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Small GTP-binding Protein, Rho, Both Increased and Decreased Cellular Motility, Activation of Matrix Metalloproteinase 2 and Invasion of Human Osteosarcoma Cells

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Rho, a member of the small GTP-binding proteins, and one of its downstream effectors ROCK (Rho-associated coiled-coil forming protein kinase) play an important role in the invasion of tumor cells. Lysophosphatidic acid (LPA) activates Rho and ROCK and promotes the organization of stress fibers and focal adhesions. However, the effect of LPA on tumor cell invasion is still controversial. In the present study, human osteosarcoma cells treated with a high concentration of LPA (high LPA) showed considerable formation of stress fibers and focal adhesions compared to the cells treated with a low concentration of LPA (low LPA). C3 (inhibitor of Rho) or Y27632 (an inhibitor of ROCK) inhibited the effects of LPA, indicating that LPA activates the Rho-ROCK pathway in the cells. In addition, Rho activation assay showed that the activation level of Rho can be altered by changing the concentration of LPA. Low LPA stimulated the motility and invasion of the cells, while high LPA reduced both. The disruption of extracellular matrix (ECM) by matrix metalloproteinase 2 (MMP2) is also critical for tumor cell invasion. MMP2 is activated by membranous type-1 MMP (MT1-MMP) and type-2 tissue inhibitor of MMP (TIMP2). High LPA suppressed the activation of MMP2 through down-regulation of MT1-MMP and TIMP2. C3 and Y27632 reversed the suppression of the activation of MMP2 and expression of MT1-MMP and TIMP2, suggesting the involvement of the Rho-ROCK pathway in ECM degradation. Tyrosine phosphorylation of focal adhesion kinase (FAK) was also required for the invasion of tumor cells to occur. Low LPA enhanced the tyrosine phosphorylation of FAK whereas high LPA reduced it. In conclusion, we suggest that Rho has a dual effect on the invasion of osteosarcoma cells by modulating the motility, the ability to degrade ECM and tyrosine phosphorylation of FAK.

Key words: Rho — Osteosarcoma — Motility — Matrix metalloproteinase and FAK

The Rho subfamily including Rho-A, -B, and -C, belongs to the small GTP-binding protein superfamily, and regulates a wide variety of cell functions, such as cell shape change and cell motility.[1)] These cellular functions are closely related to the cytoskeletal system. It has been shown that Rho activates ROCK (Rho-associated coiled-coil forming protein kinase), and that activated ROCK inhibits the myosin phosphatase, resulting in the continuous phosphorylation of myosin light chain (MLC).[2)] MLC phosphorylation induces the contraction of myosin and subsequently organizes the actin stress fibers and focal adhesions. The assembly of the actin stress fibers and the formation of focal adhesions provide protrusive and contractile forces to the cells, and play important roles in cell motility.[3)]

Cell motility is essential for malignant tumor cells to be able to invade into surrounding tissues. It has been shown that the stable transfection of Rho into hepatoma cells stimulates the organization of the actin stress fibers and the motility of the cells.[4)] The over-expression of Rho in NIH3T3 cells induces transformation of the cells and invasion and experimental metastasis *in vivo*.[5)] These studies suggest that Rho plays an important role in the acquisition of invasive phenotype for tumor cells. On the other hand, it has been demonstrated that the microinjection of constitutively activated Rho into macrophages abolishes the cell motility response to the chemoattractant.[6)] Moreover, sphingosine-I-phosphate, an activator of Rho, exerts an inhibitory effect on the motility of melanoma cells.[7)] These results suggest that Rho serves dual functions, having stimulatory and inhibitory effects on the cell motility. However, the function of Rho in cell motility and invasion and its molecular mechanism are not fully understood yet.

Focal adhesion mediates the cellular binding to extracellular matrix (ECM) of proteins through cell surface receptors called integrins. The integrins cluster at focal adhesion complex together with several other proteins, including focal adhesion kinase (FAK), vinculin, talin and paxilin.[8)] The complex transmits signals from the cell sur-

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face to the nucleus. FAK plays a critical role in controlling the signal transduction, including cell migration.[9, 10] Cultured fibroblasts isolated from FAK-deficient mice display reduced cell motility and enhanced focal adhesion formation.[11] It was also reported that an increased level of FAK expression is highly correlated with the invasion potential of human tumor cells.[12] Furthermore, it has been demonstrated that Rho activates FAK by the phosphorylation of protein.[13] These observations suggested that the modulation of cell motility by Rho may be mediated by the activation of FAK. In the present study, we investigated the effects of lysophosphatidic acid (LPA)-driven Rho activation on the cell attachment to the basement membrane, activity for degradation of the ECM, and cell motility, all critical steps of tumor cell invasion and metastasis. Furthermore, we also studied whether LPA-induced Rho activation might affect the phophorylation of FAK.

Here we show that LPA concentration correlates with the activation level of Rho and phosphorylation level of FAK. Treatment of the cells with a low concentration of LPA stimulates both cell motility and ECM degradation, whereas treatment with a high concentration of LPA inhibits both of them. Our results suggest that FAK may be involved in the regulation by Rho of cellular phenotypes related to invasion and that an optimal activation level of Rho may be required for stimulation of cell motility, ECM degradation and invasion of human osteosarcoma cells.

