Animal Cells and Systems 13: 113-118, 2009

Animal Cells
and
Systems

http://www.AnimalCells.or.kr

Keumsa Linteusan Suppresses Invasion of Cancer Cells through the Inhibition of Cellular Adhesion and MMP-9 Expression

Won-Jung Kim, Sung-Chang Hong[1], Eun-Ju Do[2], Kyungho Suk[3], Ik Jin Yun[4], and Won-Ha Lee*

School of Life Sciences and Biotechnology, Kyungpook National University, Daegu 702-701, Korea; [1]Zenitec Korea Co., Hyundai 41 Tower, Seoul 158-732, Korea; [2]Oriental Medicine Industry Support Center, Daegu Technopark, Daegu 706-828, Korea; [3]Department of Pharmacology, School of Medicine, Kyungpook National University, Daegu 702-701, Korea; [4]College of Medicine, Konkuk University, Seoul 143-702, Korea

Abstract: Extracts derived from various medical mushrooms have been reported to have antitumor and immunomodulatory properties. In order to investigate the antitumor activity of keumsa Linteusan, the water extract of *Phellinus limteus*, HT1080 cells, a human fibrosarcoma cell line, were treated with it and changes in cellular migration potential was tested *in vitro*. At a concentration range below 1,000 μg/mL, Linteusan blocked, in a dose dependent manner, the migration of cells through Matrigel as well as Boyden chamber without affecting the viability of the cells. Prolonged treatment of HT1080 cells with Linteusan suppressed TNF-α induced production of matrix metalloproteinase (MMP)-9 as well as basal level expression of MMP-2. Linteusan also affected the adhesion of the cells to fibronectin-coated surfaces. The effect of Linteusan on cell signaling pathways was also tested. Linteusan specifically affected TNF-α induced phosphorylation of AKT in a dose-dependent manner, while phosphorylation levels of ERK remained unaffected. These data indicate that Linteusan blocks the migration of HT1080 cells by affecting various processes associated with cell migration such as the expression of matrix degrading enzymes, cell adhesion, and AKT-medicated cellular signaling pathways.

Key words: Linteusan, MMP-9, cancer, migration, adhesion

INTRODUCTION

Phellinus linteus, a basidiomycetes fungus grown mainly on wild mulberry tree trunks, has been widely used as a medicinal mushroom in traditional Oriental medicine (Dai et al., 1997). The extract of *P. linteus* has been previously reported to have a immunomodulatory effect. The biological activity of it was reported to originate from its polysaccharides and proteoglycans which showed stimulatory effects on macrophages, lymphocytes, and dendrictic cells (Kim et al., 2003a; Kim et al., 2003b; Park et al., 2003; Song et al., 1995). The anti-inflammatory activity of *P. linteus*, however, has also been reported in macrophages (Kim et al., 2006a). In addition to these immunomodulatory activities, *P. linteus* exhibited antitumor activities (Han et al., 1999; Sasaki et al., 1971) through down-regulation of urikinase plasminogen activator in melanoma cells, inhibition of pulmonary metastasis in mice, and inhibition of cancer growth in prostate cancers (Fullerton et al., 2000; Guo et al., 2007; Hsieh and Wu, 2001; Shibata et al., 2004; Song et al., 2003). The molecular mechanism of these antitumor activities has not been thoroughly studied except in its effect on cell cycle progression and apoptosis in lung and prostate cancer cells (Collins et al., 2006; Zhu et al., 2007).

In the course of cancer development, metastasis is one of the major causes of cancer-related mortality (Kato et al., 2002). In contrast to its importance in cancer biology, it has been hard to effectively regulate cancer metastasis, mainly due to the lack of effective drugs. Metastasis is a complex series of events requiring cancer cell adhesion, invasion, migration, circulation through blood and lymph, infiltration into a new tissue or organ, proliferation and angiogenesis (Hannigan et al., 2005; Playford and Schaller, 2004; Stacker et al., 2002). Of these events, cancer cell migration is one of the most crucial process that require cell-to-cell and cell-to-extracellular matrix (ECM) interactions and intracellular cytoskeletal rearrangements.

