Korean J. Plant Res. 26(6):673-677(2013)
http://dx.doi.org/10.7732/kjpr.2013.26.6.673

Print ISSN 1226-3591
Online ISSN 2287-8203

Original Research Article

The Effects of Korean Cucurbitaceous Plants on the Alkaline Phosphatase Activity Associated with Sonic Hedgehog Pathway

Hwa Jin Lee*

Department of Natural Medicine Resources, Semyung University, Jecheon 390-711, Korea

Abstract - In order to examine the effects of Korean cucurbitaceous plants on sonic hedgehog pathway and growth of cancer cells with over-activated hedgehog pathway, we measured the sonic hedgehog conditioned medium (shh-CM) induced alkaline phosphatase (ALP) activity and cell viability of pancreatic cancer cell lines by treatment of cucurbitaceous plants. Among the tested cucurbitaceous plants, *Actinostemma lobatum* Maxim, *Cucumis sativus* L., *Momordica charantia* L., *Schizopepon bryoniaefolius* Maxim and *Trichosanthes kirilowii* Max, var. *japonica* Kitam showed the potent inhibitory effects (> 50 % at 20 μg/mL) on shh-CM induced ALP activity. We also evaluated the cell viability of pancreatic cancer cells treated with the cucurbitaceous plants. The tested cucurbitaceous plants showed the very weak effects on cancer cell proliferation but, *T. kirilowii* Max, var. *japonica* Kitam presented the inhibitory effect of 72.7 % on the proliferation of pancreatic cancer cells at 20 μg/mL. Taken together, we screened the effects of Korean cucurbitaceous plants on shh-CM induced ALP activity and cell viability of pancreatic cancers to search for the modulators of the hedgehog pathway leading to the inhibition of cancer cell proliferation. *T. kirilowii* Max, var. *japonica* Kitam, among the tested cucurbitaceous plants, showed the inhibitory effects on the shh-CM induced ALP activity and the proliferation of pancreatic cancer cells.

Key words - Cucurbitaceae, Alkaline phosphatase, Pancreatic cancer, *Trichosanthes kirilowii* Max, var. *japonica* Kitam, Hedgehog pathway

Introduction

There is an increased interest in the use of plant-based materials such as herbal extracts and compounds due to their various biological properties including anti-carcinogenic activities (Khan *et al*., 2008). Recent studies showed that many herbs have been used against several cancer types such as breast, lung and colon cancers (Lee and Houghton, 2005; McGovern *et al*., 2010). The extract of *Trichosanthes kirilowii* Maxim arrested the human breast carcinoma cell at G2/M phase (Jeong *et al*., 2011). The cyclic bisdemosides from *Actinostemma lobatum* Maxim showed the cytotoxicity against to cancer cells such as esophageal squamous carcinoma, lung cancer cell and gastric cancer cells (Li *et al*., 2012). In addition, flavonoids and saponin from *Gynostemma pentaphyllum* (Thunb.) Makino has been reported to show the anti-proliferative effect and apoptosis mechanism of prostate cancer cells (Cheng *et al*., 2011). Dakeng *et al*. (2012) showed that the cucurbitacin B, found in plants of the family of Cucurbitaceae, inhibited the breast cancer cells by suppression of Wnt signaling. The herbals of Cucurbitaceae are mostly known as the prostrate or climbing annual plants comprising about 125 genera and 960 species, including the melons and gourd crops like cucumbers. In Korea, the cucurbitaceous herbs have been used as the edible vegetable or the medicinal plants such as *Cucumis sativus* L., *Cucurbita moschata* Duchesne and *T. kirilowii* Maxim, which means that the cucurbitaceous plants can be safe and easily acceptable candidates for the screening of the bioactive materials from natural products.

