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Effects of the Chestnut Inner Shell Extract on the Expression of Adhesion Molecules, Fibronectin and Vitronectin, of Skin Fibroblasts in Culture

Yeon Sook Chi[1], Moon Young Heo[1], Ji Hun Chung[2], Byoung Kee Jo[2], and Hyun Pyo Kim[1]

[1]College of Pharmacy, Kangwon National University, Chunchon, Korea and [2]R & D Center, Coreana Cosmetic Co., Ltd. Cheonan, Korea

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The inner shell of the chestnut (*Castanea crenata* S. et Z., Fagaceae) has been used as an anti-wrinkle/skin firming agent in East Asia, and preliminary experiments have found that a 70% ethanol extract from this plant material can prevent cell detachment of skin fibroblasts from culture plates. In order to examine the molecular mechanisms underlying this phenomenon, its effects on the expression of adhesion molecules, such as fibronectin and vitronectin, were investigated using the mouse skin fibroblast cell line, NIH/3T3. Using fixed-cell ELISA, Western blotting and immunofluorescence cell staining, it was clearly demonstrated that the chestnut inner shell extract enhanced the expression of the cell-associated fibronectin and vitronectin. Scoparone (6,7-dimethoxycoumarin), isolated from the extract, also possessed similar properties. These findings suggest that the enhanced expression of the adhesion molecules may be one of the molecular mechanisms for how the chestnut inner shell extract preventing cell detachment and may be also responsible for its anti-wrinkle/skin firming effect.

Key words: Chestnut inner shell, *Castanea crenata* S. et Z., Fibronectin, Vitronectin, NIH/3T3 fibroblast, Scoparone.

INTRODUCTION

The interaction of a cell with its surrounding extracellular matrix regulates cell behavior and the tissue architecture. The extracellular matrix is a signaling structure essential for normal cells during adhesion, migration, survival and proliferation. Among these matrix proteins, fibronectin, a dimeric extracellular matrix protein, which contains binding sites for the cell surface receptors and for the other extracellular matrix proteins (collagens, fibrin and heparin), plays an important role in cell-matrix adhesion (Ruoslahti, 1988). Fibronectin comprised of an adhesive glycoprotein is one of prominent components of wound healing, and an alternative splicing of the fibronectin gene appears to vary with age and in other related pathologies (Brown *et al.*, 1993; Xu *et al.*, 1997). Vitronectin is another adhesion molecule with a multifunctional glycoprotein that is involved in adhesion and spreading of cells (Dahlbach *et al.*, 1986). Recent investigations have revealed that dermal wound healing is delayed in mice lacking vitronectin, suggesting its significant role in the skin structure (Jang *et al.*, 2000). Therefore, changes in the expression level of these adhesion molecules may have profound effects on the architecture of normal as well as diseased skin (Postlethwaite and Kang, 1999).

The inner shell of chestnut (*Castanea crenata* S. et Z., Fagaceae) has been used topically as an anti-wrinkle/skin firming agent for a long time in East Asia (Huh, 1966). However, this plant material was only reported to possess tyrosinase inhibitory activity (Yang *et al.*, 1999). No study on its anti-wrinkle/skin firming effect has been published. During our investigation into developing new anti-wrinkle agent from plant sources, the ethanol extract of the inner shell of chestnut (CISE) was found to prevent the detachment of NIH/3T3 skin fibroblasts from plastic culture plates after trypsin-EDTA treatment in a concentration dependent manner. This finding suggests that CISE may affect the expression level of the adhesion molecule(s), leading to altered adhesion properties and an altered skin structure. Therefore, in order to elucidate

Correspondence to: Hyun Pyo Kim, College of Pharmacy, Kangwon National University, Chunchon 200-701, Korea.
E-mail: hpkim@kangwon.ac.kr

the molecular mechanism underlying this phenomenon and the anti-wrinkle effect of CISE, the expression level of the adhesion molecules including fibronectin and vitronectin was examined.

MATERIALS AND METHODS

Chemicals

Phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM) and other cell culture reagents including fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY). Sodium deoxycholate, phenylmethylsulfonylfluoride (PMSF), aprotinin, leupeptin, pepstatin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoium bromide (MTT), Tris-HCl were purchased from Sigma. Chem. (St. Louis, MO). The goat polyclonal anti-fibronectin antibody (SC-6952) and anti-vitronectin antibody (SC-7778) were purchased from Santa Cruz Biotech. (Santa Cruz, CA).

