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Phospho-ser 15-p53 translocates into mitochondria and interacts with Bcl-2 and Bcl-xL in eugenol-induced apoptosis

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Our previous studies demonstrated that antiallergic effects of herbs such as clove and *Magnoliae Flos* (MF) resulted from the induction of apoptosis in mast cells. We here examined whether the antiallergic activity was caused by eugenol (4-allyl-2-methoxyphenol) which was one of major ingredients in the essential oils or extracts of numerous plants including clove and *Magnoliae Flos*. RBL-2H3 cells were treated with eugenol, and DNA electrophoresis, Western blotting, immunocytochemistry, confocal microscopy and immunoprecipitation were conducted. Effect of eugenol was tested using a rat anaphylaxis model. RBL-2H3 cells treated with eugenol showed typical apoptotic manifestations and translocation of p53 into mitochondria. Antisense p53 partially prevented the induction of apoptosis. Noticeably, we observed that p53 translocated into mitochondria was phosphorylated on ser 15. Phospho-ser 15-p53 physically interacted with Bcl-2 and Bcl-xL in mitochondria and its translocation into mitochondria preceded cytochrome c release and mitochondrial membrane potential (MMP) reduction. We also depicted that the survival of animals even after administration of the fatal dose of compound 48/80 might result from the decreased number of mast cells by eugenol pretreatment. In conclusion, eugenol induces apoptosis in mast cells via translocation of phospho-ser 15-p53 into mitochondria.

Keywords: apoptosis; eugenol; mast cell; Phospho-ser 15-p53.

The contribution of Yeon Suk Song to the manuscript is equal to that of Bong Soo Park.

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Introduction

Apoptosis is implicated also in a wide range of pathological conditions, including immunological diseases, allergy and cancer.[1,2] During apoptosis, cells undergo specific morphological and biochemical changes, including cell shrinkage, chromatin condensation, and internucleosomal cleavage of genomic DNA.[3] Multiple lines of evidence indicate that apoptosis can be triggered by the activation of caspase.[4] In addition, mitochondria are known to be central death regulators in response to several apoptotic stimuli.[5]

Mast cells are granule-containing secretory cells that play an essential role in immediate-type allergic and inflammatory reactions. 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.[6,7] Mast cell numbers in tissues under normal conditions are relatively constant from individual to individual, but rapidly proliferate, 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.[8]

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.[9] Therefore, inducing apoptosis of mast cells in tissue could be used therapeutically for various pathological conditions and understanding the

mechanism is important. Our previous studies represent that clinical effects of Szygium 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.[10,11]

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,[12,13] had the activity regulating mast cell death. Our observations show that eugenol induces apoptosis in RBL-2H3 cells and that phospho-ser 15-p53 translocates into mitochondria and interacts with Bcl-2 and Bcl-xL.

Materials and methods

Reagents

The following reagents were obtained commercially: Rabbit polyclonal anti-human Bcl-xL, Bcl-2, p53 (full-length) and caspase-3 and anti-horse cytochrome c, and mouse monoclonal anti-human Bax antibodies were from Santa Cruz Biotechnology (Santa Cruz, California); Mouse polyclonal anti-human Poly(ADP)-Ribose Polymerase (PARP) and rabbit polyclonal anti-human phospho p53 (Ser 15) antibodies were from Oncogene (Cambridge, MA); FITC-conjugated goat anti-rabbit and horse anti-mouse IgGs were from Vector (Burlingame, CA); HRP-conjugated donkey anti-rabbit and sheep anti-mouse IgGs were from Amersham Pharmacia Biotech (Piscataway, NJ). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide (JC-1) and Mitotracker were from Molecular Probes (Eugene, OR). p53 antisense oligonucleotide was from Calbiochem (San Diego, CA); Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). Dulbecco's modified Eagle's medium (DMEM) and Fetal bovine serum (FBS) were from Gibco (Gaithersburg, MD). Eugenol and compound 48/80 were from Sigma (St. Louis, MO); SuperSignal West Pico enhanced chemiluminescence Western blotting detection reagent was from Pierce (Rockford, IL).

Cell culture

RBL-2H3 basophilic leukemia cells were purchased from the ATCC (Rockville, Maryland). 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 Balanced Salt Solutions (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.