MATERIALS AND METHODS

Cells and reagents The human osteosarcoma cell line MNNG was obtained from ATCC (Rockville, MD). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD) in a 95% air and 5% CO_2 atmosphere at 37°C. LPA and cytochalasin D were purchased from Sigma (St. Louis, MO). C3 botulinum exotoxin, glutathione S-transferase (GST)-Rho-binding domain of Rhotekin (GST-RBD) and Anti-Rho (-A, -B, -C) Abs were purchased from Upstate Biotechnology Institute (Lake Placid, NY). Y27632, an inhibitor of p160 ROCK, was kindly provided by Yoshitomi Chemical Industry (Saitama). Anti-membranous type-1 MMP (MT1-MMP) Ab was obtained from Carbiochem (Cambridge, MA). Anti-paxillin and anti-FAK Abs were obtained from Transduction Laboratories (Lexington, KY). Anti-phosphorylated FAK and the horseradish peroxidase-conjugated secondary Abs were obtained from Biosource (Illinois, IL) and anti-actin Ab was obtained from Boehringer Mannheim (GmbH, Mannheim, Germany). Anti-type-2 tissue inhibitor of MMP (TIMP2) polyclonal Ab was generated in our own laboratory.[14]

RNA extraction and reverse-transcriptional (RT)-PCR The MNNG cells were plated at a density of 2×10^4 cells/2 ml of media in 35-mm dishes. After incubation with or without various concentrations of LPA, C3, Y27632 or Cyto D, total RNA was isolated from cells using an RNA-easy kit (Qiagen, Hilden, Germany). Two micrograms of total RNA was subjected to the reverse-transcription reaction using the Ready-to-Go cDNA synthesis kit (Amersham, Arlington Heights, IL). The sequences of primers for MT1-MMP, TIMP2 and 18S subunit of ribosomal RNA amplification have been described previously.[15–17] The PCR reaction was performed in a final volume of 50 μl for 30 cycles, consisting of a heat-denaturation step at 94°C for 1 min, a primer-annealing step at 55°C for 30 s, and an extension step at 72°C for 1 min. The PCR was performed within the linear range of amplification (confirmed by a preliminary study). The PCR products were analyzed by 1.5% agarose gel (Sigma) electrophoresis.

Western analysis Logarithmically growing cells ($2-4\times10^6$) were harvested at approximately 70% confluence and solubilized in lysis buffer [20 m*M* Tris (pH 7.4), 250 m*M* NaCl, 1.0% NP40, 1 m*M* EDTA, 50 mg/ml leupeptin, and 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 1 m*M* sodium orthovanadate and 1 m*M* NaF]. After incubation on ice for 10 min, the cells were sonicated, and clarified by centrifugation at 14 000 rpm for 30 min at 4°C. Protein quantity was determined using the Bradford protein assay kit (Bio-Rad, Hercules, CA). The samples were boiled for 5 min, and 10 μg of total proteins from each sample was run on a 4–12% gradient MOPS-polyacrylamide gel (Novex, San Diego, CA) and blotted onto nitrocellulose filters (Amersham). The filters were pretreated with TBS containing 5% dry milk and 0.05% Triton-X for 2 h at room temperature, then incubated with the appropriate primary antibodies for 2 h at room temperature. After being washed several times, the filters were incubated with the horseradish peroxidase-conjugated secondary antibody (Biosource). Following the final washing, the immunoreactivity of the blots was detected using an enhanced chemiluminescence system (Amersham).

Zymography Zymography was performed as described previously.[18] In brief, a total of 1.5×10^5 cells were seeded onto 24-well chambers and incubated for 6 h in serum-containing media. Then the cells were rinsed twice and incubated in serum-free media for an additional 24 h. After incubation, conditioned media were collected and centrifuged to remove any suspended cells or debris. The samples were mixed with sample buffer and electrophoresed on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel containing 2.5 mg/ml gelatin. The gel was washed and incubated in reaction buffer for a further 24 h at 37°C. Then, it was stained with Coomassie Blue and destained with a destaining buffer.

ELISA A total of 1.5×10^5 cells were seeded onto 24-well chambers and incubated for 6 h in serum-containing media. Then the cells were rinsed twice and incubated in

serum-free media for an additional 24 h. After incubation, conditioned media were collected and centrifuged to remove any suspended cells or debris. Levels of active and pro-form of MMP2 were measured in the conditioned media by means of ELISA (Amersham) following the manufacturer's protocol. All experiments were performed in triplicate and repeated twice.

Immunofluorescence The cells were seeded on cover slips coated with the reconstituted basement membrane Matrigel (Becton Dickinson, Bedford, MA). The cells were incubated with various agents in serum-free media for 24 h. Then the cells were washed several times with ice-cold phosphate-buffered saline (PBS), fixed for 10 min with 4% paraformaldehyde in PBS, and permeabilized in 0.1% Triton X-100. The cells were treated for 1 h at room temperature with anti-paxillin Abs. After being washed with PBS several times, the cells were incubated with FITC-conjugated anti-mouse IgG for 45 min at room temperature. Then, to stain the actin filaments, TRITC-conjugated phalloidin (Sigma) was applied to the cells at a concentration of 0.05 mg/ml in PBS for 40 min at room temperature. After several washings, the coverslips were mounted, and the cells were examined by confocal laser scanning microscopy.