Preparation of the chestnut inner shell extract and isolation of scoparone

The dried chestnut inner shell was obtained from local market (Seoul, Korea). The plant material (2 kg) was soaked in 70% ethanol at room temperature for 7 days and filtered to remove the solid material. The filtrate was evaporated in vacuo to dryness (480 g) and the dried powder (CISE) was used throughout the experiments. Scoparone was isolated from the CISE following SiO_2 column chromatography. Briefly, the dried residue (200 g) was dissolved in a small amount of methanol and poured onto a SiO_2 column. Using chloroform:methanol (97:3) as the mobile phase, repeated column chromatography yielded scoparone as colorless needles (3.1 mg). The chemical structure was identified by instrumental analysis according to a previously described method (Afek *et al.*, 1986) and compared with an authentic sample (Aldrich Chem., Milwaukee, WI).

Cell culture

Mouse NIH/3T3 skin fibroblasts that were obtained from American Type Culture Collection (Rockville, ML) were cultured initially in DMEM supplemented with 10% FBS, 2% antibiotics under 5% CO_2 at 37°C. The cells were then washed and the media was changed to DMEM containing 10% FBS pretreated with gelatin-Sepharose 4B (Pharmacia, Uppsala, Sweden) to remove the serum fibronectin and vitronectin. The cells were counted with a hemocytometer and a standard MTT assay was employed as reported previously (Mossman, 1983) in order to measure the cell proliferation and viability. The protein concentration was measured with a protein assay kit (Bio-Rad., Hercules, CA) using bovine serum albumin as a standard.

Fixed-cell enzyme-linked immunoabsorbant assay (ELISA)

A cell-based ELISA test was used according to the experimental procedures previously reported in order to determine the cell-associated adhesion molecules (Grunt *et al.*, 1992). The cells were placed in 96-well plates (1×10^4cells/well) and treated with vehicle or test compounds. The culture medium was removed after 24 h incubation. The cells were washed with PBS and fixed with 0.25% glutaraldehyde for 2 h. After washing 5 times with PBS containing 0.02% (v/v) Tween 20, the cell-fixed plates were blocked with 3% skim milk for 2 h and washed again. Fibronectin and vitronectin were detected by incubation for 1.5 h with anti-fibronectin or anti-vitronectin antibody (1:500 dilution). Anti-goat IgG peroxidase conjugate was then treated for 2 h. After adding an O-phenylenediamine based substrate kit (Sigma Chem.), the enzyme reaction was quenched with 1 M HCl and the color developed was measured at 490 nm using a microplate reader (Spectra Max, Molecular Devices, Sunnyvale, CA).

Western blotting of adhesion molecules

The cells were cultured in 15 mm dish with vehicle or test compounds for 24 h in order to measure the cell-associated adhesion molecules by Western blotting. The cells were then scraped and lysed in 0.02 M Tris buffer, pH 8.8 containing 1% deoxycholic acid, 1 mM sodium orthovanadate and protease inhibitors (1 mM PMSF, 0.16 U/ml aprotinin, 1 mM leupeptin and 1 mM pepstatin) according to previously described procedures (Klaus *et al.*, 1979). After centrifugation at 13,000 g for 10 min, the soluble part and the insoluble pellet were collected, and subjected to electrophoresis on a 6% Tris-glycine gel for fibronectin. For vitronectin, only the soluble part was run on a 4-20% gel. After transferring the protein bands to a nitrocellulose membrane, it was incubated in a blocking buffer (6% skim milk) for 1 h at room temperature. The membrane was then incubated with anti-fibronectin or anti-vitronectin antibody (1:1,000 dilution). The bands were visualized by exposure to horseradish peroxidase-conjugated secondary antibody and a DAB reagent (Vector Lab., Burlingame, CA). In order to detect β-actin, Western blotting was carried out as previously described (Chi *et al.*, 2001).