Eugenol treatment and assessment of cell viability

Twenty four hours after RBL-2H3 cells were subcultured, the original medium was removed. Cells were washed with Phosphate buffered saline (PBS) and then incubated in the same fresh medium. Eugenol from a stock solution was added to the medium to obtain 500–900 μM dilutions of the drug. Cells treated with various concentration of 500–900 μM eugenol were harvested 24 h after treatment, stained with trypan blue and then counted by a blind observer using a hemacytometer. Because the dose required for half-maximal inhibition of viability was $\sim$700 μM, this single concentration was utilized for viability assay at various time points and further assessment of apoptosis.

Hoechst staining

Cells were harvested and cell suspension was centrifuged onto a clean, fat-free glass slide with a cytocentrifuge. The samples were stained in 4 μg/ml Hoechst 33342 for 30 min at 37°C and fixed for 10 min in 4% paraformaldehyde.

DNA electrophoresis

2×10^6 cells were resuspended in 1.5 ml of lysis buffer [10 mM Tris (pH 7.5), 10 mM EDTA (pH 8.0), 10 mM NaCl and 0.5% SDS] into which proteinase K (200 μg/ml) was added. After samples were incubated overnight at 48°C, 200 μl of ice cold 5 M NaCl was added and the supernatant containing fragmented DNA was collected after centrifugation. The DNA was then precipitated overnight at $-$ 20°C in 50% isopropanol and RNase A-treated for 1 h at 37°C. The DNA from $1 \times$ or 2×10^6 cells (15 μl) was equally loaded on each lane of 2% agarose gels in Tris-acetic acid/EDTA buffer containing 0.5 μg/ml ethidium bromide at 50 mA for 1.5 h.

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 14000 revolutions per min for 15 min at 4°C. Protein concentrations of cell lysates were determined with Bradford protein assay (Bio-Rad, Richmond, CA) and 50 μg of proteins were loaded onto 7.5–15% SDS/PAGE. The gels were transferred to Nitrocellulose membrane (Amersham Pharmacia Biotech,

Piscataway, NJ) 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, Kanagawa, Japan).

Assay of mitochondrial membrane potential (MMP)

The potential-sensitive fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide JC-1 was added directly to the cell culture medium (1 μM final concentration) and incubated for 15 min. The medium was then replaced with PBS, and cells were resuspended in 10 g/ml of methanol and incubated at 37°C for 30 min. Flow cytometry to measure MMP was performed on a Epics XL (Beckman Coulter, FL, USA). Data were acquired and analyzed using EXPO32 ADC XL 4 color software. The analyzer threshold was adjusted on the FSC channel to exclude noise and most of the subcellular debris.

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.

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 cell showing condensed or fragmented nuclei in Hoechst staining and cytochrome c release in immunocytochemical staining was calculated under epifluorescence optics by a blind observer.

Confocal microscopy

To show mitochondria, cells were harvested and Mitotracker-containing medium (450 nM) was then added. 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 (Göettingen, Germany).

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 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 CaCl2, 10 mM Tris-Cl, pH 7.5, protease inhibitors). 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. 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.

p53 antisense oligonucleotide treatment

A 20-meric phosphorothioate antisense oligonucleotide reducing the expression of mutant and normal p53 protein were used: antisense primer (5′-CCCTGCTCCCCCCTGGCTCC-3′); sense strand primer (5′-GGAGCCAGGGGGGAGCAGGG-3′). Before apoptosis assay, cells were transfected as follows: Equal volumes of 1.6 μg oligonucleotide and 4 μl lipofectamine in Opti-MEM were mixed. Fifty percent confluent cells were washed with serum-free growth medium and lipofectamine reagent-oligonucleotide complexes were overlaid onto cells. After incubation of 6 h, culture medium was replaced with DMEM-10% FBS. Cells were incubated for 24 h and treated with 700 μM eugenol. Amount of apoptosis was determined by Hoechst staining.

