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Isolation of Soil Microorganisms Having Antibacterial Activity and Antimigratory Effects on Sphingosylphosphorylcholine-induced Migration of PANC-1 Cells

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To obtain soil microorganisms producing antimigratory activity which is important in controlling the metastasis of cancer cells, more than three hundreds of soil microbes were isolated from sixteen soil sources including Namsan mountain and designated as DGU1001-10338. At first, their antibiotic activities were examined by paper-disc method. More than 40 soil microbes produced compounds with antibiotic activity. Then, antimigratory activities of selected soil microorganisms were examined in a sphingosylphosphorylcholine-induced migration assay in PANC-1 cells. Six of 42 soil microorganisms having antibacterial activity also had more than 45% inhibitory activity on migration of PANC-1 cells. These results suggested that selected soil microorganisms were a useful starting point to find compounds for controlling metastasis of cancer cells.

***Key words*:** Soil microorganisms, Antibiotics, Paper disc method, Sphingosylphosphorylcholine (SPC), PANC-1 cells

INTRODUCTION

Cancer is a hyperproliferative disorder that involves transformation, dysregulation of apoptosis, proliferation, invasion, angiogenesis and metastasis (Ichikawa *et al*., 2007). Metastasis is the ability of cancer to spread from its origin to distant locations within the body and continue to grow (Chaffer and Weinberg, 2011; Fidler, 2003; Jiang and Ablin, 2011).

Invasion/metastasis is a major cause of death for cancer patients as an estimated 50% of all cancer patients may develop metastases (Fidler and Ellis, 1994). Invasion, the active translocation of neoplastic cells across tissue boundaries and through host cellular and extracellular matrix barriers, is one of the most critical steps in metastasis.

Recently, novel approaches have been tested to characterize the properties of metastastic cancer cells, such as cell elasticity or mechanical properties (Suresh *et al*., 2005; Wirtz *et al*., 2011). The clinical importance of viscoelasticity or cell stiffness was reported by Cross *et al*. (Cross *et al*., 2007). In cells from patients, the stiffness of live metastatic cancer cells taken from the body (pleural) fluids of patients with suspected lung, breast and pancreas cancer was found to be 70% less than normal tissues (Cross *et al*., 2007).

In particular, the importance of cell elasticity or viscoelasticity in several metastatic cancer cell lines has also been reported (Beil *et al*., 2003; Guck *et al*., 2005). As a particular example, sphingosylphosphorylcholine (SPC)-induced keratin phosphorylation and reorganization of human epithelial pancreatic cancer cells results in changes in the mechanical deformability of cells; this been suggested as possible pathway that facilitates easier migration and increased metastatic competence of pancreatic tumor cells (Beil *et al*., 2003).

Recently we found that transglutaminase-2 is involved in SPC-induced keratin phosphorylation and migration via JNK activation (Park *et al*., 2011). But, there few studies on compounds modulating cell elasticity or viscoelasticity of metastatic cancer cells leading to the halting of metastasis. Thus, we attempted to find the compounds for modulating cell elasticity or viscoelasticity of metastatic cancer cells from soil microorganisms.

In this study, many soil microorganisms were isolated and examined whether extracts of media of soil microorganisms suppressed SPC-induced migration of PANC-1 cells which is a model system reflecting the increased metastatic cell deformability. We found that six of more than three

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hundred chloroform extracts from soil microorganisms have inhibitory effects on SPC-induced migration of PANC-1 cells.

MATERIALS AND METHODS

Preparation of soil solutions. Several soil sources were used to isolate soil microorganisms. They were collected from several places including Namsan, Pungtaek, Asan, Samsong, and Jeju Island (Table 1). These places included soils from under the leaves, bottom of large trees, stream side soil, under the pear trees, the banks around rice fields, mountainous red clay, soil from under vegetable gardens, and under the seaside.

Collected soils were labelled as S1~S16. Soils were dried in indirect light with wind for two weeks and 1 g of each soil was put in a tube with same amounts of distilled water and then centrifuged (Table Top Centrifuge, PLC-05, 4500 RMP). Supernatants (0.2 m*l*) from centrifuged soil suspension was mixed with 1.8 m*l* of distilled water. Diluted supernatants were centrifuged again (Table Top Centrifuge, PLC-05, 4500 RMP) and used as soil solutions.

