The mechanism of apoptosis induced by eugenol in human osteosarcoma cells

Sang-Hun Shin, Jae-Hyun Park*, Gyoo-Cheon Kim*, Bong-Soo Park*, Young-Gi Gil**, Chul-Hoon Kim***

Department of Oral and Maxillofacial Surgery,
**Department of Oral Anatomy, College of Dentistry, Pusan National University, Pusan, Korea*
***Department of Anatomy, College of Medicine, Kosin University, Korea*
****Department of Oral and Maxillofacial Surgery, College of Medicine, Dong-A Medical Center, Korea*

Abstract

Eugenol is commonly used in dentistry for the sedation of toothache, pulpitis, and dental hyperalgesia. This study was performed to investigate the apoptotic effect of eugenol to human osteosarcoma (HOS) cells and the potential use of this compound in osteosarcoma cells.

Eugenol showed the apoptotic effect in HOS cells in dose- and time-dependent manner. Fragmentation and condensation of DNA were showed by TUNEL assay, Hemacolor stain and Hoechst stain. In the DNA electrophoresis analysis, cells showed DNA degradation characteristic of apoptosis with a ladder pattern of DNA fragments. Apoptosis-related factors were analyzed by western blotting. Cells treated with eugenol showed caspase-3, PARP, lamin A and DFF-45 cleavage. Eugenol treatment induced caspase-3 cleavage and activation. Cleavages of PARP, DFF-45 and lamin A were accompanied with activation of caspase triggered by eugenol in HOS cells. Though this study needs more investigations, these results suggest that eugenol induce apoptosis via caspase dependent pathway in HOS cells and eugenol may constitute a potential antitumor compound against osteosarcoma cells.

INTRODUCTION

Eugenol, the major constituent of oil of clove, is used as food flavor and fragrance agent, and is commonly used in dentistry for the sedation of toothache, pulpitis, and dental hyperalgesia[1]. Essential oils are aromatic substances found in many plants with pharmacological activity and are of fluid occurrence (although some are solid at room temperature), oily and volatile.

Apoptosis or programmed cell death is known as an important biological mechanism that contributes to the maintenance of the integrity of multicellular organism[2,3]. It is induced by a wide variety of cellular stresses such as DNA damage, UV radiation, ionizing radiation and oxidative stress[4,5], and is morphologically distinct from necrosis in many of its characteristic changes as follows; DNA fragmentation, chromatin condensation[6,7], cytoplasmic membrane blebbing, and cell shrinkage. Apoptosis involves the proteolysis of specific cellular proteins by a group of cysteine proteases known as caspases[8]. Although the pathways leading to apoptosis are not fully elucidated, several genes that play a role in the process have been identified, and some proteins have an important role in cancer[9,10].

Caspase-3 activation events preceded proteolysis of the caspase substrates DFF(DNA Fragmentation Factor)-45, lamins and Poly(ADP-ribose)polymerase(PARP) in nuclear fractions[11]. The importance of proteolytic cleavage to the ensuing morphological and molecular changes associated with apoptotic phenomena is being actively investigated.

PARP cleavage might cause dysfunctions in DNA repairs mechanisms[12]. PARP is biologically significant in

* Corresponding author

Chul-Hoon Kim
Dept. of OMFS, College of Medicine, Dong-A Medical Center, 3-1, Dongdaeshindong, Seogu, Busan, Korea
Tel: 82-51-240-5475
E-mail: bbp2000@hanmail.net

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the rejoining of DNA strand breaks[13], and damaging agents produce single or double strand breaks, which bind to the zinc finger domains of PARP raising the specific activity of this enzyme by the conformational change[14]. PARP is an abundant and highly conserved chromatin bound protein, and found only in eukaryotes[15,16], and also which catalyzes exclusively poly(ADP-ribosyl)ation of DNA binding proteins[17,18]. Poly(ADP-ribosyl)ation of nuclear proteins is posttranslational modification that occurs during apoptosis[19]. There are several studies suggesting the potential correlation between poly(ADP-ribosyl)ation of nuclear protein and internuclesomal DNA fragmentation occurring in the course of apoptosis[19].