In order to determine whether Linteusan affects the metastatic potential of cancer cells, the various cellular

*To whom correspondence should be addressed.
Tel: +82-53-950-5388; Fax: +82-53-943-6925
E-mail: whl@knu.ac.kr

responses that are associated with metastasis such as migration, secretion of matrix degrading enzymes, and adhesion to extra cellular matrix proteins, in HT1080 cells were tested. The effect of Linteusan in cellular signaling associated with cytoskeletal movements was also investigated.

MATERIALS AND METHODS

Cell culture and reagents

HT1080 cells were cultured in RPMI medium supplemented with 10% FBS and incubated at 37°C in 5% CO_2 incubator. Keumsa Sangwhang mushroom (the strain of *Phellinus linteus* KSSW01) was cultivated for 4 years by a patented process (Korean Patent 10-179725) from the Keumsa Mush & Farm Co., (Yeoju, Geyonggi-do, Korea). The dried fruiting bodies (2.5kg) were extracted with hot water (10 liter) at 110~120°C for 100~120 hr. After extraction, it was dried at 60-70°C for 24-48 hr and finally powered to obtain Keumsa Linteusan. The composition of Keumsa Linteusan is as following: moisture 11.8%, proteins 11.8%, lipids 1.1%, ash 7.7%, and carbohydrates 67.6%. Also, the β-glucan content analyzed to be 125±37.5 μg/mg in Keumsa Linteusan. Polyclonal antibodies against phospho-ERK, ERK, phospho-AKT, and AKT were purchased from Cell Signaling Technolgy Inc. (Danvers, MA, USA). Recombinant TNF-α was purchased from R&D systems (Minneapolis, MN, USA).

Measurement of invasion and migration

For the measurement of cell migration through Matrigel (Sigma, St. Luis, MO, USA), the upper part of Transwells (8 μm pore, Millipore) were coated with 100 μg/cm^2 Matrigel for 15 min at 37°C and then for 10 min at room temperature. Cells (2×10^5) were added into the upper well in the presence or absence of Linteusan and the lower wells were filled with a culture media. The plates were then incubated for 24 hr and cells in the upper side of the membrane were removed with cotton swab and the membrane was fixed in methanol and cells were stained with Hematoxylin. Pictures (100×) of the membrane were taken in 5 random fields and cell numbers were counted. Migration of cells was assessed in a 48-well microchamber (Probe Inc., Gaithersburg, MD, USA). Briefly, the lower wells were filled with 27 μL RPMI (supplemented with 10% serum) and the upper wells were filled with 50 μL of cells at a concentration of 4×10^6 cells/mL in the presence of absence of Linteusan. The two compartments were separated by a polyvinylpyrrolidone-free filter (Neuro Probe Inc) with 8-μm pores. After incubation for 24 hr at 37°C, the number of cells that had migrated into the lower wells was counted, pictures were taken and cells were counted as described above. The experiments were performed in triplicate samples.

Cell viability assay and cell adhesion assay

For the measurement of cell viability, Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) was used. Briefly, cells in 96-well plates (1×10^4/100 μL/well) were pretreated with or without Linteusan for 24 hr and 10 μL of CCK-8 solution was added into each wells. After 4 hr incubation in 37°C in 5% CO_2, absorbance at 450 nm was measured using microplate reader. For the measurement of cell adhesion, culture plates (96 well plates) were coated with 10 μg/mL fibronectin overnight. HT1080 cell that had been pretreated with or without Linteusan for 3 hr were added into each well (8×10^4 cells/well). After 30 min, unattached cells were removed by PBS washing and the CCK-8 assay was performed to measure the amount of attached cells.

