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Translocation of Phospho-ser 15-p53 in Eugenol-induced Apoptosis of in vitro Cultured Cancer Cells

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Abstract : Eugenol (4-allyl-2-methoxyphenol), a major ingredient of herbs such as clove and *Magnoliae Flos*, is known to induce apoptosis in mast cells via p53 pathway. This study was undertaken to examine the *in vivo* effect of eugenol and the molecular mechanism underlying eugenol-induced apoptosis in several cancer cells with different p53 status.

Effect of eugenol on mesenteric mast cells was tested using a rat anaphylaxis model. And TUNEL staining was conducted to observe the cells undergoing apoptosis. Several cancer cells were treated with eugenol, and Western blotting, immunocytochemistry, confocal microscopy and mitochondrial fractionation were conducted.

Eugenol induced apoptosis in mast cells of mesentery *in vivo*, decreasing the density of mast cells. Although eugenol did not increase the expression level of p53, it caused the translocation of p53 into mitochondria and subsequent release of cytochrome c. Eugenol increased the level of phospho-ser 15-p53 in several cancer cells with wild type p53 but not in the cells with mutant p53 or p53 deficient cancer cell. In cancer cells with wild type p53, p53 translocated into mitochondria was phosphorylated on ser 15.

In conclusion, eugenol induces apoptosis in cancer cells with wild type p53 via the translocation of phospho-ser 15-p53. Furthermore our data suggest that the anticancer effect on cancer cells with wild type p53 may be involved with the pharmacological effect of eugenol regulating apoptosis via a phospho-ser 15-p53 dependent fashion.

Key words : Phospho-ser 15-p53, Eugenol, Apoptosis, Mast cell

Introduction

Apoptosis, or programmed cell death, is an essential physiological process that is required for the normal development and maintenance of tissue homeostasis. However, apoptosis is implicated also in a wide range of pathological conditions, including immunological diseases, allergy and cancer (Carson and Ribeiro 1993, Ohta and Yamashita 1999). During apoptosis, cells undergoes specific morphological and biochemical changes, including cell shrinkage, chromatin condensation, and internucleosomal cleavage of genomic DNA (Wyllie et al. 1980, Williams 1991). Multiple lines of evidence indicate that apoptosis can be triggered by the activation of caspase (Thornberry et al. 1997). In addition, mitochondria are known to be central death regulators in response to several apoptotic stimuli (Green and Reed 1998).

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Mast cells are granule-containing secretory cells that play an essential role in immediate-type allergic and inflammatory reactions by releasing chemical mediators such as histamine, prostaglandins, and cytokines. The mediators from degranulating mast cells have critical function to the pathology of allergic reaction, and induce tissue remodeling, and increase of venular permeability and smooth muscle contraction of bronchus in various tissues (Hart 2001, Bachert 2002). Mast cell numbers in tissues under normal conditions are relatively constant from individual to individual, but rapidly proliferate and become activated in various pathological conditions, and processes including mastocytosis, chronic inflammatory conditions, or allergic disease. Accordingly, mast cells are rapidly eliminated from inflammatory tissues in recovery from allergic diseases. The number of mast cells in tissues was demonstrated to be regulated in part by apoptosis (Metcalfe et al. 1995, Metcalfe et al. 1997, Park et al. 2000).

Numerous studies have focused on the targeted induction of apoptosis in order to control the unlimited growth of cells. Moreover, induction of apoptosis in the activated cell may promote therapeutic efficiency in a certain case. As a matter of fact, the antiinflammatory or antiallergic effect of a certain drug was demonstrated to depend on its apoptosis-inducing effect (Fuller et al. 1995). Therefore, inducing apoptosis of mast cells in tissue could be used therapeutically for various pathological conditions and understanding the mechanism is important.