Immunoprecipitation

Cell extracts were incubated with phosphorylated p53 (Ser 15) antibody in extraction buffer at 4°C overnight. The immuno-complexes were precipitated with protein A-Sepharose beads (Sigma) for 2 h, and washed five times with extraction buffer prior to boiling in SDS sample buffer. Immunoprecipitated proteins or aliquots containing 40 μg protein were separated on SDS-polyacrylamide gels, and Western blot analysis was performed as described.

Assessment of induction of apoptosis in peritoneal mast cells by eugenol

Wistar rats 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). One hour after the injection of eugenol the mast cell degranulator compound 48/80 (8 g/kg body weight) was administered peritoneally. 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. Twenty min after treatment of compound 48/80, the mesentery was removed and 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 divided into several pieces and mounted on a glass slide. Mast cells showing metachromasia were counted in 12 microscopic fields (250× magnification) under Axiophot microscope (Zeiss, Gettingen, Germany) per each animal by a blind observer. Rats were used after our college ethics committee approved that our protocol fulfilled the guide of animal experience established by The Korean Academy of Medical Sciences.

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.

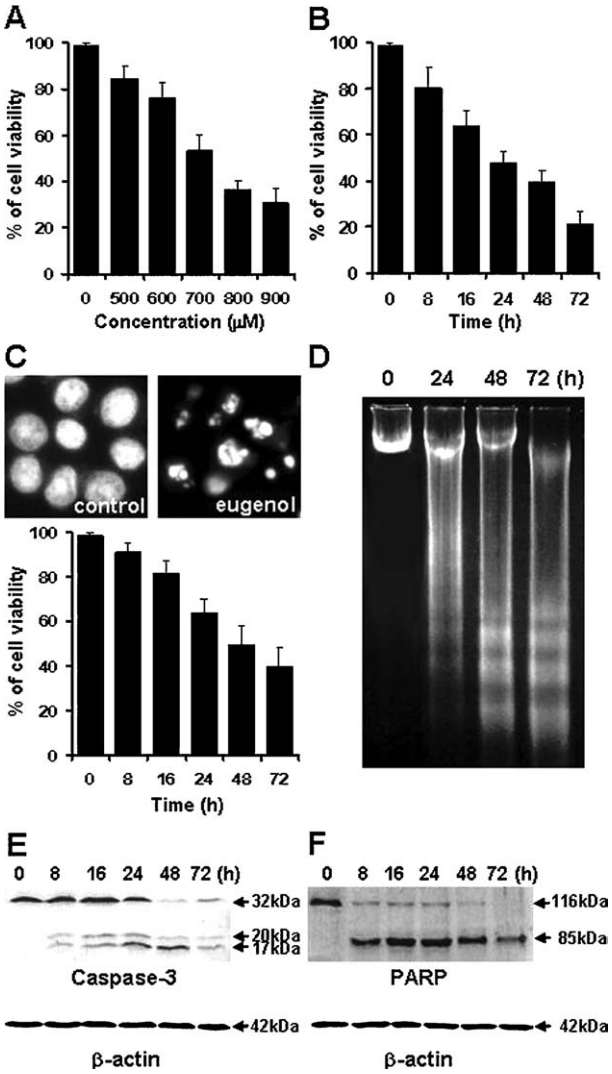


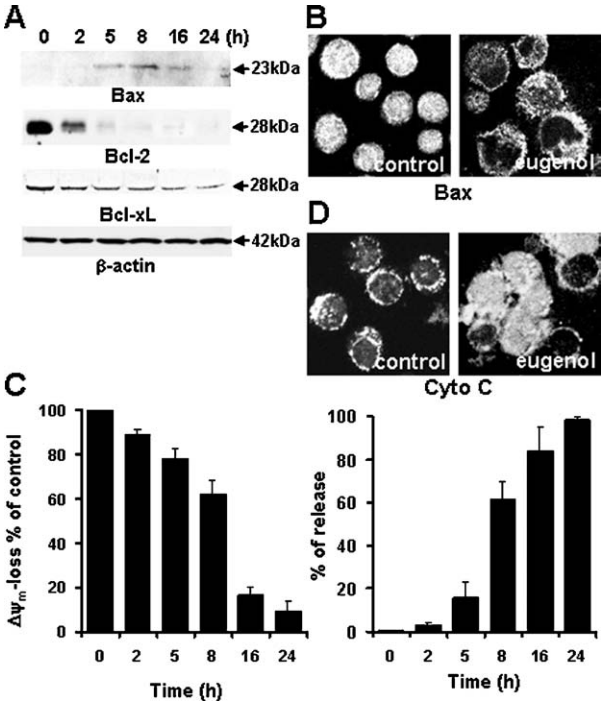
Figure 1. Eugenol induces apoptosis in RBL-2H3 cells. (A) Concentration-dependent decrease in viability (500–900 μM, $p < 0.05$). (B) Time-dependent decrease in viability (0–72 h, $p < 0.05$). (C) Demonstration of condensed and fragmented nuclei and time-dependent increase of apoptosis (0–72 h, $p < 0.05$). (D) Demonstration of DNA ladder. (E) Activation of caspase-3. (F) Demonstration of PARP 85 kDa cleavage products.