Gelatin zymogram and western blot

HT1080 cells (1×10^4/well) were incubated in 96-well plates in the presence or absence of Linteusan and/or TNF-α. The culture supernatants were collected 24 hr after activation and the gelatin zymogram analyses were performed as described previously (Kim and Lee, 2004; Lee et al., 2001). Cell lysates were obtained at various time points after activation and Western blot analysis was performed as described previously (Bae et al., 2007; Kim et al., 2006b).

Statistical analysis

Statistical significance of differences was evaluated by means of a two-sided Student's *t* test, assuming equal variances. Differences were considered significant when $P<0.05$.

RESULTS AND DISCUSSION

In order to investigate the effect of Linteusan in the migration of HT1080 cells, this study tested the migration of cells using Matrigel invasion assay in Transwell chambers. HT1080 cells migrated through the Matrigel layers and Transwell membrane without any chemoattractants (Fig. 1A). This indicates that HT1080 cell have a high level of basal migration potential. When the Matrigel invasion assay was performed in the presence of various concentrations of Lanteusan, the number of cells that migrated through the membrane decreased in a dose dependent manner (Fig. 1A). The inhibitory effect of Linteusan was confirmed using a Boyden chamber assay. The basal level migration of HT1080 was inhibited by Linteusan with a statistical significance (Fig. 1B). The inhibitory effect of Linteusan in cell migration could be due to the suppressive effect of Linteusan on cell growth (Sliva et al., 2008) or cell cycle blockage (Li et al., 2004). To test that possibility, the effect of Linteusan on cell viability was tested. As shown in Fig.

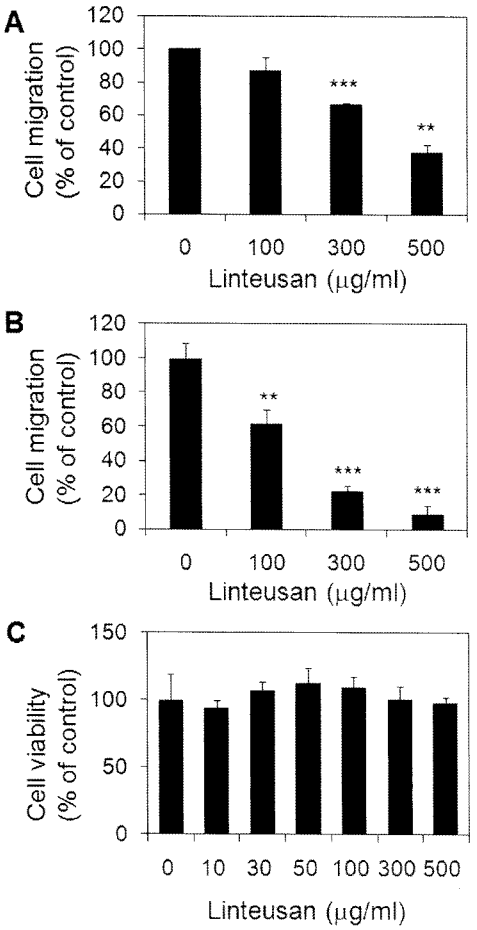

Fig. 1. Linteusan inhibits cell migration in HT1080 cells without affecting cell viability. A, The upper parts of Traswell (pore size; 8 μm) were coated with Matrigel. HT1080 cells were added into upper wells in the presence or absence of Liteusan at indicated concentrations. After 24 hr incubation, cells in the bottom of the filter were fixed, stained, and counted. B, HT1080 cells were loaded onto the upper compartments of the Boyden chamber in the presence or absence of Liteusan at indicated concentrations. After 24 hr incubation, cells in the bottom of the filter were fixed, stained, and counted. The error bars represent S.D. *P<0.05, **<0.01, and ***<0.001 when compared with samples treated without Linteusan. C, HT1080 cells were cultured in the resence of indicated concentrations of Linteusan for 24 hr. Cell viability was tested with CCK-8 assay. The error bars represent S.D.

