Participation of rhop21 in serum-dependent invasion by rat ascites hepatoma cells

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Abstract Rat ascites hepatoma cells (MM1 cells) penetrate through a cultured mesothelial cell monolayer (MCL) in the presence of fetal calf serum (FCS), but scarcely do so in its absence. Inactivation of rhop21 of MM1 cells by ADP-ribosyltransferase C3 resulted in the suppression of this serum effect on the penetration, suggesting that the serum effect was mediated by rhop21. To ascertain this assumption MM1 cells were transfected with an activated (Val^{14}) human rhoA cDNA (Neo/RhoA 1–7). The transfectants penetrated MCL extensively even in the absence of FCS and became largely independent of serum for the penetration. These results suggest that serum-induced invasion by MM1 cells is mainly mediated by rhop21.

Key words: Rhop21; Lysophosphatidic acid; Activated rhoA; Transfectant; Invasion; Rat ascites hepatoma cell

1. Introduction

Metastasis develops by a series of complex sequence of steps, among which transcellular migration of tumor cells through the host cell layer is most characteristic. The molecular mechanism of transcellular migration, however, is still largely unknown. We have developed a cell-monolayer invasion assay [1], in which tumor cells were seeded on a cultured monolayer of rat mesothelial cells (MCL) [1–4]. By counting the number of tumor cells that penetrated MCL, we quantified transcellular migration of tumor cells (in vitro invasive ability). The in vitro invasive ability of tumor cells corresponded well with their invasive capacity against the peritoneum, when the cells were implanted in the peritoneal cavity of the syngenic rats [5].

MM1 cells (a highly invasive clone from AH130 cells) penetrated MCL extensively in the presence of serum in the invasion assay medium, while in its absence, they scarcely penetrated MCL [2]. The addition of 1-oleoyl lysophosphatidic acid (LPA) into the medium was completely substituted for this serum effect, while LPA had no effect on the growth of MM1 cells [4]. Serum- or LPA-activity on inducing the tumor cell invasion was not specific to MM1 cells. The invasion of MCL by human pulmonary lung cancer (OC10) cells or mouse melanoma (B16FE7) cells also significantly depend on the presence of serum or LPA in the assay medium [4, unpublished result]. LPA is known to stimulate a number of signalling pathways under regulation of a small GTP-binding protein rhop21 [6,7,8]. These signallings appear to evoke a variety of its biological actions such as platelet activation, induction of morphological changes of neuronal cells, and proliferation of fibroblasts [7]. Recently, Takaishi et al. reported that microinjection of rho GDI, which inhibits rhop21 activity, into Swiss 3T3 cells results in suppression of cell motility, suggesting the participation of the activation of rhop21 in the motility of fibroblasts [9]. In their study the cell motility was examined using gold colloid traction assay, a measurement for phagokinetic behavior of cells. However, the molecular mechanism of transcellular migration of tumor cells through host cell monolayers remains largely unknown.

Taking advantage of our cell monolayer invasion model, we have tried to define the involvement of rhop21 in serum-dependent transcellular migration of MM1 cells. In this paper, we report that C3, which is known to selectively ADP-ribosylate rhop21, strongly suppresses the invasion by MM1 cells, and that MM1 cells transfected with Val^{14} rhoA cDNA, a point-mutated active form of rhoA, penetrate MCL extensively in the absence of serum. The transfectants became largely independent of serum for transcellular migration, suggesting that the serum effect is largely mediated by rhop21 which is essentially involved in the invasion by MM1 cells.

2. Materials and methods

2.1. Materials

Exoenzyme C3 of *Clostridium botulinum* (C3) was kindly supplied by Dr. B. Syuto (Hokkaido University, Sapporo, Japan) [10]. Purified rhoA was a generous gift from Dr. Y. Takai (Osaka University, Osaka, Japan). Activated (Val^{14}) human rhoA cDNA which has a myc epitope tag at the N-terminus [11] was kindly supplied by Dr. A. Hall (Institute of Cancer Research, London, UK). [^{32}P]NAD was purchased from Amersham.