It has been reported that the deregulations of signaling pathways in cells lead to cancerous cells with uncontrolled cell proliferation and aggressive biological actions including increased invasive and metastatic potential (Hanahan and Weinberg, 2011). The regulation of abnormal signaling pathway in cancer cells by herbal extracts or plants-oriented molecules can be the novel treatment strategies for cancer therapy. The hedgehog signaling pathway regulates the embryonic organogenesis and cellular proliferation (Ingham and McMahon, 2001). Upon binding the hedgehog protein [Sonic hedgehog (shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh)] to

*Corresponding author. E-mail : hwalee@semyung.ac.kr

receptor patched 1 (PTCH1), the signal transduces to the downstream of the pathway leading to the cell differentiation, tissue patterning and cell proliferation (Lauth *et al*., 2007). The hedgehog signaling has been known to be involved in the osteoblast differentiation, which results in increased alkaline phosphatase (ALP) activity, a marker of osteoblast differentiation (Nakamura *et al*., 1997). But the aberrant hedgehog signaling pathway has been associated with a number of human tumors. Pancreatic cancer has been recognized to be the ectopic activation of the hedgehog signaling pathway (Thayer *et al*., 2003; Xu *et al*., 2009). Therefore, it may be the worthy strategy that the herbal extracts or plant-oriented molecules control the proliferation of pancreatic cancer cells by the modulation of the hedgehog pathway. As the step of search for the herbal extracts with the antitumor activity based on inhibition of the hedgehog pathway, we assessed the sonic hedgehog-conditioned medium (shh-CM) induced alkaline phosphatase (ALP) activity in mouse mesenchymal stem cells and the cell proliferation of pancreatic cancer cells. Here, we report the effects of the cucurbitaceous plants on shh-CM induced ALP activity and cell viability of pancreatic cancer cell lines.

Materials and Methods

Plant materials

The methanol extracts of plants (99.9% methanol at 50℃, Cucurbitaceae) were provided from Professor J. H. Ryu at the College of Pharmacy, Sookmyung Women's University and the Korean plant extract bank.

Cell culture

PANC-1 and C3H10T1/2 cells (from ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle Medium that contained 10% FBS and streptomycin/penicillin (Gibco). AsPC-1 cells, which were obtained from ATCC, were grown in RPMI-1640 medium supplemented with 10% FBS.

Alkaline phosphatase (ALP) assay

C3H10T1/2 cells were plated at 5×10^3 cells/well in a 96 well plate and allowed to attach for 4 h. And then sonic hedgehog conditioned medium (shh-CM) and test materials were added. After 96 h, the cells were lysed for 15 min using 0.9% NaCl with 0.2% Triton X-100. And then cell lysates were mixed with reaction buffer (200 mM Tris-HCl pH10.5, 0.4 M 2-amino-2-methylpropanol, 8 mM $MgCl_2$) and ALP substrate (4 mM p-nitrophenyl phosphate disodium) and incubated at 37℃, in the dark, for 45 min. Absorbance at 415 nm was measured by using a microplate reader (Molecular Devices, CA, USA). The assay was performed in triplicate.

Preparation of sonic hedgehog conditioned medium

The hedgehog signaling was induced by sonic hedgehog conditioned medium (shh-CM). For the preparation of shh-CM, shh expression construct (gift from Prof. G.U. Bae at Sookmyung Women's University, Korea) was transiently transfected to HEK293 cells. The shh-producing HEK293 cells were grown to 80% confluency in DMEM containing 10% FBS. Then, the medium was replaced with DMEM containing 2% FBS, and followed by 5 days of growth. The medium was harvested and filtered through a 0.22 μm membrane. Control medium was obtained from HEK293 cells.

Cytotoxicity analysis

Cancer cells (PANC-1 and AsPC-1) were plated at a density of 3,000 cells/well in a 96 well plate. Cells were incubated with various concentrations of plant extracts for 3 days. And then 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, 5 mg/mL) was added for 4 h and lysed with DMSO. Absorbance at 570 nm was measured by using a microplate reader (Molecular Devices, CA, USA). The assay was performed in triplicate.

Statistical analysis

The results were expressed as mean ± S.D. of three experiments, and statistical analysis was performed by one way ANOVA and the Student's *t*-test. A P value of < 0.05 was considered to indicate a significant difference.