Recent studies have been demonstrated that pharmacological effects of the herbal medicine, which had been being used for treatment of allergic diseases in oriental countries (Hamasaki et al. 1996, Kim et al. 1998, Kim et al. 1999, Park et al. 2001, Na et al. 2002, Kim et al. 2003), depended on their modulation of the activity of mast cells (Hamasaki et al. 1996, Kim et al. 1998, Kim et al. 1999, Na et al. 2002). Furthermore, our previous studies proved that clinical effects of Syzygium aromaticum (L.) Merr. Et Perry (Myrtaceae) flower bud (SAFB, cloves) and *Magnoliae flos* (MF), which had been successfully used for the management of asthma and various allergic disorders in oriental countries, might depend on the pharmacological efficacy regulating of mast cell demise (Park et al. 2001, Kim et al. 2003).

We undertook this study to explore whether eugenol (4-allyl-2-methoxyphenol), which was known as one of major principles of SAFB as well as MF (Zhu 1996), had the activity regulating mast cell death. Our previous study, for the first time, demonstrated that p53 translocated into mitochondria was phosphorylated on ser 15. Furthermore we observed that phospho-ser 15-p53 physically interacted with Bcl-2 and Bcl-xL in mitochondria and its translocation into mitochondrial preceded cytochrome c release and mitochondrial membrane potential (MMP) reduction (Park et al. 2005).

This study was undertaken to examine the *in vivo* effect of eugenol and the molecular mechanism underlying eugenol-induced apoptosis in several cancer cells with different p53 status.

Materials and Methods

1. Antibodies and reagents

The following reagents were obtained commercially: Rabbit polyclonal anti-human p53 (full-length) and anti-horse cytochrome c were from Santa Cruz Biotechnology (Santa Cruz, USA); Rabbit polyclonal anti-human phospho p53 (Ser 15) antibody was from Oncogene (Cambridge, USA); FITC-conjugated goat anti-rabbit IgGs were from Vector (Burlingame, USA); Mitotracker was from Molecular Probes (Eugene, USA). Dulbecco's modified Eagle's medium (DMEM) was from Gibco (Gaithersburg, USA). Dimethyl sulfoxide (DMSO), eugenol, anti-β actin antibody, Hoe-

chst 33342, RNase A, proteinase K, aprotinin, leupeptin, PMSF, and propidium iodide were from Sigma (St. Louis, USA). TUNEL reaction mixture was from Boehringer Mannheim (Mannheim, Germany). Super-Signal West Pico enhanced chemiluminescence Western blotting detection reagent was from Pierce (Rockford, USA).

2. Assessment of induction of apoptosis in peritoneal mast cells by eugenol

Wistar rats were subdivided into 5 groups: the negative control (control rat); the experimental control (compound 48/80-treated rat); the experimental negative control (compound 48/80+saline-treated rat); eugenol+compound 48/80-treated group; and eugenol only-treated group. Four rats were used in each group. Rats were given an intraperitoneal injection of eugenol dissolved in 200 μL ethanol (10 μg/kg body weight). 1 h after the injection of eugenol the mast cell degranulator compound 48/80 (8 g/kg body weight) was administered intraperitoneally. Compound 48/80 at this concentration induced fatal shock in 100% of the experimental control in 20 min. However, 100% of eugenol-pretreated rats survived. 20 min after treatment of compound 48/80, the mesentery was removed.

To exmaine the population of mast cells, the mesentery was stained with toluidine blue. In brief, the mesentery was placed 15 min in a mast cell staining solution containing 50% ethanol, 10% formaldehyde, 5% acetic acid (v/v) and 2% toluidine blue (w/v). The mesentery was blotted dry on filter paper and was then divided into several pieces. These were mounted on a glass slide, care being taken not to fold or stretch the tissue. Mast cells showing metachromasia were counted in 12 microscopic fields (250 × magnification) under Zeiss Axiophot microscope (Gettingen, Germany) per each animal by a blind observer.

To observe the cells undergoing apoptosis, TUNEL staining was conducted. In brief, the mesentery was fixed for 30 minutes in 4% paraformaldehyde, incubated in permeabilisation solution for 2 min on ice, and labeled in TUNEL reaction mixture for 60 min at 37°C. The mesentery was observed under Zeiss Axiophot microscope per each animal by a blind observer.

