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Inhibition of Cancer Cell Migration by Compounds from Garlic Extracts

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Cell migration plays a fundamental role in cancer cell invasion and metastasis as well as in many physiological responses. Here, we screened four different sources of garlic - water extract of normal and black garlic, as well as dried normal and black garlic - for the identification of anti-invasive and anti-metastatic activity on cancer cells. Inhibition of cancer cell migration was observed in the hexane extract of dried-garlic. Inhibitory activity was further purified to near homogeneity by thin layer chromatography and named inhibitor of cancer metastasis from garlic #27 (ICMG-27). ICMG-27 completely blocked insulin-like growth factor-1 (IGF-1)-induced OVCAR-3 cell migration at 6 μg/ml. ICMG-27 completely blocked IGF-1-induced OVCAR-3 and NIH-3T3 cell migration whereas IGF-1-induced mouse embryonic fibroblast (MEF) cell migration was not affected byICMG-27. ICMG-27 inhibited all the tested IGF-1-induced cancer cell migration such as OVCAR-3, SKOV-3, and MDA-MB-231 cells. Finally, ICMG-27 could inhibit IGF-1-, lysophosphatidic acid (LPA)-, sphingosine-1-phosphate (S1P)-, leukotriene B4 (LTB4)-, and angiotensin II (AngII)-induced OVCAR-3 cell migration. These results indicate that ICMG-27 inhibits cancer cell migration by blocking essential steps in many agonists-induced cancer cell migrations. Unveiling an anti-invasive mechanism of ICMG-27 on cancer cells will provide a basis for cancer therapy.

Key words : Garlic, cancer, migration, metastasis, growth factor

Introduction

Cancer is a disease of complex etiology, defined as uncontrolled growth of cells. The transformation of normal to cancerous cell involves three distinct phases, for example, initiation, promotion, and progression [7]. During the initiation and promotion steps, cancer cells attain several cancerous features caused by genetic changes. At the end of tumorigenesis, cancer cells acquire its ability to spread to distant organs so called metastasis. The high mortality rates associated with cancer are caused by the metastasis. Indeed, metastases are the cause of 90% of cancer deaths [8]. Therefore, cancer therapies should be focused on not only tumor development but also metastasis.

It has been reported that certain dietary agents such as tomato-derived lycopene, vitamin E, and selenium may have a preventive effect for prostate cancer [18]. In particular, garlic is one of the first plant with constituents reported to possess antitumor activity and was used for treatment of tumors by Egyptians over 3500 years ago. In epidemiologic point of view, high garlic consumption is associated with protective effect on cancer. For example, high consumption of garlic (>10 g/d) was related with low prostate cancer prevalence in China [11]. It also has been reported that persons with high intake of total garlic (>24 kg/yr) had 60% reduced risk of cancer compared with low consumption (<11.5 kg/yr) on 564 patients with stomach cancer and 1131 normal controls [26]. These results suggest that garlic plays a positive role in the prevention of certain human cancers.

It is still unclear which compounds in garlic has positive role in the prevention of cancers. However, recent study has demonstrated that two major compound shows active anticancer effects. One group is lipid-soluble allyl sulfur compounds such as diallyl disulfide (DADS) and diallyl trisulfide (DATS), and the other one is the water-soluble compounds including γ-glutamyl *S*-allylcysteine group such as *S*-allylcysteine (SAC) and *S*-allylmercaptocysteine (SAMC) [21]. These compounds are not only able to suppress the skin, stomach, colon, liver, esophageal, and lung cancer growth in animal models, but also directly inhibit proliferation of various cancer cell lines derived such as from colon, lung, leukemia, skin, breast, and prostate *in vitro* [9].

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Molecular mechanism underling antitumor activity of these compounds is still unknown. Recently, it has been reported that DATS has antitumor activity by induction of apoptosis through extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK) induced down regulation of Bcl-2 [25]. Also, it has been reported that SAMC blocks colon cancer cell proliferation through activation of JNK1 and caspase-3 signaling pathways to cause apoptosis [24]. These lines of evidence indicate that may be used as effective agents in the treatment of human primary cancers.

