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Cooperation of fibronectin with lysophosphatidic acid induces motility and transcellular migration of rat ascites hepatoma cells

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Abstract

We have previously shown that the transcellular migration of rat ascites hepatoma (AH130-MM1) cells through a cultured mesothelial cell monolayer (MCL) is triggered with lysophosphatidic acid (LPA) that stimulates actin polymerization and myosin light chain phosphorylation through the activation of Rho-ROCK (Rho-kinase) cascade. When, however, the motility of MM1 cells on a glass surface was tested by phagokinetic track motility assay, LPA failed to induce the motility. Nevertheless, when the glass had been coated with fibronectin (FN), LPA could induce phagokinetic motility which was accompanied by transformation of MM1 cells to fusiform-shape and assembly of focal adhesion. β1 integrin, the counter receptor of FN, was expressed on MM1 cells. Anti-FN antibody, anti-β1 integrin antibody and cyclo-GRGDSPA remarkably suppressed LPA-induced phagokinetic motility. These antibodies suppressed LPA-induced transcellular migration through MCL, as well. These results indicate that actin polymerization and phosphorylation of myosin light chain through Rho activation are insufficient for inducing motility but the cooperative FN/β1 integrin-mediated adhesion is necessary for both the phagokinetic motility and transcellular migration of MM1 cells.

Keywords: Fibronectin; β1 integrin; Lysophosphatidic acid; Motility; Transcellular migration; Rho

1. Introduction

Invasion into surrounding tissues is a prominent phenotype of cancer cells [1–3]. We have studied molecular mechanisms of tumor cell invasion using experimental carcinomatous peritonitis by rat ascites hepatoma (AH130) cells and an in vitro model system for tumor invasion [4]. In this model system, the

Abbreviations: MCL, mesothelial cell monolayer; LPA, lysophosphatidic acid; FN, fibronectin; FCS, fetal calf serum; HA, hyaluronic acid; VN, vitronectin; CL, collagen; LM, laminin; PLL, poly-L-lysine; M-MEM, modified minimum essential medium; PBS, phosphate-buffered saline; ECM, extracellular matrix

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tumor cells were seeded on a cultured mesothelial cell monolayer (MCL) and the number of resultant invasion foci underneath the monolayer was counted and regarded to be an ability of invasion (transcellular migration) in vitro of the tumor cells. Rat ascites hepatoma cells grow in suspension in culture dishes and when implanted into the peritoneal cavity they invade the peritoneum extensively. In vitro invasive capacity of several clones obtained from the parental AH130 cells correlated well with their invasiveness in vivo [5,6], indicating that this in vitro system is a good model for invasion in vivo. Using this system, we have already clarified that certain types of tumor cells, such as invasive rat ascites hepatoma (AH130-MM1) cells, human lung cancer cells (OC10), and mouse melanoma cells (B16) require fetal calf serum (FCS) for the transcellular migration [7]. Lysophosphatidic acid (LPA), a well-characterized phospholipid, could completely substitute for the serum in inducing the transcellular migration [8].

A low molecular weight GTPase, Rho plays a pivotal role in this LPA-induced transcellular migration [9]. In short, *Clostridium botulinum* C3 exoenzyme, which specifically ADP-ribosylates and hence inhibits Rho, strongly suppressed the invasion. In addition, the introduction of dominant active rho A cDNA into MM1 cells cancelled FCS or LPA requirement in MM1 cell transcellular migration [10]. These lines of evidence support our conclusion that Rho is critical in tumor cell invasion. We have further reported LPA-induced polymerization of actin [11] and increased phosphorylation of myosin light chain through Rho-ROCK cascade [12], both of which are essential for generation of motile force in the transcellular migration.

These intracellular molecular events no doubt reflect on a series of morphological responses of MM1 cells. MM1 cells, that are plated on a glass slide extend cell surface blebbing and pseudopodia in response to LPA [13]. Since MM1 cells invade a MCL in the presence of LPA, this morphological change appeared to be sufficient for the motility of MM1 cells. However, when they were tested for their motility on a glass slide by a phagokinetic track motility assay, they were found not to move in response to LPA. In contrast, they did move in the presence of 10% FCS in the medium. In the present study, we identified a factor that cooperates with LPA in inducing the motility of MM1 cells.

2. Materials and methods

2.1. Drugs and chemicals

Bovine serum albumin (BSA) fraction V and 1-oleoyl-*sn*-lysophosphatidic acid (LPA) were purchased from Sigma, St. Louis, MO. LPA was dissolved in phosphate-buffered saline (PBS) supplemented with 0.1% BSA.

Since MM1 cells grow in suspension, poly-L-lysine (PLL) was used to adhere MM1 cells to the glass slide. PLL-coated glass slide which was supplied by Matsunami Glass, Osaka, Japan was used throughout this study.