Isolation of soil microorganisms. The 10-fold diluted soil solutions (100 μ*l*) were added into plates made from six selection media including modified Bennett medium, glycerol-arginine agar, oatmeal soil extract agar, soluble starch casein agar, glycerol-asparagine agar and starch-casein-KNO_3-agar which contained cycloheximide (100 μg/m*l*) and nystatin (100 unit/m*l*) to suppress the growth of fungi and bacteria. Components of each selected media are listed in Table 2. Then, these inoculated plates were placed in an incubator (28°C) for one week. After 1 week, soil microorganisms was selected and labeled as DGU10001-DG10338. Selected soil microorganisms were transferred to V8 agar media and cultured for 72 h at 28°C according to reported methods (Goo *et al*., 1991). Isolated soil microorganisms were maintained on V8 agar slants (per liter, V8 juice (200 m*l*), $CaCO_3$ (3 g), agar powder (20 g)).

Evaluation of antibiotic activity by paper disc agar diffusion assay. To test for antibiotics producing activities in soil microorganisms, samples were cultured in 15 m*l* of tryptic soy broth at 28°C/180 rpm for 5 days. Cultured broth of soil microbes were centrifuged at 10,000 rpm, at 4°C for 20 min and supernatants were collected. Supernatants (100 μ*l*) were added on the paper discs (8 mm) and dried for 2 hours. Discs were put on the test plate which contained either *Escherichia coli* or *Bacillus subtilis*. Test plates were placed at 4°C for 30 min and then incubated at 28°C, overnight. *E. coli* (KCCM11234) and *B. subtilis* (KCCM113160) were purchased from the Korea Culture Center of Microorganisms (KCCM) and cultured according to directions by KCCM.

Preparation of chloroform extracts from broths of soil microorganisms. Five ml of supernatants from culture broths of DGU10001-DGU10338 were mixed with the same volume of chloroform and mixtures were placed overnight. Chloroform layers were collected and evaporated with N_2 gas. Weight of samples were measured and final stock solutions with DMSO (final concentration of 10 mg/m*l*) were prepared. These stock solutions of samples were used for evaluation of antimigratory activity of samples.

Evaluation of antimigratory effects of chloroform extracts on SPC-induced migration of PANC-1 cells. Migration assays were performed using multiwell chambers (Neuroprobe, Inc. Gaithersburg, MD) coated with 10 μg/m*l* fibronectin as a chemoattractant according to reported methods (Cha *et al*., 2011). Briefly, PANC-1 cells were suspended in DMEM at 1×10^6 cells/m*l*, and a 25 μ*l* aliquot of this suspension was placed into the upper well of one chamber. Next, the aliquot was separated from the 3%-serum-containing lower well using an 8 μm polyhydrocarbon filter. After incubation for 4 hr at 37°C, the non-migrated cells on the upper surface of the membrane were scrapped off, the migrated cells on the lower surface were stained with Diff-quick and subsequently counted under five randomly chosen high-power fields (400 ×). PANC-1 cells (5×10^4 cells per well) were treated with 5 μM of SPC with or without chloroform extracts of the DGU samples for 1 hour.

Statistical analysis. The data are expressed as the means ± S.E.M. of at least three independent experiments performed in triplicate. A p value < 0.05 was considered significant.

RESULTS

Isolation of soil microorganisms. We collected 16 soil samples from various places and isolated several soil microorganisms by selective media for streptomycetes containing antibiotics such as nystatin and cycloheximide (Goo *et al*., 1991; Kuester and Williams, 1964). Some selective

Table 1. List of soil sources used

Soil No.	Places	Soil No.	Places
S1	Namsan 1st pl.	S9	Chungnam
S2	Namsan 2nd pl.	S10	Pungtaek 1st pl.
S3	Namsan 3rd pl.	S11	Asan si 1st pl.
S4	Namsan 4th pl.	S12	Pungtaek 2nd pl.
S5	Namsan 5th pl.	S13	Asan si 2nd pl.
S6	Namsan 6th pl.	S14	Samsong pl.
S7	Sangrokwon	S15	Jeju 1st pl.
S8	Children Park	S16	Jeju 2nd pl.

