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2-Hydroxycinnamaldehyde Inhibits SW620 Colon Cancer Cell Growth Through AP-1 Inactivation

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Abstract. Cinnamaldehyde derivatives isolated from *Cinnamomum cassia* have been widely used for treating dyspepsia, gastritis, and inflammatory disease as well as cancer. To investigate the anti-tumor activities of several cinnamaldehyde derivatives, we compared the inhibitory effect of cinnamaldehyde derivatives on cell growth and AP-1 transcriptional activity in SW620 human colon cancer cells since AP-1 is a transcriptional factor implicated to control cancer cell growth. Among the derivatives, 2'-hydroxycinnamaldehyde (HCA) most significantly inhibited cancer cell growth and AP-1 transcriptional activity in a dose-dependent manner with an IC_{50} value of 12.5 and 9 μg/ml, respectively. In further studies on the mechanism, we found that consistent with the inhibitory effect on cell growth, HCA dose-dependently (0 – 20 μg/ml) inhibited DNA binding activity of AP-1 accompanied with down regulation of c-Jun and c-Fos expressions. HCA also induced apoptotic cell death as well as expression of the apoptosis-regulating gene caspase-3, but inhibited the anti-apoptosis regulating gene bcl-2 in a dose-dependent manner. These results suggested that HCA has the most potent inhibitory effect against human colon cancer cell growth, and AP-1 may be an important target of HCA.

Keywords: 2'-hydroxycinnamaldehyde (HCA), AP-1, colon cancer, cell growth inhibition

Introduction

The cinnamaldehyde derivatives isolated from the stem bark of *Cinnamomum cassia* have been shown to have various activities such as anti-angiogenic activity and chemopreventive activity (1 – 3). 2'-Hydroxycinnamaldehyde (HCA), a cinnamaldehyde derivative, was reported to have an inhibitory effect on farnesyl protein transferase activity and also to inhibit cell proliferation of several human cancer cell lines including breast, leukemia, ovarian, and lung tumor cells (4). However, little is known about the structure-activity effects and putative mechanism of cancer cell growth inhibition by cinnamaldehyde derivatives in colon cancer cells.

AP-1 is an inducible eukaryotic transcription factor containing products of the jun and fos oncogene families (5). The inducible AP-1 complexes are composed of Jun-Jun or Jun-Fos dimers. When stimulated, AP-1 binds to transactivation promoter region TREs [12-*O*-tetradecanoylphorbol-13-acetate (TPA) response elements] and induces transcription of several genes involved in cell proliferation, metastasis, and metabolism (6). Many stimuli are able to induce AP-1 activity, including the phorbol ester TPA and epidermal growth factor (EGF), which are strong inducers of cellular transformation of many different cell types and animal models (7). Increased AP-1 activity is associated with malignant transformation and cancer development by UV radiation, growth factors, phorbol esters, and transforming oncogenes (8). Oncoproteins, such as v-Src or Ha-Ras (9), are also potent inducers of AP-1 activity. The AP-1 family, c-Fos and c-Jun, contains cysteine residues in their DNA-binding domains. The DNA binding domain of human c-Jun processes two functionally important cysteine residues. Several phytochemicals such as curcumin, capsaicin, resveratrol, and green tea catechins have been shown to suppress AP-1 activation (10). An AP-1 blockade has been shown to interfere with

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the transmission of proliferative signals induced by peptide growth factors as well as steroid growth factors such as estrogens (11). These results suggest that chemopreventive agents specifically targeting AP-1 inactivation could be promising agents for the treatment of several cancers.

Therefore, in the present study, we investigated the structure and activity relationship among cinnamaldehyde derivatives in SW620 colon cells and the possible mechanism of cell growth inhibition.

Materials and Methods

Materials

A series of cinnamaldehyde derivatives (Fig. 1) was synthesized according to the procedure described elsewhere (3). Chemical reagents were purchased from Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise mentioned.