Fibronectin (FN) from bovine plasma and hyaluronic acid (HA) were purchased from Sigma, vitronectin (VN) from bovine plasma and collagen (CL) type I, type IV from Koken, Tokyo, Japan and laminin (LM) from Chemicon, Temecula, CA. Cyclo-GRGDSPA was a gift from Asahi Glass, Yokohama, Japan. C3 was provided from Dr. R. Komagome, Hokkaido University, Faculty of Veterinary Medicine.

Hamster anti-rat CD29 (integrin β1-chain) monoclonal antibody (Ha2/5) and mouse anti-rat CD61 (integrin β3-chain) monoclonal antibody (F11) were purchased from Pharmingen, San Diego, CA. Anti-bovine FN polyclonal antibody was purchased from Yagai, Yamagata, Japan.

2.2. Cells and culture conditions

Rat mesothelial cells were isolated from Donryu rat mesentery and cultured in modified MEM (Nissui, Tokyo, Japan) containing 2-fold amino acids and vitamins (M-MEM) supplemented with 10% FCS. When mesothelial cells grew confluent, MCL was used for the transcellular migration assay. Cells of MM1, which is a highly invasive clone isolated from parental rat ascites hepatoma (AH130) cells, were cultured in suspension in M-MEM supplemented with 10% FCS.

2.3. Phagokinetic track motility assay

Cell motility was determined on the basis of phagokinetic tracks on a gold particle-coated glass slide. Briefly, uniform carpets of colloidal gold particles were prepared on glass slides with a slight modification of the method previously described by Albrecht-Buehler [14]. The glass slides were further coated with FCS or ECM components, when necessary. The slides were then placed in 35-mm tissue culture dishes containing 2 ml of culture medium, and then 1.2×10^5 MM1 cells that had been washed once with M-MEM were added to each dish together with 10% FCS or 25 μM LPA and incubated at 37°C for 7 h in a CO_2 incubator. When necessary, MM1 cells were preincubated with anti-integrin antibodies or cyclo-GRGDSPA at 4°C for 10 min, or FN-coated or FCS-coated glass slide surface was preincubated with anti-FN antibody at 4°C for 10 min. The area swept out by MM1 cells was measured with an image analyzer (Olympus XL-20, Tokyo, Japan). The cell motility was expressed as the total area of gold particles swept out by 100 cells for 7 h.

2.4. Flow cytometry

MM1 cells were washed twice with M-MEM. Suspended cells (1×10^6 cells) were incubated on ice for 1 h with the mixture of primary antibody solution (10 μg/ml) in 1% BSA in PBS and normal mouse IgG or normal hamster IgM (100 μg/ml). Control samples were incubated with normal mouse IgG or normal hamster IgM. Cells were washed once with 1% BSA in PBS and then incubated with FITC-conjugated anti mouse IgG (Cappel, Durham, NC) or hamster IgM (Pharmingen, San Diego, CA) on ice for 45 min. After washing once with PBS, the cells were fixed in 1 ml of 0.5% paraformaldehyde and analyzed with a flow cytometer (EPICS Profile Analyzar, Coulter, Miami, FL).

2.5. Observation of morphology of MM1 cells

MM1 cells were stimulated with or without 25 μM LPA in M-MEM and incubated on FN-coated glass slide for 1 h at 37°C in a CO_2 incubator. (This glass slide had not been coated with colloidal gold particles.) The cells were fixed in 10% formalin to observe their shape under a phase contrast microscope (Olympus IX70, Tokyo, Japan). For scanning microscopy, the cells were fixed for 2 h in 2.5% glutaraldehyde followed by 1 h postfixation with 1% osmium tetroxide and rinsed with PBS. The samples were serially dehydrated in graded ethanol and critical-point dried in CO_2 in a Hitachi HCP-1 (Hitachi, Japan). The samples were then sputter-coated with gold-pallidium in Emscope SC500 (Meiwa, Osaka, Japan) and viewed at 5 V on a scanning electron microscope JSM-840 (Jeol, Tokyo, Japan).

2.6. Immunofluorescence

MM1 cells were stimulated with or without 25 μM LPA in M-MEM and incubated on FN-coated glass slide for 1 h at 37°C in a CO_2 incubator. The cells were fixed in 5% formalin in PBS and permeabilized with 1% Triton X-100 in PBS for 10 min, and then blocked with 1% BSA in PBS for 60 min. The cells were incubated with an anti-vinculin antibody (VIN-11-5, Sigma, St. Louis, MO) (1:100) in 1% BSA in PBS for 60 min, washed extensively, and then incubated with a Rhodamine-conjugated secondary antibody (BioSource, Camarillo, CA) (1:50) for a further 60 min. The cells were examined under a fluorescence microscope (Olympus BX50, Tokyo, Japan).