As we mentioned above, metastasis covers 90% of causality of cancer patients' death indicating that anti-metastatic agents are required for efficient cancer therapy. Recent studies have provided evidence that sarcoma cell migration was inhibited by aged garlic extract (AGE) although exact molecular nature of corresponding agents in AGE was not known [12]. However, it has been reported that SAMC potently reduced the ability of prostate cancer cell in matrigel invasion through the enhancement of E-cadherin expression [3]. Down regulation of E-cadherin is characteristic of the epithelial-mesenchymal transition, which is early key step in metastasis [16]. However, it is still ambiguous by which agents suppress metastasis of cancer cells from garlic extracts.

Despite the abundant *in vitro* evidence for anti-cancer effect of garlic extracts, anti-metastatic effect of garlic extract or its compounds is not well studied. In this study, we provide novel anti-metastatic activity from hexane fractions of dried garlic. Nearly pure compound of this agent inhibited variety of cancer cell migration. In addition, this compound blocked various stimuli-induced migration of OVCAR-3 breast cancer cell migration.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, and antibiotics were purchased from Hyclone Laboratories Inc (Logan, UT, USA). Anti-phospho-Akt (Ser473) and anti-phospho-Akt (Thr308) were purchased from Cell Signaling Technology (Boston, MA, USA). IRDye700- and IRDye800-conjugated rabbit/mouse secondary antibodies were obtained from Li-COR Bioscience (Lincoln, NE, USA). IGF-1, lysophosphatidic acid (LPA), and angiotensin II (AngII) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Leukotriene B4 (LTB4) was obtained from Cayman (Ann Arbor, MI, USA). Sphingosine-1-phosphate was purchased from Biomol (Plymouth, PA, USA). Chloroform, methanol, ethanol, n-butanol, and hexane were purchased from Fisher Scientific, Ltd (Pittsburgh, USA). All other reagents with high quality were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Plant materials

Garlic was cultivated in Changyoung, Korea. Cloves were purchased from a local supplier. The black garlic was manufactured by New Green Company (Changyoung, Korea) under following steps: normal raw garlic was incubated at 75°C with 70% relative humidity for 2 weeks. A voucher specimen (accession number PDRL-10) has been deposited in the Plant Drug Research Laboratory of Pusan National University (Busan, Korea).

Activity-guided fractionation of garlic extracts

Four different garlic preparations were made. For example, water was used to extract and black garlic. In addition, dried normal or black garlic was directly used to identify anti-tumor activity. Four groups of sample were further partitioned with hexane (H), chloroform (C), ethanol (E), methanol (M), and *n*-butanol (B). The upper layer suspension was filtered and evaporated under reduced pressure at 45°C and then lyophilized. Powder form of sample was dissolved with DMSO for activity analysis or dissolved in chloroform and then applied to thin layer chromatography for further separation. Separated compound was visualized with iodine, numbered, and marked with pencil. Silicas in marked area was scrapped with razor blade and dissolved in chloroform. Samples are evaporated and lyophilized until use.

Determination of a single compound by NMR spectra

The purity of isolated active compound was determined by the ^{1}H, ^{13}C, Dept, HSQC, HMBC NMR spectra in $CDCl_3$. NMR spectra showed two primary [δC 14.06 (C-1), 14.26 (C-2')], thirteen secondary [δC 20.56 (C-17), 31.5 (C-16), 25.62 X 2 (C-11, 14), 27.21 (C-8), 29.10-29.58 (C-4-7), 24.98 (C-3), 34.39 (C-2), 25.0 (C-3), 60.2 (C-1')], six tertiary [δC 127.12 - 131.97 (C-9, 10, 12, 13, 15, 16)], and one quaternary carbons [δC 173.93 (C-1)], indicating that isolated compound is nearly single compound.

Cell culture

Mouse embryonic fibroblast (MEF) cells which are established as previously reported [1]. MEF cells, NIH-3T3, and MDA-MB-231 breast cancer cells were cultured in DMEM supplemented with 10% (v/v) FBS and penicillin/streptomycin, and maintained at 37°C in 5% CO_2. OVCAR-3 and SKOV-3 cells were cultured in RPMI medium.