3. Cell culture

RBL-2H3 basophilic leukemia cell lines were purchased from the ATCC (Rockville, USA). RBL-2H3 cells were maintained at 37°C with 5% CO_2 in air atmosphere in DMEM with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, and supplemented with 15% FBS. Apoptosis inducing activity of eugenol was tested in several different cancer cell lines. p53-deficinet cells (PC-3), cells with mutant p53 (p815 and HT-29) and cells with wild type p53 (K562, A549, MCF-7) were used. PC-3, p815, HT-29, K562, A549 and MCF-7 cell lines were also purchased from the ATCC (Rockville, USA).

4. Immunofluorescent staining

Cells were cytocentrifuged and fixed for 10 min in 4% paraformaldehyde, incubated with each primary antibody for 1 h, washed 3 each for 5 min, and then incubated with FITC-conjugated secondary antibody for 1 h at room temperature. Cells were mounted with PBS.

5. Photomicrography and cell counting

Cells were observed and photographed under Zeiss Axiophot microscope (Gettingen, Germany). Total cell number, at least 300 cells from each experiment, was counted under DIC optics and the cells showing condensed or fragmented nuclei in Hoechst staining were calculated under epifluorescence optics by a blind observer.

6. Western blot analysis

Cells (2×10^6) treated with eugenol were washed twice with ice-cold PBS, resuspended in 200 μL ice-cold solubilizing buffer [300 mM NaCl, 50 mM Tris-Cl (pH 7.6), 0.5% TritonX-100, 2 mM PMSF, 2 μL/mL aprotinin and 2 μL/mL leupeptin] and incubated at 4 ˚C for 30 min. The lysates were centrifuged at 14,000 revolutions per min for 15 min at 4˚C. Protein concentrations of cell lysates were determined with Bradford protein assay (Bio-Rad, Richmond, USA) and equivalent amounts were loaded onto 7.5～15% SDS/PAGE. The gels were transferred to Nitrocellulose membrane (Amersham Pharmacia Biotech, UK) and reacted with each antibody. Immunostaining with antibodies was performed using SuperSignal West Pico enhanced chemiluminescence substrate and detected with LAS-3000PLUS (Fuji Photo Film Company, Japan). Equivalent protein loading was confirmed by Ponceau S staining.

7. Confocal microscopy

To show mitochondria, cells were harvested and the prewarmed (37˚C) and Mitotracker-containing medium (450 nM) was then added. Cells were incubated at 37 ˚C for 15 min, replaced with fresh prewarmed medium and incubated at 37˚C for 30 min. Immunofluorescent staining using antibodies to p53 (whole or phosphorylated) were undertaken as above. Fluorescent images were observed and analyzed under Zeiss LSM 510 laser-scanning confocal microscope (Gettingen, Germany).

8. Mitochondrial fractionation

Mitochondria were prepared by sucrose density gradients as described. Briefly, cells (5×10^7) were washed in TD buffer (135 mM NaCl, 5 mM KCl, 25

