Ethacrynic Acid Inhibits Sphingosylphosphorylcholine-Induced Keratin 8 Phosphorylation and Reorganization via Transglutaminase-2 Inhibition

Hyun Jung Byun1, Kyung Jin Kang1, Mi Kyung Park1, Hye Ja Lee1, June Hee Kang1, Eun Ji Lee1, You Ri Kim1, Hyun Ji Kim1, Young Woo Kim1, Kyung Chae Jung2, Soo Youl Kim2 and Chang Hoon Lee1,*

1College of Pharmacy, Dongguk University, Seoul 100-715, 2National Cancer Center, Goyang 410-769, Republic of Korea

Abstract
Sphingosylphosphorylcholine (SPC) is significantly increased in the malicious ascites of tumor patients and induces perinuclear reorganization of keratin 8 (K8) filaments in PANC-1 cells. The reorganization contributes to the viscoelasticity of metastatic cancer cells resulting in increased migration. Recently, we reported that transglutaminase-2 (Tgase-2) is involved in SPC-induced K8 phosphorylation and reorganization. However, effects of Tgase-2 inhibitors on SPC-induced K8 phosphorylation and reorganization were not clearly studied. We found that ethacrynic acid (ECA) concentration-dependently inhibited Tgase-2. Therefore, we examined the effects of ECA on SPC-induced K8 phosphorylation and reorganization. ECA concentration-dependently suppressed the SPC-induced phosphorylation and perinuclear reorganization of K8. ECA also suppressed the SPC-induced migration and invasion. SPC induced JNK activation through Tgase-2 expression and ECA suppressed the activation and expression of JNK in PANC-1 cells. These results suggested that ECA might be useful to control Tgase-2 dependent metastasis of cancer cells such as pancreatic cancer and lung cancers.

Key Words: Sphingosylphosphorylcholine, Transglutaminase-2, Keratin-8 phosphorylation and reorganization, Ethacrynic acid, Migration, Invasion

INTRODUCTION
Metastasis is the ability of cancer cells to spread from its origin to distant locations within the body and to continue its growth (Valastyan and Weinberg, 2011). The high mortality rates associated with cancer are caused by the metastatic spread of tumor cells away from the site of their origin (Park et al., 2013a). In fact, metastases are the cause of 90% of cancer deaths (Steeg, 2006). Therefore, several researchers are trying to develop new anti-metastatic compounds. Recently, novel approaches have been proposed to characterize the properties of metastatic cancer cells, such as cell elasticity or mechanical properties (Beil et al., 2003; Suresh, 2007). The clinical importance of viscoelasticity or cell stiffness was reported by 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; Rolli et al., 2010). For example, sphingosylphosphorylcholine (SPC)-induced keratin phosphorylation and reorganization of human epithelial pancreatic cancer cells combined with the resulting changes in viscoelasticity of the cells have been suggested as a possible pathway that facilitates the migration and increased metastatic competence of pancreatic tumor cells (Beil et al., 2003; Rolli et al., 2010).

Keratin reorganization is achieved through phosphorylation of specific serine residues in keratin by MAP kinases, such as ERK, p38, and c-jun N-terminal kinase (JNK) (Ku et al., 2002; Park et al., 2011; Busch et al., 2012). Several compounds such as sphingosylphosphorylcholine (SPC) and leukotriene B4 induces keratin 8 (K8) phosphorylation and reorganization in PANC-1 cells (Beil et al., 2003; Park et al., 2011). We also showed that BLT2 and transglutaminase-2 (Tgase-2) are involved in K8 phosphorylation and reorganization (Park et al., 2011; Park et al., 2012).

Tgase-2 is a multifunctional protein. In addition to catalyzing Ca2+-dependent transamidation reactions, it can bind...
and hydrolyze GDP/GTP (Mhaouty-Kodja, 2004). The selective expression of Tgase-2 in chemoresistant and metastatic cancer cells, such as pancreatic, lung and ovarian tumors makes it a promising therapeutic target (Verma et al., 2006; Chhabra et al., 2009; Park et al., 2013b). However, studies on Tgase-2 inhibitors modulating metastasis of cancers were not enough. We screened inhibitory effects of some compounds on Tgase-2.