The nuclear lamina is a meshwork of intermediate filaments, localized on the inner aspect of the inner nuclear membrane, that forms from polymerization of proteins called lamins[20,21,22]. Nuclear breakdown leading to the formation of apoptotic bodies has been postulated to involve degradation of nuclear structural proteins, such as lamins A/C and B[23]. Although nuclear segmentation occurs during the maturation of polymorphonuclear leukocytes (neutrophils), its mechanism is not known. Cleavage of lamins may interfere of the nuclear envelope[24]. The nuclear lamina is a network of intermediate filaments composed of lamin subunits[25]. Structural proteins of the nuclear matrix such as lamins are also key substrates for caspase[26,27]. Contrasting with the early cleavage of lamins, the nuclear envelope persists until the late stage of apoptosis in vivo and in vitro.

DFF is one of the major endonucleases responsible for internucleosomal DNA cleavage during apoptosis[28]. Understanding the regulatory checkpoints involved in safeguarding non-apoptotic cells against accidental activation of this nuclease is as important as elucidating its activation mechanisms during apoptosis.

This study was performed to investigate the apoptotic effect of eugenol on human osteosarcoma (HOS) cells and a potential use of this compound in the osteosarcoma cells.

MATERIALS AND METHODS

1. Cell culture

HOS cell line was purchased from the ATCC (Rockville, Maryland). Cells were maintained at 37℃ with 5% CO_2 in air atmosphere in DMEM with 4 mM L-glutamine, 1.5 g/ l sodium bicarbonate, 4.5 g/ l glucose and 1.0 mM sodium pyruvate supplemented with 10% FBS.

2. Eugenol Treatment

Eugenol was dissolved in the serum free medium prior to use and exposed to cells at 37℃ for the desired times. Cells were cultured for more than 48 hours in tissue culture petridishes prior eugenol the cells washed three times with phophate buffered saline (PBS).

3. Cell viability assay

Cell viability was measured by hemocytometer using the trypan blue dye exclusion. Trypsinized cells were incubated with 0.4% trypan blue solution (Sigma) for 10 minutes, and more than 300 cells were scored on a hemocytometer. Viable and nonviable cells were counted by inverted microscopy.

4. Morphological Assessment of Apoptosis

1) Nuclear staining with hoechst 33258

Cells were washed with cold PBS and incubated with 4 μg / ml of Hoechst 33258, a DNA-binding fluorescent dye, for 30 minutes at 37℃. After that fixed in 4% paraformaldehyde for 10 minutes. The morphological characteristics of apoptotic cells were identified with the aid of a fluorescent microscope (ECLIPSE E800, Nikon, Tokyo, Japan) with excitation at 540 nm. The cells with fragmented and/or condensed nuclei were classified as apoptotic cells.

2) Hemacolor staining

Cell suspesions were loaded into a cytospin chamber and centrifuge at 500 rpm for 2 minutes. Air-dry slides for at least 5 minutes. Cells were fixed Hemacolor fixative solution. Cells were stained by dipping the slides 10 seconds in Hemacolor red reagent, and then counterstained by dipping the slides 10 seconds in Hemacolor blue reagent. Slides were rinsed off excess dye and allowed to air-dry. To quantify apoptosis, preparations were examined under 40x magnification. A minimum of three fields containing around 100 cells were analysed.

3) TUNEL assay

To detect DNA breaks in situ, the TUNEL assay was

employed with an TUNEL reaction mixture kit (Boehringer Mannheim, Germany). After tubercidin treatment, cells were washed twice with PBS, fixed in 4% paraformaldehyde for 10 minutes and applied permeabilisation solution for 2 minutes at 4℃, and washed again with PBS. This was followed by *in situ* end labeling according to the manufacturer's instructions. Apoptotic cells were detected under a fluorescent microscope with excitation at 450 nm.

5. 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 ㎍/ml) was added. After samples were incubated overnight at 48℃, 200 ㎕ 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℃ in 50% isopropanol and RNase A-treated for 1 hour at 37℃. A loading buffer containing 100 mM EDTA, 0.5% SDS, 40% sucrose, and 0.05% bromophenol blue were added at 1:5 (v/v). Separation was achieved in 2% agarose gels in Tris-acertic acid/EDTA buffer (containing 0.5 ㎍/ml ethidium bromide) using 50 mA for 1.5 hours.