Results and Discussion

The effects of cucurbitaceous plants on shh-CM induced ALP activity

The hedgehog signaling has been known to be involved in the osteoblast differentiation (Tian *et al*., 2012). It has been

shown earlier that sonic hedgehog (shh) is able to induce alkaline phosphatase (ALP) activity, a marker of osteoblast differentiation (Nakamura *et al*., 1997). The screen was carried out by measurement of ALP activity induced with shh in C3H10T1/2 mouse mesenchymal stem cells (MSC). The shh conditioned medium (shh-CM) was prepared as described in the section of Materials and Methods to differentiate the MSC with shh protein. To investigate whether the test materials affect the hedgehog pathway, the extracts of cucurbitaceous herbals were added to the MSC with shh-CM for 4 days. The cyclopamine, the inhibitor of the shh signaling pathway, was used as the positive control for the inhibition of ALP activity induced by shh signal (94.5% inhibition at 5 μM). As shown in Table 1, nine kinds of cucurbitaceous herbs were examined at concentration of 20 μg/mL. Among the tested herbal extracts, *A. lobatum, C. sativus, M. charantia, S. bryoniaefolius* and *T. kirilowii* var. *japonica* exhibited the potent inhibitory effects (> 50% at 20 μg/mL) on shh-CM induced ALP activity in MSC. In addition, the stems and leaves of *T. kirilowii* var. *japonica* demonstrated 85.6% inhibitory effect whereas the fruit pulps of that showed the below 10% inhibition with the same concentration treatment. This result indicates that *A. lobatum, C. sativus, M. charantia, S. bryoniaefolius* and *T. kirilowii* var. *japonica* are involved in the modulation of shh signaling pathway although the exact target protein or mechanism is not elucidated.

Table 1. The inhibitory effects of cucurbitaceous plants on sonic hedgehog conditioned medium induced ALP activity in C3H10T1/2 mouse mesenchymal stem cells

Botanical names of plants	Parts[z]	Inhibition (%)[y]
Actinostemma lobatum	W	87.5
Cucumis sativus	S, L	74.8
Cucurbita moschata	W	< 10
Gynostemma pentaphyllum	W	< 10
Lagenaria leucantha	S, F	15.4
Momordica charantia	W	64.8
Schizopepon bryoniaefolius	W	50.4
Trichosanthes kirilowii	S, L	47.4
Trichosanthes kirilowii var. *japonica*	FP	< 10
Trichosanthes kirilowii var. *japonica*	S, L	85.6

[z]W:whole plant, S: stems, L: leaves, F: fruit, FP: fruit pulps.
[y]at concentration of 20 μg/mL.

The effects of cucurbitaceous plants on cell viability of pancreatic cancer cells

The other method of the screen for the inhibition of shh signaling pathway can be the treatment of the herbal extracts to cancer cells activated shh signal pathway and the measurement of the cell viability of cancer cells, because the shh signaling pathway has been involved in cell proliferation in cancer cells. In this study, the effects of the cucurbitaceous herbs on

Table 2. Inhibition of cucurbitaceous plants on proliferation of pancreatic cancer cells

Botanical names of plants	Parts[x]	Inhibition (%)[w]	
		AsPC-1 cells	PANC-1 cells
Actinostemma lobatum	W	41.1	10.9
Cucumis sativus	S, L	24.1	8.1
Cucurbita moschata	W	26.1	13.8
Gynostemma pentaphyllum	W	17.0	12.3
Lagenaria leucantha	S, F	0.3	0.2
Momordica charantia	W	5.2	9.5
Schizopepon bryoniaefolius	W	12.4	3.6
Trichosanthes kirilowii	S, L	20.0	23.9
Trichosanthes kirilowii var. *japonica*	FP	15.3	0.5
Trichosanthes kirilowii var. *japonica*	S, L	57.2	72.7

[x]W:whole plant, S: stems, L: leaves, F: fruit, FP: fruit pulps.
[w]at concentration of 20 μg/mL.

the cancer cell proliferation were observed by using the MTT assay in human pancreatic cancer cells, AsPC-1 and PANC-1 which have been known to be the ectopic activation of hedgehog signaling pathway (Tsuda *et al*., 2006). The extracts of cucurbitaceous plants demonstrated the very weak inhibitory effects (< 30% inhibition at 20 μg/mL) against the proliferation of AsPC-1 cells except for the extracts of *A. lobatum* and *T. kirilowii* var. *japonica* (used parts: the stems and leaves) with 41.1% and 57.2% inhibition, respectively (Table 2). *T. kirilowii* var. *japonica* (used parts: the stems and leaves) exhibited the most potent inhibition of the proliferation of PANC-1 cells (72.7% inhibition at 20 μg/mL) while the fruit pulps of *T. kirilowii* var. *japonica* did not (Table 2). This result demonstrates that the extract of *T. kirilowii* var. *japonica* (used parts: the stems and leaves) among the tested cucurbitaceous herbs suppresses the proliferation of human pancreatic cancer cells AsPC-1 and PANC-1, which means that *T. kirilowii* var. *japonica* can control the cell viability of cancer cells activated hedgehog signaling pathway.