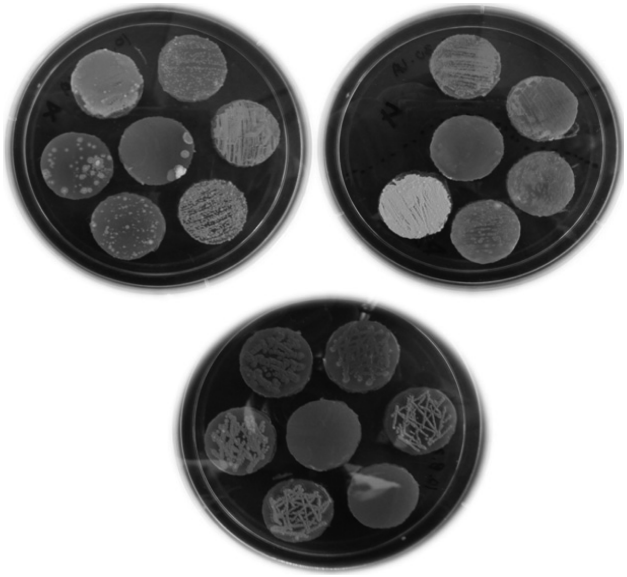

Fig. 1. Images of soil microorganisms on V8 agar plugs isolated from S1~S16 soil sources.

media used for this purpose are shown in Table 1. Soil microorganisms were transferred to V8 agar plugs and growth was confirmed in V8 agar plugs (Fig. 1). After confirmation of growth in V8 agar plugs, we maintained each soil microorganism in a cap tube containing V8 agar and labeled them as DGU10001 to DGU10338.

Examination of antibacterial producing capacities of DGU10001-10338. Each DGU10001-10338 colony was cultured in 5 m*l* of tryptic soy broth (TSB) for 3 days, then cultured broths were centrifuged and 100 μ*l* of the supernatant was used to examine antibacterial activity against *E. coli* and *B. subtilis*. Forty-two DGU strains showed antibacterial activities (Table 2). For example, antibacterial inhibition zones of DGU strains are shown in agar test plates containing *B. subtilis* (Fig. 2). Some of the DGU strains such as DGU10107 had strong antibacterial activity against both *B. Subtilis* and *E. coli*.

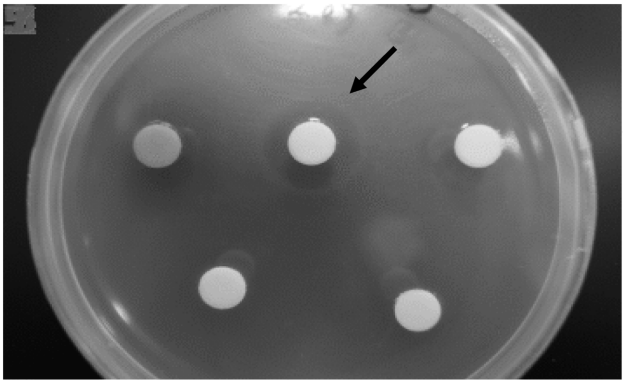

Fig. 2. Antibacterial activity of DGU10009. Antibacterial activity was evaluated by the paper disc method.

A number of soil microorganisms having antibacterial activities were classified with soil sources. More than six of the soil microorganisms from S1, S4, S8, and S13 soil samples were found to have antibacterial activities against *B.subtilis* or *E. coli* (Fig. 3A). Many soil microorganisms having antibacterial activity were isolated from glycerol-asparagine media (M5) (Fig. 3B).

