Food Sci. Biotechnol. Vol. 16, No. 3, pp. 457 ~ 462 (2007)

Food Science and Biotechnology

Potent HAT Inhibitory Effect of Aqueous Extract from Bellflower (*Platycodon grandiflorum*) Roots on Androgen Receptor-mediated Transcriptional Regulation

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Abstract Histone acetyltransferase (HAT) is a family of enzymes that regulate histone acetylation. Dysfunction of HAT plays a critical role in the development of cancer. Here we have screened the various plant extracts to find out the potent HAT inhibitors. The bellflower (*Platycodon grandiflorum*) root have exhibited approximately 30% of the inhibitory effects on HAT activity, especially p300 and CBP (CREB-binding protein) at the concentration of 100 μg/mL. The cell viability was decreased approximately 52% in LNCaP cell for 48 hr incubation. Furthermore, mRNA level of 3 androgen receptor target genes, PSA, NKX3.1, and TSC22 were decreased with bellflower root extract treatment (100 μg/mL) in the presence of androgen. In ChIP assay, the acetylation of histone H3 and H4 in PSA promoter region was dramatically repressed by bellflower root treatment, but not TR target gene, D1. Therefore, the potent HAT inhibitory effect of bellflower root led to the decreased transcription of AR target genes and prostate cancer cell growth with the repression of histone hyperacetylation.

Keywords: bellflower root, histone acetyltransferase, androgen receptor

Introduction

Prostate cancer is second only to lung cancer in the western centuries, and it rates in South Korea appear to be steadily rising like in other Asian countries over the last few years (1, 2). Prostate cancers are typically treated with hormone therapy aimed at blocking testosterone signaling at the androgen receptor (AR) (3). AR is activated by androgen to regulate prostate gland development and prostate cancer growth (4, 5). AR acetylation is a key posttranslational modification regulating growth control in human prostate cancer (6). The AR is acetylated by p300, P/CAF, and TIP60 and acetylation of the AR regulates co-regulator recruitment and growth properties of the receptors in cultured cells and *in vivo*. AR acetylation mimic mutants convey reduced apoptosis and enhanced growth properties correlating with altered promoter specificity for cell-cycle target genes (7).

Recent studies implicate alteration in chromatin structure by histone hyperacetylation/deacetylation as playing an important role in eukaryotic gene transcription, carcinogenesis, and the therapy of cancer (8). The reversible process of histone acetylation is controlled by two classes of enzyme, histone acetyltransferase (HAT), and histone deacetylase (HDAC), which catalyze the addition and removal, respectively, of acetyl groups and lysine residues in protein (9). Dysfunction of the balance between the acetylation and deacetylation states of histones is often associated with the manifestation of cancer (10). The antitumor effects of HDAC inhibitors are well documented and some of them are the subjects of clinical trials (11).

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Received July 12, 2006; accepted August 13, 2006

The HAT inhibitors may also possess therapeutic potential. The groups which have HAT activity are identified the P300/cAMP response element binding protein(CREB)-binding protein (CBP) family, TAF250, p300/CBP-associated factor (PCAF), hGCN5, and TIP60 (9, 12). These coactivators, TIP60, P300, and PCAF enhance the inherent transcriptional activity of the AR by direct receptor acetylation and up-regulate rate by histone acetylation of AR target genes. A limited number of HAT inhibitors have been described such as synthetic peptide-CoA conjugate (p300 and PCAF) (13), isothiazolone (PCAF) (9), polyprenylated benzophenone from *Garcinia indica* fruit rind (14), curcumin from *Curcuma longa* (p300) (10), and anacardic acid from cashew nut shell liquid (15).

We screened 500 kinds of extract from edible plants for examination of the HAT inhibitory effect. Hot water extract from the root of *Platycodon grandiflorum* (commonly known as bellflower) exhibited the highest HAT inhibitory effects.

The bellflower root has been used as a food and a folk remedy for bronchitis, asthma, pulmonary tuberculosis, inflammatory disease, diabetes, and as a sedatives (16-19). The extract of bellflower root exhibits anticancer and antioxidant activities in human cancer cell lines (19). In the present study, we demonstrate that the aqueous extract of bellflower root is a potent inhibitor of HAT activity, especially for p300 and CBP and could be developed a potential use of antineoplastic therapeutics throughout inhibition of cancer cell growth.

