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ATTENUATION BY CYCLIC PHOSPHATIDIC ACID OF PERITONEAL METASTASIS OF AZOXYMETHANE-INDUCED INTESTINAL CANCERS IN WISTAR RATS

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The effect of cyclic phosphatidic acid, a unique analogue of lysophosphatidic acid, on the induction of bombesin-enhanced peritoneal metastases from intestinal adenocarcinomas induced by azoxymethane was investigated in male Wistar rats. Rats were given 10 weekly injections of azoxymethane (7.4 mg/kg body weight, s.c.) and of bombesin (40 μg/kg body weight, s.c.) every other day from the start of the experiment, and from week 16, they received injections of cyclic phosphatidic acid (3 or 6 mg/kg body weight, s.c.) every other day until the end of the experiment in week 45. Cyclic phosphatidic acid at both dosages significantly decreased the incidence of bombesin-enhanced cancer metastases to the peritoneum but had little or no effect on the location, histologic type, depth of involvement or infiltrating growth patterns of the tumors. Cyclic phosphatidic acid at either dose decreased significantly the incidence of lymphatic vessel invasion of adenocarcinomas and the activity of RhoA protein in the tumors, both of which were enhanced by bombesin. Our findings indicate that cyclic phosphatidic acid inhibits cancer metastasis through inhibition of RhoA protein activation.

Key words: *metastasis; cyclic phosphatidic acid; azoxymethane; intestinal cancer; RhoA*

Lysophosphatidic acid (LPA), a lipid mediator generated by activated platelets, has various effects on numerous types of cells,[1] causing LPA-stimulated cell motility and migration in various cells.[2–5] Therefore, the metastatic capabilities of various types of cancer cells can be enhanced by a receptor-driven cellular process initiated by LPA.[6,7] We previously found that 1-oleoyl LPA induces transmonolayer migration (*in vitro* invasion) of rat ascites hepatoma MM1 cells and the morphological changes leading to this migration.[8–10]

Cyclic phosphatidic acid (1-palmitoyl-*sn*-glycerol 2,3-cyclic phosphate [C16:0-cPA]; cPA) is a unique analogue of LPA.[11–13] cPA has a cyclic phosphate at the C-2 and C-3 positions of glycerol and was isolated originally from the true slime mold *Physarum polycephalum*. We found in *in vitro* that cPA suppressed invasion by MM1 cells dose dependently, without affecting proliferation.[13]

We have previously developed a new metastatic animal model,[14] in which administration of the gastrointestinal peptide bombesin significantly increased the incidence of metastasis to the peritoneum from intestinal adenocarcinomas induced by azoxymethane (AOM). In our study, we used this animal metastasis model to investigate the effects of cPA on the development of peritoneal metastases from intestinal adenocarcinomas induced by AOM.

MATERIAL AND METHODS

Animals

One hundred fifteen 6-week-old male Wistar rats, purchased from Japan SLC (Shizuoka, Japan), were used in this experiment. The rats were housed in suspended, wire-bottomed metal cages in our animal quarters at controlled temperature (20 to 22°C) and humidity (30 to 50%), on a 12 hr–12 hr light-dark cycle. Regular chow pellets (Nihon-Nosan, Yokohama, Japan) and normal tap water were supplied *ad libitum* until the end of the experiment at week 45.

Experimental design

Rats were randomly divided into 6 groups of 15 or 20 rats each. Each group was given weekly injections (7.4 mg/kg body weight, s.c.) of AOM (Sigma Chemical Co., St. Louis, MO) in 0.9% NaCl solution for the subsequent 10 weeks, and received one of the following treatments until the end after week 45: group 1 (control group), vehicle (olive oil) alone; group 2, bombesin alone; groups 3 and 4, bombesin and cPA 3 and 6 mg/kg body weight, respectively and groups 5 and 6, cPA 3 and 6 mg/kg body weight without bombesin, respectively.