Activation assay of Rho Activation assay of Rho was performed as described previously.[19] MNNG cells were washed twice with PBS and incubated in fresh DMEM without serum for 6 h; various concentrations of LPA were added and the cells were harvested at 30 min after LPA addition. The cell pellet was lysed in 50 m*M* Tris-HCl buffer, pH 7.5, containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 m*M* NaCl, 10 m*M* $MgCl_2$, 10 μg/ml each of leupeptin and aprotinin, and 1 m*M* PMSF. Cell lysates were clarified by centrifugation at 13 000×*g* at 4°C with glutathione beads (Amersham) coupled with GST protein alone or bacterially expressed GST-RBD fusion protein (Upstate Biotechnology Institute). The beads were washed four times with 50 m*M* Tris-HCl buffer, pH 7.5, containing 1% Triton X-100, 0.1% SDS, 500 m*M* NaCl, 10 m*M* $MgCl_2$, 10 μg/ml each of leupeptin and aprotinin, and 1 m*M* PMSF. Activated Rho bound to beads or total Rho in cell extracts was detected by western blotting using a polyclonal antibody against Rho (-A, -B, -C) (Upstate Biotechnology Institute). Rho activity is indicated by the amount of RBD-bound Rho normalized to the amount of Rho in whole cell lysates.

Adhesion assay The adhesion assay was performed as previously described.[20] Briefly, Matrigel was incubated and dried in each well of a 24-well culture plate and the plates were then incubated with 3% bovine serum albumin (BSA) in PBS to block nonspecific binding and washed with PBS. MNNG cells were incubated with or without various concentrations of LPA (5–50 μM) for 24 h. The cells were detached from dishes and resuspended in DMEM containing 0.1% BSA with or without LPA. The cells (5×10^4 cells/well) were allowed to attach for 1 h at 37°C, and adherent cells were then counted with a Coulter counter (Beckman Coulter, Inc., Fullerton, CA). All experiments were performed in triplicate and repeated twice.

Invasion assay The invasion assay was performed as described previously.[21, 22] Polycarbonate filters, 8 μm in pore size (Costar, Cambridge, UK), were coated with 25 μg of basement membrane Matrigel (Becton Dickinson), dried under a hood, and placed in Boyden chambers. The lower chambers were filled with conditioned media obtained by incubating NIH3T3 cells for 24 h in DMEM containing 0.005% ascorbate and 0.1% BSA. MNNG cells were incubated with or without various concentrations of LPA for 24 h. Then, 3×10^5 viable cells suspended in DMEM supplemented with 0.1% BSA were placed in the upper compartments of the Boyden chamber with or without LPA. After incubation for 5 h at 37°C in a 95% air and 5% CO_2 atmosphere, the filters were removed, fixed with methanol and stained with hematoxylin and eosin. The cells that had migrated to the lower surface of the filters were counted in eight randomly selected microscopic fields (×400) per filter. All experiments were performed in triplicate and repeated three times.

Phagokinetic track assay The cover slips were coated with colloidal gold as described previously.[23] Briefly, 9 ml of 14.5 m*M* $AuCl_4H$ and 30 ml of 36.5 m*M* Na_2CO_3 were added to 55 ml of H_2O, and the mixture was heated in a glass beaker. Immediately after reaching the boiling point, the solution was removed from the heat, and 9 ml of 0.1% formaldehyde solution was added. Colloidal gold formed within 1 min. The solution was poured into 35-mm tissue culture dishes coated with bovine serum albumin. After incubation for 45 min, the solution was removed and the dishes were washed extensively with PBS. For the cell motility assay, the cells were seeded on colloidal gold-coated dishes at a density of 5×10^3 cells per dish in 1 ml of serum-free medium and incubated for 24 h. We randomly selected fifty cells and the cell motility was evaluated by measuring areas free of gold particles.

Statistical analysis All statistical analyses were carried out according to Student's *t* test.

RESULTS

Effect of LPA on the formation of stress fibers and focal adhesions in MNNG cells LPA is commonly known to induce the formation of focal adhesions and actin stress fibers through the activation of Rho.[24] To determine the effect of LPA treatment on MNNG cells, we incubated the cells with various concentrations of LPA on Matrigel-coated coverslips. Since phalloidin binds to actin fiber and paxillin aggregates to focal adhesions, we performed immunofluorescence histochemistry with phalloi-

din and anti-paxillin antibody to observe the formation of actin fiber and focal adhesions, respectively. These experiments were performed under serum-free conditions to avoid possible interference. The untreated MNNG cells did not show formation of stress fibers (Fig. 1a). Focal adhesions were observed at the periphery of the cells (Fig. 1b). The cells incubated with a low concentration of LPA (low LPA) (5 μM) displayed a considerably greater degree of spreading. The cells also developed focal adhesions primarily at the edge of the cells and a relatively small amount of stress fibers was observed (Fig. 1, c and d). On the other hand, when the cells were incubated with a high concentration of LPA (high LPA) (25 μM), large amounts of stress fibers and focal adhesions were formed (Fig. 1, e and f). These results suggest that the effects of LPA on the formation of stress fibers and focal adhesions may differ according to the concentration of LPA.