Materials and Methods

Reagents and construction RPMI-1640 medium, antibiotics and fetal bovine serum was purchased from Gibco

BRL (Grand Island, NY, USA). HAT activity colorimetric assay kit was from Biovision (Mountain View, CA, USA). Easy-spin total RNA extraction kit and Maxime RT premix kit was from Intron (Seoul, Korea). The antibodies were purchased from Upstate Biotechnology (Charlottesville, VA, USA). Effectene transfection reagent was from Quagen (Valencia, CA, USA). PGL3-PSA construct was a kind gift from Dr. Kyung-Sup Kim (Yonsei Univ.). Protein A/G PLUS agarose bead was from Santa Cruz (Santa Cruz, CA, USA).

Preparation of aqueous extract for screening potent HAT inhibitors Various plants including bellflower root was purchased from Gyeongdong Market, Seoul, Korea and from plant extract bank in Plant Diversity Research Center, Daejeon, Korea. To prepare the sample, each sample (5 g dry weight) was blanched, homogenized at 600 rpm with Ultra-Turrax (IKA-Lab, Staufen, Germany), and centrifuged at 10,000×g for 30 min. After lyophilization of the supernatant, dried and powdered sample was refluxed with 4 volumes of distilled water for 2 hr. The extraction was centrifuged at 3,000×g, evaporated in vacuum, and then lyophilized.

Immunoprecipitation and HAT activity assay We used HeLa nucleic extract which contains various HAT proteins for determination of HAT activity. HeLa cell nuclear extract was prepared as described previously (20). HAT activity assays were performed using nuclear extracts following the manufacturer's protocol. Immunoprecipitation (IP) were performed using anti-p300, anti-CBP, anti-P/CAF, and anti-BRG1 with HeLa nuclear extracts. Precleared HeLa nuclear extract (50 μL) were incubated with 2 mL of antibodies overnight with Protein A/G PLUS agarose beads at 4°C. Immunoprecipitation were collected, and washed with HAT assay buffer (50 mM pH 8.0 Tris, 10% glycerol, 0.1 mM EDTA) and then performed HAT activity assay. The percentage of the HAT inhibitory activity value for each sample was calculated as compared with the control activity.

Cell culture Human prostate cancer cell line LNCaP cells were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were grown in RPMI 1640 with L-glutamine supplemented with 10% heat-inactivated FBS, 1% antibiotics and antimycotics. All cultures were maintained in humidified chamber at 37°C with 5% CO_2.

Reporter assay Prostate-specific antigen (PSA) is considered to be the most sensitive biochemical marker available for monitoring the presence of the prostatic disease, particularly prostate cancer, and response to therapy (21). The expression of PSA is primarily activated by androgen receptor at the transcription level (22). LNCaP cells were seeded at 5×10^5 per well in 6-well culture plates and incubated overnight with RPMI 1640 containing 10% FBS. At 90% confluence, a 0.5 μg of pGL3-PSA and 1 ng of pGL3-control vector containing SV40 DNA/6 wells were used for transfection into LNCaP cells for luciferase assays. The medium was replaced with phenol free RPMI 1640 containing 10% charcoal-stripped fetal bovine serum. Medium was then changed with 50 nM of the synthetic androgen R1881 (Perkin-Elmer, Waltham, MA, USA) treatment and plant extracts (100 μg/mL) was added 6 hr after R1881 treatment.

Cell viability test LNCaP cells were seeded at 1×10^4 per well in 96-well culture plates and incubated overnight with RPMI 1640 containing 10% FBS. The cell then treated with serum-free medium containing hot water extracts from bellflower root (final concentration 100 mg/mL) for 48 hr. After 48 hr, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) solution in PBS was added and the plates were incubated for 2 hr. At the end of the incubation, the medium was removed and the formed blue formazan was solubilized with DMSO. Absorbance was measured at 570 nm with background subtraction at 630 nm.

