAP, which is simultaneous with paxillin focal accumulation and activation of signaling through MAP kinases [16]. We have also indicated that cell adhesion resulting in focal adhesion formation is indispensable for A II to induce paxillin tyrosine phosphorylation and to modulate the paxillin complex formation with p190 and RasGAP, which are activated by A II stimulation [29]. In short, our data suggest that cell adhesion to ECMs is essential for A II to prevent the overactivation of MAP kinase through regulating the paxillin-GAPs complexes formation at focal adhesion.

It has been reported that A II type 2 (AT2) receptors antagonize the growth effects of AT1 receptors [30] by inhibiting MAP kinase activity [31]. However, AT1 receptor is dominantly expressed but the expression of AT2 receptor is negligible in mature kidney. AT2 receptor has a relatively weaker inhibitory effect on MAP kinase activity via the mediation of non-specific serine/threonine phosphatase 2A [31], whereas p190 and RasGAP are specific inhibitor of Rho and Ras, respectively.

It is possible that the suppressive effects of cell adhesion to ECMs on cell growth may influence on the progression of some glomerular diseases and experimental nephritis. For example, the massive accumulation of fibronectin observed in mesangial lesion of diabetic rats [32] and the transient accumulation of fibronectin and type I collagen in mesangium in the course of Thy-1.1 nephritis [33] may partly be related to the depletion of mesangial cells. Future work will be directed towards further investigation on the relationship between signals from other growth factors and cell adhesions to ECMs.

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