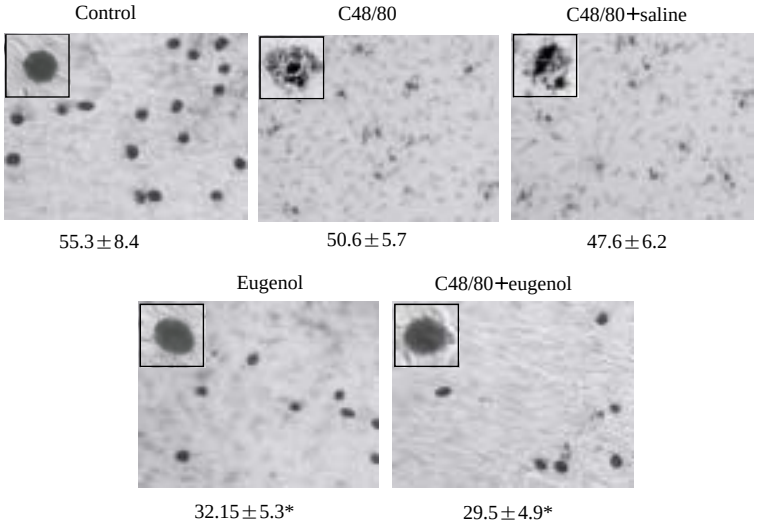

Fig. 1. Eugenol injection into rat peritoneal cavity reduced the density of mast cells. Micrographs showing the density of the mesenteric mast cell. The number below each micrograph is the mean±SD of the means of the mast cell number in 250 microscopic field (obtained from 12 microscope slides per each animal). Four animals were used for each group. Eugenol decreased the density of mast cells compared to the control (*, $p < 0.05$). Analyzed by nonparametric Kruskall-Wallis test. Insets demonstrate the higher magnification of representative mast cells from each group (original magnification × 800). Although most mast cells are degranulated in compound 48/80 treated group, eugenol pretreatment prevented degranulation. Original magnification, × 250

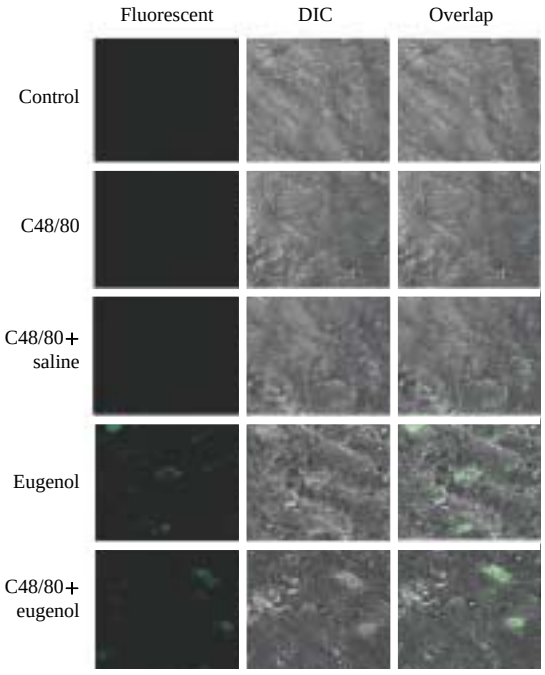

Fig. 2. Microscopy of the flat mounted rat mesentery. Left panels, fluorescent micrographs. Middle panels, micrographs under DIC optics. Right panels, overlap images. Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) positive cells are observed in eugenol treated group. Original magnification × 800

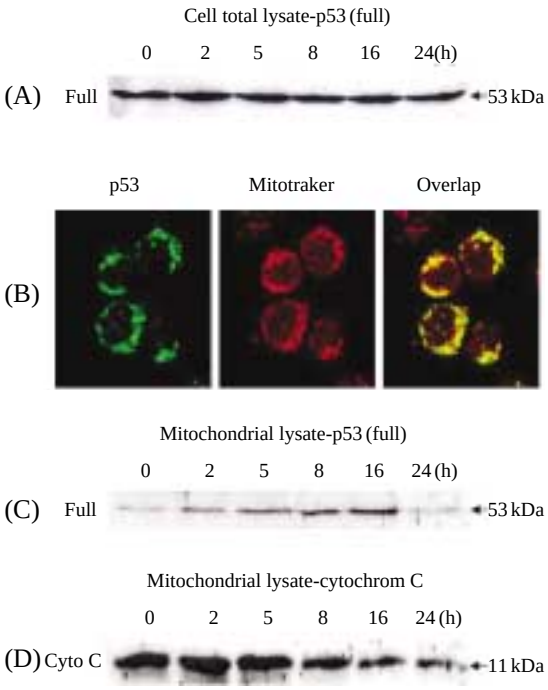

Fig. 3. Eugenol treatment induced the translocation of p53 into mitochondria of RBL-2H3 cells, which preceded the release of cytochrome c from mitochondria. (A) Eugenol treatment did not show significant changes in the level of p53 at various time points. (B & C) Translocation of p53 into mitochondria after eugenol treatment. (B) Confocal microscopy and (C) Western blot using the antibody to full-length p53. The amount of full length p53 in mitochondrial fraction was increased in a time dependent fashion. (D) The time-dependent decrease of cytochrome c in mitochondrial fraction showing the release of cytochrome c into cytosol.