6. Western blot analysis

For protein analysis, cells were lysed with RIPA buffer (10 mM Tris/HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride) on ice for 1 hour. Lysates were clarified by centrifugation at 12000 rpm for 15 minutes at 4℃, that the supernatant was obtained. The protein contents of the lysate were determined using the Bio-Rad Protein Assay (Bio-Rad laboratories Hercules, CA). The 70 ㎍ protein was mixed with equal volume of sample buffer (10 mM Tris/HCl, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol). After heating, the protein was resolved on polyacrylamide SDS gels and transferred to nitrocellulose membrane. After transfer, equal loading was confirmed by Ponceau S staining. The membranes were blocked with blocking reagent (5% non-fat milk, 0.05% Tween 20 in TNE buffer, pH 7.5) for 1 hour and then the membranes were incubated with primary antibody. The membranes were incubated for 1 hour with the corresponding secondary antibody, diluted in the above blocking reagent. After three final washes, the membranes were treated with chemiluminescence reagent (ECL, Amersham Pharmacia Biotech Inc, San Francisco, CA). All the procedures were done at room temperature.

RESULT

Cell Viability

Eugenol treatment of HOS cells decreased cell viability in a dose-dependent (Fig. 1) and time-dependent decreased manner (Fig. 2). Fig. 1 showed 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, 5.0 mM and 10.0 mM eugenol treated groups showed 91.7%, 83.1%, 56.6%, 25.3%, 13.2% and 8.4% survival rates. At 2 mM of eugenol, the viability dropped to 84.8%, 53%, 25.3% and 5% of the control on treatment times 8, 16, 24 and 48 hours, respectively.

Morphological change

Morphology of apoptotic cells stained with Hoechst 33258 was shown in Fig. 3. Untreated normal cells showed homogeneous staining of their nuclei. Apoptotic cells represented irregular staining of their nuclei as a result of chromatin condensation and nuclear fragmentation. Fig. 4 showed morphology of apoptotic cells determined by TUNEL assay in HOS cells. Control cells showing negative reaction, cells treated with eugenol for 24 hours showed positive reaction. Fig. 5 showed morphology of apoptotic cells determined by Hemacolor assay in HOS cells. Live cells stain dark blue cytomembrane and nuclei. Apoptotic cells stain dark blue nuclei and necrotic cells stain pink.

DNA fragmentation

DNA fragmentation was evidently demonstrated by DNA electrophoresis (Fig. 6). Cells treated with eugenol showed DNA degradation characteristic of apoptosis with a ladder pattern of DNA fragments. Fig. 6 showed DNA ladder pattern treated with 2 mM eugenol in HOS cells. DNA ladder patterns appeared at 16 hours.

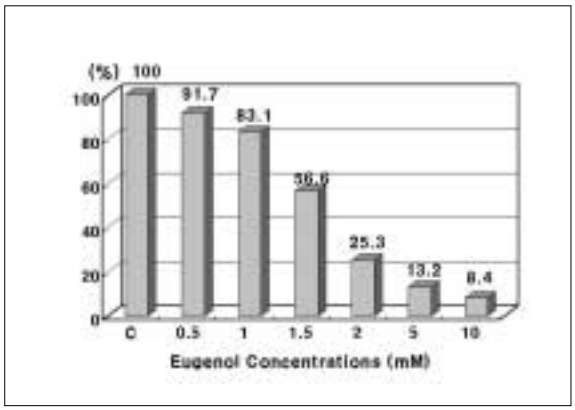


Fig. 1. Effect of different eugenol concentrations on cell viability in HOS cells. Cell members were measured by trypan blue assay after different concentrations of eugenol treatment for 24 hours.