We next addressed the question of whether the activation of Rho is involved in the LPA-induced formation of stress fibers and focal adhesions in MNNG cells. We examined the effect of botulinum C3 exotoxin (C3) (5 μg/ml), a selective inhibitor of Rho, on the LPA-treated cells. Treatment with C3 almost completely blocked the high-LPA-induced formation of stress fibers and focal adhesions (Fig. 1, g and h). The C3 treatment also inhibited the low-LPA induced formation of stress fibers and focal adhesions (data not shown). It is known that Rho induces actin fiber organization through p160ROCK,[2] and that a recently synthesized peptide Y27632, selectively inhibits the activation of p160ROCK.[25] When the cells were treated with high-LPA plus Y27632 (10 μM), the formation of stress fibers and focal adhesions induced by high LPA was also inhibited (data not shown). These data suggest that the formation of stress fibers and focal adhesions by both low LPA and high LPA might be mediated by the signaling pathway from Rho to p160ROCK in MNNG cells.

Activation of Rho by low and high LPA To investigate the activation level of Rho by low and high LPA, we next measured the intracellular levels of the GTP-bound, active form of Rho by the pull-down assay system.[19] As shown in Fig. 2, the level of GTP-bound Rho was elevated dose-dependently after the addition of LPA. Virtually no Rho was detected bound to agarose beads with GST alone. The

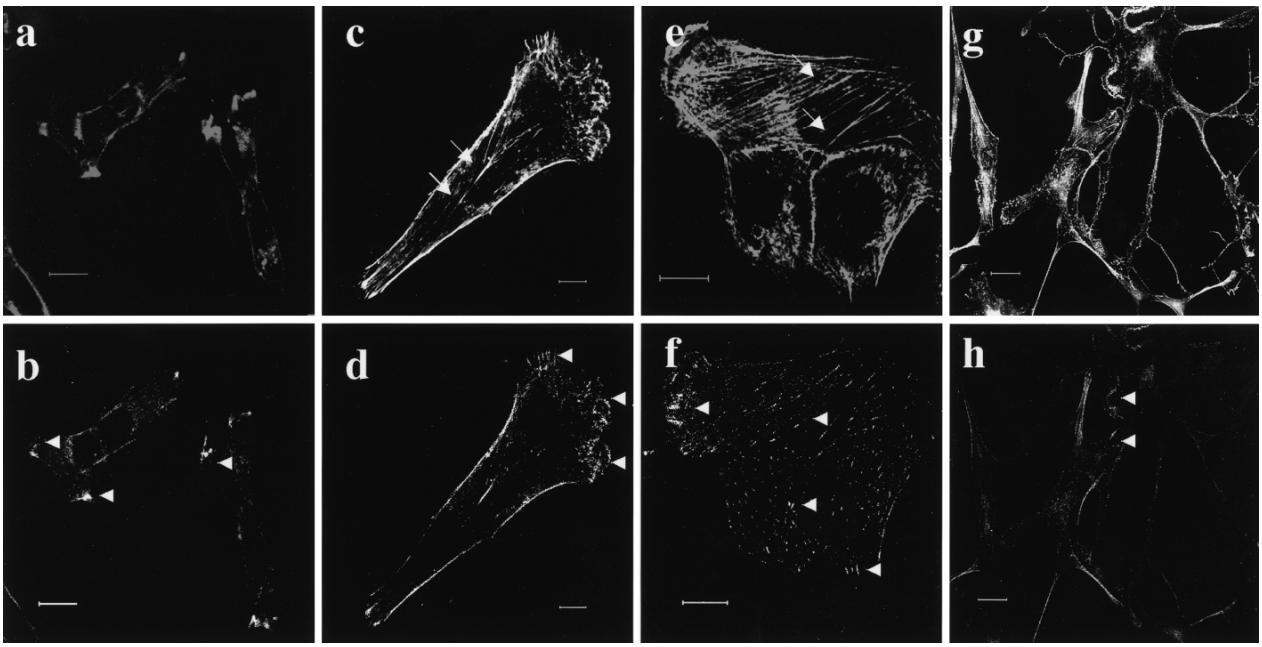

Fig. 1. Modulation of the formation of actin stress fibers and focal adhesions of MNNG cells after treatment with LPA. The cells were seeded and allowed to spread on Matrigel-coated cover slips with the indicated concentrations of LPA with or without botulinum exotoxin C3 for 24 h. Then, the cells were stained with rhodamine-phalloidin (a, c, e, g), or immunostained with antibodies to paxillin (b, d, f, h) to detect actin stress fibers or focal adhesions, respectively. Arrows indicate stress fibers and arrowheads indicate focal adhesions. Scale bars, 20 μm.

values of relative Rho activity after low and high LPA treatment were increased about 1.6- and 2.6-fold compared to serum-starved control cells, respectively. These results demonstrate that LPA stimulates the activation of Rho in a dose-dependent manner.