Ethacrynic acid (ECA) is a diuretic that inhibits cellular ion flux that leads to an increase in intracellular Na concentrations (Fig. 1A) (Vivas and Chiaraviglio, 1989; Li and El-Mallakh, 2004). ECA is used rarely as a diuretic because other potent agents have been introduced. Nevertheless, ECA still has a place in the modern practice of medicine (Wall et al., 2003; Han et al., 2005). ECA showed new pharmacological activities independent of diuretic activity. For example, ECA suppressed the all-retinoic acid-induced monocye chemoattractant protein-1 production and is known to reduce the retinoid-induced ear edema in mice (Kim et al., 2010).

In this study, we found that ECA inhibited Tgase-2 and confirmed the involvement of Tgase-2 in SPC-induced K8 phosphorylation and reorganization. Our finding suggested the possibility that ECA might be used as antimetastatic drugs.

**MATERIALS AND METHODS**

**Material**

D-erythro SPC was obtained from Matreya (Pleasant Gap, PA, USA). The phosphospecific antibody to detect K8 Ser431 was purchased from Abcam (Cambridge, UK). The anti-Tgase-2 antibody was supplied by Labvision Corporation-NeoMarkers (Thermo scientific, Fremont, CA, USA). Peroxidase-labeled secondary antibodies and lentiviral shRNA were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa Fluor 594 goat anti-mouse antibody was obtained from Molecular Probes, Inc. (Eugene, OR, USA).

**Cell culture**

The human pancreatic carcinoma cell line, PANC-1 (ATCC CRL 1469), was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM), penicillin-streptomycin (10,000 IU/ml and 10,000 μg/ml, respectively), and sodium pyruvate (1 mM). The PANC-1 cells were maintained in medium containing 10% (v/v) fetal calf serum (FCS). The cells were incubated at 37°C in a humidified atmosphere containing 10% CO2. The cells were washed twice in serum-free DMEM and incubated in serum-free DMEM 18 hours before the respective experiments.

**In vitro Tgase-2 inhibition assay**

The inhibitory effect of each compound was determined by measuring the incorporation of [1,4-14C]putrescine into succinylated casein (Park et al., 2013a). Following 10 min of pre-incubation of 2.5 milliunits (mU) of Tgase-2 from the guinea pig liver with each concentration of chemicals in 0.1 ml of reaction buffer solution without 10 mM CaCl2, we added 0.4 ml of substrate solution containing 5 mg of succinylated casein and 100 μCi of [1,4-14C] putrescine. After further incubation at 37°C for 1 h, the reaction was terminated by the addition of 4 ml of cold (4°C) 7.5% (w/v) TCA. TCA-insoluble precipitates were collected in GF/A glass fiber filters (Millipore Co.), washed with cold 5% (w/v) TCA, dried and assessed for incorporation of radiolabel using a scintillation counter (Beckman Coulter Co.). The resultant data represent the means of three independent experiments.

**Western blot**

The PANC-1 cells were harvested and lysed in 50 mM Tris-Cl (pH7.5), 150 mM NaCl, 1% triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, sterile solution and protease inhibitors (Gendepot, Barker, TX, USA) (Park et al., 2011). The protein concentrations of the supernatants were determined using Coomassie Plus (Pierce Biotechnology Inc., Rockford, IL, USA), as recommended by the manufacturer. The protein lysates were loaded onto a precast 4% to 12% polyacrylamide gradient gel (Invitrogen, Carlsbad, CA, USA). The membranes were blocked in 5% non fat milk and probed with the appropriate primary antibodies such as anti-Tgase-2, keratin-8, and JNK. After incubation with the primary antibody, the membranes were washed with TBS+0.1% Tween 20 and incubated with the appropriate peroxidase-conjugated secondary antibodies followed by development with a chemiluminescence substrate (Pierce Biotechnology Inc., Rockford, IL, USA) and exposure to X-ray film (Kodak, Rochester, NY, USA).