1C, Linteusan treated at concentration as high as 500 μg/mL failed to affect cell viability within a 24 hr time span. The cytotoxic effect of *P. linteus* extracts observed in previous publication required a relatively long-term incubation such as 92 hr(Shin et al, 2007). This data clearly indicates that Lineusan has inhibitory effects on the migration of HT1080 cells in short-term treatment condition.

The migration of cells through extracellular matrix (ECM) requires the involvement of multiple cellualar activities such as degradation of ECM using matrix degrading enzymes, firm adhesion to ECM, reorganization of cellular cytoskeletal network for the accommodation of cell movement, etc. In order to investigate the underlying mechanism responsible for the inhibition of cell migration, the effect of Linteusan on these activities was tested.

First, the effect of Linteusan in the production of matrix degrading enzyme was tested. As shown in Fig. 2, pretreatment with Linteusan affected both the basal level and TNF-α-induced expression of MMPs in a dose dependent manner (Fig. 2B). It is interesting that the secretion of MMP-2, whose expression is not known to be affected by the activation levels of cell, is also inhibited by Linteusan in a dose dependent manner. The viability of cells was not significantly affected by long-term (48 hr) treatment of Linteusan (95.1±8.0 and 90.0±1.9% viability when treated with 300 or 500 μg/mL of Linteusan, respectively, in the presence of 1 ng/mL TNF-α). These data indicate that the presence of Linteusan has an inhibitory effect on matrix degrading activity of cells. This effect of Linteusan in MMP expression could be responsible for the decrease in cell migration through ECM.

The effect of Linteusan on cell adhesion was then tested in culture dish that had been coated with fibronectin. As shown in Fig. 3, the presence of Linteusan resulted in a dose-dependent decrease in cell adhesion. The adhesion assay was performed in the absence of TNF-α to mimic the experimental situation used in cell migration (Fig. 1). This indicates that Linteusan decreases the interaction between

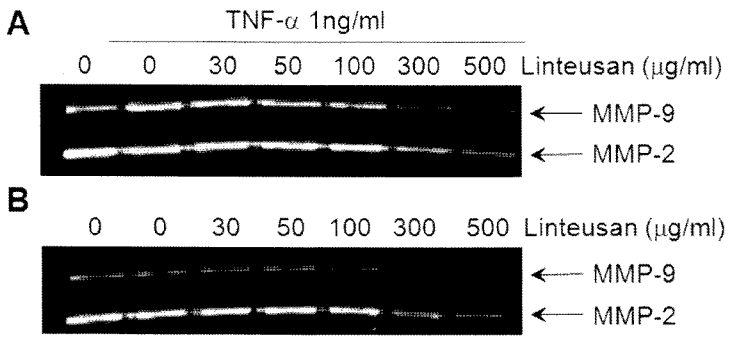

Fig. 2. Linterusan reduces the expression of MMP-2 and MMP-9 in HT1080 cells. HT1080 cells were pretreated with 0-500 μg/mL M Linteusan overnight and stimulated with (A) or without (B) 1 ng/mL of TNF-α. The culture supernatants were collected 24 hr after activation and subjected to gelatin zymogram.

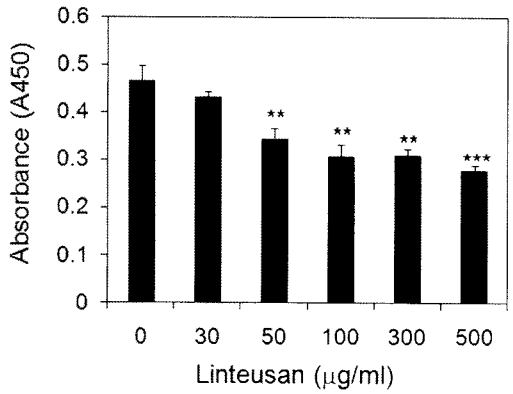

Fig. 3. Linteusan inhibits adhesion of HT1080 cells to ECM proteins. HT1080 cell that had been pretreated with indicated concentrations of Linteusan for 3 hr were added into Culture plates that had been coated with 10 μg/mL fibronectin. After 30 min, the amounts of attached cells were measured using CCK-8 assay. The error bars represent S.D *P<0.05, **<0.01, and ***<0.001 when compared with samples treated without Linteusan.