2.7. Transcellular migration assay

The assay procedure for in vitro capacity of transcellular migration of MM1 cells was essentially the same as described in our previous report [15]. Briefly, 2.0×10^5 MM1 cells were washed once with M-MEM and seeded over MCL in 35-mm dishes together with 25 μM LPA. Three hours after incubation at 37°C in a CO_2 incubator, the supernatant was removed and the resultant monolayer was fixed in situ with 10% formalin. The number of penetrated single MM1 cells and MM1 cell colonies (collectively called invasion foci) was counted under a phase contrast microscope in 16 independent visual fields. The invasive capacity was expressed as the number of invasion foci per square centimeter.

3. Results

3.1. FCS, but not LPA, induced phagokinetic motility of MM1 cells

The effect of LPA or FCS on the motility of MM1 cells was tested using a phagokinetic track motility assay. As shown in Table 1, phagokinetic motility of MM1 cells did not occur in M-MEM, but the addition of 10% FCS to the medium induced the motility of MM1 cells, resulting in the migrated area of $165.1 \pm 8.6 \times 10^2$ μm^2/100cells. In sharp contrast, phagokinetic motility hardly occurred by the addition of 25 μM LPA which is sufficient to substitute for FCS in inducing the transcellular migration (Table 1). This indicates LPA is not equivalent to FCS in triggering phagokinetic motility, while it is in inducing transcellular migration. So we tested the phagokinetic motility on a glass slide coated with 10% FCS (FCS-coated glass slide). MM1 cells showed a considerable motility on an FCS-coated glass slide by the addition of LPA (Table 1). This motility-inducing effect of LPA was dose-dependent (1–50 μM, data not shown) as was seen in the transcellular migration of MM1 cells [8]. These results suggest that FCS contains a certain factor(s) that collaborates with LPA to induce the phagokinetic motility of MM1 cells. This factor was heat-labile, because the activity in FCS was inactivated to 10% of the untreated FCS by heating at 100°C for 10 min. The activity was adsorbed to DEAE-Sepharose. Gel filtration using Superdex 200 pg indicated that the factor was of molecular weight of over 60 kDa (data not shown).

3.2. LPA induced the phagokinetic motility on FN-coated glass surface

Since the above results indicate that a specific adhesion on FCS-coated glass slide is required for the induction of motility by LPA, we considered that the relevant factor in FCS might be an adhesion molecule. So we tested the effect of extracellular matrix (ECM) components as candidates of the factor in FCS. Among several ECM components tested, LPA could induce phagokinetic motility of MM1 cells on a glass slide that had been coated with bovine FN (FN-coated glass slide) (Fig. 1). LPA-induced motility on an FN-coated glass slide was almost comparable to that on an FCS-coated glass slide. The activity of FN was highest at the coating concentration of 10 μg/ml which was comparable to FN concentration in 10% FCS [16] and decreased at 2 μg/ml and 50 μg/ml. Bovine VN also showed the activity, but much less active than FN. CL type I, CL type IV, LM and HA did not exhibit any appreciable activity (Fig. 1A). When MM1 cells were not stimulated with LPA on an FN-coated glass slide, the motility was negligible (Fig. 1).

These results suggest that the major factor in FCS that cooperates with LPA is FN. Actually, FN was adsorbed to DEAE-Sepharose, and its molecular weight is approximately 230 kDa, satisfying the characters of the serum factor. To confirm the role of FN in the motility of MM1 cells, we tested the effect of anti-FN antibody on LPA-induced phagokinetic motility on an FCS-coated glass slide (Fig. 2). Anti-FN antibody suppressed the phagokinetic motility to 21.6% of the control. The anti FN-antibody also suppressed the phagokinetic motility on an FN-coated

Table 1
Effects of FCS and LPA on phagokinetic motility and transcellular migration

Addition to serum-free medium	Concentration	Phagokinetic motility (total area migrated[a] $\times 10^{-2}$ μm^2 (100 cells))		Transcellular migration (number of invasion foci[a]/cm^2)
		On uncoated glass slide	On FCS-coated glass slide	
None		1.8 ± 0.4	1.9 ± 0.8	35 ± 6
FCS	10%	165.1 ± 8.6[b]	ND	1736 ± 123[b]
Oleoyl-LPA	25 μM	2.1 ± 0.7	134.7 ± 13.9[b]	2258 ± 180[b]

Phagokinetic motility of MM1 cells was assayed on FCS-coated or uncoated gold particle-glass slide. ND, not determined
[a] Mean ± S.D. of at least 3 determinations.
[b] $P < 0.0001$ compared with none.