Immunofluorescence cell staining

In order to detect the cell-associated fibronectin by an immunofluorescence technique, the cells were cultured on a microscopic slide (76×26 mm) until they reached 80% confluence and then treated with vehicle or test compounds for 24 h. After washing with PBS, cells were

fixed with ice-cold methanol:acetone (1:1) at -20°C for 10 min, followed by blocking with 3% skim milk in PBS. The slides were incubated with anti-fibronectin antibody, followed by treatment with FITC-conjugated secondary antibody for 30 min. Each slide was observed by fluorescence microscope (Olympus Optical Co., Japan).

Statistical analysis

All results are presented as an arithmetic mean ± S.D. The statistical significance was evaluated using a one-way ANOVA. All experiments were carried out at least twice and they gave similar results.

RESULTS

As shown in Fig. 1A, a treatment with CISE slightly increased the viability of the cells, as checked by MTT assay for 24 h incubation period (10.0% increase at 200 μg/ml). The total number of cells decreased slightly (13.2% and 5.0% decrease at 100 and 200 μg/ml, respectively). However, the changes in these two parameters were not statistically significant compared to the vehicle-treated control at 10-200 μg/ml. In contrast, CISE markedly prevented the detachment of the NIH/3T3 fibroblasts from the plastic surface after trypsin-EDTA treatment (68.3% inhibition at 200 μg/ml) in a concentration dependent manner. These results suggest that CISE can prevent cell detachment without significantly affecting the cell proliferativity and viability. When the concentrations of the cell-associated fibronectin and vitronectin were measured by a fixed-cell ELISA technique, CISE increased the amounts of fibronectin as well as vitronectin in a concentration dependent manner between 10-200 μg/ml (Fig 1B). The maximum fibronectin expression level was observed when the cells were treated with 200 μg/ml of CISE (55.5% increase compared to the vehicle-treated control). In the case of vitronectin, a 47.9% increase was achieved at a maximum at 200 μg/ml compared to the vehicle-treated control.

In the Western blotting experiment used to measure the adhesion molecules directly, the fibronectin expression level from the soluble part and the insoluble pellet was examined after deoxycholate treatment. For measuring vitronectin, the soluble part was only examined. As shown in Fig. 2A, CISE treatment clearly increased the cell-associated fibronectin and vitronectin expression level. However, CISE did not enhance β-actin expression. These results were well correlated with the above ELISA results. The immunofluorescence technique also confirmed that CISE increased the cell-associated fibronectin concentration in the fibroblasts (Fig. 2B).

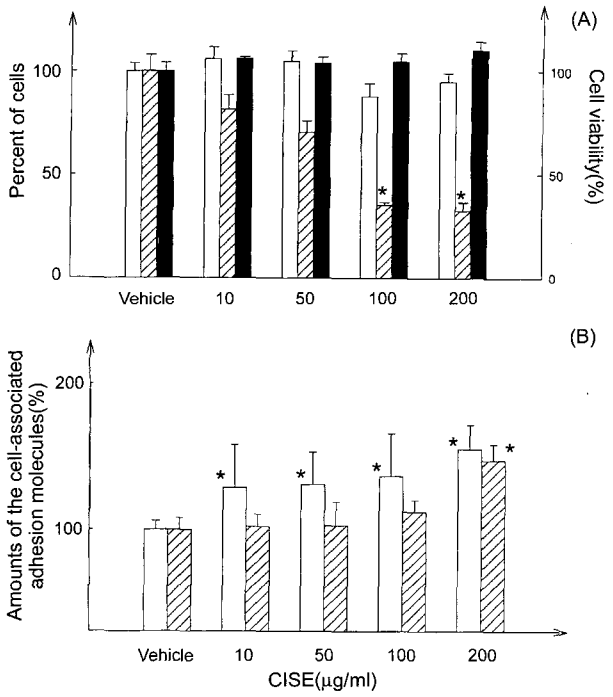

Fig. 1. Effects of CISE on the expression of the adhesion molecules in NIH/3T3 fibroblasts. (A) Effect on the detachment of NIH/3T3 cells by trypsin-EDTA treatment. The cells were incubated with the vehicle or CISE for 24 h and the total number of cells was counted by a hemocytometer (□). In a separate set of experiments, 0.05% trypsin-EDTA was treated for 10 min and numbers of the detached cells were counted by hemocytometer (▨). A viability of the total cells was determined by a MTT assay (■). In contrast to no significant difference in total number of cells and viability between in the vehicle-treated and the CISE-treated cells, a significant decrease in the numbers of detached cells after CISE treatment was observed in a concentration-dependent manner. (B) Effect on the cell-associated adhesion molecules. The amounts of fibronectin (FN, □) and vitronectin (VN, ▨) were measured by a fixed-cell ELISA according to the procedure described in materials and methods. n = 3, *: $P < 0.005$, significantly different from the vehicle-treated cells.