Reverse transcription and polymerase chain reaction (RT-PCR) LNCaP cells were seeded at 5×10^5 per well in 6-well culture plates and incubated overnight with RPMI 1640 containing 10% FBS. At 90% confluence, the medium was replaced with phenol free RPMI 1640 containing 10% charcoal-stripped fetal bovine serum for 48 hr. R1881 and bellflower root extract were added in the same manner. Followed by overnight incubation, total RNA was isolated by Easy-spin total RNA extraction kit and RT-PCR was performed using Maxime RT-premix kit. GAPDH was used as internal control. Primer sequences used in this study were as follows: for the NKX 3-1, 5'-AGC CGCTCACGTCCTTCCTCATCC-3' and 5' -GGGGCCCGGTGCTCAGCTCGTCG TTCT-3'; for the TSC22, 5'-ATTTTTCTCTATTAGTTCTTTGATTTG-3' and 5'-GACTTGATAATAGCTCCTCTGGT-3'; for the PSA, 5'-GCCCACCCAGG AGCCAGCACT-3' and 5'-GGCC CCCAGAATCACCCGAGCAG-3'. Primers for the GAPDH amplification were 5'-CGCGGGGCTCTCCAG AACATCATCC-3' and 5'-CTCCGACGCCTGCTTCAC CACCTTCTT-3'. The reaction mixture was heated at 94°C for 2 min, 27 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, and extension at 94°C for 5 min. Amplification products were electrophoresed through a 1.5 % agarose gels.

ChIP assays For ChIP assays, we first isolated chromatin as described (23). In brief, approximately 2×10^9 LNCaP cells in 150-mm dishes were first treated with PBS containing 1% formaldehyde for 10 min, washed twice with PBS and incubated with 100 mM Tris (pH 9.4)-10 mM dithiothreitol (DTT) at 30°C for 15 min. The cells were then rinsed twice with PBS and resuspended in 600 μL of SolA buffer (10 mM Hepes (pH 7.9), 0.5% NP-40, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) by pippeting. After a short spin (5 min at 800×g), the pellets were resuspended in SolB (20 mM Hepes (pH 7.9), 25% glycerol, 0.5% NP-40, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA) containing protease inhibitors by vigorous pippeting to extract nuclear proteins. After centrifugation at 15,700×g for 30 min, the nuclear pellets were resuspended in IP buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl and protease inhibitors) and sonicated to break chromatin into fragments

with an average length of 0.5-1 kb. The ChIP assays were then performed with indicated antibodies essentially as described but omitting SDS in all buffers (24). Primers used for ChIP analysis: for PSA, 5'-CATGTTCACATTA GTACACCTTGCC-3' and 5'- TCTCAGATCCAGGCTT GCTTACTGRC-3'; for D1, 5'-GGAGGCCAAGGCGG GTAGGTCATCT-3' and 5'-CCGGGTCAGGGGAAGGAG TCAGGTCA-3'.

Results and Discussion

Identification of anti-HAT acitivity from aqueous extract of bellflower root To isolate anti-HAT activity containing substances, 500 kinds of extract from edible plants were examined for the HAT inhibitory effect. Hot water extract (100 μg/mL) from *Epimedium koreanum* (Barrenwort) (27.5%), the root of *P. grandiflorum* (bellflower root) (31.5%), and *Diospyros kaki* (Persimmon leaf, 23.1%) showed the higher inhibitory effects compare to saline control (Table 1). Among those extracts, extracts of the bellflower root exhibited the highest HAT inhibitory effects.

Repression of AR-mediated transcriptional activation by bellflower root To examine whether anti-HAT activity containing extracts modulate AR-mediated transcription, we first wished to perform the reporter assay using an androgen-dependent reporter construct (pGL3-PSA) bearing ARE (androgen responsive element). Toward this goal, we used LNCaP cell line, which is an androgen-sensitive prostate cancer line derived from a lymph node metastasis in a human subject. After transfection, the cells were either left untreated and treated with the synthetic androgen R1881, and then added *C. bursapastoris*, *E. koreanum*, bellflower root, and *D. kaki* exracts, respectively. The cells were lysed 16 hr later and luciferase activities were determined. A representative results is shown in Fig. 1, it is evident that bellflower root extract treatment decreased luciferase activity by 50% compare with R1881 alone, there by suggesting that anti-HAT activity containing bellflower root repress the AR-mediated transcriptional activation.