Bombesin (Peptide Institute, Osaka, Japan, 40 μg/kg body weight) and cPA(3 and 6 mg/kg body weight) were prepared as suspended in olive oil and injected in a volume of 1 ml/kg body weight s.c. every other day. Bombesin and cPA were injected between 2 and 3 p.m. at various sites from the start of the experiment and from week 16 until the end of the experiment at week 45. cPA was synthesized by the method of Murakami-Murofushi *et al.*[11,12]

Colonic tumors induced in rats usually cannot be observed before week 15. Therefore, we started the injections of cPA from week 16. The dosages of cPA used in our study were based on our previous *in vitro* results. Inhibition of invasion by cPA is dose-dependent and more than 93.8% inhibition was achieved using a concentration of 25 μM.[13]

Sample collection and histologic observation

Rats that survived more than 35 weeks were included in the study; the first tumor was found in the colon of a rat in group 2 in

Abbreviations: AOM, azoxymethane; cPA, cyclic phosphatidic acid; GTP, guanidine triphosphate; LPA, lysophosphatidic acid; PMSF, phenylmethylsulforyl fluoride; SDS, sodium dodecyl sulfate.

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TABLE I – BODY WEIGHT AND INCIDENCE OF INTESTINAL TUMORS IN AOM-TREATED RATS

Group number	Treatment[1]	Body weight (g)		Effective number	Number of rats with intestinal tumors (%)
		Initial	Week 45		
1	Control	118 ± 3	310 ± 16	14	10 (71)
2	Bombesin	116 ± 2	340 ± 13	20	18 (90)
3	Bombesin + cPA (3.0 mg/kg body weight)	120 ± 3	322 ± 15	20	19 (95)
4	Bombesin + cPA (6.0 mg/kg body weight)	119 ± 2	339 ± 9	19	18 (95)
5	cPA (3.0 mg/kg body weight)	118 ± 2	283 ± 14	20	15 (75)
6	cPA (6.0 mg/kg body weight)	117 ± 2	321 ± 12	20	12 (60)

[1]Treatment: Each group was given weekly injections (7.4 mg/kg body weight, s.c.) of AOM (Sigma Chemical Co., St. Louis, MO) in 0.9% NaCl solution for the subsequent 10 weeks, and received one of the following treatments until the end after week 45: group 1 (control group), vehicle (olive oil) alone; group 2, bombesin alone; groups 3 and 4, bombesin and cPA 3 and 6 mg/kg body weight, respectively; groups 5 and 6, cPA 3 and 6 mg/kg body weight without bombesin, respectively. Bombesin, (Peptide Institute, Osaka, Japan, 40 μg/kg body weight) and cPA(3 and 6 mg/kg body weight) were prepared as suspended in olive oil and injected in a volume of 1 ml/kg body weight s.c. every other day. Bombesin and cPA were injected between 2 and 3 p.m. at various sites from the start of the experiment and from week 16 until the end of the experiment at week 45.

TABLE II – LOCATIONS AND HISTOLOGIC TYPES OF INTESTINAL TUMORS IN AOM-TREATED RATS

Group number	Treatment[1]	Number of intestinal tumors	Location (%)		Histologic type (%)	
			Small intestine	Large intestine	Adenoma	Adenocarcinoma
1	Control	16	2 (13)	14 (88)	5 (31)	11 (69)
2	Bombesin	26	4 (15)	22 (85)	4 (15)	22 (85)
3	Bombesin + cPA (3.0 mg/kg body weight)	31	1 (3)	30 (97)	6 (19)	25 (81)
4	Bombesin + cPA (6.0 mg/kg body weight)	23	3 (13)	20 (87)	4 (17)	19 (83)
5	cPA (3.0 mg/kg body weight)	22	2 (9)	20 (91)	3 (14)	19 (86)
6	cPA (6.0 mg/kg body weight)	19	2 (11)	17 (89)	6 (32)	13 (68)

[1]For explanation of treatments, see Table I.