2.2. Cells and cell culture

Rat mesothelial cells were isolated from Donryu rat mesentery and cultured in modified MEM containing 2-fold amino acid and vitamins (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% FCS, as reported previously [1–5]. MM1 cells which were cloned from cultured AH130 cells were maintained in modified MEM supplemented with 10% FCS.

2.3. Cell-monolayer invasion assay

The assay procedure of in vitro invasive capacity of tumor cells was described in our previous reports [1–5]. Briefly, when mesothelial cells from rat mesentery had grown to confluency, the culture medium was removed and 2×10^5 MM1 cells in 2 ml MEM with or without 10% FCS were seeded on MCL. After 20 h, the medium was removed and the cells were fixed with 10% formalin. The number of penetrated single tumor cells and tumor cell colonies (invasion foci) was counted under a phase contrast microscope.

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2.4. ADP-ribosylation by C3

MM1 cells were treated with predetermined concentrations of C3 (10 μg/ml) in the culture medium supplemented with 10% FCS for 4 h followed by washing with MEM. To make sure that the cellular rhop21 was ADP-ribosylated, the amount of unribosylated rhop21 in the lysates of C3 pretreated cells was determined as described previously [12]. Briefly, the lysates of cells that had been treated with C3 were incubated in 0.1 ml of 100 mM Hepes/NaOH at pH 7.5 containing 10 mM DTT, 0.1 mM $MgCl_2$, 10 mM [^{32}P]NAD and 1 μg C3 at 30°C. After 20-min incubation, the reaction was terminated by adding 12 μl of 100% (w/v) trichloroacetic acid. Resultant precipitates were dissolved in 40 μl of 62 mM Tris-HCl (pH 6.7) containing 3% SDS, 5% 2-mercaptoethanol, 5% glycerol and 0.001% Bromophenol blue to subject to SDS-PAGE (12% polyacrylamide gel). The gel was dried and autoradiographed at −80°C on a HyperfilmTM-MP (Amersham). The amount of C3 used in Table 2 was more than 5 times larger than those in Table 1. This is only because of the difference of the specific activity of C3 in each preparation of the enzyme.

2.5. Transfection

The pRC/CMV-RhoA plasmid that encodes the activated (Val^{14}) human rhoA cDNA was subcloned into the drug-selectable pRC/CMV vector (Invitrogen). MM1 cells (1×10^7 cells/ml) were transfected with the pRC/CMV-RhoA plasmid (60 μg/ml) by electroporation using a Bio-Rad Gene Pulser. Electroporation conditions were as follows: capacitance 25 μF, field strength 2.5 kV/cm. After the transfection, the cells were grown for 48 h, followed by selection with G418 (120 μl/ml). Seven clonal transfectants were isolated from the resistant cells by limiting dilution (Neo/RhoA 1–7). Neo/only transfectant was obtained from MM1 cells transfected with the pRC/CMV plasmid (Neo/only).

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR of the Val^{14}rhoA mRNAs was carried out as follows. The first strand cDNA was synthesized from 2.5 μg of total RNA using Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase and oligo $(dT)_{18}$ primer (Pharmacia Bio.) under incubation at 37°C for 1 h. The polymerase chain reaction (PCR) was conducted by the addition of 35 μl or PCR reaction mixture containing 50 pmol of sense primer (myc epitope tag): 5′-AAGCTGATCTCCGAGGAGGA-3′, 50 pmol of antisense primer (nucleotide position of human rhoA 568–587): 5′-TCTTCAGGTTTCACCGGCTC-3′ and 2.5 units of TaKaRa Ex Taq DNA polymerase (TaKaRa Biomedicals) with 30 thermal cycles, 94°C for 30 s, 60°C for 2 min, 72°C for 1 min 30 s. A sequence in the neo gene was amplified by PCR using a primer set: sense (nucleotide position 750–770) 5′-CAAGATGGATTGCACGCAGGT-3′ and antisense (nucleotide position 1308–1328) 5′-GATATTCGGCAAGCAGGCATC-3′. The PCR products were electrophoresed on 3% agaroseX gel (Wako Pure Chemical Industries) and stained with ethidium bromide.