Results

Eugenol induces apoptosis in RBL-2H3 cells

Cells treated with various concentrations of eugenol (500–900 μM) were harvested 24 h after treatment, stained with trypan blue and then counted using a hemacytometer. Eugenol reduced viability of RBL-2H3 cells in a concentration dependent manner (Figure 1A). Because the dose required for half-maximal inhibition of viability was ~700 μM, this single concentration was utilized for viability assay at various time points (0, 8, 16, 24, 48 and 72 h after treatment). Eugenol reduced viability of RBL-2H3 cells in a time dependent manner (Figure 1B). We also studied whether eugenol could decrease the viability of primary cultured mast cells. In parallel with the result of RBL-2H3 cell line, eugenol decreased the viability also in the bone marrow-derived mast cells dose- and time-dependently (data not shown). RBL-2H3 cells displayed condensed and fragmented nuclei and the percentage of dead or dying cells, as determined by nuclear morphology, was increased in a time-dependent manner (Figure 1C). DNA ladder was also demonstrated and activation of caspases-3 was shown (Figure 1D and E). Eugenol treatment also showed the PARP 85 kDa cleavage product (Figure 1F). These

Figure 2. Mitochondrial pathway is involved. (A) Increase of Bax-to-Bcl-2/Bcl-xL ratios (5–16 h). (B) Ttranslocation of Bax into mitochondria. (C) Time-dependent reduction of MMP (5–24 h, $p < 0.05$). (D) Eugenol caused the release of cytochrome c from mitochondria into the cytosol in a time-dependent manner compared to the control (5–24 h, $p < 0.05$).

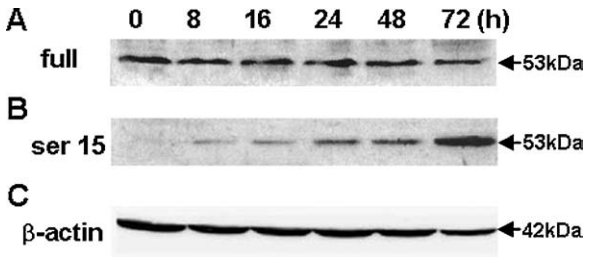


data reveal that eugenol induces apoptosis in RBL-2H3 cells.

Eugenol induces apoptosis in RBL-2H3 cells via mitochondrial pathway

We also examined whether mitochondrial events were involved in eugenol-induced apoptosis. Alterations in protein contents of the proapoptotic factor Bax and antiapoptotic factors Bcl-2 and Bcl-xL were assayed by Western blot analysis. Bax level was shown to increase at 5 to 16 h after eugenol treatment and thereafter it decreased (Figure 2A). On the other hand, the expression level of antiapoptotic factors Bcl-2 or Bcl-xL remained only at the early time points (0–2 h) and decreased at later time points (8–24 h). Immunofluorescent staining showed that Bax was concentrated in a punctate pattern after treatment with eugenol, which assumed that Bax had translocated into mitochondria (Figure 2B). Loss of mitochondrial membrane potential ($\Delta\Psi m$) is known to be a common event in many pathways of apoptosis induction. In this study, the potential-sensitive fluorescent probe JC-1 was employed to detect loss of $\Delta\Psi m$. Membrane potential was reduced after treatment with eugenol (Figure 2C). In

Figure 3. Eugenol treatment significantly increased phosphorylation of p53 on serine 15. (A) Western blot analysis of full-length p53 and (B) phospho-ser 15-p53.



the immunofluorescent study, cytochrome c in the control cell was found in a punctate pattern, in keeping with its normal mitochondrial location. However, it had assumed a diffuse distribution in the eugenol-treated cells, which supported that eugenol led to the release of cytochrome c from mitochondria into the cytosol (Figure 2D). These data support that eugenol induces apoptosis in RBL-2H3 cells via mitochondrial pathway.