TABLE III. – INCIDENCE AND GRADES OF PERITONEAL METASTASIS OF INTESTINAL ADENOCARCINOMAS IN AOM-TREATED RATS

Group number	Treatment[1]	Number of rats with adenocarcinoma	Number of rats with peritoneal metastasis (%)	Grade of metastasis (%)[2]		
				P1	P2	P3
1	Control	9	0 (0)	0 (0)	0 (0)	0 (0)
2	Bombesin	17	15 (88)[3]	7 (47)	1 (6)	7 (47)
3	Bombesin + cPA (3.0 mg/kg body weight)	19	1 (5)[4]	0 (0)	0 (0)	1 (100)
4	Bombesin + cPA (6.0 mg/kg body weight)	18	3 (17)[4]	0 (0)	0 (0)	3 (100)
5	cPA (3.0 mg/kg body weight)	12	0 (0)	0 (0)	0 (0)	0 (0)
6	cPA (6.0 mg/kg body weight)	12	1 (8)	0 (0)	0 (0)	1 (100)

[1]For explanation of treatments, see Table I. –[2]Grade of metastasis: P_1'-metastatic nodules detectable over the peritoneum near the primary cancer; P_2'-a few metastatic nodules detectable over the peritoneum far from the primary cancer; P_3'-many metastatic nodules detectable over the peritoneum far from the primary cancer. –[3]Significantly different from the value for group 1 at $p < 0.001$. –[4]Significantly different from the value for group 2 at $p < 0.001$.

week 34. Rats were killed when they became moribund due to intestinal obstruction, ascites, or large tumor, and all surviving rats were killed at the end of week 45. The internal organs of all animals killed during the experimental period or in week 45 were carefully examined. The large and small intestines were opened, pinned flat on a cork mat and fixed with buffered picric acid-formaldehyde solution. Tumors and anatomical regions suspected of lesions were excised and embedded in paraffin. 5 μm-thick sections were cut to expose the central part of the tumor or the stalk, when present, and were stained with hematoxylin and eosin. Moreover, flat mucosa from the fixed intestine with no visible tumor was cut into 3 mm-wide strips, which were embedded in paraffin. Thin sections were prepared and examined microscopically for tumor foci. Histological scoring was done in number-coded specimens without the knowledge of the treatment applied.

Histology of intestinal tumors

Colonic tumors induced by AOM were classified as either adenocarcinomas or adenomas. Adenocarcinomas were histologically defined as lesions in which neoplastic cells had penetrated the muscularis mucosae to invade the submucosa or deeper layers. In contrast, adenomas were defined as lesions with neoplastic cells confined to the mucosal layer. Adenocarcinomas were further classified into well-differentiated or mucinous carcinomas as described earlier.[15]

Peritoneal metastases

Peritoneal metastasis was defined as the macroscopic presence of cancer cells in the extracolonic peritoneum. The grades of peritoneal metastases from intestinal adenocarcinomas to the extracolonic peritoneum were classified according to the criteria of the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus in Japan,[16] as follows: P1, metastatic nodules detectable only over the peritoneum near the primary cancer; P2, few metastatic nodules detectable over the peritoneum far from the primary cancer and P3, many metastatic nodules detectable over the peritoneum far from the primary cancer.

Infiltrating growth pattern of adenocarcinomas

Based on the same classification,[16] the predominant patterns of infiltrating growth into the surrounding tissue were classified as follows: α, tumor shows expanding growth and a distinct border to the surrounding tissue; β, tumor growth and borders are between

those of types α and γ; and γ, tumor infiltrates the surrounding tissue.

Assay for activated RhoA

About 20–30 mg of the colorectal tumors were quickly frozen in liquid nitrogen after removal and stored at −85°C. Guanosine triphosphate (GTP)-bound cellular RhoA was determined using a Rho pull-down assay as previously described.[17] Densitrometric analysis was performed using NIH (version 1.61) imaging software.

Statistical analysis

Results were analyzed using the chi-square test, Fisher's exact probability test[18] or 1-way analysis of variance with Dunn's multiple comparison, where appropriate.[19] Data are expressed as mean ± SE. Differences were considered significant at a p value of less than 0.05.

RESULTS

Effects of cPA on tumor induction

On week 45, rats that received bombesin with and without cPA had a slight but not significant increase in body weight compared to control rats (Table I).

In control group 1 (vehicle), intestinal tumors were found in 10 (71%) of 14 rats examined (Table I). In group 2 (bombesin alone), the incidence of intestinal tumors was slightly, but not significantly, higher than that in group 1. Combined administration of bombesin and cPA (at both doses) had no significant effect on the incidence of intestinal tumors compared to that in group 2. The incidence of tumors in rats treated with cPA alone (groups 5 and 6) was not significantly different from that in group 1.