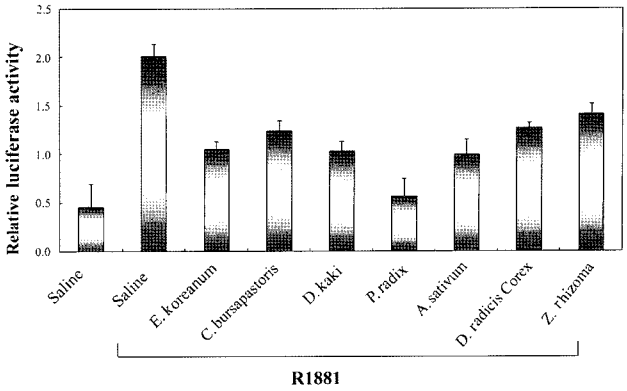

Fig. 1. Repression of AR-mediated transcriptional activation by aqueous extract of bellflower roots. LNCaP cells were transiently transfected with pGL3-PSA and 1 ng of SV40 DNA. After 24 hr, cell was treated with or without R1881 and then added 100 μg/mL samples 6 hr later. Mean values were calculated from 3 independent experiments.

Table 1. Inhibitory effect of water extracts from various plants on HAT activity

Scientific name	HAT inhibitory activity (%)[1)]
Allium tuberosum L.	8.9
Epimedium koreanum	27.5
Pinus densiflora	8
Zedoariae rhizoma	19.5
Houttuyniae herba	13.4
Dictamni radicis Cortex	19.8
Rhei rhizoma	ND
Anemarrhenae rhizoma	2
Platycodon grandiflorum (root)	31.5
Whasabia japonica	6.1
Coriandrum sativum L.	3.4
Allium sativum	19.3
Carthamus tinctorius	ND
Juglas sinensis	8.7
Diospyros kaki	23.1

[1]HAT inhibitory activity (%): The percentage of the HAT inhibitory activity value for each sample was calculated as compared with the control activity. The final concentration of each sample in assay mixture was 100 μg/mL.

Inhibition of HAT activity for p300 and CBP by bellflower root It has been demonstrated that P300, CBP, P/CAF, and BRG1 protein complex possess HAT activity (12). To examine the inhibitory effects of bellflower root on enzyme-specific HAT activity, immunoprecipitation was performed with HeLa nuclear extract using anti-p300, CBP, P/CAF, and BRG1 antibodies. We observed exhibited the inhibitory effects on p300 and CBP specifically, approximately 30% at the concentration 100 μg/mL with the hot water extract from bellflower root (Fig. 2).

These results led us to find out the hot water extract of bellflower root possessed inhibitory effects toward p300 and CBP. The bellflower root is known to be a medicinal plant for bronchitis, asthma, and pulmonary tuberculosis, hyperlipidemia, diabetes, and inflammatory diseases (18). Several studies have been reported the antitumor activity of the water or organic extracts from bellflower root (16, 18, 19).

The effect of bellflower root extract on prostate cancer cell growth As we expected, cell viability was decreased in a dose-dependent manner (Fig. 3). At the 200 mg/mL concentration, the MTT reduction was approximately 52% in LNCaP cell for 48 hr incubation. Cytotoxicity is one of the major therapeutic targets of anti-tumor activity (25). It has been reported that the AR acetylation mimics promoted cell survival and growth of prostate cancer cells and in nude mice and augmented transcription of a subset of

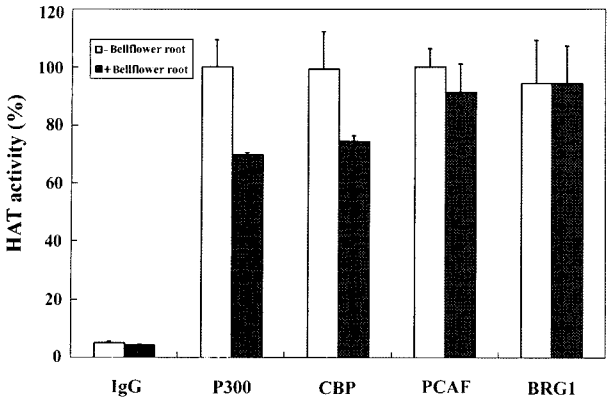

Fig. 2. Inhibition of HAT activity for p300 and CBP by bellflower roots. Histone acetyltrasferase assays were performed with IP product using anti-p300, CBP, P/CAF, and BRG1. The concentration of extract is 100 μg/mL for anti-p300, anti-CBP, P/CAF, and BRG1. The result represents the mean±SD.