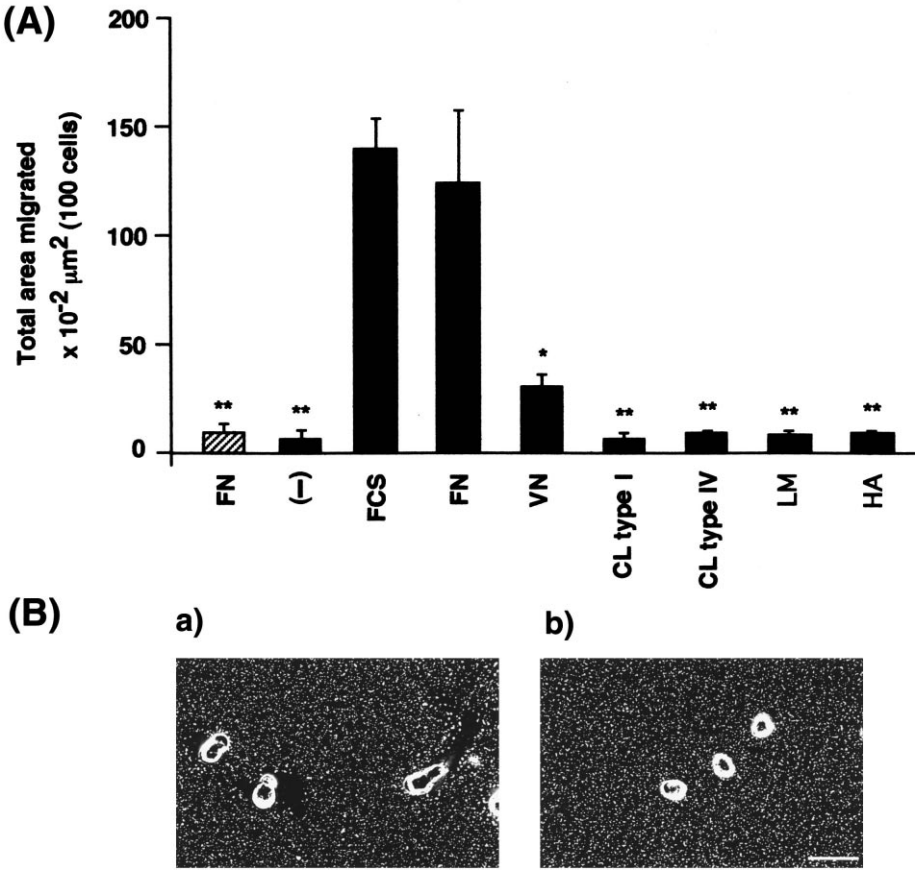

Fig. 1. Effect of extracellular matrix (ECM) components on lysophosphatidic acid (LPA)-induced phagokinetic motility of MM1 cells. (A) Phagokinetic motility of MM1 cells was assayed on FCS or following ECM component-coated glass slide as described in Section 2: 10 μg/ml fibronectin (FN), 20 μg/ml vitronectin (VN), 2 μg/ml collagen (CL) type I, 2 μg/ml CL type IV, 10 μg/ml laminin (LM), 10 μg/ml hyaluronic acid (HA) or without coating (−). The cell motility in the presence (filled bars) or absence (hatched bar) of LPA is shown. Error bars indicate the S.D. of triplicate determinations. $*P < 0.001$ compared with FCS, $**P < 0.0001$ compared with FCS. (B) LPA induced phagokinetic motility on an FN-coated glass slide (a). In the absence of LPA, phagokinetic motility of MM1 cells hardly occurred on an FN-coated glass slide (b). Scale bar: 25 μm.

glass slide almost completely (Fig. 2). Non-immunized corresponding immunoglobulin had no inhibitory effect.

3.3. Expression on MM1 cells of integrins capable of binding to FN

Since FN requires specific integrins to function as an adhesion molecule, we examined the expression of integrins on the MM1 cell surface by flow cytometry. As shown in Fig. 3, integrin β1-chain was expressed but integrin β3-chain was not. Anti-β1 integrin antibody suppressed the phagokinetic motility on an FCS-coated glass slide to 16.7% of the control and almost completely on an FN-coated glass slide (Fig. 2). Non-immunized corresponding immunoglobulin showed no inhibitory effect. The effects of RGD-containing peptide that interferes with the interaction between FN and cells was tested. Cyclo-GRGDSPA suppressed the phagokinetic motility both on FCS- and FN-coated glass slides almost completely. (Fig. 2).

3.4. Morphological response of MM1 cells on FN to LPA

MM1 cells that had adhered to a glass slide, when stimulated by LPA, extended cell surface blebbing and often formed pseudopodia (Fig. 4a,e). However, the phagokinetic motility was not observed (Table 1).