The location of intestinal tumors and the distribution of adenomas and adenocarcinomas did not differ significantly among the 6 groups (Table II).

Effects of cPA on peritoneal metastases

In group 1 (vehicle, control), no peritoneal metastases were found in 9 rats with intestinal adenocarcinoma (Table III). In group 2, administration of bombesin significantly increased the incidence of peritoneal metastasis compared to that in group 1. Peritoneal metastases were found in 15 (88%) of the 17 tumor-bearing rats in group 2 (Fig. 1). Concomitant administration of bombesin and cPA at 3 mg/kg (group 3) and 6 mg/kg (group 4) significantly decreased the incidence of peritoneal metastases compared to that in group 2. However, combined administration of bombesin and cPA had no significant effects on the grade of peritoneal metastases. Treatment with cPA alone at either dosage (groups 5 and 6) had no significant effect on the incidence of peritoneal metastases compared to that in group 1.

Effects of cPA on lymphatic vessel invasion

In group 1 (control), no lymphatic vessel invasion was found in 11 adenocarcinomas (Table IV). However, administration of bombesin (group 2) significantly increased the incidence of lymphatic vessel invasion; it was found in 16 (73%) of 22 adenocarcinomas. Concomitant administration of bombesin and cPA at either dosage (groups 3 and 4) significantly decreased the incidence of lymphatic vessel invasion compared to that in group 2.

Effects of cPA on RhoA activity

Administration of bombesin (group 2) significantly increased the activity of GTP-bound RhoA compared to values in the control group 1 (Fig. 2, Table V). Combined administration of bombesin and cPA at 3 mg/kg body weight (group 3) and 6 mg/kg body weight (group 4) significantly decreased the bombesin-induced activity of GTP-bound RhoA. However, bombesin alone and combined bombesin and cPA had little or no influence on total RhoA.