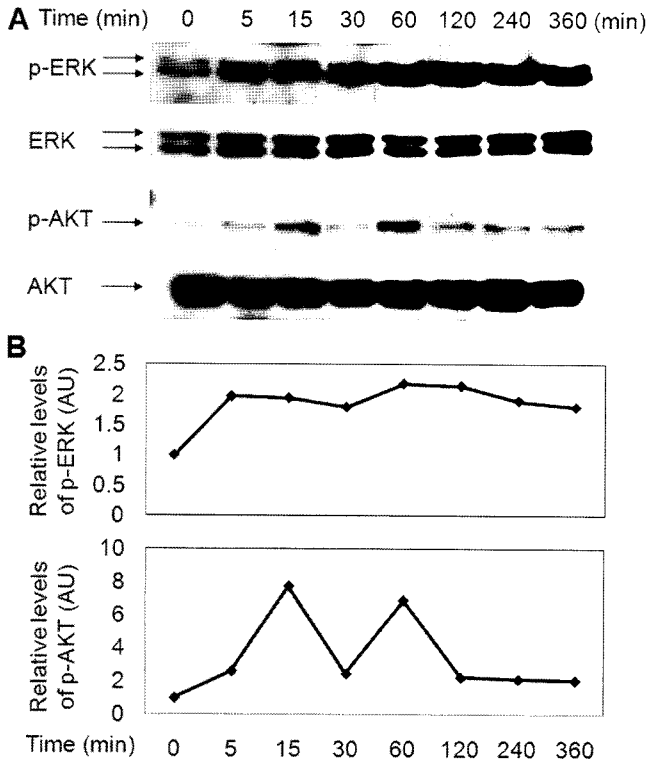

Fig. 4. TNF-α treatment induces phosphorylation of ERK and AKT in HT1080 cells. A, HT1080 cells were stimulated with 1 ng/mL of TNF-α for indicated times points, and the cell lysates were subjected to Western blot analysis using polyclonal antibodies against phospho-ERK, ERK, phospho-AKT, and AKT. B, Band intensities in panel A were measured using a densitometer and the measured values of phospho-proteins were normalized with that of corresponding total protein. AU: arbiturary unit

ECM and HT1080 cells and, as a result, reduces the activating signals induced by interaction between ECM and integrins, the cellular receptors involved in the interaction with ECM proteins.