Measurement of cancer cell migration

Cancer cells were grown and starved serum for 6 hr before plating on ChemoTx chamber. Cells were detached with trypsin-EDTA and washed with serum-free DMEM. For migration assay, bottom side of ChemoTx membrane was coated with type I collagen for 30 min and 2×10^4 serum-starved cells in 100 μl volume were placed on top side of ChemoTx membrane. Migration was induced by placing the cell over-laid ChemoTx membrane on top of serum-free medium containing IGF-1 (50 ng/ml) or IGF-1 with garlic extracts (10 μg/ml) for 3 hr. ChemoTx membrane was fixed with 4% paraformaldehyde and non-migrated cells on top side of membrane were removed by gently wiping with cotton swab. Membrane was stained with DAPI and migrated cells were counted under the fluorescent microscope at 20× magnitude (Axiovert 200).

Western blotting

Cell lysates were subjected to 8-15% gradient polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Membranes were incubated with the indicated primary antibodies and IRDye-conjugated secondary antibodies, and protein bands were visualized using an Infrared image analyzer (Li-COR Bioscience).

Statistical analysis

For analysis of migration and invasion, results are expressed as the mean±S.D. of three independent experiments (n=3 for each experiment). When comparing two groups, an unpaired Student's t-test was used to assess differences. P-values less than 0.05 were considered significant and indicated as * and P-values higher than 0.05 were considered insignificant and indicated as **.

Results and Discussion

Garlic, a widely used herbal vegetable, has been suggested as an anti-cancer agent for several decades in epidemiological studies [21]. However, the exact molecular mechanism underlying garlic extracts exert its effect on anti-cancer properties are still unknown. In the present study, we provide novel activity which suppresses the migration of cancer cells. To screen the novel compound from garlic that specifically suppresses metastasis of cancer cells, we initially prepared two different forms of garlic such as normal garlic and black garlic which is made from normal garlic by fermentation at high temperature. During this process, it is believed that certain chemical compounds are changed as noticed by changes in color, smell, and taste. Both types of garlic were either dried or extracted with water to make four different types of garlic sources. Extraction of garlic sources with organic solutions such as ethanol, chloroform, hexane, and methanol showed different effect on the stimulation or inhibition of cancer cell migration. As shown in Fig. 1A, fractions extracted with water could not enhance the migration of cancer cells indicating that there are no water-soluble compounds that stimulate cancer cell migration. By contrast, there was strong inhibitory activity of cancer cell migration in hexane-soluble form of water extract and this activity was significantly increased by black garlic (Fig. 1B). These results indicate that some inhibitory compound is generated during the conversion of garlic to black garlic. In contrast to the water extract of both normal and black garlic, some extracts of dried garlic showed strong stimulatory activity of cancer cell migration. As shown in Fig. 1A, ethanol extract of dried normal garlic showed strong stimulatory activity of cancer cell migration, whereas ethanol extract of dried black garlic did not show stimulatory activity of cancer cell migration. This result indicates that some stimulatory compound in normal garlic is changed to inactive form during the conversion of normal garlic to black garlic. Likewise, hexane extract of normal garlic or ethanol extract of black garlic significantly inhibited IGF-1-induced cancer cell migration. Since our primary purpose was to identify novel compound that inhibit cancer cell metastasis, we decided to select hexane extract of dried normal garlic (HEDG) which inhibits IGF-1-induced cancer cell migration and does not strongly stimulate cancer cell migration (Fig. 1). However, further separation of HEDG fraction by thin layer chromatography also showed both stimulatory and inhibitory activities of cancer cell migration (Fig. 2). Among the thin layer chromatographic fractions, #27 fraction (inhibitor of cancer metastasis from garlic #27, named