Effect of LPA on invasion, cell adhesion to Matrigel and chemokinesis of MNNG cells To examine whether treatment of the cells with LPA would affect the invasiveness of the tumor cells, *in vitro* invasion assay was carried out. As can be seen in Fig. 3a, treatment with low LPA (5 and 10 μM) stimulated the invasion of the cells to 1.2- and 1.5-fold of that of the control, respectively. However, when the cells were treated with high LPA (25 and 50 μM), the number of invaded cells was reduced to about 50% and 35% of that of the control cells, respectively. These results suggest that LPA treatment might have both a stimulatory and an inhibitory effect on the invasion of the cells. To determine whether the effect of LPA on invasion is due to altered adhesion, we examined the effect of LPA on cell adhesion to Matrigel. However, the adhesiveness of the cells to Matrigel was not affected by any concentration of LPA examined (Fig. 3b). Since one of the critical steps of invasion of the tumor cells is cell motility, we next examined the effect of LPA treatment on the motility of MNNG cells. Cell motility can be random (chemokinesis) or directed towards concentration gradients of various attractants (chemotaxis). Gold colloid phagokinetic assay, which is correlated with the chemokinesis of cells, demonstrated that treatment with low LPA significantly stimulated the chemokinesis of the cells, while treatment with high LPA inhibited the chemokinesis when compared to untreated cells (Fig. 3c). Similar results were obtained from chemotaxis assay (data not shown). This suggests that stimulation and inhibition of cell motility by treatment with low and high LPA, respectively, may result in alteration of the invasion phenotype of the cells.

Effect of LPA on ECM degradation of MNNG cells Enzymatic degradation of ECM is another important step in tumor cell invasion.[26] It has been shown that matrix metalloproteinase-2 (MMP2) plays a critical role in the degradation of ECM, and that the expression of MMP2

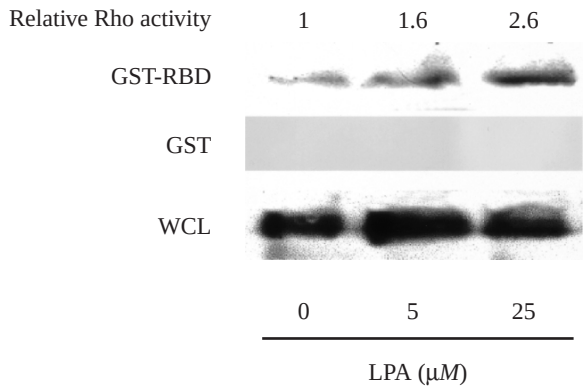

Fig. 2. Activation of cellular Rho in response to stimulation with low and high LPA. MNNG cells were incubated in the absence of serum for 6 h, and subsequently cultured with 5 and 25 μM LPA for 30 min. The cell lysates were then incubated with GST or GST-RBD beads. After extensive washing, the bound protein and total Rho in the whole cell lysates (WCL) were analyzed by western blotting with a polyclonal anti-Rho (-A, -B, -C) antibody. Rho activity was indicated by the amount of RBD-bound Rho normalized to the amount of Rho in WCL. Values represent Rho activity relative to serum-starved control cells.

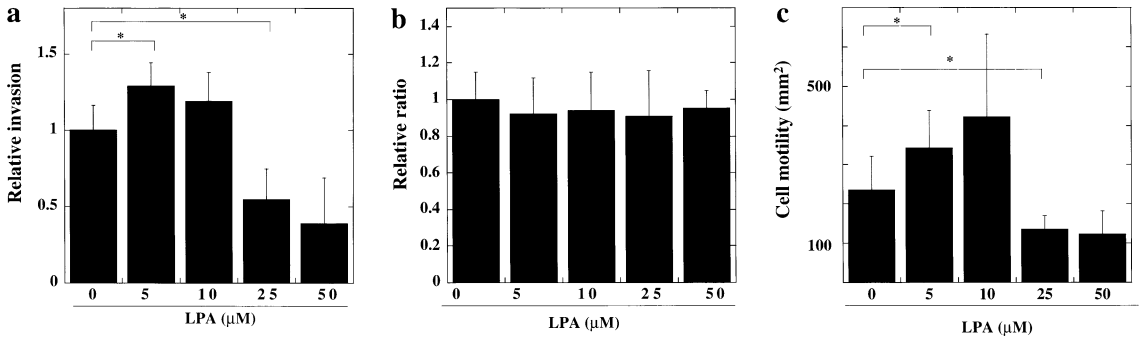

Fig. 3. Analyses of invasion and cell motility of the MNNG cells. a, *In vitro* invasion assay of MNNG cells. The cells were incubated with various concentrations of LPA and were allowed to migrate for 5 h through polycarbonate filters coated with 25 μg of Matrigel. The invasion was measured as described under "Materials and Methods." Bars, standard deviation (SD) from three independent experiments. *, $P<0.05$ versus control. b, Adhesion assay of MNNG cells. The cells were treated with the indicated concentrations of LPA for 24 h and attachment of cells to Matrigel was determined as described under "Materials and Methods." c, Phagokinetic track assay of MNNG cells. The cells were plated on cover-slips coated with colloidal gold. After incubation for 24 h, cell motility was evaluated by measuring areas free of gold particles. Bars, SD from three independent experiments. *, $P<0.05$ versus control.

closely correlates with the metastatic abilities of various tumors.[27] Thus, we examined the effect of LPA on MMP2 activation in MNNG cells. ELISA analysis showed that treatment of the cells with high LPA (25 and 50 μM) reduced the expression of the active form of MMP2 compared to control cells, whereas treatment of the cells with low LPA (5 and 10 μM) enhanced the expression of the active form of MMP2 ($P<0.05$) (Fig. 4a). Expression of the pro-form of MMP2 was not altered by the LPA treatment (Fig. 4b). Similar results were obtained from gelatin zymography (data not shown). We next co-treated the cells with LPA plus C3 or Y27632. As shown in Fig. 4c, treatment of the cells with 25 μM LPA suppressed the activation of MMP2, but the cells incubated with 25 μM LPA plus C3 or Y27632 showed restored expression of the active form of MMP2. These results suggest that the activation of Rho induced by high-LPA treatment was linked to the inhibition of the activation of MMP2.