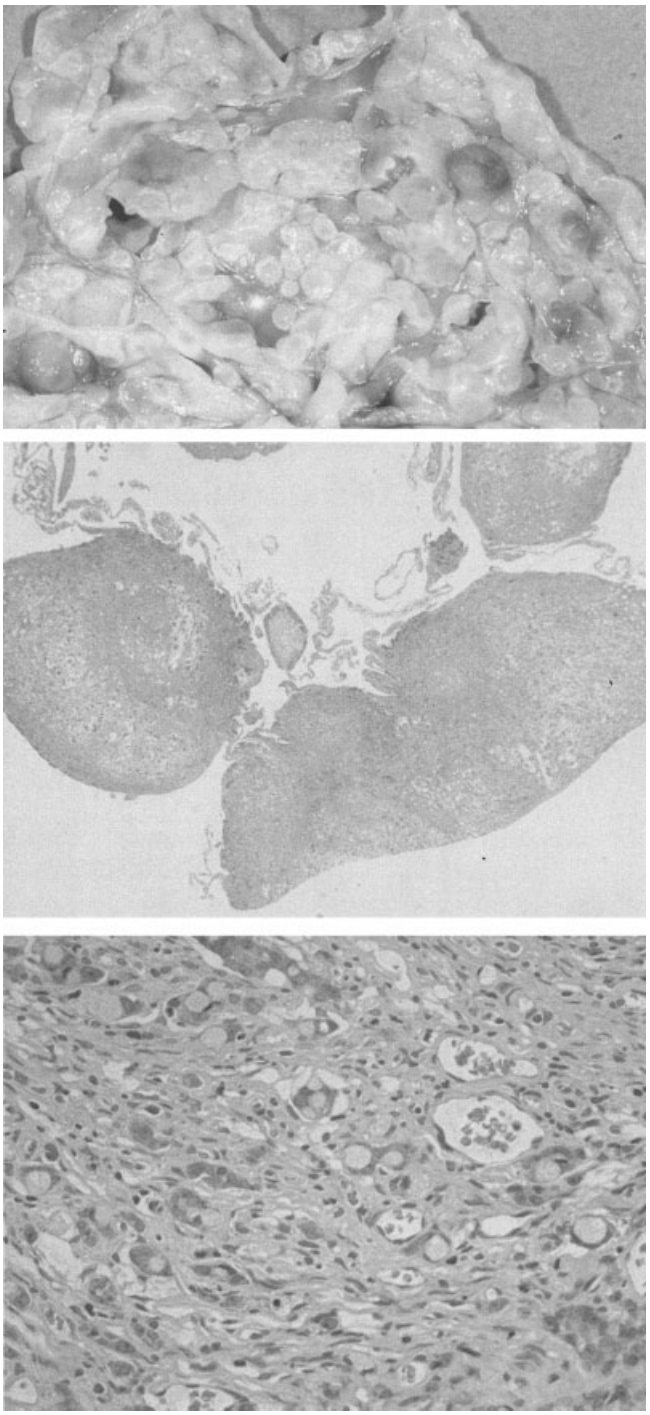

FIGURE 1 – Induction of peritoneal metastases from intestinal cancers by bomesin. (*a*) Macrophotograph showing many metastatic nodules on the omentum. (*b*,*c*) Microphotographs of peritoneal metastases. Original magnifications: (*a*) ×2. (*b*) ×5. (*c*) ×100.

DISCUSSION

Our present results confirm that bombesin enhances peritoneal metastases and lymphatic vessel invasion of intestinal adenocarcinomas induced by AOM, and establish that cPA attenuates the bombesin-enhanced peritoneal metastases and

TABLE IV – EFFECTS OF BOMBESIN AND, cPA ON THE LOCATION, HISTOLOGIC TYPE, DEPTH OF INVOLVEMENT, INFILTRATING GROWTH PATTERN AND VESSEL INVASION OF INTESTINAL ADENOCARCINOMAS

Group number	Treatment[1]	Number of adenocarcinomas	Location (%)		Histology (%)		Depth of involvement (%)		Infiltration growth pattern[b] (%)[2]			Vessel invasion	
			Small intestine	Large intestine	Well differentiated	Mucinous	Submucosa or muscle layer	Subserosa or deeper	Alpha	Beta	Gamma	Venous	Lymphatic
1	Control	11	2 (18)	9 (82)	4 (36)	7 (64)	10 (91)	1 (9)	5 (45)	2 (19)	4 (36)	0 (0)	0 (0)
2	Bombesin	22	4 (18)	18 (82)	10 (46)	12 (54)	10 (46)	12 (54)	4 (18)	4 (18)	14 (64)	0 (0)	16 (73)[3]
3	Bombesin + cPA (3.0 mg/kg body weight)	25	1 (4)	24 (96)	8 (32)	17 (68)	19 (76)	6 (24)	11 (44)	10 (40)	4 (16)	0 (0)	2 (8)[4]
4	Bombesin + cPA (6.0 mg/kg body weight)	19	3 (16)	16 (84)	6 (32)	13 (68)	12 (63)	7 (37)	5 (26)	3 (16)	11 (58)	0 (0)	2 (11)[4]
5	cPA (3.0 mg/kg body weight)	19	2 (11)	17 (89)	11 (58)	8 (42)	13 (68)	6 (32)	5 (26)	5 (26)	9 (47)	0 (0)	0 (0)
6	cPA (6.0 mg/kg body weight)	13	2 (15)	11 (85)	4 (31)	9 (69)	5 (38)	8 (62)	1 (8)	2 (15)	10 (77)	0 (0)	0 (0)

[1]For explanation of treatments, see Table I. – [2]Pattern of infiltrating growth: alpha-tumor showed expanding growth and a distinct border proximate to surrounding tissue; beta-this category is between alpha and gamma types; gamma-tumor showed infiltrating growth and an indistinct border proximate to surrounding tissue. – [3]Significantly different from the value for group 1 at $p < 0.001$. – [4]Significantly different from the value for group 2 at $p < 0.001$.