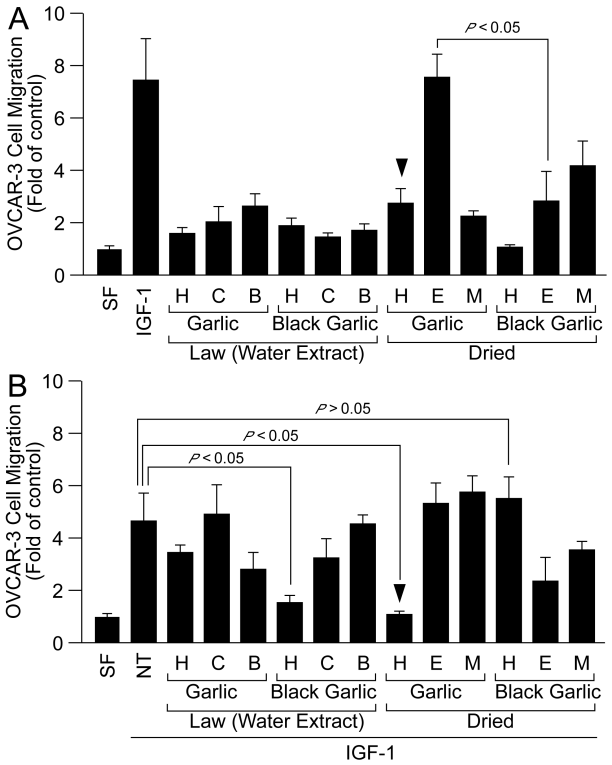

Fig. 1. Identification of anti-migrative activity of cancer cells from hexane extract of dried garlic. Garlic was fermented at high temperature to make black garlic as described in "Materials and Methods" Both garlic and black garlic were either extracted with water or dried. Water extracts, dried garlic, or dried black garlic were further extracted with hexane (H), chloroform (C), *n*-butanol (B), or methanol (M). (A) OVCAR-3 cancer cells were stimulated with each fraction (10 μg/ml) for 3 hr and then measured migration of cancer cells as described in "Materials and Methods" (B) Each fraction was added prior to stimulation with IGF-1 (50 ng/ml) and IGF-1-induced cancer cell migration was measured. Arrows indicate fraction that inhibits IGF-1-induced cancer cell migration but does not stimulate cancer cell migration. Data are the mean±S.D. of three independent experiments (n=3 for each experiment). When comparing two groups, an unpaired Student's t-test was used to assess differences. P-values less than 0.05 were considered significant and P-values higher than 0.05 were considered insignificant.

ICMG-27) was revealed to single compound as judged by the analysis of NMR spectra (Fig. 2C), and strongly inhibited IGF-1-induced cancer cell migration (Fig. 2B). However, ICMG-27 did not stimulate cancer cell migration (Fig. 2A). Therefore, ICMG-27 seems to be a single compound that inhibits IGF-1-induced OVCAR-3 cell migration. However, the molecular identity of ICMG-27 is still ambiguous.