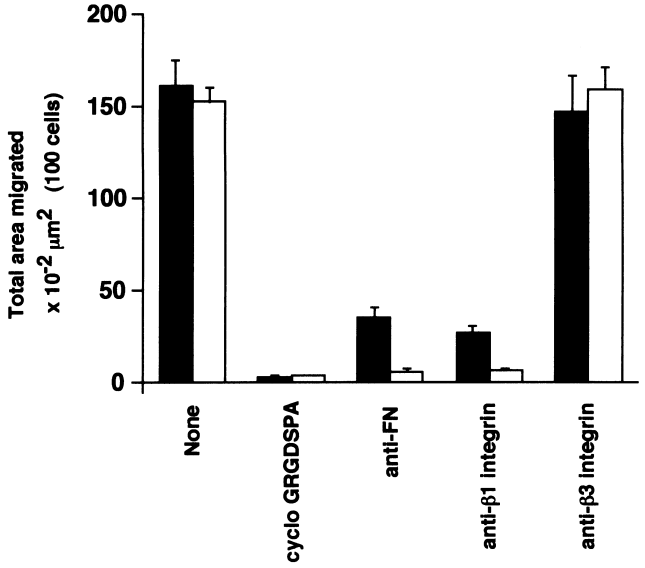

Fig. 2. Inhibitory effects of RGD peptide and antibodies on LPA-induced phagokinetic motility. LPA-induced phagokinetic motility of MM1 cells was assayed on an FCS-coated (filled bars) or FN-coated (open bars) glass slide as described in Section 2. The concentrations of RGD peptide and antibodies used in this study were: cyclo-GRGDSPA, 100 μg/ml; anti-FN antibody, 50 μg/ml; anti-β1 integrin antibody, 15 μg/ml; and anti-β3 integrin antibody, 15 μg/ml. Error bars indicate the S.D. of triplicate determinations.

In contrast, when MM1 cells on an FN-coated glass slide were stimulated by LPA, they showed a unidirectionally extended fusiform-shape with the nucleus localized in one end (Fig. 4c,g). Phagokinetic motility was seen in this condition (Fig. 1) and the fusiform-shaped cells indeed migrated with the nuclear end as the moving front under a phase contrast microscope. In the absence of LPA, MM1 cells adhered to an FN-coated glass slide and flattened (Fig. 4b,f), but did not move (Fig. 1). When MM1 cells that had been pretreated with C3 exoenzyme to inactivate Rho GTPase were seeded on an FN-coated glass slide and stimulated by LPA, no fusiform-shaped cells nor the motility was observed (Fig. 4d), indicating Rho participates in the morphological change leading to the motility.

Then, we stained the MM1 cells that adhered to an FN-coated glass slide for vinculin-containing focal adhesions in the presence or absence of LPA. As shown in Fig. 5b, clustered vinculin stains were seen on the fusiform-shaped cells in the presence of LPA. Whereas, no vinculin stain was observed on flattened cells in the absence of LPA (Fig. 5a). These results suggest the activation of Rho is essential for FN-dependent focal adhesion assembly which is necessary for cell migration.

3.5. *Inhibition of transcellular migration by MM1 cells with anti-FN or anti-β1 integrin antibody*

As described above, MM1 cells invade a MCL in the presence of LPA. If FN is involved in this process, the invasion must be suppressed by the inhibition of the interaction of cells with FN. To test this possibility, anti-FN or anti-β1 integrin antibody was added to the medium of the transcellular migration assay. As shown in Fig. 6, LPA-induced transcellular migration of MM1 cells was remarkably suppressed either by anti-FN antibody or by anti-β1 integrin antibody.

4. Discussion

We have established an in vitro invasion model to

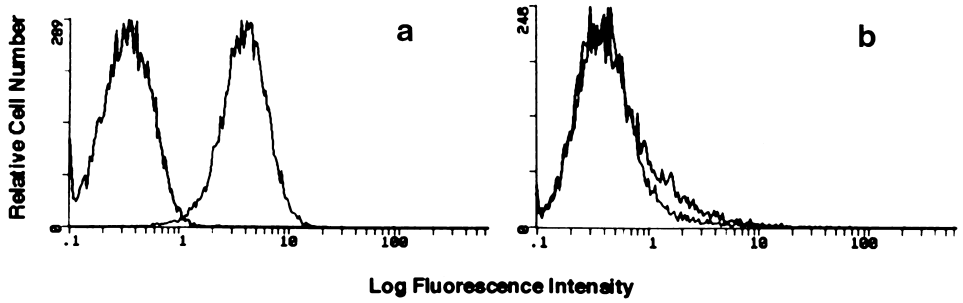

Fig. 3. Fluorescence histograms demonstrating the expression of β1 integrins and β3 integrins on MM1 cells obtained by flow cytometry. Control profiles were obtained by omitting primary antibodies. Anti-β1 integrin (a) and anti-β3 integrin antibodies (b) were used, respectively.