**Confocal microscopy**

PANC-1 cells were grown on coverslips and fixed 24 hours later with fresh 4% paraformaldehyde, pH 7.0, for 10 min at room temperature. Fixed cells were permeabilized with a 10 min wash in 0.1% Triton X-100 at room temperature followed by several washes in PBS with 3% bovine serum albumin (PBS/BSA). The phosphospecific antibody detecting K8

---

**Fig. 1.** ECA induced Tgase-2 expression in Panc1 cells. (A) The structure of ethacrynic acid. (B) Expression level of Tgase-2 in PANC-1 cell stimulated with the indicated concentration of ECA for 30 min. *p<0.05 was considered statistically significant.
Ser431 (Abcam, Cambridge, MA, USA) primary antibody was incubated with coverslips overnight at 4°C (Park et al., 2011). Excess antibody was removed with four washes in PBS/BSA. Species-specific second antibodies conjugated to goat anti-rabbit IgG antibody (Alexa Fluor 488, 1:500 Molecular Probes) or goat anti-mouse IgG antibodies (Alexa Fluor 594, 1:500, Molecular Probes) were then reacted with the coverslips for 1 hour at room temperature followed with four washes in PBS/BSA. The final samples were mounted onto slides and visualized using a Zeiss Axiophot confocal microscope.

**Migration**

Migration of PANC-1 cells through 8-μm size-limited pores was assessed in response to SPC according to Park's report (Park et al., 2011). PANC-1 cells (5×10⁴ cells per well) were treated with the indicated concentrations of SPC for 1 hour. PANC-1 cells plated in the upper chamber were allowed to migrate for 5 hours to establish the temporal kinetics of migration. The transwell membranes were then fixed and stained with Diff-Quik® staining kit (Kobe, Japan). Membranes were then reacted with the coverslips for 1 hour at room temperature followed with four washes in PBS/BSA. The final samples were mounted onto slides and visualized using a Zeiss Axiophot confocal microscope.

**Invasion assay using transwell plates**

Cell invasion was studied using matrigel-coated (0.5 lg/ml) transwell inserts, as described previously (Park et al., 2013a). Trypsinised cells were suspended in serum-free medium, and 2×10⁵ cells were added to the upper chamber of the transwell inserts. Medium with 10% serum was added to the lower chamber. After a 16 h incubation with PANC-1 cells, the non-migrated cells on the upper surface of the membrane were removed, and the cells on the lower surface were stained using the Hema 3 staining system (Fisher Scientific, Houston, TX, USA), photographed (200× magnification) and counted in 10 randomly selected fields. All experiments were repeated at least three times with two replicates each.

**Statistical analysis**

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

**RESULTS**

**ECA inhibits transglutaminase-2**

We have shown that Tgase-2 is involved in SPC-induced K8 phosphorylation and reorganization by JNK activation leading to migration of metastatic pancreatic cancer cells (Park et al., 2011). Therefore, Tgase-2 inhibitor may be effective for metastasis treatment. To obtain a Tgase-2 Inhibitor, we firstly screened a single compound library comprised of used drugs and natural extracts. We found that ECA has a concentration-dependent Tgase-2 inhibitory effect (Fig. 1B).

**ECA suppressed the SPC-induced K8 phosphorylation and reorganization in PANC-1 cells**

In previous report, cystamine (CTM), a well-known Tgase inhibitor, suppressed the SPC-induced K8 phosphorylation and reorganization. (Park et al., 2011). So we examined whether ECA, a newly found Tgase-2 inhibitor, could suppress the SPC-induced K8 phosphorylation and reorganization. SP induced phosphorylation of serine 431 of K8 and ECA concentration dependently inhibited the SPC-induced K8 phosphorylation (Fig. 2A). SPC also induced ring like perinuclear reorganization of K8 in PANC-1 cells and Tgase-2 is involved in this event. ECA inhibited the SPC-induced perinuclear reorganization of K8 (Fig. 2B).