Evaluation of antimigratory effects of chloroform extracts from 42 DGU strains on SPC-induced migration of PANC-1 cells. Forty-two DGU strains having antibacterial activity were cultured and 3 m*l* of media were extracted with chloroform. Chloroform extracts were dissolved in DMSO and examined whether they could suppress SPC-induced migration the functional result of regulating viscoelasticity and keratin reorganization in PANC-1 cells. Six of the 42 samples showed more than a 45% antimigratory effect on SPC-induced migration (Fig. 4A). Six samples showed dose-dependent inhibition against SPC-induced migration of PANC-1 cells (Fig. 4B). In particular, DGU10047, DGU10070a (derived from DGU10070), and DGU10120 strains showed good dose-dependent inhibition of SPC-induced migration in PANC-1 cells.

Table 2. Lists of media used in isolation of soil microorganisms

No	Media	Constituents (%)
M1	Modified Bennett agar	Yeast extract, 0.1; Beef extract, 0.1; Casein hydrolysate, 0.2; 1 M Potassium phosphate buffer (pH 7.0), 3 (v/v); 50% (w/v) glucose, 2; Trace salt, 1; Agar, 2.
M2	Glycerol-arginine agar	Glycerol, 2; Arginine, 0.25; NaCl, 0.1; $CaCO_3$, 0.01; $MgSO_4$ $7H_2O$, 0.01; $FeSO_4$ $7H_2O$, 0.01; Agar, 2.
M3	Oatmeal soil extract agar	Oat meal agar, 2; Soil extract, 50 (v/v)
M4	Soluble starch casein agar	Soluble starch, 1; Casein (in NaOH), 0.1, K_2HPO_4, 0.05; $MgSO_4$, 0.05; Agar, 2.
M5	Glycerol-asparagine agar	Glycerol, 1; Asparagine, 0.1; K_2HPO_4, 0.1; Agar, 2.
M6	Starch-casein-KNO_3-agar	Starch, 1; Casein, 0.03; KNO_3, 0.02; NaCl, 0.02; K_2HPO_4, 0.02; $MgSO_4$ $7H_2O$, 0.005; $CaCO_3$, 0.002; $FeSO_4$ $7H_2O$, 0.001; Agar, 1.8.

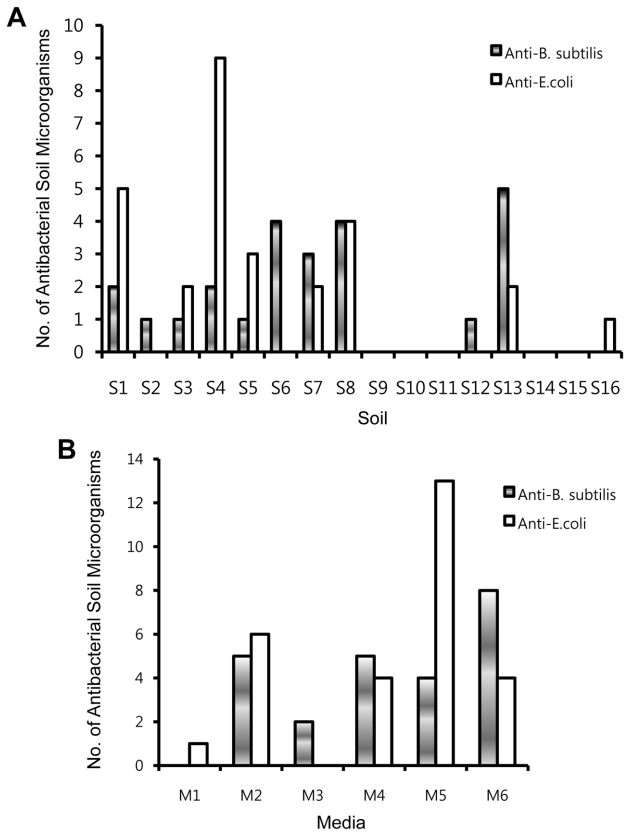

Fig. 3. Analysis of soil microorganisms having antibacterial activity by soil sources and selection media. (A) Classification of soil microorganisms having antibacterial activity by soil sources (S1~S16; details are listed in Table 1). (B) Classification of soil microorganisms having antibacterial activity by selection media (M1~M6; details are listed in Table 2). (A~B) Anti-*B. subtilis* represents soil microorganisms having antibacterial activity against *B. Subtilis*. Anti-*E. coli* represents soil microorganisms having antibacterial activity against *E. coli*.