3. Results

3.1. Diminished invasion by MM1 cells pretreated with C3

To test whether the invasion by MM1 cells is mediated by rhop21, we examined the effects of pretreating MM1 cells by C3 on their transcellular migration. C3 is known to selectively ADP-ribosylate and inactivate rhop21. MM1 cells were preincubated with 0–10 μg/ml of C3 for 4 h, washed and then their invasive ability was measured by the cell-monolayer invasion assay. The transcellular migration of MM1 cells was inhibited by the pretreatment with C3 in the dose-dependent manner (Table 1). The inhibition of the migration was almost 90%, when the cells were treated with 10 μg/ml of C3 for 4 h. This treatment of C3 hardly affected the growth of MM1 cells during the period of invasion assay (data not shown). To confirm that rhop21 in MM1 cells was actually ADP-ribosylated by the addition of C3 into the culture medium, the amount of ADP-ribosylated rhop21 in the lysates of treated MM1 cells was determined by examining the incorporation of [^{32}P]ADP into residual rhop21 by adding C3 to the lysate. Incorporation of [^{32}P]ADP into 22 kDa band corresponding to rhoA p21 was found to be reduced in the lysate of treated cells compared with the untreated control (Fig. 1), indicating ADP-ribosylation of rhop21 by C3 added extracellularly.

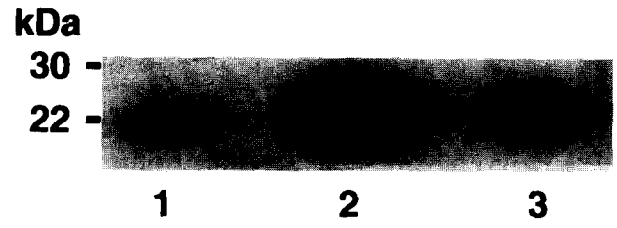

Fig. 1. ADP-ribosylation of rho protein by C3. Purified rhoA and cell lysates of MM1 cells were subjected to ADP-ribosylated by C3 (10 μg/ml) in the presence of [^{32}P]NAD. The labeled proteins were analyzed by SDS-PAGE and autoradiography. Lane 1: purified rhoA; lane 2: MM1 cells pretreated without C3; lane 3: MM1 cells pretreated with C3 (10 μg/ml).

3.2. Val^{14}rhoA expression in transfected cell clones

To confirm the participation of rhop21 in transcellular migration, MM1 cells were transfected with the pRC/CMV-Val^{14}RhoA plasmid. Seven independent clones (Neo/RhoA 1–7) were isolated and characterized. RT-PCR using the Val^{14}rhoA specific primers spanning over a DNA fragment of 452 bp indicated that Val^{14}rhoA mRNA was expressed in all

Table 1
Effect of C3 on in vitro invasion by MM1 cells

C3 (μg/ml)	Number of invasion foci/cm^2
None	2100 ± 185[1]
0.5	995 ± 88
1	918 ± 81
3	613 ± 54
10	229 ± 21

[1] Mean ± S.D. of at least 2 determinations.
MM1 cells (1×10^5/ml) were preincubated with indicated concentrations of C3 in the culture medium supplemented with 10% FCS for 4 h. After washed, 2×10^5 MM1 cells in 2 ml MEM supplemented with 10% FCS were seeded on MCL.