The effects of *T. kirilowii* var. *japonica* on the shh-CM induced ALP activity and the cell viability of pancreatic cancer cells

The extract of *T. kirilowii* var. *japonica* (used parts: the stems and leaves) among the tested cucurbitaceous herbs showed the most potent inhibitory activities on the shh-CM induced ALP activity and the proliferation of pancreatic cancer cells. To confirm the inhibitory activity of *T. kirilowii* var. *japonica*, we measured the shh-CM induced ALP activity and the proliferation of pancreatic cancer cells at various concentrations. As shown in Fig. 1A, *T. kirilowii* var. *japonica* inhibited the ALP activity dose dependently while the only shh-CM treated group showed the activated ALP activity. In addition, *T. kirilowii* var. *japonica* suppressed the proliferation of AsPC-1 and PANC-1 cells at 20 and 50 μg/mL (Fig. 1B). These results suggest that the *T. kirilowii* var. *japonica* (used parts: the stems and leaves) may regulate the sonic hedgehog pathway and modulate the proliferation of the hedgehog pathway related cancer cells.

Taken together, the cucurbitaceous plants were screened for the inhibitor of pancreatic cancer cell proliferation by modulation of sonic hedgehog signaling pathway. The stems and leaves of *T. kirilowii* var. *japonica* presented potent inhibitory effects on the shh-CM induced ALP activity in MSC, which means the stems and leaves of *T. kirilowii* var. *japonica* might regulate the sonic hedgehog signaling pathway. Moreover, the proliferation of pancreatic cancer cells (AsPC-1 and PANC-1) with aberrant activated hedgehog signaling pathway was controlled by the stems and leaves of *T. kirilowii* var. *japonica*. Therefore, this study could provide the valuable sources for the modulators of pancreatic cancer proliferation and sonic hedgehog signaling pathway.

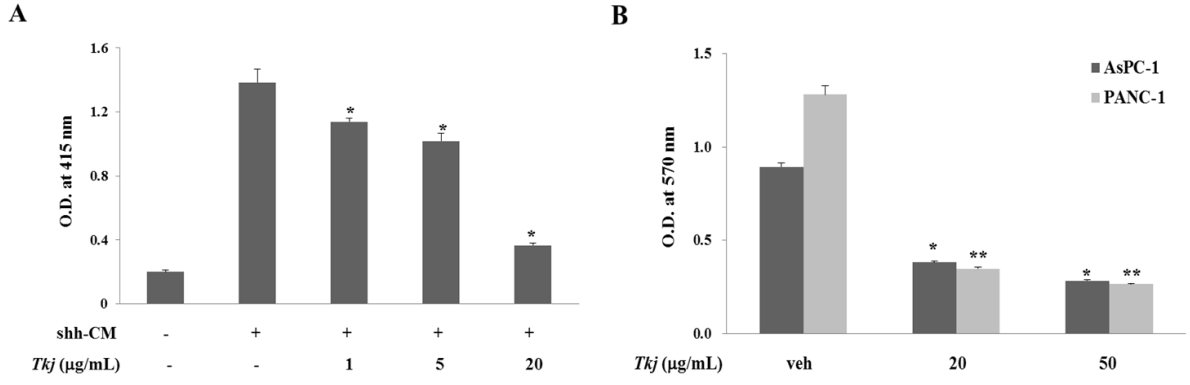

Fig. 1. (A) The effect of *T. kirilowii* var. *japonica* (used parts: stems and leaves) on the sonic hedgehog conditioned medium induced ALP activity in C3H10T1/2. The values are expressed as the means ± S.D. of three individual experiments. * $p < 0.05$ indicate significant differences from the shh-CM alone. *Tkj*: the stems and leaves of *T. kirilowii* var. *japonica* (B) The effect of *T. kirilowii* var. *japonica* (used parts: stems and leaves) on the proliferation of pancreatic cancer cells AsPC-1 and PANC-1. The values are expressed as the means ± S.D. of three individual experiments. * and ** $p < 0.05$ indicate significant differences from the vehicle treatment (*AsPC-1 cells and ** PANC-1, respectively). *Tkj*: the stems and leaves of *T. kirilowii* var. *japonica*.