FIGURE 2 – RhoA activation by bombesin and its suppression by cPA in intestinal tumors (Western blotting).

lymphatic vessel invasion of adenocarcinomas. These findings suggest that cPA has antimetastatic activity. LPA, a lipid mediator generated by activated platelets, has various effects on numerous types of cells.[1] In a previous report using cell monolayer invasion model *in vitro*, we found that LPA is a potent inducer of cancer invasion.[20] The low molecular-weight GTPase, RhoA, plays an important role in the LPA-induced transmonolayer migration, as indicated by the fact that the *Clostridium botulinum* C3 exoenzyme,[21] which specifically ADP-ribosylates and inhibits Rho, strongly suppressed the transmonolayer migration.[22] We previously reported that LPA induced polymerization of actin[13] and increased phosphorylation of the myosin light chain *via* the Rho-Rho-associated kinase ROCK cascade.[23] Recent studies have shown that LPA activates Rho and ROCK, and that the LPA/Rho signaling cascade plays important role in cell motility and metastasis.[2,24–27]

The exact mechanisms by which cPA attenuates bombesin-enhanced peritoneal metastases from intestinal cancers remains unclear. However, the following activities of cPA might be contribute to its antimetastatic activity: 1) antimitogenic regulation of the cell cycle,[11] 2) regulation of Ca^{2+} release,[11] 3) regulation of actin rearrangement[28] and 4) inhibition of apoptosis. However, we previously reported that proliferation of MM1 cells was not affected by cPA in the range of concentrations (12.5–50 μM) that effectively suppressed tumor invasion.[13] Our present results show that administration of cPA in rats slightly increases the cancer incidence in the submucosa and musclaris layer and that of tumors exhibiting infiltrative pattern α; however these differences were not statistically significant. The present results also show that cPA significantly increases the lymphatic vessel invasion of the tumors. Using the *in vitro* transmonolayer migration system, we found that cPA suppresses cancer cell invasion.[13] These results suggest that the inhibition of cancer cell invasion may be closely related to the inhibition of peritoneal metastases by cPA.

We previously showed that through elevation of the intracellular cAMP concentration in cancer cells cPA inhibited the LPA-induced actin polymerization essential for morphological changes leading to transmonolayer migration.[13] Other cAMP-elevating agents, such as dibutyryl cAMP, forskolin (an adenyl cyclase activator) and cholera toxin, also suppress tumor cell invasion. These results indicate that the inhibition by cPA might be mediated by an elevation in cAMP levels. cAMP exerts dramatic effects on the cytoskeletal architecture: elevation of cAMP concentration in cells induces loss of action stress fibers, focal adhesions, rounding of cells and detachment from the underlying substratum.[29–31] However, the molecular mechanism by which cPA inhibits LPA-induced invasion remains to be elucidated in future studies. Recent studies have reported that cAMP inactivates GTP-bound, active Rho.[32–36] Using the

TABLE V – EFFECTS OF BOMBESIN WITH AND WITHOUT cPA ON GTP-BOUND RHOA ACTIVITY AND TOTAL RHOA ACTIVITY

Group number	Treatment[1]	Arbitrary unit	
		GTP-bound RhoA ($\times 10^3$)	Total RhoA ($\times 10^5$)
1	Control	14.9 ± 1	29.1 ± 1.3
2	Bombesin	31.3 ± 3.6[2]	33.4 ± 4.3
3	Bombesin + cPA (3.0 mg/kg body weight)	6.1 ± 2.6[3]	34.2 ± 3.9[3]
4	Bombesin + cPA (6.0 mg/kg body weight)	5.2 ± 2.3[3]	33.0 ± 3.9

[1]For explanation of treatments, see Table I.– [2]Significantly different from the value for group 1 at $p < 0.01$.– [3]Significantly different from the value for group 2 at $p < 0.001$.

Rho pull-down assay, Mukai *et al*.[8] found that elevation of cAMP was mediating the inhibition of RhoA activation and the consequential morphological response. In our *in vivo* study; however, the effects of elevation of cAMP by forskolin and cPA treatment of the control tumor on the activation of RhoA could not be examined and cAMP levels in control *vs*. the cPA-treated metastatic tissue could not be determined. Although we found that cPA attenuates the bombesin-induced increase in RhoA protein activity, we could not estimate the amount of fully activatable RhoA present in the tissue using GTP-gamma-S because the amount of tumor tissue were small and we had already exhausted all of the tumor tissue in the previous assay.

In conclusion, our present results show that cPA inhibits peritoneal metastases from intestinal adenocarcinomas *via* inhibition of RhoA activity.