Table 2
In vitro invasion by Val^{14}rhoA transfectants

Cell	Number of invasion foci/cm^2	
	FCS (−)	FCS (+)
MM1	38 ± 21[1]	2427 ± 102
Neo/only	43 ± 16	2618 ± 64
Neo/RhoA-1	859 ± 107	4335 ± 126
Neo/RhoA-2	360 ± 76	3975 ± 149
Neo/RhoA-3	1007 ± 53	4007 ± 269
Neo/RhoA-4	3211 ± 330	4791 ± 252
Neo/RhoA-5	922 ± 144	5226 ± 217
Neo/RhoA-6	3541 ± 176	4887 ± 292
Neo/RhoA-7	392 ± 79	4929 ± 530
Neo/RhoA-3 pretreated with C3*	ND[2]	201 ± 14

[1] Mean ± S.D. of at least 2 determinations.
[2] Not done.
MM1 cells (2×10^5/dish) were seeded on MCL in the presence or absence of FCS in the assay medium.
*Neo/RhoA-3 cells (1×10^5/ml) were preincubated with 50 μg/ml of C3 for 24 h. After washed, 2×10^5 cells were seeded on MCL in the presence of FCS in the assay medium.

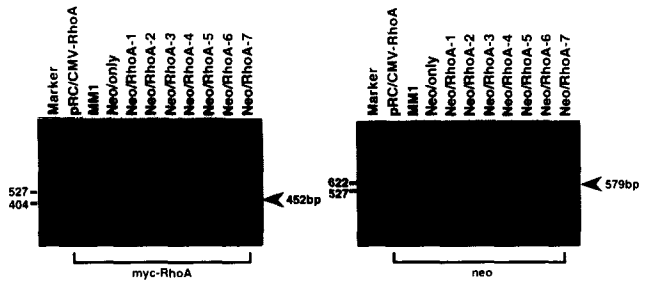

Fig. 2. Detection of Val[14]rhoA mRNA and neo mRNA by RT-PCR. Val[14]rhoA and neo specific primers were prepared as described in section 2. 452 bp and 579 bp bands corresponding to Val[14]rhoA and neo, respectively, are indicated by arrows. PCR products were electrophoresed on 3% agaroseX gel and detected by ethidium bromide staining.

of these Neo/RhoA clones, but not in parental MM1 cells or Neo/only (Fig. 2). Neo mRNA was expressed in both Neo/RhoA clones and Neo/only (Fig. 2).

3.3. *In vitro invasive ability and morphology of Val[14]RhoA transfectants*

Transcellular migration of Val[14]rhoA transfectants were examined by the cell-monolayer invasion assay. As shown in Table 2, all of the Neo/RhoA clones invaded extensively in the absence of FCS in the assay medium, although the parental MM1 cell and Neo/only scarcely invaded. In the presence of FCS, however, all the cell lines were highly invasive. Thus the serum dependency of MM1 cell invasion was greatly reduced by the transfection of Val[14]rhoA cDNA (Table 2).

In contrast to parental MM1 cells and Neo/only which grew in suspension, Neo/RhoA clones adhered to culture dishes with a scattered morphology (Fig. 3A,B,C).

To ascertain whether the transfected Val[14]rhoA does function in the transfectants or not, Neo/RhoA-3 was treated with 50 μg/ml of C3 for 24 h. The treated cells completely rounded up and the cell morphology appeared to be reversed to that of the original MM1 cells or Neo/only (Fig. 3D). The invasive ability of the C3-treated Neo/RhoA-3 was found to be greatly reduced (Table 2).

4. Discussion

In this paper, C3, which specifically inactivated rhop21, was shown to inhibit the transcellular migration of MM1 cells through MCL. Introduction of C3 into fibroblasts such as Swiss 3T3 cells is reported to result in disappearance of stress fibers and focal contacts [13]. Therefore, the inhibition of the migration of MM1 cells by C3 may be mediated by the destruction of these intracellular structures. This assumption is supported by our previous result that cytochalasin B, which disrupts actin filaments [14], strongly suppressed the transcellular migration of MM1 cells [15] and our recent finding that serum- or LPA-induced F-actin increase in MM1 cells was completely suppressed by prior treatment with C3 (unpublished result). C3 is reported to inhibit spontaneous and chemoattractant-induced motility of neutrophils [16] and phagokinesis of Swiss 3T3 cells tested by gold colloid traction assay, as well [9]. Taken together, rhop21 appears to participate in cell locomotion like the transcellular migration as well as in cell motility.