**ECA suppressed the SPC-induced migration and invasion of PANC-1 cells**

The expected final outcome of SPC-induced reorganization of the keratin network in PANC-1 cells is increased migratory properties (Beil et al., 2003). Therefore, in previous report, we demonstrated that Tgase-2 is involved in the SPC-induced migration of PANC-1 cells by CTM and gene silencing (Park et al., 2011). SPC treatment induced the increased migration and invasion of PANC-1 cells (Fig. 3). ECA concentration dependently inhibited the SPC-induced migration and invasion of PANC-1 cells (Fig. 3). No cytoxic effects of ECA were observed in our experimental setting of migration and invasion.

**ECA suppressed the SPC-induced JNK activation and expression**

Tgase-2 is involved in SPC-induced K8 phosphorylation via JNK activation (Park et al., 2011). So, we examined whether ECA suppressed the Tgase-2-dependent JNK activation. SPC treatment increased the phosphorylation of JNK and ECA treatment suppressed the phosphorylation and expression of JNK (Fig. 4A).

**Fig. 2.** ECA suppressed the SPC-induced K8 phosphorylation and reorganization. (A) Effect of ECA on SPC-induced K8 phosphorylation. The PANC-1 cells were treated with various amounts of ECA and with or without SPC (5 μM). (B) Confocal microscopic examination of the effect of ECA on SPC-induced K8 phosphorylation of PANC-1 cells. PANC-1 cells were treated with ECA (5 μM) 30 min and SPC (5 μM) for 1 hr. Immunostaining was performed using K8-Ser431 (green).

http://dx.doi.org/10.4062/biomolther.2013.066
DISCUSSION

Metastatic cancer cells are reported to have unique mechanical characteristics, such as soft stiffness and elasticity (Cross et al., 2007). Keratins are one of the main intermediate filaments that control the mechanical characteristics of cells (Bordeleau et al., 2008). This study focused on ECA, Tgase-2 inhibitor modulating the SPC-induced keratin phosphorylation and reorganization in PANC-1 cells that controls the viscoelasticity and migratory properties of cancer cells.

MAP kinase is involved in keratin reorganization through the phosphorylation of keratin (Ku et al., 2002; Park et al., 2011; Busch et al., 2012), but there are few studies on the other proteins affecting keratin reorganization, except plectin (Cheng et al., 2008). Recently, we reported that Tgase-2 is involved in SPC-induced keratin reorganization via JNK activation (Park et al., 2011). Tgase-2 mediates the metastasis and chemoresistance of several cancer cells and is a new and interesting target (Kim, 2011). However, effective Tgase-2 inhibitors are not yet available to clinical application although several approaches revealed promising Tgase-2 inhibitors (Lai et al., 2008; Lee et al., 2013; Park et al., 2013a). So, we examined inhibitory effects of some drugs on Tgase-2 since drug can be easily applicable to cancer treatment. We found that ECA concentration-dependently inhibited the Tgase-2 (Fig. 1B). The inhibitory mechanism of ECA against Tgase-2 is not clear but the molecular structure of ECA contains an exo-methylene group conjugated to a carbonyl group (Fig. 1A). This electrophilic “eneone” moiety can alkylate thiol groups in proteins or glutathione via a Michael-type addition reaction (Han et al., 2005). Interestingly, one of key residues of Tgase-2 is cysteine residue at 277th amino acid (Lee et al., 1993). Thus, ECA might modify critical thiol residues in 277th Tgase-2.

The results showed that ECA suppressed the phosphorylation of K8 and perinuclear keratin reorganization (Fig. 2). These observations confirmed that Tgase-2 is involved in SPC-induced K8 phosphorylation and perinuclear reorganization of K8 (Park et al., 2011).

SPC-induced keratin phosphorylation and reorganization...
led to increased migration of PANC-1 cells and Tgase-2 inhibition by ECA suppressed the SPC-induced migration and invasion (Fig. 3). ECA is known to have diverse effects such as glutathione-S-transferase inhibition and thiol-adduct formation. So these diverse effects also might be involved in inhibition of migration and invasion. However, to our knowledge, we could not find reports about suppressing the migration of cancer cells via GST inhibition. However, thiol-adduct formation of ECA might contribute to inhibition of Tgase-2 since Tgase-2 has cystein residue at 277th in active site. In previous paper, we showed that SPC induced migration of PANC-1 cells via Tgase-2 expression (Park et al., 2011). Therefore, ECA might suppress the SPC-induced migration by inhibition of Tgase-2.