REFERENCES

1. Tokumura A. Physiological and pathophysiological roles of lysophosphatidic acids produced by secretory lysophospholipase D in body fluids. Biochim Biophys Acta 2002;1582:18–25.
2. Panetti TS. Differential effects of sphingosine 1-phosphate and lysophosphatidic acid on endothelial cells. Biochim Biophys Acta 2002; 1582:190–6.
3. Svetlov SI, Sautin YY, Crawford JM. EDG receptors and hepatic pathophysiology of LPA and S1P: EDG-ology of liver injury. Biochim Biophys Acta 2002;1582:251–6.
4. Zhou D, Luini W, Bernasconi S, Diomede L, Salmona M, Mantovani A, Sozzani S. Phosphatidic acid and lysophosphatidic acid induce haptotactic migration of human monocytes. J Biol Chem 1995;270: 25549–56.
5. Chettibi S, Lawrence AJ, Stevenson RD, Young JD. Effect of lysophosphatidic acid on motility, polarisation and metabolic burst of human neutrophils. FEMS Immunol Med Microbiol 1994;8:271–81.
6. Sliva D, Mason R, Xiao H, English D. Enhancement of the migration of metastatic human breast cancer cells by phosphatidic acid. Biochem Biophys Res Commun 2000;268:471–9.
7. Fishman DA, Liu Y, Ellerbroek SM, Stack MS. Lysophosphatidic acid promotes matrix metalloproteinase (MMP) activation and MMP-dependent invasion in ovarian cancer cells. Cancer Res 2001;61: 3194–9.
8. Mukai M, Nakamura H, Tatsuta M, Iwasaki T, Togawa A, Imamura F, Akedo H. Hepatoma cell migration through a mesothelial cell monolayer is inhibited by cyclic AMP-elevating agents via a Rho-dependent pathway. FEBS Lett 2000;484:69–73.
9. Imamura F, Mukai M, Ayaki M, Akedo H. Y-27632, an inhibitor of rho-associated protein kinase, suppresses tumor cell invasion via regulation of focal adhesion and focal adhesion kinase. Jpn J Cancer Res 2000;91:811–6.
10. Ayaki M, Mukai M, Imamura F, Iwasaki T, Mammoto T, Shinkai K, Nakamura H, Akedo H. Cooperation of fibronectin with lysophosphatidic acid induces motility and transcellular migration of rat ascites hepatoma cells. Biochim Biophys Acta 2000;1495:40–50.
11. Murakami-Murofushi K, Kaji K, Kano K, Fukuda M, Shioda M, Murofushi H. Inhibition of cell proliferation by a unique lysophosphatidic acid, PHYLPA, isolated from *Physarum polycephalum*: signaling events of antiproliferative action by PHYLPA. Cell Struct Funct 1993;18:363–70.
12. Murakami-Murofushi K, Mukai M, Kobayashi S, Kobayashi T, Tigyi G, Murofushi H. A novel lipid mediator, cyclic phosphatidic acid (cPA), and its biological functions. Ann NY Acad Sci 2000;905:319–21.
13. Mukai M, Imamura F, Ayaki M, Shinkai K, Iwasaki T, Murakami-Murofushi K, Murofushi H, Kobayashi S, Yamamoto T, Nakamura H, Akedo H. Inhibition of tumor invasion and metastasis by a novel lysophosphatidic acid (cyclic LPA). Int J Cancer 1999;81:918–22.
14. Iishi H, Tatsuta M, Baba M, Yamamoto R, Taniguchi H. Enhancement by bombesin of colon carcinogenesis and metastasis induced by azoxymethane in Wistar rats. Int J Cancer 1992;50:834–9.
15. Iishi H, Tatsuta M, Baba M, Uehara H, Nakaizumi A. Ornithine decarboxylase inhibitor attenuates bombesin enhancement of intestinal carcinogenesis and metastasis induced by azoxymethane. Int J Cancer 1994;58:533–7.
16. Japanese Research Society for Cancer of the Colon and Rectum. General rules for clinical and pathological studies on cancer of the colon, rectum and anus, 6th ed. Tokyo: Kanahara Shoten, 1998.
17. Ren XD, Kiosses WB, Schwartz MA. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMBO J 1999;18:578–85.
18. Siegel S. Nonparametric statistics for the behavioral sciences. New York: McGraw-Hill, 1965.
19. Miller RG, Jr. Simultaneous statistical influence. New York: McGraw-Hill, 1966.
20. Imamura F, Horai T, Mukai M, Shinkai K, Sawada M, Akedo H. Induction of *in vitro* tumor cell invasion of cellular monolayers by lysophosphatidic acid or phospholipase D. Biochem Biophys Res Commun 1993;193:497–503.
21. Sekine A, Fujiwara M, Narumiya S. Asparagine residue in the rho gene product is the modification site for botulinum ADP-ribosyltransferase. J Biol Chem 1989;264:8602–5.
22. Imamura F, Shinkai K, Mukai M, Yoshioka K, Komagome R, Iwasaki T, Akedo H. Rho-mediated protein tyrosine phosphorylation in lysophosphatidic-acid-induced tumor-cell invasion. Int J Cancer 1996;65: 627–32.
23. Itoh K, Yoshioka K, Akedo H, Uehata M, Ishizaki T, Narumiya S. An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. Nat Med 1999;5:221–5.
24. Matsumoto Y, Tanaka K, Harimaya K, Nakatani F, Matsuda S, Iwamoto Y. Small GTP-binding protein, Rho, both increased and decreased cellular motility, activation of matrix metalloproteinase 2 and invasion of human osteosarcoma cells. Jpn J Cancer Res 2001; 92:429–38.
25. Ai S, Kuzuya M, Koike T, Asai T, Kanda S, Maeda K, Shibata T, Iguchi A. Rho-Rho kinase is involved in smooth muscle cell migration through myosin light chain phosphorylation-dependent and -independent pathways. Atherosclerosis 2001;155:321–7.
26. Panetti TS, Nowlen J, Mosher DF. Sphingosine-1-phosphate and lysophosphatidic acid stimulate endothelial cell migration. Arterioscler Thromb Vasc Biol 2000;20:1013–9.
27. Manning TJ, Jr, Parker JC, Sontheimer H. Role of lysophosphatidic acid and rho in glioma cell motility. Cell Motil Cytoskeleton 2000; 45:185–99.
28. Fischer DJ, Liliom K, Guo Z, Nusser N, Virag T, Murakami-Murofushi K, Kobayashi S, Erickson JR, Sun G, Miller DD, Tigyi G. Naturally occurring analogs of lysophosphatidic acid elicit different cellular responses through selective activation of multiple receptor subtypes. Mol Pharmacol 1998;54:979–88.
29. Lamb NJ, Fernandez A, Conti MA, Adelstein R, Glass DB, Welch WJ, Feramisco JR. Regulation of actin microfilament integrity in living nonmuscle cells by the cAMP-dependent protein kinase and the myosin light chain kinase. J Cell Biol 1988;106:1955–71.
30. Lampugnani MG, Giorgi M, Gaboli M, Dejana E, Marchisio PC. Endothelial cell motility, integrin receptor clustering, and microfila-