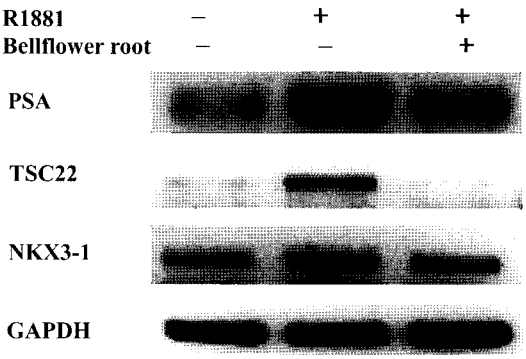

Fig. 4. Anti-HAT activity of bellflower roots suppress agonist-stimulated activation of AR target genes. RT-PCR analysis of PSA, NKX3-1, and TSC22 was performed 16 hr after treatment of bellflower root (100 μg/mL) with or without R1881.

growth control target gene promoters. Our data suggested that modulation of AR acetylation by HAT inhibitors control aberrant cellular growth *in vivo*.

Anti-HAT activity of bellflower root suppress agonist-stimulated activation of AR target genes Androgen receptor (AR) expression is observed by primary prostate cancer and can be detected throughout hormone sensitive and hormone refractory cancers (21, 22). Several AR coactivators such as the CBP family, TIP60, and P300 possessing HAT activity have shown to enhance AR activities (26). To investigate the effect of bellflower root extract on the expression of endogeneous AR target gene, we selected 3 kinds of AR target genes including PSA, NK3 transcription factor locus 1 (NKX 3-1), and TSC22, previously reported by our group (5, 20). As expected, mRNA expressions of AR target genes are robustly enhanced by R1881 treatment. As shown in Fig. 4, bellflower root extract (100 μg/mL) generally repressed mRNA expression of PSA, NKX 3-1, and TSC22. NKX 3.1 is one of the earliest markers for prostate development and its continuously expressed at all stages during prostate development and in adulthood. NKX 3.1 mRNA is expressed in LNCaP cells and upregulated by androgen (28). Taken together, we conclude that anti-HAT activity of bellflower root extract repress the expression of AR target genes through inhibition of coactivator dynamics which is essential for AR-mediated transcriptional activation.

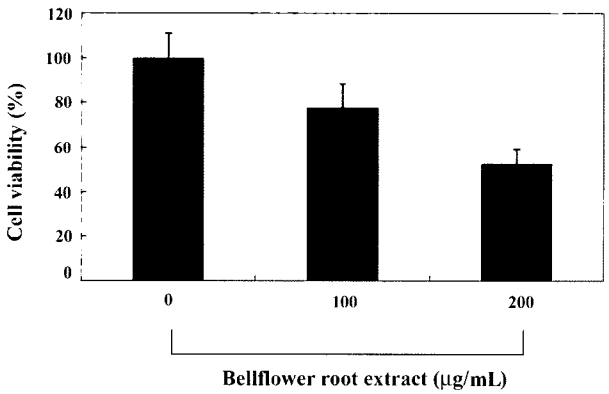

Fig. 3. The effect of bellflower root extract on prostate cancer cell growth. LNCaP cells were seeded at 1×10^4 per well in 96-well culture plates and incubated overnight with RPMI 1640 containing 10% FBS. The cell then treated with serum-free medium containing hot water extracts from edible plants (final concentration 100 μg/mL) for 48 hr. The result represents the mean±SD.