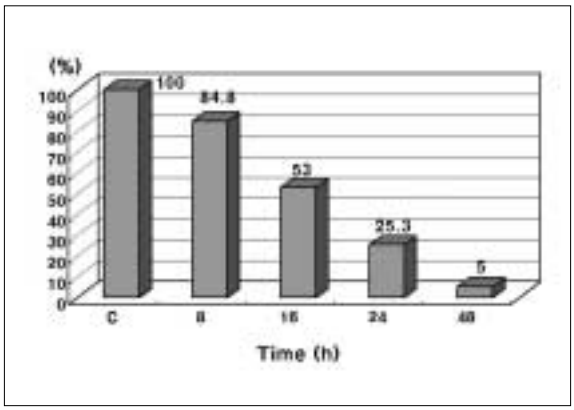


Fig. 2. Time course of eugenol on cell viability in HOS cells. Cells were treated with 2 mM eugenol for indicated time periods and then, cell numbers measured by trypan blue assay.

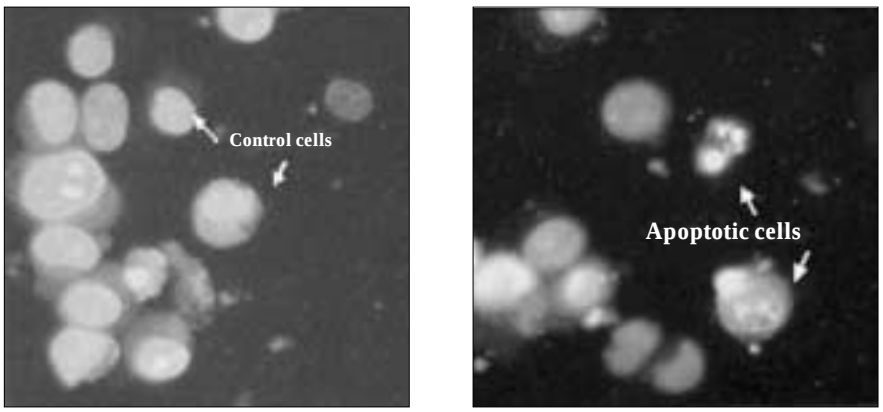


Fig. 3. Morphology of apoptotic cells stained with Hoechst 33258 in HOS cells induced by eugenol. Control cells showing negative reaction (left panel), cells treated with eugenol for 24 hours show positive reaction (right panel).

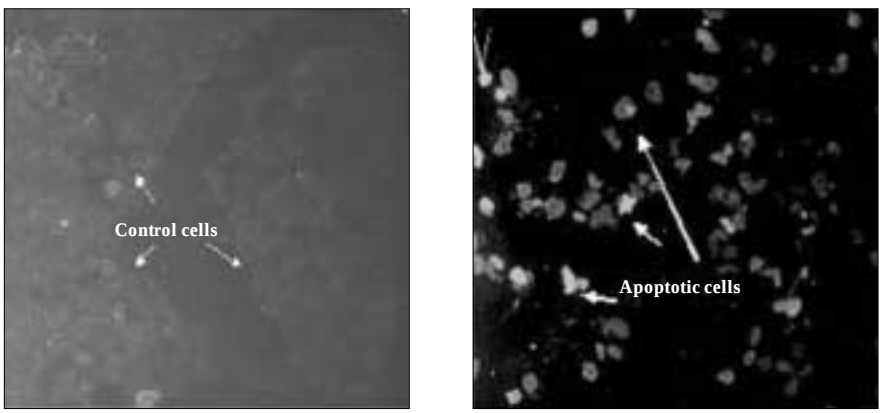


Fig. 4. TUNEL assay in HOS cells. Control cells show negative reaction (left panel), cells treated with eugenol for 24 hours show positive reaction (right panel).