mM Tris-Cl, pH 7.6) and allowed to swell for 10 min in ice-cold hypotonic CaRSB buffer (10 mM NaCl, 1.5 mM $CaCl_2$, 10 mM Tris-Cl, pH 7.5). Cells were Dounce-homogenized with 60 strokes, and MS buffer (210 mM mannitol, 70 mM sucrose, 5 mM EDTA, 5 mM Tris, pH 7.6) was added to stabilize mitochondria (2 mL of 2.5 × per 3 mL of homogenate). After removing nuclear contaminants (2 × 3,000 rpm for 15 min), the supernatant was layered over a 1 ~ 2 M sucrose step gradient (in 10 mM Tris, pH 7.6, 5 mM EDTA, 2 mM dithiothreitol, protease inhibitors) and spun at 4°C for 30 min at 22,000 rpm (110,000 g maximum). Mitochondria were collected at the 1 ~ 1.5 M interphase by lateral suction through the tube, washed (in 4 volumes of MS buffer, 15,000 rpm), resuspended in a final volume of 200 μL of MS buffer, and used for all assays.

9. Effect of eugenol on several cell lines with different p53 status

Apoptosis inducing activity of eugenol was tested in several different cell lines with different p53 status. p53-deficinet cells (PC-3), cells with mutant p53 (p815 and HT-29) and cells with wild type p53 (K562, A549, MCF-7) were used.

10. Statistical analysis

The results of the experimental and control groups were tested for statistical significance by the nonparametric Kruskal-Wallis test. For *in vitro* experiment, four independent experiments were carried out. Statistical results were expressed as the mean ± the standard deviation of the means obtained from triplicates of each independent experiment. In all cases, a p value estimated less than 0.05 was considered significant.

Results

1. Eugenol injection into rat peritoneal cavity decreased the density of mesenteric mast cells

Not only eugenol pretreatment prevented the degranulation of mast cells by compound 48/80, it also decreased the density of mesenteric mast cells. These data support that the survival of animals even after administration of the fatal dose of compound 48/80 might at least partly result from the decreased number of mast cells by eugenol pretreatment (Fig. 1).

2. Eugenol induced apoptosis in mesenteric mast cells

To examine that the decreased number of mast cells in the mesentery resulted from the induction of apoptosis, TUNEL assay was conducted. Numerous TUNEL positive cells were observed in eugenol treatment groups without pretreatment of compound 48/80 (Fig. 2).