Tgase-2 is involved in SPC-induced JNK activation and ECA, Tgase-2 inhibitor, suppressed the JNK activation in PANC-1 cells (Fig. 4A). Especially, ECA also suppressed the JNK expression (Fig. 4A). These results suggested that ECA inhibited JNK expression via Tgase-2 inhibition.

Our findings confirmed the role of ECA as a Tgase-2 inhibitor in the suppression of SPC-induced K8 phosphorylation and reorganization of PANC-1 cells via JNK (Fig. 4B). Therefore, ECA might be helpful in modulating the Tgase-2 involved metastasis of cancer cells such as pancreatic cancers, and lung cancers.

ACKNOWLEDGMENTS

This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) and funded by the Korean government (MEST) (No. 2012053532) and the Research Program for New Drug Target Discovery (2011-0030173).

REFERENCES

Beil, M., Micoulet, A., von Wichert, G., Walther, P., Om-ary, M. B., Van Veldhoven, P. P., Gern, U., Wolff-Hieber, E., Eg-germann, J., Waltenberger, J., Adler, G., Spatz, J. and Seufferlein, T. (2003) Sphinsolysophosphorylcholine regulates keratin network, architecture and visco-elastic properties of human cancer cells. Nat. Cell Biol. 5, 803-811.

Bordeleau, F., Bessard, J., Sheng, Y. and Marceau, N. (2008) Keratin contribution to cellular mechanical stress response at focal adhesions as assayed by laser tweezers. Biochem. Cell Biol. 86, 352-359.

Busch, T., Armacck, M., Eiselcr, T., Joodi, G., Temme, C., Jansen, J., von Wichert, G., Omary, M. B., Spatz, J. and Seufferlein, T. (2012) Keratin 8 phosphorylation regulates keratin reorganization and migration of epithelial tumor cells. J. Cell Sci. 125, 2145-2159.

Cheng, C. C., Lai, T. S., Liu, Y. H., Ho, C. C., Chao, W. T., Pei, R. J., Hsu, Y. H., Yeh, K. T., Ho, L. C., Tsai, M. C. and Lai, Y. S. (2008) The influence of plectin deficiency on stability of cytokeratin 18 in hepatocellular carcinoma. J. Mol. Histol. 39, 209-216.

Chhabra, A., Verma, A. and Mehta, K. (2009) Tissue transglutaminase promotes or suppresses tumors depending on cell context. Anti-cancer Res. 29, 1909-1919.

Cross, S. E., Jin, Y. S., Rao, J. and Gimzewski, J. K. (2007) Nanome-chanical analysis of cells from cancer patients. Nat. Nanotechnol. 2, 766-783.

Han, Y., Englert, J. A., Delude, R. L. and Fink, M. P. (2005) Ethacrynic acid inhibits multiple steps in the NF-kappaB signaling pathway. Shock 23, 45-53.

Kim, K. M., Noh, M. S., Kim, S. H., Park, M. K., Lee, H. J., Kim, S. Y. and Lee, C. H. (2010) Ethacrynic acid and citral suppressed the all trans retinoid-induced monocyte chemoattractant protein-1 production in human dermal fibroblasts. Biomol. Ther. 18, 71-76.

Kim, S. Y. (2011) Transglutaminase 2: a new paradigm for NF-kappaB involvement in disease. Adv. Enzymol. Relat. Areas Mol. Biol. 78, 161-195.

Ku, N. O., Azhar, S. and Omary, M. B. (2002) Keratin 8 phosphorylation by p38 kinase regulates keratin filament reorganization: modulation by a keratin 1-like disease causing mutation. J. Biol. Chem. 277, 10775-10782.