Changes in the expression of MT1-MMP and TIMP2 by LPA treatment Since the activation of MMP2 is mainly regulated by MT1-MMP and TIMP2,[28] we also investigated the effect of LPA treatment on the expression of MT1-MMP and TIMP2 in MNNG cells. Western blot analysis showed that treatment with high LPA (25 μM)

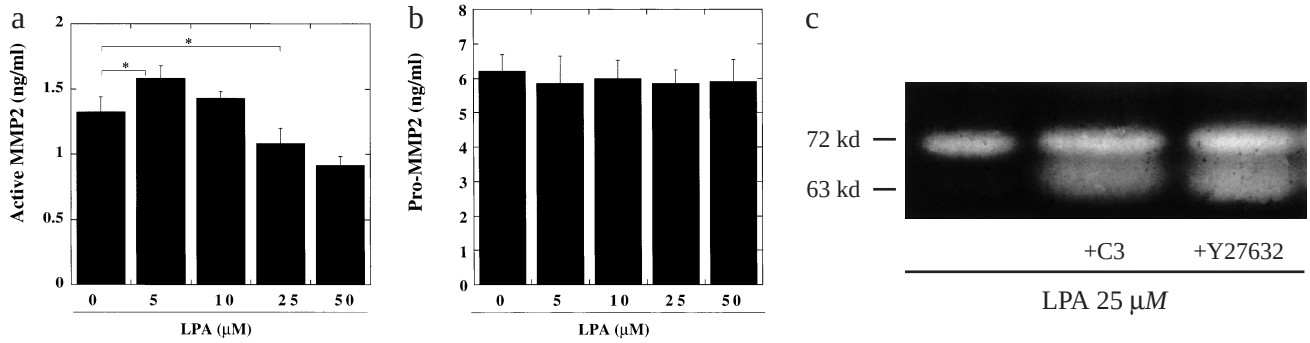

Fig. 4. Effect of LPA on activation of MMP2. MNNG cells were treated with the indicated concentrations of LPA for 48 h. After the treatment, the conditioned medium was harvested and subjected to ELISA analysis as described under "Materials and Methods." a, Levels of active MMP2 expression in MNNG cells. Bars, SD from two independent experiments. ∗, $P<0.05$. b, Levels of pro-MMP2 expression MNNG cells. Bars, SD from two independent experiments. c, Effect of Rho and ROCK inhibitors on LPA-suppressed MMP2 expression. MNNG cells were co-treated with 25 μM LPA plus C3 (5 μg/ml) or Y27632 (10 μM) for 48 h and the conditioned medium was harvested and analyzed by zymography as described above. The latent (72 kDa) and active (63 kDa) forms of MMP-2 are indicated.

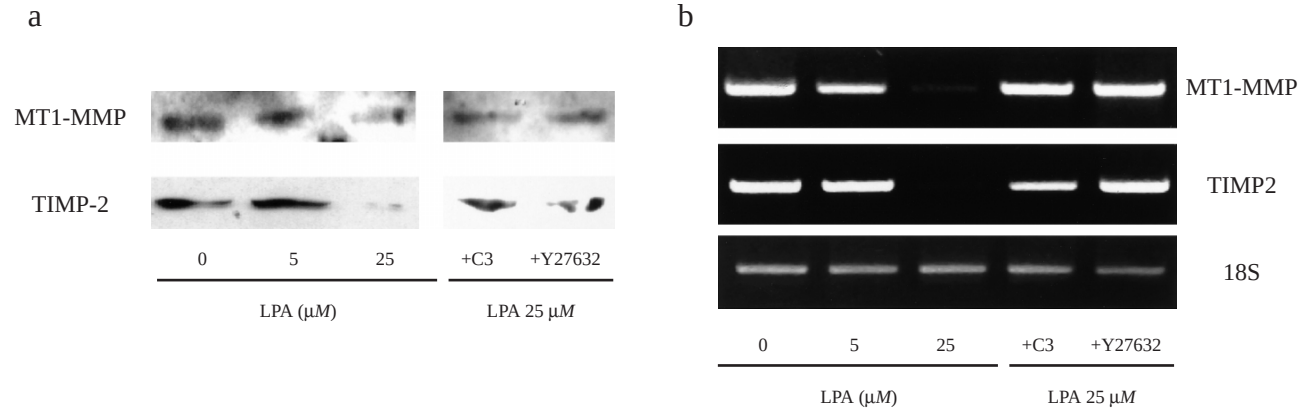

Fig. 5. Effect of LPA, C3 and Y27632 treatment on the expression of MT1-MMP and TIMP2. a, Western blot analysis of MT1-MMP and TIMP2 expression. MNNG cells were incubated with the indicated concentrations of LPA, C3 and Y27632 for 24 h and the cell lysates were subjected to immunoblotting with anti-MT1-MMP and anti-TIMP2 Abs. b, RT-PCR analysis of the expression of MT1-MMP and TIMP2 mRNAs in MNNG cells co-treated with LPA plus C3 or Y27632. After treatment for 24 h, total RNA was extracted and 2 μg of total RNA was reverse-transcribed. The cDNA was then subjected to PCR and electrophoresed on a 1.5% agarose gel. Ribosomal 18S RNA amplification served as an internal control.