We have already found that MM1 cells require the presence of FCS in the culture medium to exert their transcellular migration [2], and that LPA can be substituted for the serum [4]. Serum or LPA is reported to induce in fibroblasts the rapid formation of actin stress fibers and the assembly of focal adhesion plaques through rhop21 pathway [8,13]. These observations and the above results with C3 lead to speculate that the migration-inducing effect of FCS is mediated by rhop21 activation. To test this possibility, we introduced human Val[14]rhoA cDNA, a constitutively active form of rhoAp21, into MM1 cells and tested whether the transfectants migrate through MCL even in the absence of FCS. As a result, the transfectants exerted extensive transcellular migration without adding FCS in the assay medium (Table 2). Furthermore, they became mostly independent of serum for the migration, suggesting that FCS triggers the migration by signalling largely through rhop21 activation.

During the preparation of this manuscript, Michiels et al. reported using a similar invasion model to ours that the introduction of active rac (a small G protein in the rho subfamily) conferred to non-invasive lymphoma BW5147 cells an ability to migrate through a fibroblast monolayer [17]. As described, rhop21 essentially contributes to the migration of MM1 cells through MCL. Additionally, we observed that suppression of the migration of tumor cells by knocking out rhop21 activity using C3 was not specific to MM1 cells but was seen with the other tumor cells ([4], unpublished result). The reason for the preferential participation of rac or rho in different experimental systems is not clear at present. The small G protein necessary for the transcellular migration of tumor cells might be different depending on the pair of tumor and host cells. Alternatively, the activation of both rac and rho may be necessary for tumor cells to locomote, as suggested that cdc42-rac-rho sequence may function in cell locomotion [18,19]. The rac activation system leading to induction of transcellular migration might be

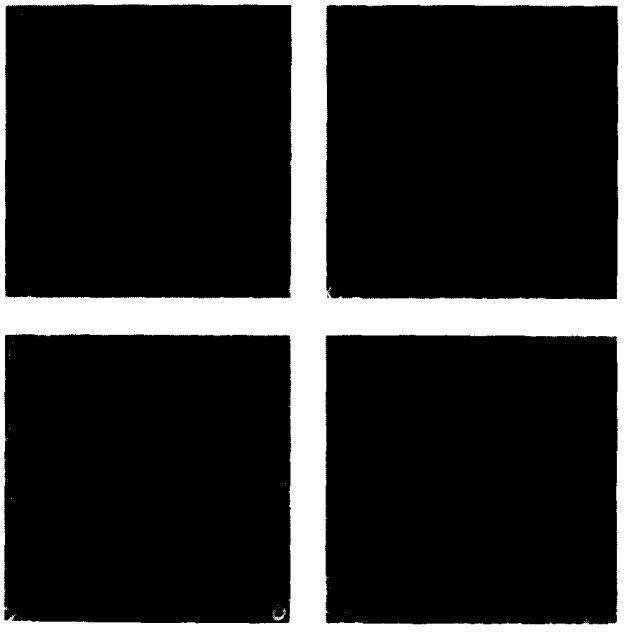

Fig. 3. Morphology of Val[14]rhoA transfectant. (A) MM1 cells. (B) Neo/only cells. (C) Neo/RhoA-3 cells. (D) C3 treated Neo/RhoA-3 cells.

deficient in lymphoma cells, whereas the rho activation system is silent in MM1 cells in the absence of serum. In Michiels' experiment, BW5147 cells were non-invasive in the presence of serum in the assay medium. It is interesting to see whether BW5147 cells and active rac-transfectants migrate through the fibroblast monolayer even in the absence of serum or not. This may give a better understanding of the relation between rac and rho. Although further investigation will be required, our results presented here will give critical information to understand the signalling of transcellular migration of tumor cells.

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