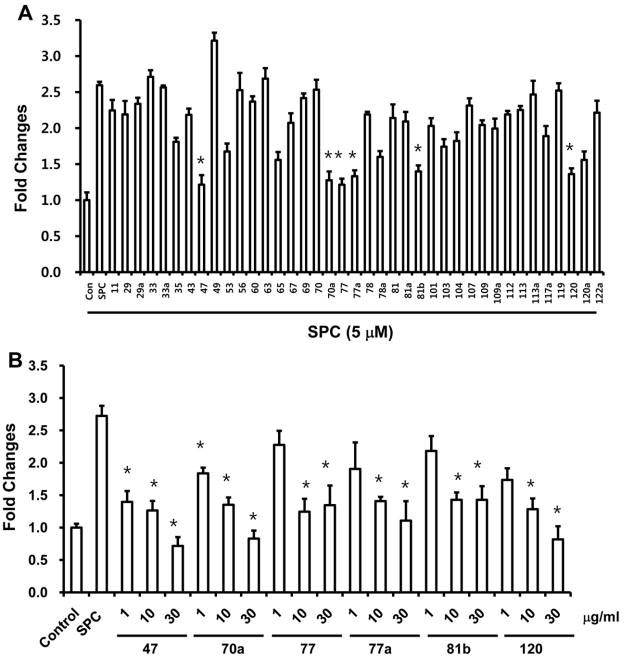

Fig. 4. Antimigratory effects of soil microorganisms on SPC-induced migration of PANC-1 cells. (A) Screening of 42 DGU strains having antibacterial activities for inhibition of SPC-induced migration of PANC-1 cells using boyden chamber assays (details in Methods). (B) Dose-dependency of chloroform extracts of DGU10047, 10070a, 10077, 10077a, 10081b, and 10120 in SPC-induced migration of PANC-1 cells.

DISCUSSION

Metastasis is a major issue for antitumor therapy and 50% of all deaths of cancer patients are due to metastases. Recently, the physical properties of metastatic cancer cells were reported to be different with those of normal cells or even nonmetastatic cancer cells (Guck *et al*., 2005). These observations were also proved in clinical metastatic cancer cells obtained from ascites of cancer patients (Cross *et al*., 2007). Suresh proposed novel ways of controlling the physical properties of cancer cells (Suresh *et al*., 2005) and we attempted to find compounds that controlled the physical properties of metastatic cancer cells. Thus, we isolated soil microorganisms using selective media for actinomyces and used SPC-induced migration as screening system for finding the compounds controlling the physical properties of metastatic cancer cells. It is already well-established that SPC-induced migration of PANC-1 cells occurs via regulation of viscoelasticity and keratin reorganization (Beil *et al*., 2003).

Forty-two DGU strains from a total of 338 had antibacterial activities (Table 3). Twenty DGU strains were isolated from the glycerol-asparagine agar plate which is a well-known selection media for streptomycetes (Kuester and Williams, 1964). These results suggested that many strains from glycerol-asparagine agar plate might belong to streptomycetes.

In considering the soil sources, many of soil microorganisms having antibacterial activity were found in S4, S8, and S13 soil sources (Fig. 3A). S4 was collected under fallen leaves of trees from Namsan in Seoul. S8 was collected from the flower garden of the campus at Dongguk University at Seoul and S13 is collected from a vegetable garden of Asan city. These results suggested that soil under the fallen leaves and gardens such as flower and vegetable garden are good source for soil microorganisms having antibacterial activity.