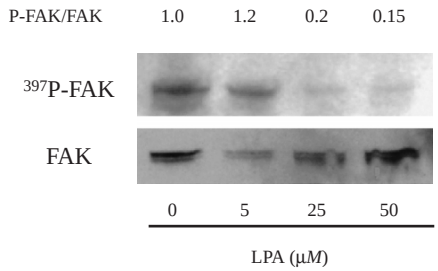

Fig. 6. Effect of LPA on tyrosine phosphorylation of FAK. MNNG cells were incubated with the indicated concentrations of LPA for 24 h and the cell lysates were subjected to western blotting using anti-FAK and anti-phosphorylated FAK Abs. The anti-phosphorylated FAK Ab reacted with the tyrosine residue at 397 aa, the autophosphorylation site of FAK. The ratios of tyrosine-phosphorylated FAK to FAK were determined by scanning the intensity of each band on the blots.

reduced the expression of MT1-MMP and TIMP2 compared to controls by approximately 20% and 10%, respectively (Fig. 5a). Consistent with the western blot results, RT-PCR analysis also demonstrated that treatment with high LPA reduced mRNA expression of MT1-MMP and TIMP2 in the cells (Fig. 5b). The levels of MT1-MMP and TIMP2 expression were restored by co-treatment with 25 μM LPA plus C3 or Y27632 (Fig. 5, a and b).

The effect of LPA on tyrosine phosphorylation of FAK

To examine the effect of LPA treatment on FAK phosphorylation in MNNG cells, the cells were challenged with various concentrations of LPA for 24 h, and the cell lysates were immunoblotted with anti-FAK or anti-phosphorylated FAK. Since autophosphorylation at Y397 in FAK is necessary for the FAK-dependent motility of human breast cancer cells,[29] we used the anti-phosphorylated FAK Ab which reacts to Y397 in FAK. Densitometric analysis revealed that treatment of the cells with low LPA slightly stimulated the expression of tyrosine-phosphorylated FAK. However, only a faint expression of tyrosine-phosphorylated FAK was observed after incubation of the cells with high LPA (Fig. 6).

DISCUSSION

It has been reported that the invasion of tumor cells requires the activation of the small GTP-binding protein Rho.[4, 5, 30] Meanwhile, several other reports have demonstrated that the activation of Rho could reduce the invasiveness and cell motility of tumor cells and mouse macrophages.[6, 7] These contradictory observations may suggest that the activation of Rho has dual effects on tumor cell invasion.

To investigate this possibility, we used LPA, a strong and specific activator of Rho. It is known that low LPA (less than 1.0 μM) exhibits biological activities such as neurite retraction of neuroblastoma cells and platelet aggregation.[31] However, it was also reported that high LPA but not low LPA is required for stimulation of invasion of rat ascites hepatoma cells.[32] Thus, we examined the effects both of low and high LPA on human osteosarcoma cells, MNNG. Treatment of the cells with high LPA caused the formation of a large amount of stress fibers and focal adhesions compared to the cells treated with low LPA. Botulinum exotoxin C3, which is a specific inhibitor of Rho, abrogated the organization of stress fibers and focal adhesions induced by the LPA treatment. In addition, Rho activation assay clearly demonstrated that the relative Rho activity of MNNG cells after high-LPA treatment was stronger than that after low-LPA treatment. These results suggest that LPA-induced formation of stress fibers and focal adhesions was due to the activation of Rho and that LPA stimulates the activation level of Rho in a dose-dependent manner.

Recent studies revealed that Rho and ROCK play essential roles in migration of rat ascites hepatoma cells and that C3 and Y27632 suppress the migration.[33] These observations indicate that inhibition of activation of Rho may result in the reduction of invasion of the cells. In this study, we further investigated the correlation of low and high activation levels of Rho and invasion of the cells. Treatment with low LPA stimulated the invasion of the cells *in vitro*, whereas treatment with high LPA significantly reduced the invasion of the cells. These data suggest that the invasive phenotype of the cells may be precisely controlled by the activation levels of Rho induced by LPA. In other words, there seems to be an optimal activation level of Rho for the stimulation of tumor cell invasion.