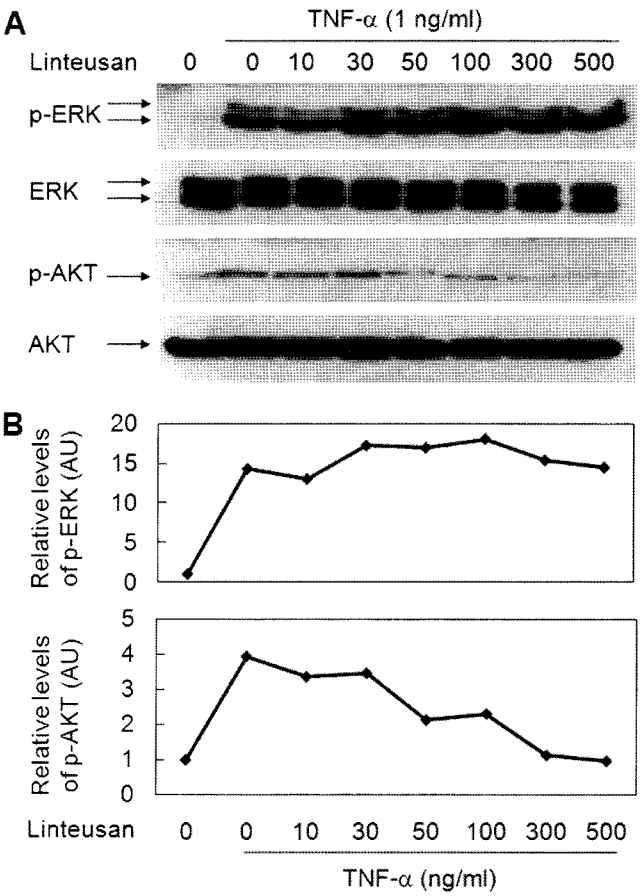

Fig. 5. Linteusan inhibits phosphorylation of AKT, but not ERK in HT1080 cells stimulated with TNF-α. HT1080 cells were pretreated with indicated concentrations of Linteusan for 3 hr and stimulated with 1 ng/mL of TNF-α for 15 min. Total cell lysates were obtained and subjected to Western blot analysis using polyclonal antibodies against phospho-ERK, ERK, phospho-AKT, and AKT. B, Band intensities in panel A were measured using a densitometer and the measured values of phospho-proteins were normalized with that of corresponding total protein. AU: arbiturary unit

It is well known that cancer cells are under constant influence from their microenvironment and inflammation is closely associated with cancer development and metastasis (Karin and Greten, 2005; Maeda and Omata, 2008; Schwartsburd, 2003). Cancer cells are stimulated through their interaction of cells with ECM or interaction with inflammatory cytokines that constitute the cancer microenvironment. Activation signals induced by these interactions generate intracellular signaling that facilitate further transformation of cells and the acquisition of migratory potential that are essential in cancer metastasis (Sethi et al., 2008; Shacter and Weitzman, 2002). In order to investigate the effect of Linteusan in cellular signaling associated with cell migration, the activation status of PI3K/AKT and ERK was tested in cells stimulated with TNF-α. Activation of PI3K/AKT and ERK has been shown to be involved in the migration of various cell types including cancer cells (Gentilini et al., 2007; Saxena et al.,

2007; Ye et al., 2008). TNF-α induced phosphorylation of both ERK and AKT. Phosphorylation of ERK started 5 min after stimulation and the phosphorylation status was maintained up to three hr. Phosphorylation of AKT, however, occurred in a biphasic manner: the first wave of stimulation occurred within 15 min after treatment and subsided within 30 min after stimulation; and the second wave of stimulation peaked around 60 min after stimulation (Fig. 4). The effect of Linteusan in the activation of ERK or AKT was then tested at 15 min after stimulation. As shown in Fig. 5, Linteusan dose dependently inhibited AKT phosphorylation while the phosphorylation status of ERK was not changed. These data indicate that Linteusan specifically inhibits the activation of PI3K in HT1080 cells stimulated with TNF-α.

These data provide a first demonstration of the inhibitory activity of Linteusan on cancer cell migration. The molecular mechanism underlying the migration inhibitory activity of Linteusan appears to come from its inhibitory effect on the production of matrix degrading enzymes, inhibition of cell adhesion to ECM proteins, and inhibition of PI3K activation.

ACKNOWLEDGMENTS

This research was supported by Kyungpook National University Research Fund, 2007.

REFERENCES

Bae E, Kim WJ, Kang YM, Suk K, Koh EM, Cha HS, Ahn KS, Huh TL, and Lee WH (2007) Glucocorticoid-induced tumour necrosis factor receptor-related protein-mediated macrophage stimulation may induce cellular adhesion and cytokine expression in rheumatoid arthritis. *Clin Exp Immunol* 148: 410-418.

Collins L, Zhu T, Guo J, Xiao ZJ, and Chen CY (2006) Phellinus linteus sensitises apoptosis induced by doxorubicin in prostate cancer. *British J Cancer* 95: 282-288.

Dai D, Chen J, and Xu L (1997) [Quantitative analysis of E-cadherin expression and clinicopathologic evaluation in gastric cancer]. *Zhonghua Yi Xue Za Zhi* 77: 668-671.