Finally, the effect of scoparone was examined. When measured by fixed-cell ELISA, this compound weakly increased the amounts of fibronectin and vitronectin (Fig. 3A). Scoparone increased the cell-associated fibronectin concentration by 10.4% and 10.4% at 10 and 100 μM, respectively but it was not statistically significant compared to the vehicle-treated control. The same compound significantly increased the cell-associated vitronectin level by 20.8% at 100 μM. In this experiment, scoparone exhibited weak cytotoxicity against NIH/3T3 cell at 100 μM (20.1% cytotoxicity by MTT assay). When Western blotting technique was used to examine the expression of these adhesion molecules, the same compound enhanced the cell-associated fibronectin and vitronectin expression at 0.1-100 μM (Fig. 3B).

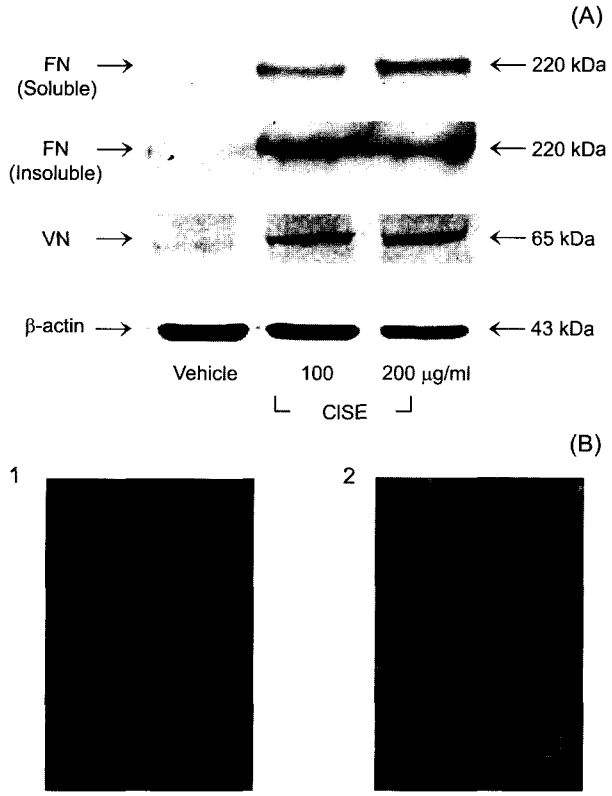

Fig. 2. Effects of CISE on the expression of the adhesion molecules in NIH/3T3 fibroblasts by western blotting and immunofluorescence. (A) Effect on the cell-associated adhesion molecules by western blotting. The same amount of protein (10 mg) was loaded on each lane. (B) Immunofluorescence staining against fibronectin. Vehicle-treated cells (1), CISE-treated cells (2, 200 mg/ml). Note: Substantially increased fluorescence was observed in the CISE-treated cells (× 100).