Lai, T. S., Liu, Y., Tucker, T., Daniel, K. R., Sane, D. C., Toone, E., Burke, J. R., Strittmattter, W. J. and Greenberg, C. S. (2008) Identification of chemical inhibitors to human tissue transglutaminase by screening existing drug libraries. Chem. Biol. 15, 960-976.

Lee, K. N., Arnold, S. A., Birkichler, P. J., Patterson, M. K. J., Fraij, B. M., Takeuchi, Y. and Carter, H. A. (1993) Site-directed muta-genesis of human tissue transglutaminase: Cys-277 is essential for transglutaminase activity but not for GTPase activity. Biochim. Biophys. Acta 1202, 1-6.

Lee, S. H., Kim, N., Kim, S. J., Song, J., Gong, Y. D. and Kim, S. Y. (2013) Anti-cancer effect of a quinoline derivative GKI3 as a transglutaminase 2 inhibitor. J. Cancer Res. Clin. Oncol. 139, 1279-1294.

Li, R. and El-Mallah, R. S. (2004) Differential response of bipolar and normal control lymphoblastoid cell sodium pump to ethacrynic acid. J. Affect. Disord. 80, 11-17.

Mhauty-Kodja, S. (2004) Ghaptha/tissue transglutaminase 2: an energ G protein in signal transduction. Biol. Cell 96, 363-367.

Park, M. K., Jo, S. H., Lee, H. J., Kang, J. H., Kim, Y. R., Kim, H. J., Lee, E. J., Koh, J. Y., Ahn, K. O., Jung, K. C., Oh, S. H., Kim, S. Y. and Lee, C. H. (2013a) Novel suppressive effects of cardamomin on the activity and expression of transglutaminase-2 lead to blocking the migration and invasion of cancer cells. Life Sci. 92, 154-160.

Park, M. K., Lee, H. J., Shin, J., Noh, M., Kim, S. Y. and Lee, C. H. (2011) Novel participation of transglutaminase-2 through c-Jun N-terminal kinase activation in sphingosylphosphorylcholine-induced keratin reorganization of PANC-1 cells. Biochim. Biophys. Acta 1811, 1021-1029.

Park, M. K., Park, Y., Shim, J., Lee, H. J., Kim, S. and Lee, C. H. (2012) Novel involvement of leukotriene B(4) receptor 2 through ERK activation by PP2A down-regulation in leukotriene B(4)-induced keratin phosphorylation and reorganization of pancreatic cancer cells. Biochim. Biophys. Acta 1823, 2120-2129.

Park, M. K., You, H. J., Lee, H. J., Kang, J. H., Oh, S. H., Kim, S. Y. and Lee, C. H. (2013b) Transglutaminase-2 induces N-cadherin expression in TGF-beta1-induced epithelial mesenchymal transi-tion via c-Jun-N-terminal kinase activation by protein phosphatase 2A down-regulation. Eur. J. Cancer 49, 1692-1705.

Roli, C. G., Seufferlein, T., Kemkemer, R. and Spatz, J. P. (2010) Impact of tumor cell cytoskeleton organization on invasiveness and migration: a microchannel-based approach. PLoS One 5, e8726.

Suresh, S. (2007) Biomechanics and biophysics of cancer cells. Acta Biomater. 3, 413-438.

Steeg, P. S. (2006) Tumor metastasis: mechanistic insights and clinical challenges. Nat. Med. 12, 895-904.

Valastyan, S. and Weinberg, R. A. (2011) Tumor metastasis: molecular insights and evolving paradigms. Cell 147, 275-292.

Verma, A., Wang, H., Manavathi, B., Fok, J. Y., Mann, A. P., Kumar, R. and Mehta, K. (2006) Increased expression of tissue transglu-taminase in pancreatic ductal adenocarcinoma and its implications in drug resistance and metastasis. Cancer Res. 66, 10525-10533.

Vivas, L. and Chiaraviglio, E. (1989) Central effect of agents which sensitize patient. Brain Res. 527, 201-206.

Walf, G. C., Bigner, D. and Craig, S. (2003) Ethacrynic acid and the sulfa-sensitive patient. Arch. Intern. Med. 163, 116-117.