Fullerton SA, Samadi AA, Tortorelis DG, Choudhury MS, Mallouh C, Tazaki H, and Konno S (2000) Induction of apoptosis in human prostatic cancer cells with beta-glucan (Maitake mushroom polysaccharide). *Molecular Urology* 4: 7-13.

Gentilini D, Busacca M, Di Francesco S, Vignali M, Vigano P, and Di Blasio AM (2007) PI3K/Akt and ERK1/2 signalling pathways are involved in endometrial cell migration induced by 17beta-estradiol and growth factors. *Molecular Human Reproduction* 13: 317-322.

Guo J, Zhu T, Collins L, Xiao ZX, Kim SH, and Chen CY (2007) Modulation of lung cancer growth arrest and apoptosis by Phellinus Linteus. *Molecular Carcinogenesis* 46: 144-154.

Han SB, Lee CW, Jeon YJ, Hong ND, Yoo ID, Yang KH, and Kim HM (1999) The inhibitory effect of polysaccharides isolated from Phellinus linteus on tumor growth and metastasis. *Immunopharmacology* 41: 157-164.

Hannigan G, Troussard AA, and Dedhar S (2005) Integrin-linked kinase: a cancer therapeutic target unique among its ILK. *Nature Reviews* 5: 51-63.

Hsieh TC and Wu JM (2001) Cell growth and gene modulatory activities of Yunzhi (Windsor Wunxi) from mushroom Trametes versicolor in androgen-dependent and androgen-insensitive human prostate cancer cells. *International J Oncology* 18: 81-88.

Karin M and Greten FR (2005) NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 5: 749-759.

Kato R, Ishikawa T, Kamiya S, Oguma F, Ueki M, Goto S, Nakamura H, Katayama T, and Fukai F (2002) A new type of antimetastatic peptide derived from fibronectin. *Clin Cancer Res* 8: 2455-2462.

Kim BC, Choi JW, Hong HY, Lee SA, Hong S, Park EH, Kim SJ, and Lim CJ (2006a) Heme oxygenase-1 mediates the anti-inflammatory effect of mushroom Phellinus linteus in LPS-stimulated RAW264.7 macrophages. *J Ethnopharmacology* 106: 364-371.

Kim GY, Oh YH, and Park YM (2003a) Acidic polysaccharide isolated from Phellinus linteus induces nitric oxide-mediated tumoricidal activity of macrophages through protein tyrosine kinase and protein kinase C. *Biochemical and Biophysical Research Communications* 309: 399-407.

Kim GY, Park SK, Lee MK, Lee SH, Oh YH, Kwak JY, Yoon S, Lee JD, and Park YM (2003b) Proteoglycan isolated from Phellinus linteus activates murine B lymphocytes via protein kinase C and protein tyrosine kinase. *International Immunopharmacology* 3: 1281-1292.

Kim WJ, Bae EM, Kang YJ, Bae HU, Hong SH, Lee J, Park JE, Kwon BS, Suk K, and Lee WH (2006b). Glucocorticoid-induced TNFR family-related protein (GITR) mediates inflammatory activation of macrophages that can destabilize atherosclerotic plaques. *Immunology* 119, 421-429.

Kim WJ and Lee WH (2004) LIGHT is expressed in foam cells and involved in destabilization of atherosclerotic plaques through induction of matrix metalloproteinase-9 and IL-8. *Immune Network* 4: 116-122.

Lee WH, Kim SH, Lee Y, Lee BB, Kwon B, Song H, Kwon BS, and Park JE (2001) Tumor necrosis factor receptor superfamily 14 is involved in atherogenesis by inducing proinflammatory cytokines and matrix metalloproteinases. *Arteriosclerosis, Thrombosis, and Vascular Biology* 21: 2004-2010.

Li G, Kim DH, Kim TD, Park BJ, Park HD, Park JI, Na MK, Kim HC, Hong ND, Lim K. *et al.* (2004) Protein-bound polysaccharide from Phellinus linteus induces G2/M phase arrest and apoptosis in SW480 human colon cancer cells. *Cancer Letters* 216: 175-181.