Acknowledgment

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2012R1A1A3013645).

Literature Cited

Cheng, T.C., J.F. Lu, J.S. Wang, L.J. Lin, H.I. Kuo and B.H. Chen. 2011. Antiproliferation effect and apoptosis mechanism of prostate cancer cell PC-3 by flavonoids and saponins prepared from Gynostemma pentaphyllum. J. Agric. Food Chem. 59:11319-11329.

Dakeng, S., S. Duangmano, W. Jiratchariyakul, Y. U-Pratya, O. Bögler and P. Patmasiriwat. 2012. Inhibition of Wnt signaling by cucurbitacin B in breast cancer cells: reduction of Wnt-associated proteins and reduced translocation of galectin-3-mediated β-catenin to the nucleus. J. Cell. Biochem. 113: 49-60.

Hanahan, D. and R.A. Weinberg. 2011. Hallmarks of cancer: the next generation. Cell 144:646-674.

Ingham, P.W. and A.P. McMahon. 2001. Hedgehog signaling in animal development: paradigms and principles. Genes Dev. 15:3059-3087.

Jeong, S.M., M.K. Jeong, S.G. Ko, Y.K. Choi, J.H. Park and C.Y. Jun. 2011. Effect of arresting MCF-7 human breast carcinoma cell at G2/M phase of *Trichosanthes kirilowii*. Kor. J. Ori. Physiol. pathol. 25:857-862 (in Korean).

Khan, N., F. Afaq and H. Mukhtar. 2008. Cancer chemoprevention through dietary antioxidants: progress and promise. Antioxid. Redox Signal. 10:475-510.

Lauth, M., A. Bergström, T. Shimokawa and R. Toftgård. 2007. Inhibition of GLI-mediated transcription and tumor cell growth by small-molecule antagonists. Proc. Natl. Acad. Sci USA. 104:8455-8460.

Lee, C.C. and P. Houghton. 2005. Cytotoxicity of plants from Malaysia and Thailand used traditionally to treat cancer. J. Ethnopharmacol. 100:237-243.

Li, W., J. Cao, Y. Tang, L. Zhang, Q. Xie, H. Shen and Y. Zhao. 2012. Cyclic bisdesmosides from *Actinostemma lobatum* MAXIM (Cucurbitaceae) and their *in vitro* cytotoxicity. Fitoterapia 83:147-152.

McGovern, P.E., M. Christofidou-Solomidou, W. Wang, F. Dukes, T. Davidson and W.S. El-Deiry. 2010. Anticancer activity of botanical compounds in ancient fermented beverages. Int. J. Oncol. 37:5-14.

Nakamura, T., T. Aikawa, M. Iwamoto-Enomoto, M. Iwamoto, Y. Higuchi, M. M. Pacifici, N. Kinto, A. Yamaguchi, S. Noji, K. Kurisu and T. Matsuya. 1997. Induction of osteogenic differentiation by hedgehog proteins. Biochem. Biophys. Res. Commun. 237:465-469.

Thayer, S.P., M.P. di Magliano, P.W. Heiser, C.M. Nielsen, D.J. Roberts, G.Y. Lauwers, Y.P. Qi, S. Gysin, C.Fernandez-del Castillo, V. Yajnik, B. Antoniu, M. McMahon, A.L. Warshaw and M. Hebrok. 2003. Hedgehog is an early and late mediator of pancratic cancer tumorigenesis. Nature 425:851-856.

Tian, Y., Y. Xu, Q. Fu and Y. Dong. 2012. Osterix is required for sonic hedgehog-induced osteoblastic MC3T3-E1 cell differentiation. Cell Biochem. Biophys. 64:169-176.

Tsuda, N., S. Ishiyama, Y. Li, C.G. Ioannides, J.L. Abbruzzese and D.Z. Chang. 2006. Synthetic microRNA designed to target glioma-associated antigen 1 transcription factor inhibits division and induces late apoptosis in pancreatic tumor cells. Clin. Cancer Res. 12:6557-6564.

Xu, F.G., Q.Y. Ma and Z. Wang. 2009. Blockade of hedgehog siganling pathway as a therapeutic strategy for pancreatic cancer. Cancer Lett. 283:119-124.

(Received 11 October 2013 ; Revised 26 November 2013 ; Accepted 29 November 2013)