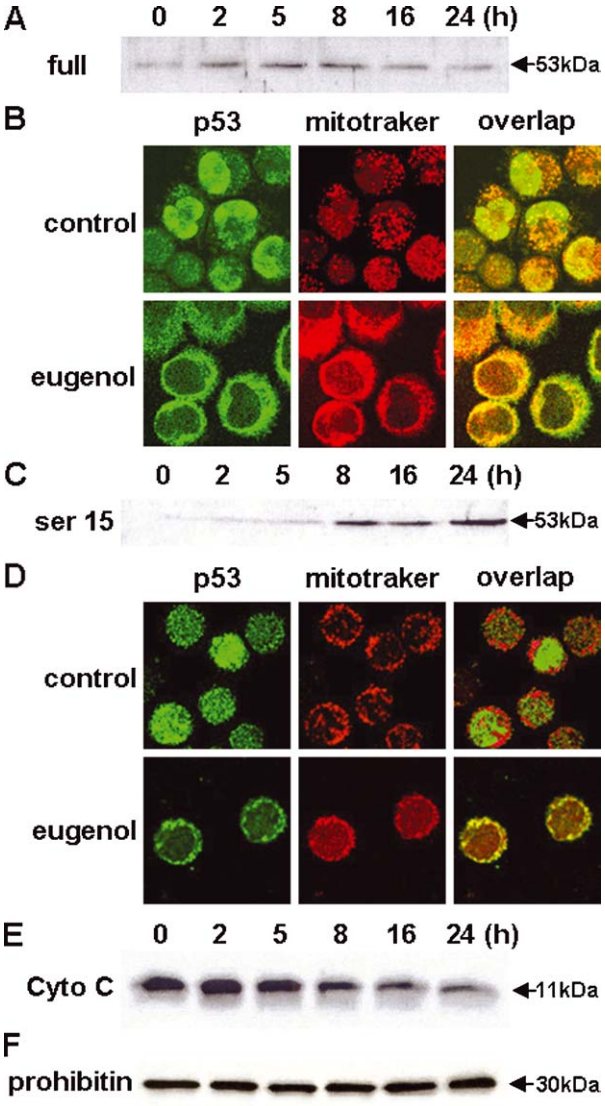
Eugenol causes significantly increased phosphorylation of p53 on serine 15

Next, we determined whether p53 was implicated in eugenol-induced apoptosis of RBL-2H3 cells. Western blot analysis using the antibody to full-length p53 elucidated no prominent changes in the amount of expressed p53 (Figure 3A). However, western blot analysis using phosphorylation site-specific antibodies showed that eugenol treatment caused significantly increased phosphorylation of p53 on serine 15 at the C-terminus (Figure 3B).

Translocation of phopho-ser 15-p53 into mitochondria precedes the reduction of MMP and the release of cytochrome c from mitochondria

We asked further whether p53 translocated into mitochondria. Western blot analysis and confocal microscopy using the antibody to full-length p53 demonstrated that eugenol treatment caused the translocation of p53 into mitochondria (Figure 4A and B). Importantly, Western blot analysis and confocal microscopy using phosphorylation site-specific antibodies showed that eugenol induced the translocation of phospho-ser 15-p53 into mitochondria (Figure 4C and D). The translocation of phospho-ser 15-p53 into mitochondria became evident within 2 h after eugenol treatment (Figure 4C). The reduction of MMP and the release of cytochrome c from mitochondria were not evident until 5 h after eugenol treatment (Figures 2C, D and 4E). These data supports that the translocation of

Figure 4. Translocation of phopho-ser 15-p53 into mitochondria preceded the release of cytochrome c from mitochondria. For western blotting, mitochondrial fraction was used. (A–D) Translocation of p53 into mitochondria. (A–B) Western blot (A) and confocal microscopy (B) using the antibody to full-length p53. (C–D) Western blot (C) and confocal microscopy (D) using the antibody to anti-phospho p53 (ser 15). (E) The time-dependent decrease of cytochrome c in mitochondria.