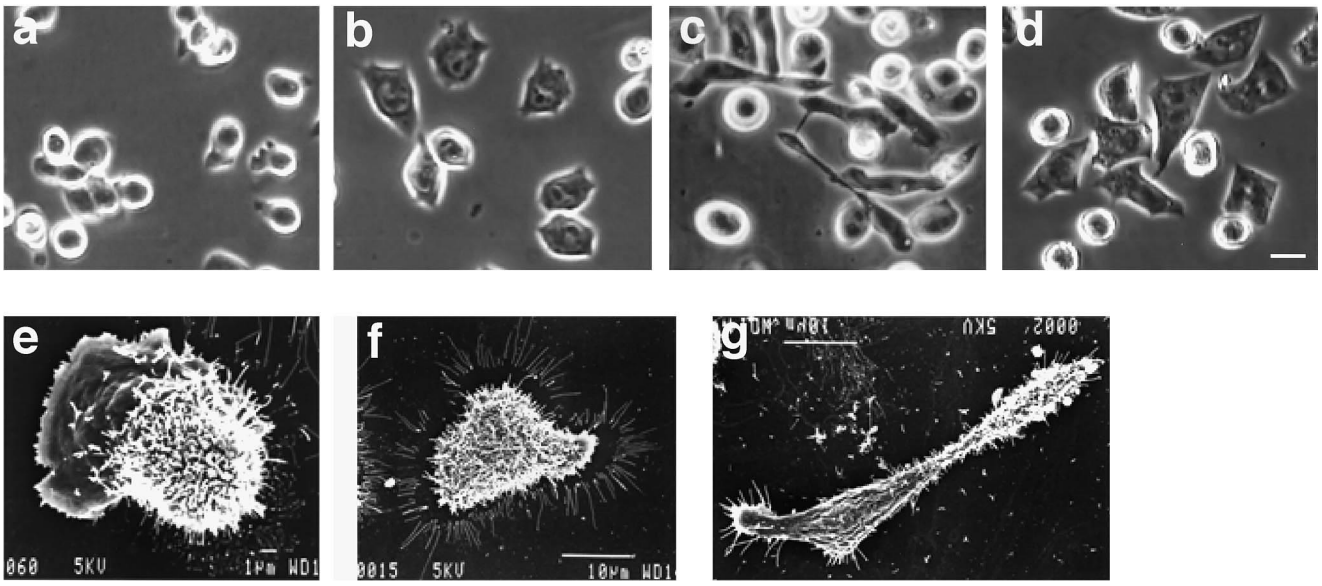

Fig. 4. Morphological response of MM1 cells to LPA. MM1 cells on a glass slide were stimulated with LPA (a,e). MM1 cells on an FN-coated glass slide were cultured without LPA (b,f). MM1 cells on an FN-coated glass slide were stimulated with LPA (c,g). C3-treated MM1 cells were plated on an FN-coated glass slide and stimulated with LPA (d). Phase contrast micrographs (a–d), scanning electron micrographs (e–g). (e) A cell with pseudopodium. (f) A flattened cell. (g) A fusiform-shaped cell. Scale bar: 10 μm.

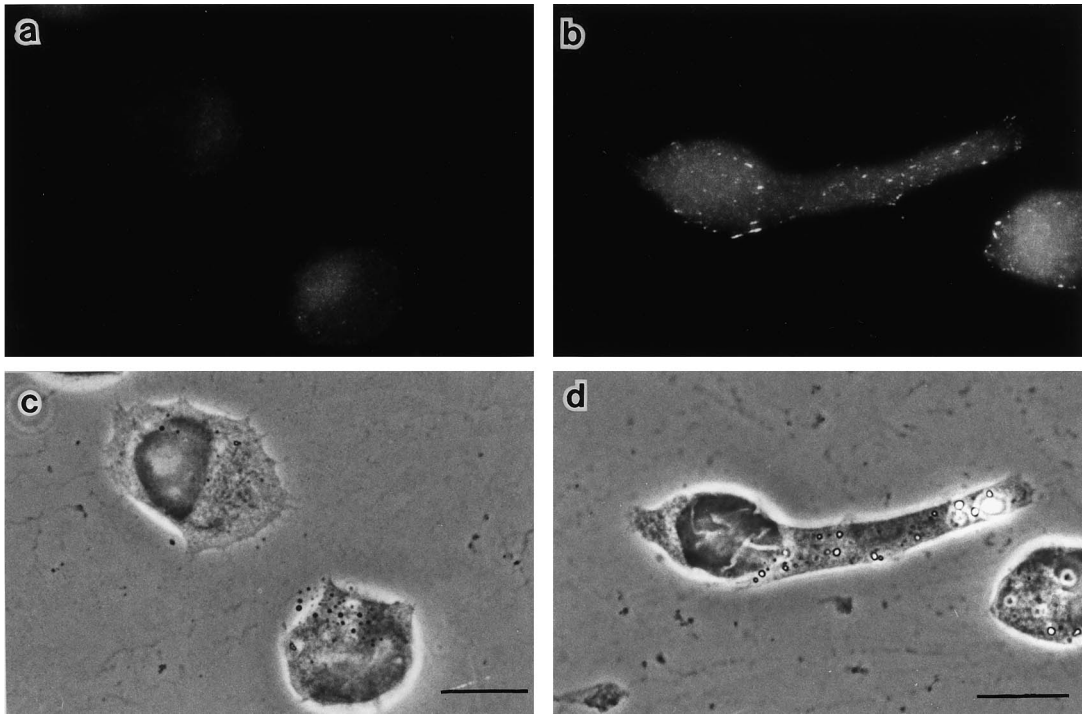

Fig. 5. Assembly of focal adhesions. MM1 cells on an FN-coated glass slide were cultured without LPA (a,c). MM1 cells on an FN-coated glass slide were stimulated with LPA (b,d). Cells were stained with vinculin. Immunofluorescence micrographs (a,b), phase contrast micrographs (c,d). Scale bar: 10 μm.