ment organization are inhibited by agents that increase intracellular cAMP. Lab Invest 1990;63:521–31.
31. Glass WF II, Kreisberg JI. Regulation of integrin-mediated adhesion at focal contacts by cyclic AMP. J Cell Physiol 1993;157:296–306.
32. Kreisberg JI, Ghosh-Choudhury N, Radnik RA, Schwartz MA. Role of Rho and myosin phosphorylation in actin stress fiber assembly in mesangial cells. Am J Physiol 1997;273:F283–8.
33. Laudanna C, Campbell JJ, Butcher EC. Elevation of intracellular cAMP inhibits RhoA activation and integrin-dependent leukocyte adhesion induced by chemoattractants. J Biol Chem 1997;272: 24141–4.
34. Busca R, Bertolotto C, Abbe P, Englaro W, Ishizaki T, Narumiya S, Boquet P, Ortonne JP, Ballotti R. Inhibition of Rho is required for cAMP-induced melanoma cell differentiation. Mol Biol Cell 1998;9: 1367–78.
35. Lang P, Gesbert F, Delespine-Carmagnat M, Stancou R, Pouchelet M, Bertoglio J. Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. EMBO J 1996;15:510–9.
36. Dong JM, Leung T, Manser E, Lim L. cAMP-induced morphological changes are counteracted by the activated RhoA small GTPase and the Rho kinase ROKa. J Biol Chem 1998;273:22554–62.