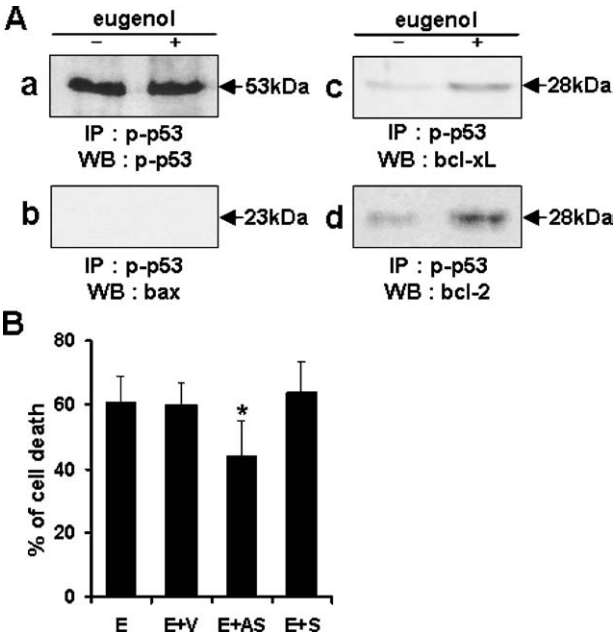


phospho-ser 15-p53 into mitochondria preceded MMP reduction and cytochrome c release.

Phospho-ser 15-p53 physically interacts with Bcl-2 in mitochondria

To prove that phospho-ser 15-p53 physically interacted with antiapototic factors Bcl-2 or Bcl-xL, Western blot analysis after immunoprecipitation were undertaken,

Figure 5. Phospho-ser 15-p53 physically interacted with Bcl-2 and Bcl-xL in mitochondria. For western blotting, mitochondrial fraction was used. (A) Western blot assays after immunoprecipitation with phospho-ser 15-p53 showing that phospho-ser 15-p53 interacted both with Bcl-2 and Bcl-xL but not with Bax. (B) Prevention of apoptosis by p53-specific antisense oligonucleotide transfection ($p < 0.05$). E, eugenol. V, vehicle. AS, antisense. S, sense.

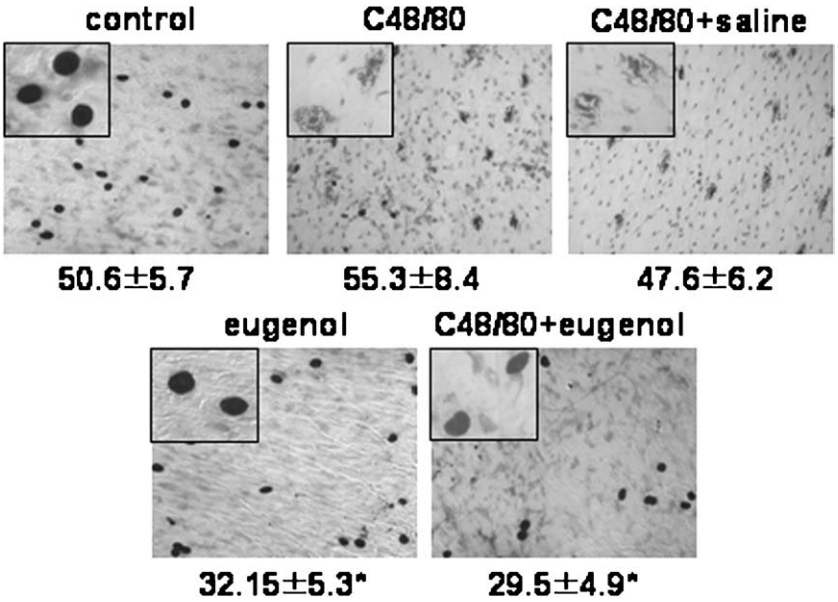


which elucidated that phospho-ser 15-p53 interacted both with Bcl-2 and Bcl-xL. On the other hand, phospho-ser 15-p53 did not interact with Bax (Figure 5A). To the end of this *in vitro* study using RBL-2H3 cells, we examined whether blocking p53 prevented the induction of eugenol-induced apoptosis in mast cells. Expectedly, p53-specific antisense oligonucleotide prevented eugenol-induced apoptosis of RBL-2H3 cells (Figure 5B). These data suggest that phospho-ser 15-p53 plays a pivotal role in eugenol-induced apoptosis of RBL-2H3 cells.