We examined which steps of the invasive phenotypes, including adhesion, motility and ECM degradation, were altered by LPA treatment. We first carried out adhesion assay using MNNG cells. None of the concentrations of LPA affected the adhesiveness of the cells. Therefore, we next examined the effect of LPA treatment on MNNG cell motility by a phagokinetic track assay. The motility of the cells treated with low LPA increased to 2 times that of the control, while cells treated with high LPA showed reduced cell motility. It is known that cells have to polarize morphologically for migration[34] and that microinjection of constitutively activated Rho into mouse macrophages abrogated the polarization of the cells,[2] resulting in the inhibition of cell migration. These results suggest that the reduction of cell motility after high-LPA treatment might be due to the inhibition of polarization of the cells, which was induced by an excess activation of Rho and subsequent over-expression of stress fibers and focal adhesions. When the cells were treated with C3 plus low LPA, the cell motility was also inhibited (data not shown). There-

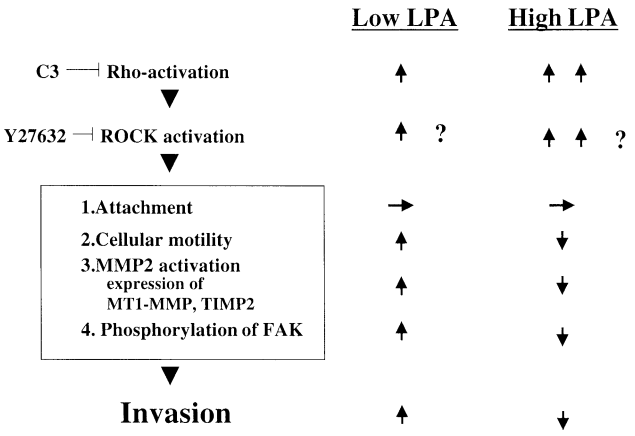

Fig. 7. Summary of effects of low- and high-LPA treatment on MNNG cells.

fore, the activity of Rho should be precisely regulated in order for cells to migrate effectively.

Disruption of the ECM and basement membrane by proteinases is another critical step in tumor cell invasion.[28] Since MMP2 activation plays an important role in enzymatic destruction of ECM by tumor cells,[27] we examined the effect of LPA treatment on the activation of MMP2. Results of ELISA analysis revealed that treatment of MNNG cells with low LPA increased the expression of the active form of MMP2. On the other hand, treatment with high LPA decreased the expression of the activated MMP2. However, we could not detect any significant difference in the expression of pro-form of MMP2 after the LPA treatment. Therefore, the inhibition of MMP2 activation may be another reason for the reduction of invasion of the cells treated with high LPA. The expression level of MT1-MMP, which plays a major role in the activation of MMP2,[35] was also reduced after high-LPA treatment. MMP2 is also known to bind to its natural inhibitor, TIMP2, and it has been shown that TIMP2 is required for the activation of MMP2.[36] In our study, TIMP2 expression was also reduced by treatment with high LPA. These results suggest that treatment with high LPA may decrease the amount of active MMP2 by reducing the expression of MT1-MMP and TIMP2, resulting in the inhibition of cell invasion.

It has been reported that MMP2 activation and MT1-MMP expression were decreased by the organization of the stress fibers in human palmar fascial fibroblasts.[37] Cytochalasin D binds to actin filaments and disrupts the formation of stress fibers and focal adhesions in human mesangial cells, inducing the activation of MMP2.[18] These observations suggest that the formation of stress fibers and focal adhesions might cause the inactivation of MMP2. Therefore, we analyzed whether the Rho-ROCK pathway is involved in the activation of MMP2. When MNNG cells were co-treated with 25 μM LPA plus C3, the formation of stress fibers and focal adhesions was disrupted and the expression of MT1-MMP and TIMP2 and the activation of MMP2 were restored. In addition, Y27632 also effectively restored the expression of MT1-MMP and TIMP2 and induced activation of MMP2. Therefore, we concluded that, in MNNG cells, the Rho-ROCK pathway may be involved in the activation of MMP2 via regulation of the expression of MT1-MMP and TIMP2.

Recent study has demonstrated that changes in phosphorylation of several focal adhesion proteins, including FAK, play important roles in cell adhesion, migration and invasion.[38, 39] LPA stimulates the tyrosine phosphorylation of FAK through the activation of Rho.[13] Furthermore, tyrosine phosphorylation of FAK in rat ascites hepatoma cells by high LPA (25 μM) was implicated in the regulation of invasion of the cells.[32] However, the dose-response effect of LPA on tyrosine phosphorylation of FAK was not known. In the present study, treatment of the cells with low LPA increased phosphorylation of FAK, while high LPA reduced the phosphorylation of FAK. In addition, co-treatment with low LPA plus herbimycin A, a tyrosine kinase inhibitor, reduced the invasion of the cells (data not shown). These results suggest that tyrosine phosphorylation of FAK might be involved in the invasion of MNNG cells, which was stimulated or inhibited by LPA treatment and subsequent Rho activation. However, further study is needed to determine the role of the LPA-Rho-FAK pathway in tumor cell invasion. The variety of effects of low- and high-LPA treatment on MNNG cells is summarized in Fig. 7.

In conclusion, our data indicate that treatment with low LPA stimulates the invasion and motility of MNNG cells, whereas treatment with high LPA inhibits the invasion through the over-formation of stress fibers and focal adhesions. This inhibited invasion may be due to the loss of cell motility, the inactivation of MMP2 and the dephosphorylation of FAK. The cell motility, ECM degradation and the cell invasion of human osteosarcoma cells may be precisely and simultaneously regulated by the activation level of Rho.

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