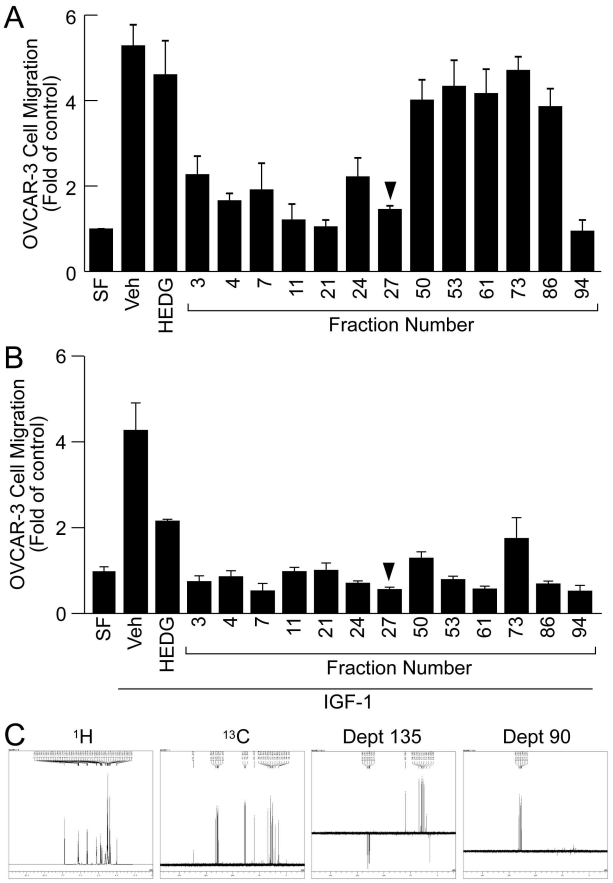

Fig. 2. Isolation of single compound inhibiting OVCAR-3 cancer cell migration. Hexane extract of dried garlic (HEDG) was further separated on thin layer chromatography and fractionated. (A) Effect of each fraction on OVCAR-3 cell migration was measured as described in "Materials and Methods" (B) Effect of each fraction on IGF-1-induced OVCAR-3 cancer cell migration was measured. Arrows indicate fractions that inhibit IGF-1-induced OVCAR-3 cell migration but does not stimulate OVCAR-3 cancer cell migration. Data are the mean±S.D. of three independent experiments (n=3 for each experiment). P-values less than 0.05 were considered significant and P-values higher than 0.05 were considered insignificant. (C) ^{1}H (500 MHz in $CDCl_3$), ^{13}C, Dept 135 and Dept 90 NMR (100 MHz in $CDCl_3$) spectrum of ICMG-27.

It has been reported that active ingredients in garlic that suppress cancer cell proliferation are SAC and SAMC [2,3,10,24]. Indeed, the calculated IC_{50} of SAMC and SAC were 13.1 mg/ml and 0.33 μg/ml, respectively [3]. However, IC_{50} of ICMG-7 is significantly different from that of SAMC and SAC. For example, the IC_{50} of ICMG-27 on IGF-1-induced cancer cell migration was 32.7 ng/ml (Fig. 3A). In addition, ICMG-27 was isolated from hexane extract of dried normal garlic indicating that ICMG-27 is lipid-soluble

compound. However, SAMC and SAC are water-soluble compounds. Migration of cells is often inhibited by the suppression of cell proliferation. However, the effect of ICMG-27 is not the case since addition of ICMG-27 (10 μg/ml) to the growing cells did not affect the proliferation of OVCAR-3 cells (Fig. 3B). Therefore, it seems likely that ICMG-27 is novel compound that uniquely inhibits cancer cell migration.

Although it is still unknown about molecular target of ICMG-27, the target of ICMG-27 seems to be cell-type specific. For example, ICMG-27 significantly inhibited IGF-1-induced OVCAR-3 cell migration (Fig. 3C) whereas ICMG-27 could not inhibit IGF-1-induced MEF cell migration (Fig. 3D). In addition, IGF-1-induced migration of NIH-3T3 cell was completely blocked by ICMG-27 (Fig. 3E). Given these results, it is possible that the molecular target of ICMG-27 is differentially expressed in different cell types. Phosphatidylinositol 3-kinase (PI3K) and Akt signaling pathway plays an essential role in a variety of growth factor-induced cell migration [5,13,17,19,20]. Especially, activation of Akt is required for many different types of cell migration [4,14,15]. However, pretreatment of ICMG-27 did not attenuate IGF-1-induced activation of Akt (Fig. 3E) indicating that ICMG-27 inhibits IGF-1-induced OVCAR-3 cell migration by affecting downstream of PI3K/Akt pathways. Since IGF-1 induced similar extent of cell migration in different cell types and ICMG-27 could not inhibit certain type of cell migration, it is reasonable that ICMG-27 does not directly inhibit IGF-1 signaling pathway, instead it has different target that suppresses IGF-1 signaling pathway downstream of PI3K/Akt.

Circulating level of IGF-1 has been shown to be associated with various cancer development and metastasis [6,22,23]. Likewise, stimulation of OVCA-3, SKOV-3, and MDA-MB-231 cells with IGF-1 significantly induced the migration (Fig. 4A) indicating that IGF-1 signaling plays an essential role in a variety of cancer cell migration. ICMG-27 attenuated IGF-1-induced migration of all the tested cancer cells such as OVCAR-3, SKOV-3, and MDA-MB-231 cells. Therefore, it is likely that almost all the tested cancer cells express target protein of ICMG-27 thereby inhibit cancer cell migration. Therefore, ICMG-27 seems to have broad spectrum of target cancer cell lines for the inhibition of migration and metastasis. In addition to the effect on growth factor-induced cancer cell migration, ICMG-27 also inhibited various agonists-induced cancer cell migrations. For example, lysophosphatidic acid (LPA), sphingosine-1-phosphate (S1P),