Eugenol injection into rat peritoneal cavity decreases the density of mesenteric mast cells

To extend our understanding of the pharmacological activity of eugenol, an *in vivo* study was conducted. 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 (Figure 6).

Figure 6. Micrographs showing the density of the mesenteric mast cell. Eugenol decreased the density of mast cells compared to the control ($p < 0.05$). 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).



Discussion

Although numerous studies showed that individual herbal medicines had various pharmacological activities, *e.g.* antiallergic, antipyretic, analgesic, antiinflammatory and anticancer effects, action mechanism of most herbal medicines remains elusive. For exploration of their pharmacological mechanism, the total extracts[10,11] or the identified active compounds,[14,15] were used. Our previous studies showed that pharmacological mechanisms of total extracts such as SAFB and MF depended on their modulation of the activation or demise[10,11] of mast cells. We, in the past studies, used eugenol, a naturally occurring phenolic compound used as a food flavor and fragrance agent and the main component of oil of clove and the essential oils or extracts of numerous plants.[12,13] Those studies demonstrated that eugenol inhibited immediate hypersensitivity by inhibition of histamine release from mast cells *in vivo* and *in vitro.*[16,17] 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.

Current evidence suggests that p53 induces apoptosis by a multitude of molecular pathways. p53 can mediated 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.[18,19] However, it was known that p53 mediate apoptosis in the absence of any gene transcription or translation.[20,21] A few past studies delineated that translocation of p53 into mitochondria influenced the downstream events.[20] Moreover, 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.[21] Increased expression of p53, its translocation into mitochondria and subsequent downstream events such as reduction of MMP and cytochrome c release were also observed in our study.

However, the role of p53 in mitochondria had not been dissected in detail before a recent study. The study showed that targeting p53 to mitochondria was sufficient to launch apoptosis.[22] Furthermore, it 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 survival 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. Thus, we assume that p53 tranlocated into mitochondria after eugenol treatment interacts with Bcl-2 or Bcl-xL but not with Bax, which promote apoptosis as explained above. However, the molecular mechanism explaining the role of p53 in

mitochondria is still largely unknown. Considering that elucidating the role of p53 out of nucleus is an upcoming important challenging task,[23] further future studies are needed.

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. Although p53 phosphorylation has been shown to influence p53 activity under physiological conditions, the phosphorylation status of p53 targeted into mitochondria in apoptosis has not been determined to date. We, for the first time, revealed that the phosphorylated p53 was translocated into mitochondria in eugenol-induced apoptosis, and presented that p53, which moved into mitochondria, was phosphorylated on serine 15 at the C-terminus.

Furthermore, we observed that phospho-ser 15-p53 interacted with Bcl-2 and Bcl-xL but not with Bax. Those data extend our understanding of the posttranslational status of p53 targeted into mitochondria in apoptosis and suggest that the presence of phospho-ser 15-p53/Bcl-2 or Bcl-xL as well as the absence of phospho-ser 15-p53/Bax triggers the downstream events in eugenol-induced apoptosis in mast cells.

Our *in vivo* data also suggest 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. Moreover, we observed that considerable percentage of peritoneal mast cells was TUNEL-positive after eugenol treatement (data unshown). Therefore we assume that antiallergic effect of eugenol is in part depend its apoptosis inducing efficacy in mast cells.

Taken collectively, eugenol induces apoptosis in mast cells via translocation of phospho-ser 15-p53 into mitochondria. The antiallergic effect of herbal medicines such as SAFB and MF may depend on the pharmacological efficacy of eugenol regulating apoptosis of mast cells via a phospho-ser 15-p53 dependent fashion.

Acknowledgments

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