The treatment of bellflower root led to the reduced histone acetylation CBP/P300 acetylates both histones and nonhistone proteins to regulate their activity. Several studies have reported that both AR and histones can be acetylated *in vitro* by p300, P/CAF, and TIP60 (14, 29-31). A recent study indicates that AR is also acetylated in the presence of the HDAC inhibitor TSA or DHT in a prostate cancer cell line (25, 31). Interestingly, the acetylation of AR was shown to enhance p300 binding and the histone acetylation, whereas unacetylated AR show reduced p300 binding and reduced transcription of AR target genes. These results suggest that acetylation of AR and histone may influence the decision of transcriptional level of target genes during prostate cancer cell growth.

Because bellflower root inhibits p300/CBP HAT activity *in vitro* and repress the transcription of AR target genes, we next wished to determine whether the treatment of bellflower root led to the reduced histone acetylation in the promoter region of AR target gene by using ChIP assay (Fig. 5). ChIP assay is the technique to analyze *in vivo* interactions of protein with genomic DNA. The chromatin associated or DNA binding protein, and changes in histone modifications could be analyzed by ChIP assay. For this purpose, LNCaP cells were treated with or without R1881 or bellflower root, either individually or in combination. Three days later, the cells were processed for ChIP assays for the anti-AcH3 and AcH4. To examine whether the anti-HAT activity of bellflower root is specific for AR target genes, we also designed the primer for the promoter region of deiodinase (D1), TR (thyroid hormone receptor) target gene, as a

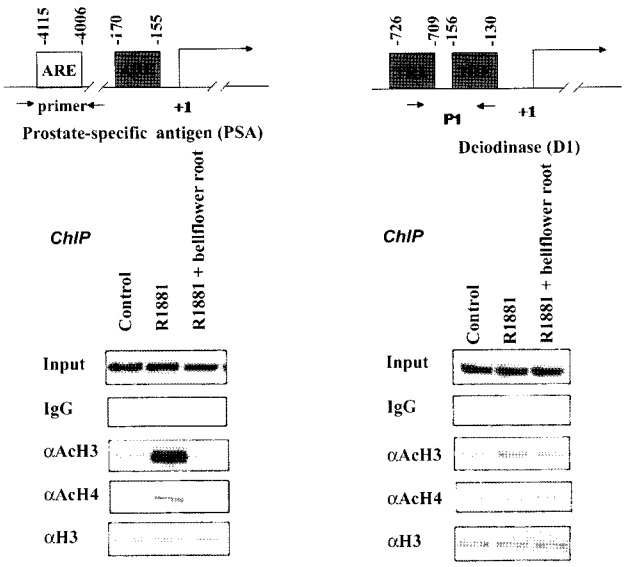

Fig. 5. The treatment of bellflower roots led to the reduced histone acetylation. LNCaP cells were treated with or without R1881 or bellflower root, either individually or in combination. Three days later, the cells were processed for ChIP assays for the anti-AcH3 and AcH4.

negative control. Input was used as a control of total DNA for PCR effectiveness. As expected, the acetylation of histone H3 and H4 was dramatically increased by R1881 specifically in the AR target gene, PSA. ChIP analysis in Fig. 5 revealed that the treatment of bellflower root had no effect on the histone acetylation of TR target gene, D1. However, an increased acetylation of histone H3 and H4 was observed although the overall level of histone H3 was not changed. These results convincingly establish bellflower root as a potent inhibitor of p300/CBP activity *in vitro* and *in vivo.*

Collectively, the potent HAT inhibitory effect of bellflower root generally led to the decreased transcription of AR target genes and cancer cell growth with the repression of histone hyperacetylation.

Several studies strongly suggested that the AR acetylation is involved in the regulation of prostate cancer cell growth and apoptosis. The fact that acetylation mimic mutants of the AR enhance prostate cancer cell growth, and that expression of AR coactivators in prostate cancer tissues correlates with AR expression, prostate cancer progression, and recurrence suggest that acetylation of the AR may contribute to the development of androgen-independent prostate cancer (6, 25). Inhibition of the AR acetylation process and AR-coactivator binding is likely to lead the development of new therapeutic drugs for prostate cancer.

Acknowledgments

This work was supported by a grant (Code #20070301034007) from BioGreen 21 Program, Rural Development Administration; a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, (A05-0125-AA0718-05N1-00010A), Korea; Yonsei University Research Fund of 2005; a faculty research grant of Yonsei University College of Medicine for 2005 (to H.-G. Yoon).

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