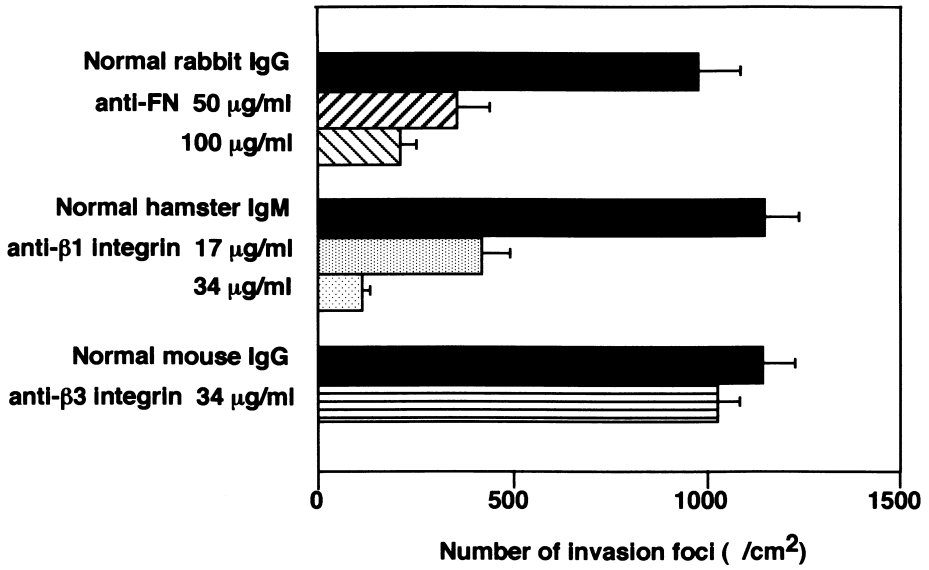

Fig. 6. Inhibitory effects of antibodies on LPA-induced transcellular migration. LPA-induced transcellular migration of MM1 cells was assayed as described in Section 2. The invasive capacity was expressed as the number of invasion foci per square centimeter. Error bars indicate the S.D. of triplicate determinations.

clarify the mechanism of transcellular migration of cancer cells. In this system, ascites hepatoma MM1 cells transmigrate through a cultured MCL. The transmigration occurred in the presence of FCS, but scarcely did in the absence of FCS [7]. LPA could completely substitute for FCS in inducing the transcellular migration [8].

So we first considered that LPA stimulation would be sufficient for MM1 cell motility. However, when cell motility was tested by a phagokinetic track motility assay, MM1 cells scarcely moved in the presence of LPA, while they did in the presence of FCS (Table 1). However LPA evoked the phagokinetic motility of MM1 cells, when tested on an FCS-coated glass slide. Since MM1 cells did not move on an FCS-coated glass slide without LPA, these results suggest that the cooperation of a certain factor(s) in FCS with LPA is sufficient for inducing the phagokinetic motility of MM1 cells, but either one of the two alone is not.

To determine this serum factor that cooperates with LPA, we tested a variety of extracellular matrix components by coating a glass slide with them to see whether the material tested could induce MM1 cell phagokinetic motility in the presence of LPA (Fig. 1A). The result revealed that FN was a candidate for the major factor in FCS capable of inducing the phagokinetic motility in cooperation with LPA. In the absence of LPA, FN could not induce the motility (Fig. 1). In the absence of FN, although MM1 cells efficiently adhered to a glass slide, LPA could not induce the motility (Fig. 1), indicating that the specific adhesion is necessary for LPA induction of the motility. The activity of FN was highest at the coating concentration of 10 μg/ml. This result is consistent with the report of Palecek et al. [17] on the effect of FN concentration on CHO cell motility.

Participation of FN was confirmed by using anti-FN antibody that suppressed LPA-induced phagokinetic motility on both FCS- and FN-coated glass slides (Fig. 2). The counter receptors of FN consist of α3β1, α4β1, α5β1, αvβ1, αvβ3, αvβ6, and αIIbβ3 integrins [18]. MM1 cells expressed β1- but not β3-chain (Fig. 3). We do not know at present which α-chain expresses on MM1 cells, because anti-rat integrin α-chain antibody is not available. RT-PCR analysis revealed the expression of α1- and αv-chain in MM1 cells. The addition of anti-β1 integrin antibody inhibited the LPA-induced phagokinetic motility on both FCS- and FN-coated glass slides (Fig. 2). These results again indicate that the interaction of β1 integrin and FN is critically involved in LPA-induced phagokinetic motility of MM1 cells.