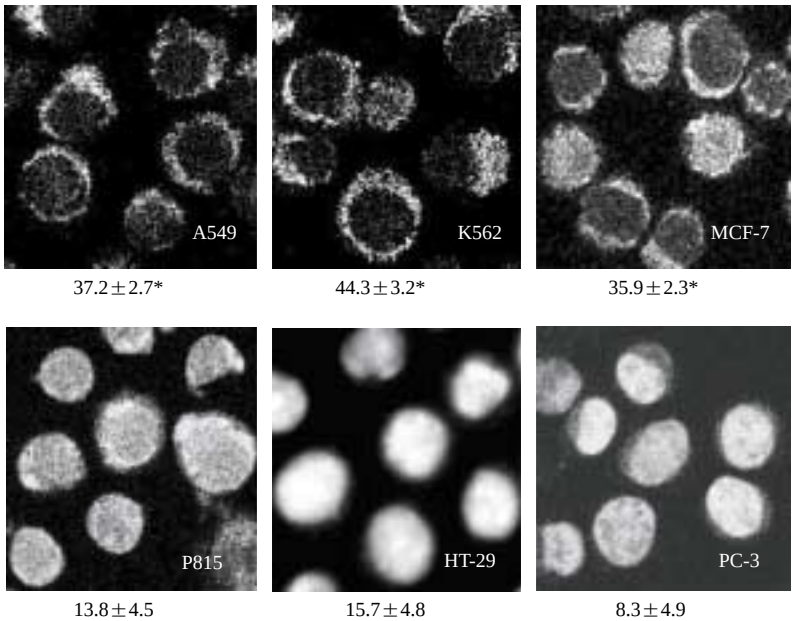

Fig. 4. Eugenol induced apoptosis and the translocation of p53 into mitochondria in cells having wild type p53. Eugenol induced apoptosis and the translocation of p53 in those cells having wild type p53 (A549, K562 and MCF-7) (* $p<0.05$). In cells having mutant type p53 (p815 and HT-29), apoptosis was induced but not significantly ($p>0.05$) and the translocation of p53 was not observed. Eugenol did not induce apoptosis in the p53-deficient cancer cells (PC-3) ($p>0.05$) and the translocation of p53 was not observed. The number below each micrograph is the mean ± SD of the means of apoptotic cells as determined by Hoechst staining. Four independent assays were performed and data shown are the mean ± SD of the means obtained from triplicates of each experiment. Analyzed by nonparametric Kruskall-Wallis test.

3. Eugenol induced the translocation of p53 into mitochondria of RBL-2H3 cells, which preceded the release of cytochrome c from mitochondria

Although significant alteration in the level of full length p53 was not observed, its translocation into mitochondria was observed. Western blot analysis of mitochondrial lysate showed that eugenol induced the translocation of full length p53 into mitochondria. The translocation of full length p53 into mitochondria became evident within 2 h after eugenol treatment. The release of cytochrome c from mitochondria was not evident until 5 h after eugenol treatment. These data supports that the translocation of p53 into mitochondria preceded cytochrome c release (Fig. 3).

4. Eugenol induced apoptosis and the translocation of p53 into mitochondria in cancer cells with wild type p53

Eugenol induced the translocation of p53 into mitochondria in cells with wild type p53. Eugenol induced the translocation of p53 and apoptosis in those cells with wild type p53 (A549, K562 and MCF-7). In cells with mutant type p53 (p815 and HT-29), apoptosis was slightly induced and the translocation of p53 was not observed. Eugenol did not induce the translocation of p53 into mitochondria and apoptosis in the p53-deficient cancer cells (PC-3) (Fig. 4).

5. Eugenol increased phospho-ser 15-p53 and induced the translocation of phospho-ser 15-p53 into mitochondria in cancer cells with wild type p53

Western blot was conducted using total cell lysate, which showed the increased level of phospho-ser 15-p53 in cells with wild type p53 (A549, K562 and MCF-7) (Fig. 5A). Confocal microscopy proved that phospho-ser 15-p53 was translocated into mitochondria in those cells (Fig. 5B).