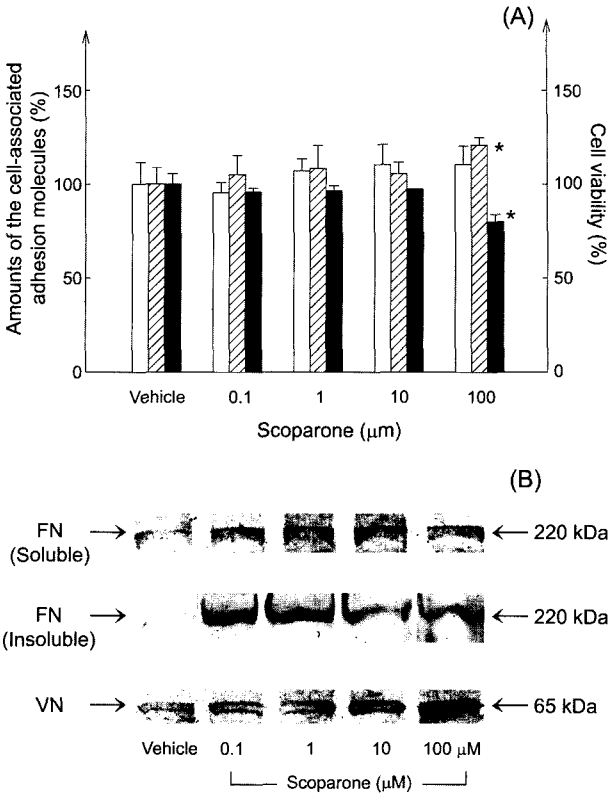

Fig. 3. Effects of scoparone on the expression of the adhesion molecules in NIH/3T3 fibroblasts. (A) Fixed-cell ELISA of the cell-associated adhesion molecules. Fibronectin (□), vitronectin (▨), and viability by MTT treatment (■). (B) Western blotting of the cell-associated adhesion molecules. The same amount of protein (10 mg) was loaded on each lane. Fibronectin (FN), vitronectin (VN). n = 3, *: $P < 0.005$, significantly different from the vehicle-treated cells.

DISCUSSION

This study has clearly shown that CISE enhances the expression of adhesion molecules including fibronectin and vitronectin without affecting β-actin expression level. Three different techniques; fixed-cell ELISA, Western blotting and immunofluorescence staining confirmed these results. In an initial experiment, CISE exhibited significant cytotoxicity against the NIH/3T3 fibroblasts at concentrations ≥ 500 μg/ml. However, CISE concentrations tested in this study were limited to within 200 μg/ml, indicating that all the results obtained here were not related to the cytotoxic effect against NIH/3T3 cells. Therefore, it is evident that CISE specifically enhanced the cell-associated expression of fibronectin and vitronectin in the skin fibroblasts. A similar example of increased adhesion molecule synthesis was reported with tretinoin (all-trans-retinoic acid), which also enhanced fibronectin synthesis after a 10 week topical application on ultraviolet radiated-photoaged mouse skin (Schwartz and Klingman, 1995). Recently, an eupolin extract using a traditional herbal treatment for skin burns was found to stimulate the expression of adhesion complexes and fibronectin (Phan *et al.*, 2000).

There are various families of adhesion molecules, i.e. selectins, integrins, and immunoglobulin superfamilies, such as intercellular adhesion molecule-1, etc. Among these molecules, fibronectin and vitronectin are major adhesion molecules possessing important biological activities including a cell-cell interaction and a cell-matrix interaction. Although there have been many contradictory results concerning the age-related and disease-related changes in these adhesion molecules (Pieraggi *et al.*, 1984; Shevitz *et al.*, 1986; Takeda *et al.*, 1992; Kumazaki *et al.*, 1993), they are deeply associated with the regulation of the skin structure in the normal as well as in the diseased state (Postlethwaite and Kang, 1999).

The inner shell of the chestnut is known to contain considerable amounts of tannins due to its bitter taste. While ellagic acid was recently identified in CISE by HPLC analysis (data not shown), scoparone might be the first compound isolated from this plant material. This compound was shown to enhance the expression of the

adhesion molecules, fibronectin and vitronectin from the present investigation, whereas ellagic acid did not (data not shown). It is important to note that the maximum enhancing activity of scoparone was far less than that of the whole extract, suggesting the existence of other compound(s) that participate in this biological effect. However, it is thought that scoparone may be, at least, one of the active principles of CISE that enhance the expression of the adhesion molecules, although unknown constituents may possess a higher activity. The detailed molecular mechanism for the increased expression of the adhesion molecules by this plant material is unclear. The possible changes in the expression level and/or affinity of the receptors for these adhesion molecules could not be completely excluded. In addition, the effects on the signal transduction pathway such as phospholipase D through RalA (Ladeda *et al.*, 2001) need to be further investigated. An *in vivo* study is currently under investigation to confirm the *in vitro* findings of this study.

In conclusion, CISE was found to enhance fibronectin and vitronectin expression without affecting the β-actin expression level in NIH/3T3 skin fibroblasts. It was also demonstrated that an isolated coumarin derivative from this extract, scoparone (6,7-dimethoxycoumarin), exhibited similar properties. These effects may be partly responsible for the detachment resistance of the fibroblasts from the plastic surfaces, and are possibly associated with the anti-wrinkle/skin firming effect of this plant material.

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