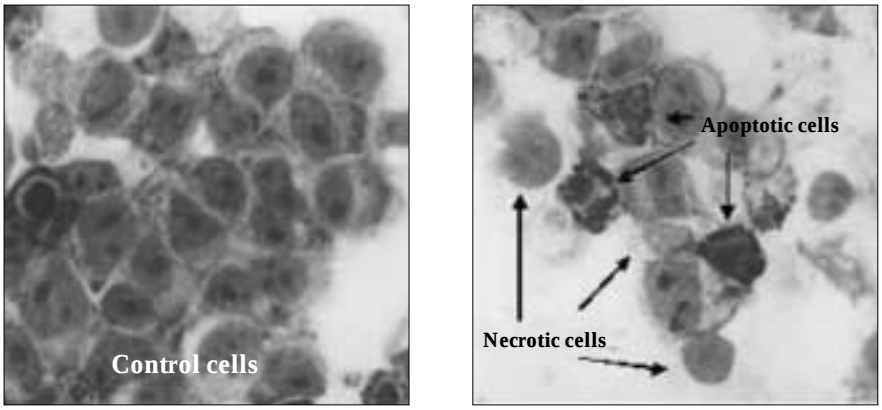


Fig. 5. Morphology of apoptotic cells stained with Hemacolor in HOS cells induced by eugenol. Shrunk cells and dark blue nuclei were observed in eugenol-treated cells (right panel) in contrast to control cells (left panel).

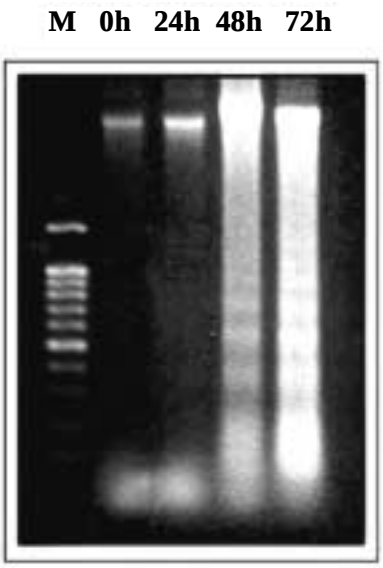


Fig 6. Time course of 2 mM eugenol on DNA fragmentation patterns in HOS cells.

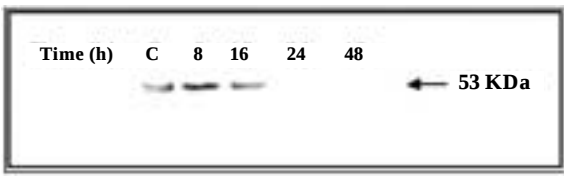


Fig. 7. Western blotting analysis of p53 at various time points after 2 mM eugenol treatment.

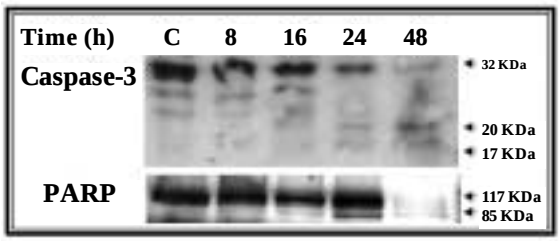
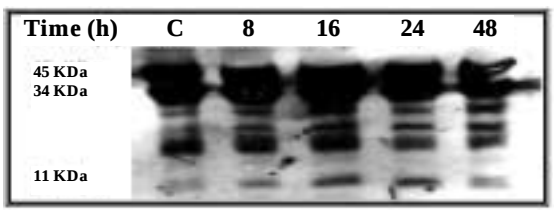
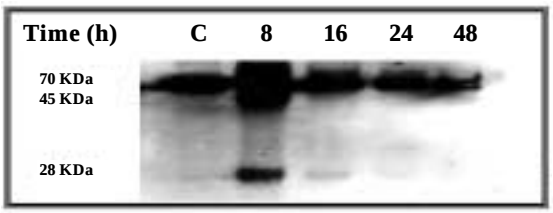


Fig. 8. Western blotting analysis of Caspase-3 and PARP at various time points after 2 mM eugenol treatment.



DFF45



Lamin A

Fig. 9. Western blotting analysis of DFF45 and lamin A at various time points after 2 mM eugenol treatment.

Western blot

Western blots were used to measure protein expression level of several genes related to apoptosis. The expression level of p53 was incleased at 8hours and then decreased (Fig. 7). Fig. 8 showed eugenol induced Caspase-3 activity and PARP degradations at 16 hours. Caspase-3 was cleaved 32 kDa, 20 kDa and 17 kDa. PARP is cleaved 116 kDa and 85 kDa. Eugenol treatment caused decreases in the cytosolic DFF-45. There was the appearance of eugenol-induced 32 and 11 kDa bands first observed at 8 hours (Fig. 9). And also, eugenol administration resulted in a cleavage of lamin A in nuclear fractions. The 45 and 28 kDa lamin A fragments were visible 8 hours after eugenol treatment (Fig. 9).