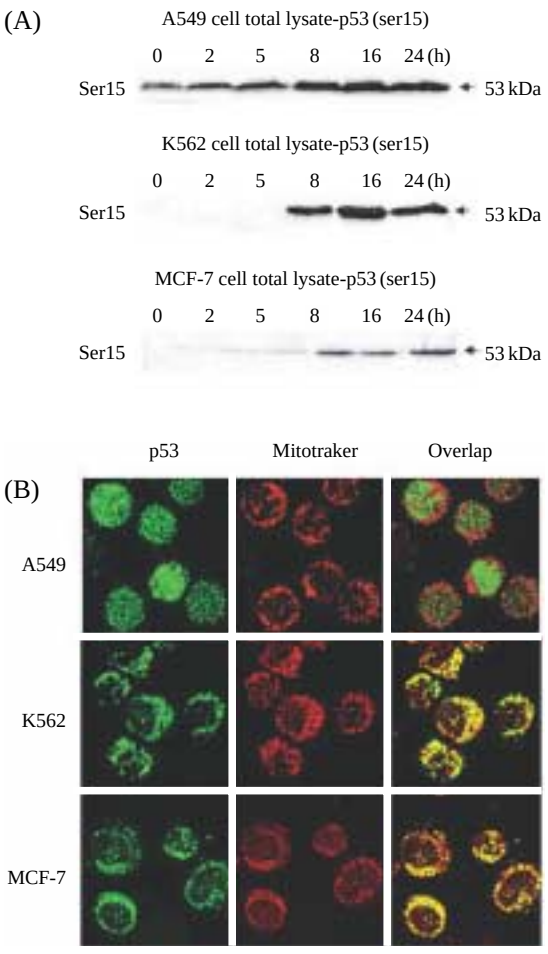

Fig. 5. (A) Western blots demonstrating the time-dependent increase of phospho-ser 15-p53 in total cell lysate after eugenol treatment. (B) Eugenol induced the translocation of phospho-ser 15-p53 into mitochondria of the cells with wild type p53 (A549, K562 and MCF-7).

Discussion

Herbal medicine use is on the rise. The greater acceptance of herbal medicine by healthcare professionals and the public results partly from lively addressing

of their pharmacological mechanism. Numerous studies showed that individual herbal medicines had various pharmacological activities, e.g. antiallergic, antipyretic, analgesic, antiinflammatory and anticancer effects.

Irrespective of numerous *in vitro* and *in vivo* studies, action mechanism of most herbal medicines remains elusive. For exploration of their pharmacological mechanism, the total extracts (Kim et al. 1998, Kim et al. 1999, Park et al. 2001, Na et al. 2002, Kim et al, 2003) or the identified active compounds (Huang et al. 1991, Huang et al. 1994, Rao et al. 1995, Lin et al. 1997, Arbiser et al. 1998, Cao and Cao 1999, Bai et al. 2003) were used. Previous studies showed that pharmacological mechanisms of total extracts such as SAFB and MF depended on their modulation of the activation (Kim et al. 1998, Kim et al. 1999) or demise (Park et al. 2001, Kim et al. 2003) of mast cells. Eugenol is a naturally occurring phenolic compound used as a food flavour and fragrance agent and the main component of oil of clove and the essential oils or extracts of numerous plants (IARC Monographs 1985, Zhu 1996). It have been identified that eugenol inhibited immediate hypersensitivity by inhibition of histamine release from mast cells *in vivo* and *in vitro* (Kim et al. 1997, Shin et al. 1997). In this context, we undertook the present study, which showed that eugenol induced apoptosis of mast cells *in vitro* and *in vivo*. These data suggest that clinical effects of SAFB and MF for the management of asthma and various allergic disorders may partly result from apoptosis-inducing activity in mast cells by eugenol.

It is now widely appreciated that agents capable of inducing apoptosis in cancer cells potentially lead to the development of mechanism-based prevention and treatment approaches for cancer. Natural products also contain a variety of chemopreventive compounds that have been shown to prevent the development of malignancies (Rao et al. 1995, Lin et al. 1997) or to inhibit angiogenesis (Huang et al. 1991, Huang et al. 1994, Cao and Cao 1995, Arbiser et al. 1998, Bai et al. 2003). The data of the present study suggest the possibility of chemoprevention and chemotreatment by eugenol. However, since resistance of cancer cells to chemotherapeutic agents is correlated with p53 stataus and furthermore, p53 plays an important role in eugenol-induced apoptosis, p53 status of cancer cells is the primarily important factor in considering eugenol as chemopreventive or chemotreatment agent. Although our study was conducted in a limited type of cancer cells, eugenol evidently induced apoptosis only in cancer cells with wild type p53. Our understanding obtained from this study may be useful for development for cancer prevention and/or therapy.

Current evidence suggests that p53 induces apoptosis by a multitude of molecular pathways (Harris 1996, Levine 1997, Gottlieb and Oren 1998). The p53 can mediate apoptosis by transcriptional activation of proapoptotic genes like the BH3-only proteins Noxa and Puma, Bax, p53 AIP1, Apaf-1, and PERP and by transcriptional repression of Bcl-2 an IAPs (Nakano and Vousden 2001, Johnstone et al. 2002). In addition, it was known that p53 could mediate apoptosis in the absence of any gene transcription or translation. And several studies support that translocation of p53 into mitochondria influences the downstream events (Caelles et al. 1994, Wagner et al. 1994). A study elucidated that p53 protein trafficked to mitochondria and that the translocation of p53 to mitochondria was rapid and preceded changes in MMP, cytochrome c release and procaspase-3 activation (Marchenko 2000). Furthermore, a recent study showed that targeting p53 to mitochondria was sufficient to launch apoptosis (Mihara et al. 2003). Also, in our study, the translocation of p53 into mitochondria and a subsequent downstream event such as cytochrome c release were observed.