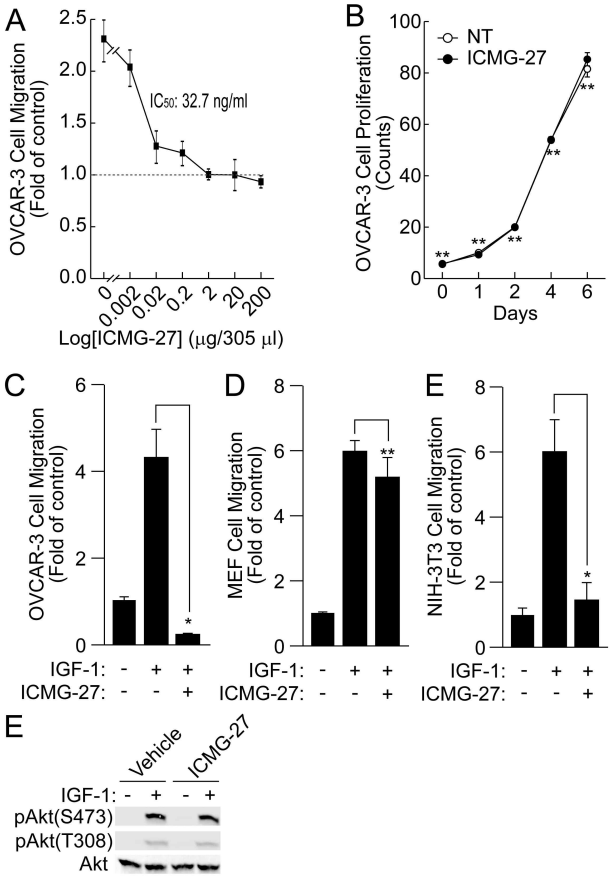

Fig. 3. Cell type-specific inhibition of migration by ICMG-27. (A) OVCAR-3 cells were pretreated with ICMG-27 as indicated concentration prior to stimulation with IGF-1. Basal migration was indicated with dashed line. (B) OVCAR-3 cells were grown in presence or absence of ICMG-27 (10 mg/ml) and cell number was counted by trypan exclusion assay. Data are the mean±S.D. of three independent experiments (n=3 for each experiment). P-values higher than 0.05 were considered insignificant and indicated as **. OVCAR-3 cells (C), MEF cells (D), and NIH-3T3 cells (E) were stimulated with IGF-1 (50 ng/ml) in the presence or absence of ICMG-27 (10 μg/ml) and measured migration. Data are the mean±S.D. of three independent experiments (n=3 for each experiment). P-values less than 0.05 were considered significant and indicated as * and P-values higher than 0.05 were considered insignificant and indicated as **. (E) OVCAR-3 cells stimulated with IGF-1 in the presence or absence of ICMG-27 (10 μg/ml). Activation of Akt was verified by western blotting with phospho-specific antibodies against phospho-Ser473 and phospho-Thr308, respectively.

leukotriene B4 (LTB4), and angiotensin II (AngII) significantly induced the OVCAR-3 cell migration and the induction of migration was also completely blocked by