DISCUSSIONS

Numerous in vitro and in vivo studies have been conducted to assess the pharmacological and toxic effects of eugenol. At the cellular level, eugenol inhibits cell migration[29,30,31], prostaglandin synthesis[30], cell respiration[31] and mitochondrial activity[29,31]. It causes a change in enzyme activity and an alteration of the cell membrane[32]. In addition, eugenol stimulates the neutrophils, which release superoxidizing free radicals produced through oxidant-mediated mechanisms and causes lung damage[33,34]. In particular, they were more cytotoxic against cancer cells compared with normal fibroblasts and their cytotoxicity was significantly suppressed by sodium ascorbate or β-carotene[35].

Apoptosis is a major type of physiological or pathological cell death, which is important in the control of cell numbers during development and proliferation and in the removal of damaged cells that threaten homeostasis[2]. The induction of apoptosis by the natural phytochemicals in malignant cells validates a promising strategy for human cancer chemoprevention[36,37]. antitumor agents also induce apoptosis in some cancer cells both in vitro and in vivo, indicating that apoptosis plays a very important role in cancer chemotherapy[38].

In this study, eugenol produces a significant dose-dependent decrease in cell proliferation and induces an apoptotic-type cell death. Eugenol-induced apoptosis was confirmed by a variety of methods such as trypan blue exclusion assay, Hoechst staining, TUNEL assay, Hemacolor staining and DNA fragmentation. Caspase cleavage can directly inactivate protein function.

The cellular functions affected by the caspases include the apoptotic pathway, cell cycle and growth regulating pathways, a the maintenance of cell structure. Proteolysis of caspase substrates within these pathways modifies protein function in distinct ways[39]. Substrates can be directly activated, directly inactivated, or can modulate the function of other proteins as a result of cleavage[40]. Caspase cleavage can directly inactivate protein function[39,40,41]. The caspases have cleverly targeted the proteins within signaling pathways that will assist in their purpose to destroy the cell[42,43,44]. Thus death receptor mediated caspase cleavage of substrates can engage the mitochondria to amplify the pro-apoptotic signal[45]. Caspase-3 had been perceived as the principle enzyme responsible for cleaving PARP[46]. PARP plays the active role of "nick sensor" during DNA repair and apoptosis, when it synthesizes ADP-ribose from NAD+ in the presence of DNA strand breaks. Moreover, PARP becomes a target of apoptotic caspases, which originate two proteolytic fragments of 89 kDa and 24 kDa[47]. In this study, eugenol treatment induced Caspase-3 cleavage and activation. Intact 32 kDa Caspase-3 and its 20 kDa and 17 kDa cleaved products are indicated. Intact PARP protein 116 kDa and 85 kDa cleaved product. The activated caspase-3 led cleavage of the PARP.

Author found proteolysis of the casapase-6 substrate lamin A, whose cleavage has been reported to be necessary for complete condesation of DNA during apoptosis. The caspase-3 substrate DFF45[48] is cleaved during eugenol-induced apoptosis. DFF is comprised of DFF45 and DFF40 subunits. Its celavage by caspase-3 results in the liberation of the active DFF40, the major nuclease implicated in caspase-dependent DNA fragmentation.

In apoptotic process p53 plays a key role in apoptotic cell death and expression of p53 tumor suppressor gene product was increased, suggesting the onset of p53 pathway[49]. P53 determines the cell regulation in response to DNA damage and other cellular stresses[50,51]. Under DNA damage and other stimulation the wild type p53 level rapidly increases in the cell and this increases level is required for the functioning of p53 as a guardian of the genome. In this study, eugenol increased the expression of p53, early time which is consistent with others' reports.

In summary, these results showed that eugenol induces apoptosis of the HOS cells, provide further evidence for eugenol as an anticancer compound. By activating caspase-3, eugenol may play an important role in the antitumor activity on HOS cells.

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