Maeda S and Omata M (2008). Inflammation and cancer: role of nuclear factor-kappaB activation. *Cancer science* 99: 836-842.

Park SK, Kim GY, Lim JY, Kwak JY, Bae YS, Lee JD, Oh YH, Ahn SC, and Park YM (2003) Acidic polysaccharides isolated from Phellinus linteus induce phenotypic and functional maturation of murine dendritic cells. *Biochemical and Biophysical Research Communications* 312: 449-458.

Playford MP and Schaller MD (2004) The interplay between Src

and integrins in normal and tumor biology. *Oncogene* 23: 7928-7946.

Sasaki T, Arai Y, Ikekawa T, Chihara G, and Fukuoka F (1971) Antitumor polysaccharides from some polyporaceae, Ganoderma applanatum (Pers.) Pat and Phellinus linteus (Berk. et Curt) Aoshima. *Chemical & Pharmaceutical Bulletin* 19: 821-826.

Saxena NK, Sharma D, Ding, X, Lin S, Marra F, Merlin D, and Anania FA (2007) Concomitant activation of the JAK/STAT, PI3K/AKT, and ERK signaling is involved in leptin-mediated promotion of invasion and migration of hepatocellular carcinoma cells. *Cancer Research* 67: 2497-2507.

Schwartsburd PM (2003) Chronic inflammation as inductor of pro-cancer microenvironment: pathogenesis of dysregulated feedback control. *Cancer Metastasis Reviews* 22: 95-102.

Sethi G, Sung B, and Aggarwal BB (2008). TNF: a master switch for inflammation to cancer. *Front Biosci* 13: 5094-5107.

Shacter E and Weitzman SA (2002). Chronic inflammation and cancer. *Oncology (Williston Park, NY* 16, 217-226, 229; discussion 230-212.

Shibata Y, Kurita S, Okugi H, and Yamanaka H (2004) Dramatic remission of hormone refractory prostate cancer achieved with extract of the mushroom, Phellinus linteus. *Urologia Internationalis* 73: 188-190.

Shin JY, Lee S, Bae IY, Yoo SH, Lee HG (2007) Structural and biological study of carboxymethlated Phelinus linteus polysaccharides. *J Agricultural and Food Chemistry* 55: 3368-3372

Sliva D, Jedinak A, Kawasaki J, Harvey K, and Slivova V (2008) Phellinus linteus suppresses growth, angiogenesis and invasive behaviour of breast cancer cells through the inhibition of AKT signalling. *British J Cancer* 98: 1348-1356.

Song KS, Cho SM, Lee JH, Kim HM, Han SB, Ko KS, and Yoo ID (1995) B-lymphocyte-stimulating polysaccharide from mushroom Phellinus linteus. *Chemical & Pharmaceutical Bulletin* 43: 2105-2108.

Song YS, Kim SH, Sa JH, Jin C, Lim CJ, and Park EH (2003) Anti-angiogenic, antioxidant and xanthine oxidase inhibition activities of the mushroom Phellinus linteus. *J Ethnopharmacology* 88: 113-116.

Stacker SA, Achen MG, Jussila L, Baldwin ME, and Alitalo K (2002) Lymphangiogenesis and cancer metastasis. *Nature Reviews* 2: 573-583.

Ye M, Hu D, Tu L, Zhou X, Lu F, Wen B, Wu W, Lin Y, Zhou Z, and Qu J (2008) Involvement of PI3K/Akt signaling pathway in hepatocyte growth factor-induced migration of uveal melanoma cells. *Investigative Ophthalmology & Visual Science* 49: 497-504.

Zhu T, Guo J, Collins L, Kelly J, Xiao ZJ, Kim SH, and Chen CY (2007) Phellinus linteus activates different pathways to induce apoptosis in prostate cancer cells. *British J Cancer* 96: 583-590.

[Received January 23, 2009; accepted June 16, 2009]