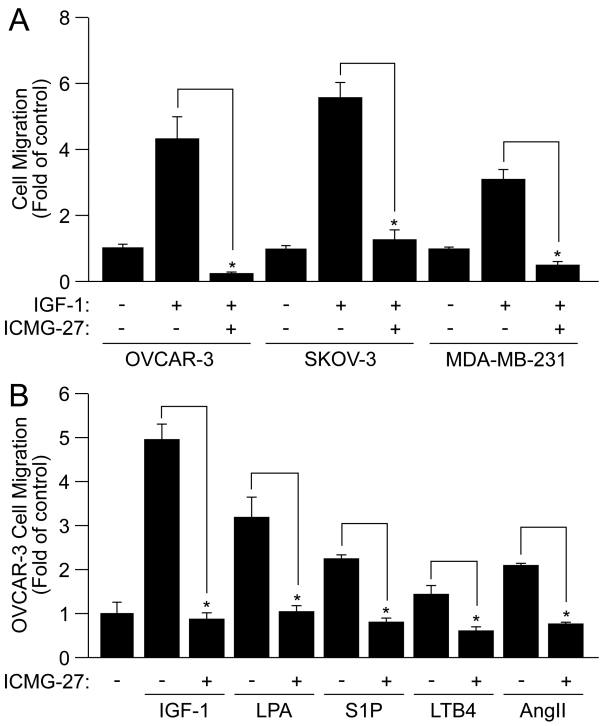

Fig. 4. Inhibition of various agonists-induced migrations of various cancer cells. (A) OVCAR-3, SKOV-3, and MDA-MB-231 cells were stimulated with IGF-1 (50 ng/ml) in the presence or absence of ICMG-27 (10 μg/ml) and measured migration (B) OVCAR-3 cells were stimulated with IGF-1 (50 ng/ml), LPA (10 μM), S1P (100 nM), LTB4 (100 nM), and AngII (10 μM) in the presence or absence of ICMG-27 (10 μg/ml) followed by measuring migration as described in "Materials and Methods" Data are the mean±S.D. of three independent experiments (n=3 for each experiment). P-values less than 0.05 were considered significant and indicated as *.

ICMG-27 (Fig. 4B). However, it is notable that IGF-1-induced OVCAR-3 cell migration was much higher than by those G protein coupled receptor (GPCR) agonists. These results indicate that ICMG-27 modulates critical cellular target protein(s) that are involved in the growth factor- or GPCR-induced cancer cell migration. This target protein(s) might be not downstream of IGF-1 signaling pathway since ICMG-7 was not effective on IGF-1-induced MEF cell migration (Fig. 3D).

In summary, use of garlic as food or dietary supplement for the chemoprevention or treatment of cancer has been shown remarkably effective. In this study, we have demonstrated novel anti-metastatic compound in hexane extract in dried garlic and successfully isolated as inhibitor of cancer metastasis from garlic (ICMG-27). ICMG-27 inhibited virtually all the tested cancer cells by modulating target protein(s) which are not activated by IGF-1 signaling pathway. Further characterization of ICMG-27 will provide alternative therapeutics for cancer metastasis.

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초록 : 마늘추출물에 의한 암세포의 이동 저하

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세포의 이동은 많은 생리적 반응뿐만 아니라 암 세포 침윤과 전이에 중요한 역할을 수행한다. 본 연구에서는 마늘이 암세포의 이동에 미치는 영향을 확인하기 위해, 표준 마늘과 흑마늘을 준비하고 이들을 각각 물을 이용하여 추출하거나 건조하여 추출한 추출물 4 종류를 이용하여 항침윤성과 항전이성에 대해 조사하였다. 실험결과, 암세포의 이동은 건조 후 헥산으로 추출한 분획에 암세포의 이동 억제 활성이 관찰되었다. 이 분획을 박막 크로마토그래피를 이용하여 분리정제하였으며, 이를 inhibitor of cancer metastasis from garlic #27 (ICMG-27)이라 명명하였다. ICMG-27 (6 ug/ml)을 세포에 처리하였을 때, IGF-1에 의한 OVCAR-3와 NIH-3T3 세포의 이동을 억제함을 확인하였다. 그러나 ICMG-27은 mouse embryonic fibroblast (MEF) 세포에서 IGF-1에 의한 이동에는 영향을 주지 않았다. 이러한 ICMG-27은 OVCA-3, SKOV-3와 MDA-MB-231 세포와 같은 암세포에서 모두 IGF-1에 의한 이동을 억제함을 관찰하였다. 마지막으로 세포이동을 일으키는 인자에 따른 ICMG-27의 영향을 확인한 것으로, IGF-1, lysophosphatidic acid (LPA), sphingosine-1-phosphate (S1P), leukotriene B4 (LTB4) 그리고, angiotensin Ⅱ (AngⅡ)에 의한 OVCAR-3 세포의 이동을 모두 억제하였다. 이러한 결과를 바탕으로, ICMG-27은 암세포의 이동을 유도하는 많은 인자들에 의한 필수적인 단계를 차단함으로써, 암세포의 이동을 억제하는 것을 확인 할 수 있었으며, ICMG-27에 의한 암세포의 항 침윤 메커니즘의 규명은 암환자의 치료에 기초적인 발판을 제공할 것입니다.