Finally, we examined the antimigratory effects of the DGU strains containing antibacterial activity on SPC-induced migration of PANC-1 cells and six strains had antimigratory effects (Fig. 4A, 4B). DGU10047, DGU10070a (derived from DGU10070), and DGU10120 strains exhibited good

Table 3. Antibacterial activities of soil microorganisms expressed as a diameter of the inhibition zone

[1]Soil M.	[2]Test M.		Soil M.	Test M.		Soil M.	Test M.		Soil M.	Test M.	
DGU10	[3]B	[4]E	DGU10	B	E	DGU10	B	E	DGU10	B	E
1	0	0	32	0	0	63	12	0	94	0	0
2	0	0	33	0	11	64	0	0	95	0	0
3	0	0	34	0	0	65	15	14	96	0	0
4	0	0	35	16	0	66	0	0	97	0	0
5	0	0	36	0	0	67	0	14	98	0	0
6	0	0	37	0	0	68	0	0	99	0	0
7	0	14	38	0	0	69	0	16	100	0	0
8	0	0	39	0	0	70	0	25	101	0	14
9	0	0	40	16	0	71	0	0	102	0	0
10	0	0	41	0	0	72	0	0	103	0	18
11	0	0	42	18	0	73	0	0	104	0	12
12	0	0	43	16	0	74	0	0	105	0	19
13	0	0	44	0	0	75	0	0	106	0	0
14	0	0	45	0	15	76	0	0	107	24	20
15	0	0	46	0	0	77	15	0	108	0	0
16	0	0	47	0	10	78	14	0	109	20	13
17	0	0	48	0	0	79	0	0	110	24	12
18	0	0	49	19	0	80	0	0	111	26	10
19	0	0	50	0	0	81	0	14	112	22	10
20	0	0	51	0	0	82	0	0	113	22	10
21	0	0	52	0	12	83	0	0	114	0	0
22	0	0	53	0	16	84	0	0	115	0	0
23	0	0	54	0	0	85	0	0	116	12	0
24	0	0	55	0	17	86	0	0	117	16	0
25	0	0	56	0	18	87	0	0	118	0	0
26	0	0	57	0	14	88	0	0	119	14	0
27	0	0	58	16	0	89	0	0	120	11	0
28	0	0	59	0	16	90	0	0	121	16	0
29	14	12	60	0	0	91	0	0	122	14	0
30	0	0	61	0	0	92	0	0	123	0	0
31	0	0	62	12	14	93	0	0	124	0	0

[1]Soil M: Soil microorganisms isolated from several soil sources; [2]Test M: Microorganisms for used in antibacterial test; [3]B: Bacillus subtilis; [4]E: *Escherichia coli.*

dose-dependent of inhibition in SPC-induced migration (Fig. 4B). SPC is a unique sphingolipid and found in high amounts in ovarian cancer, atopic dermatitis and Nieman-Pick Disease (Kim *et al*., 2008a; Rodriguez-Lafrasse and Vanier, 1999; Xu *et al*., 2000). SPC has many biological effects in both inflammation and cancer (Choi *et al*., 2010; Kim *et al*., 2010; Park *et al*., 2011). SPC-induced migration of PANC-1 cells by regulating keratin reorganization and viscoelasticity leads to increased deformability of PANC-1 cells (Beil *et al*., 2003; Park *et al*., 2011; Rolli et al., 2010).

To our knowledge, no compounds have been found to modulate the physical properties of metastatic cancer cells or SPC-induced migration of PANC-1 cells. Therefore, further study to identify the key principles of soil microorganisms having the antimigratory effects on the SPC-induced migration is ongoing. Chloroform extracts of soil microorganisms having antimigratory activity might be expected to suppress the process of keratin reorganization since these processes are involved in SPC-induced migration.

Our study suggests that some compounds in culture broths of several strains can be new types of antimigatory compounds which might be used to modulate the keratin reorganization leading to the control of viscoelasticity or deformabilioty of cells. Streptomyces isolated from soil produced several compounds having antitumor effects or antimetastatic activity (Kim *et al*., 2008b; Zhao *et al*., 2005). Our newly found DGU strains could be useful sources for new compounds to fight against metastasis.

In altogether, we isolated 338 soil microorganisms from 16 different soil samples and isolated 42 soil microorganisms having antibacterial activity; 6 of those 42 soil microorganisms had good antimigratory activity to SPC-induced migration of PANC-1 cells. Further study demonstrated that three soil microorganisms showed good dose-dependent effects in this assay suggesting that these compounds may be capable of controlling the physical properties of cancer

cells such as viscoelasticity or deformability.

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