A tripeptide, RGD, located within the FN mole-

cule is the recognition site for the attachment of cells to this protein [19]. The cyclic peptide, cyclo-GRGDSPA has reported to be more potent than a linear RGD peptide in inhibiting FN-mediated adhesion and lung metastasis of B16 melanoma cells [20]. Cyclo-GRGDSPA remarkably suppressed the phagokinetic motility of MM1 cells, suggesting that RGD site was involved in the motility (Fig. 2).

When cells are plated on an ECM-coated surface, complex series of events are set in motion that contribute to the establishment of focal adhesion and actin stress fibers. Adhesion also affects additional actin-based structures, such as microspikes, membranous ruffles, and morphological changes involved in cell spreading and motility [21,22]. Numerous proteins present at the cytoplasmic face of focal adhesions are considered to be structural components of focal adhesions, including cytoskeletal proteins, such as vinculin and talin [23]. When examined by vinculin-staining, LPA stimulation of MM1 cells that had adhered to an FN-coated glass slide resulted in a formation of focal adhesions (Fig. 5b). Without LPA, the cells remained flattened on an FN-coated glass slide and no focal adhesion resulted (Fig. 5a). Formation of FN-mediated focal adhesions on fusiform-shaped cells appears essential for motility, since the transformation to fusiform-shape and focal adhesions did not occur by LPA stimulation on FN-uncoated glass slide. The fusiform-shape, the motile form of MM1 cells, was not observed even in the presence of LPA on an FN-coated glass slide, when the cells were pretreated with C3 to inactivate Rho GTPase (Fig. 4d). Taken together, the motility of MM1 cells appears to be induced by a coordination of Rho-dependent generation of motile force and formation of focal adhesions.

The transcellular migration of MM1 cells through a MCL was also inhibited by the addition of anti-FN or anti-β1 integrin antibody to the transcellular migration assay system, indicating that the interaction of FN and β1 integrin also contributes to the transcellular migration (Fig. 6). Nomura et al. reported that highly malignant rat ascites hepatoma cells (AH66F) expressed LFA-1 on the cell surface and that a part of the adhesion of AH66F cells to mesothelial cells is due to LFA-1/ICAM-1 interaction [24]. However, MM1 cells (AH130) did not express LFA-1.

LPA is a platelet-derived cytokine and is present in serum at concentrations of 2–20 μM which also mediates mitogenesis and chemotaxis [25,26]. Kundra et al. showed that LPA stimulated the chemotaxis of NIH M17 fibroblasts [27]. Stam et al. reported that transcellular migration of human T-lymphoma cells through a rat embryonic fibroblast monolayer required FCS that could be replaced by LPA or sphingosine-1-phosphate (S1P) [28]. Unfortunately, however, they did not refer to any participation of cell adhesion molecules in their system. Recently, Sakai et al. found that β1 integrin was required for LPA-induced motility of fibroblastic cells derived from mouse embryonic stem cells and that cells expressing β1 integrins with mutations in conserved cytoplasmic domain showed a poor motility in response to LPA. This study indicated that the cytoplasmic domain of integrin interacts with outside-in signaling pathways [29].

S1P and LPA are structurally related lipid mediators that act on distinct G-protein-coupled receptors to evoke similar biological responses [30]. In our study, the effective concentrations of LPA in inducing both the transcellular migration and phagokinetic motility are relatively high compared with those reported for other cells [27–29]. Edg2 and Edg4 as LPA receptors and Edg1, Edg3, Edg5 as S1P receptors have been isolated and characterized [30]. The sequence similarity between them suggested that LPA at high doses may act through S1P receptors. Although LPA receptor in MM1 cells has not yet been characterized, S1P was not found to substitute for LPA in the present study.

We reported elsewhere that stimulation with LPA of MM1 cells in suspension caused a transient increase of tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin [9]. Maximal phosphorylation was observed 10 min after addition of LPA and then it was decreased. Inhibitors of tyrosine kinases, such as genistein and herbimysin A, suppressed both LPA-induced tyrosine phosphorylation and transcellular migration [9]. In contrast, when MM1 cells adhered to FN-coated glass slide were stimulated with LPA, the increase of tyrosine phosphorylation of FAK and paxillin continued for as long as 60 min (unpublished observation). These observations suggest that the outside-in signals from FN/β1 integrin may influence LPA-induced sig-

nals leading to cell motility and transcellular migration.

In conclusion, our results demonstrated that costimulation of MM1 cells with LPA and FN/β1 integrin induced phagokinetic motility and transcellular migration. We have elucidated Rho-regulated signaling triggered by LPA, actin organization and myosin light chain phosphorylation which generate motile force of MM1 cells [11,12]. How FN/β1 integrin signaling crosstalks with the LPA signal in inducing the cell motility is the subject of our further study. The clarification of the role of FN/β1 integrin in LPA-induced cell motility contributes to the understanding of the molecular mechanism of cancer invasion.

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