The role of p53 translocated into mitochondria was recently dissected in detail (Mihara et al. 2003, Park et al. 2005). A study proved that p53 formed a specific complex with Bcl-2 and Bcl-xL. Through structure/

function analysis the study proved that the interaction between p53 and Bcl-2 or Bcl-xL abolished the function of Bcl-2 and Bcl-xL as survial factors. The study also showed that p53/Bax complex was not detected, and explained that the absence of p53/Bax complex promoted apoptosis judging from that mitochondrial p53 and Bax were proven to promote apoptosis in a functionally synergistic manner.

p53 has a short half-life, and the proapoptotic function of p53 is achieved by increased expression at the transcriptional level and by post-translational stabilization of the protein by escaping from ubiquintin-dependent degradation. Phosphorylation of p53 at multiple sites is the main post-translational modification that is regulated by several different protein kinases depending on types of cells and extracellular stimuli.

Taken collectively, eugenol induces apoptosis of mast cells via a phospho-ser 15-p53 dependent fashion.

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Eugenol에 의해 세포자멸사를 보이는 세포 배양된 다양한 암세포들에서의 phospho-ser 15-p53의 이동

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간추림 : 신이(*Magnoliae flos*)나 정향(clove) 같은 풀잎의 주성분인 eugenol (4-allyl-2-methoxyphenol)은 p53 단백질 경로를 통해서 비만세포의 세포자멸사(apoptosis)를 유도한다고 알려져 있다. 본 연구는 eugenol의 생체내에서의 효과와 다른 유형의 p53 단백질을 발현하는 여러 가지의 암세포에서의 eugenol 유도 세포자멸사의 분자생물학적인 기전을 알기 위해서 수행되었다.

배안 비만세포에 대한 eugenol의 효과를 알기 위해서 쥐을 이용한 아나필락시스 실험을 시행하였고 세포자멸사를 일으키는 비만세포를 관찰하기 위해서는 TUNEL 염색법을 사용하였다. 그리고 여러 가지의 암세포들에 eugenol을 적용한 후, Western blot 분석, 세포면역화학염색, 공초점레이저주사현미경 검경, 사립체분리 등을 시행하였다.

생체내에서의 실험에서 eugenol은 창자간막(mesentery)의 비만세포에 세포자멸사를 유도하여, 비만세포의 밀집도를 감소시켰다. 비록 eugenol이 p53의 발현을 증가시키지는 못했지만, eugenol은 p53을 사립체로 이동시키고, 이동된 후 연이어서 cytochrome c를 사립체에서 세포질로 유리시키는 것이 관찰되었다. 그리고 eugenol은 wild type p53을 가지고 있는 암세포들에서는 phospho-ser 15-p53을 증가시켰으나, mutant p53 혹은 p53 결핍 암세포들에서는 phospho-ser 15-p53의 증가는 없었다. 더 나아가서 wild type p53을 가지고 있는 암세포에서 사립체로 이동한 p53이 ser 15에 인산화되는 것이 관찰되었다.

본 연구는 eugenol이 wild type p53을 가진 암세포에서 phospho-ser 15-p53의 세포내 위치이동을 통해 세포자멸사를 유도하다는 증거를 제시하였다. 더 나아가서 wild type p53을 가진 암세포의 항암효과는 phospho-ser 15-p53의 의존적 방법을 통한 세포자멸사를 유도하는 eugenol의 약리학적 효과가 관계할 수 있다는 것을 추정할 수 있다.

찾아보기 낱말 : phospho-ser 15-p53, eugenol, 세포자멸사, 비만세포

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