Cell culture

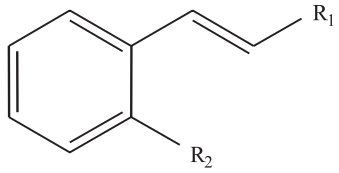
SW620 human colon cells were obtained from the American Type Culture Collection (Cryosite; Lane Cove NSW, Australia). Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin, and fetal bovine serum were purchased from Gibco Life Technologies (Rockville, MD, USA). SW620 human colon cells were grown in RPMI1640 with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2 humidified air.

Cell viability assay

To determine the appropriate dose that is not cytotoxic to the cells, SW620 human colon cells, obtained from the American Type Culture Collection, were treated with HCA. The cell growth inhibition was evaluated in cells cultured for 24, 48, and 72 h by the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, MTT was dissolved in culture medium without phenol red at a concentration of 10 μg/ml, and 10 μl of this solution was then added to cells cultured for the designed time period. After 4 h, cultures were removed from the incubator and the formazan crystals dissolved by adding 100 μl solubilization solution (0.04 N HCl in isopropanol) were quantified by measuring light absorbance at 570 nm. Viable cells were also counted after trypan blue staining.

Detection of apoptosis

SW620 cancer cells (2.5×10^5 cells/cm^2) were cultured on a chamber slide (Lab-Tak II chamber slider system; Nalge Nunc Int., Naperville, IL, USA), fixed in 4% paraformaldehyde, membrane-permeabilized by exposure for 30 min to 0.1% Triton X-100 in phosphate-buffered saline at room temperature. Cells were then exposed to DAPI solution. TUNEL assays were performed by using the in situ Cell Death Detection Kit (Roche Diagonostics GmbH, Mannheim, Germany) according to manufacturer's instructions. Apoptotic cells were determined by the morphological changes after 4,6-diamino-2-phenylindole (DAPI) staining under



Compound	R_1 group	R_2 group	Name
1	COH	2'-OH	2'-hydroxycinnamaldehyde (HCA)
2	$COOCH_3$	2'-OH	3-(2'-methylcarboxyphenyl)-2-propenal
3	CH_2OH	2'-OH	3-(2'-hydroxyphenyl)-2-propenal
4	COH	2'-OCH_3	3-(2'-methoxyphenyl)-2-propenal
5	COH	2'-$OC(O)CH_3$	3-(2'-*O*-acetylphenyl)-2-propenal
6	COH	2'-OCH_2Ph	3-(2'-*O*-benzylphenyl)-2-propenal
7	COH	2'-OCH_2Ph-4-OCH_3	3-[2'-*O*-(4-methoxybenzyl)-phenyl]-2-propenal
8	COH	2'-OCH_2Ph-4-CH_3	3-[2'-*O*-(4-methylbenzyl)-phenyl]-2-propenal

Fig. 1. Chemical structures of HCA (2'-hydroxycinnamaldehyde) and cinnamaldehyde derivatives.

fluorescence microscopic observation (DAS microscope, 200X; Leica Microsystems, Inc., Deefield, IL, USA). Total number of cells in a given area was determined by using DAPI nuclear staining. TUNEL-positive stained cells were counted for quantification of apoptotic cell death. Percentage of apoptotic cell death was expressed by the ratio of the number of TUNEL-positive cells/DAPI-stained cells.

Gel electromobility shift assay

Gel shift assays were performed according to the manufacturer's recommendations (Promega, Madison, WI, USA). Briefly, cells treated with HCA for 1 h (1×10^6 cells/ml) were washed twice with 1× PBS, followed by the addition of 1 ml of PBS, and the cells were scraped into a cold Eppendorf tube. Cells were spun down at 15,000 × *g* for 1 min, and the resulting supernatant was removed. Solution A (50 mM HEPES, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, and 0.5% Nonidet P-40) was added to the pellet in a 2:1 ratio (v/v) and allowed to incubate on ice for 10 min. Solution C (solution A + 10% glycerol and 400 mM KCl) was added to the pellet in a 2:1 ratio (v/v) and vortexed on ice for 20 min. The cells were centrifuged at 15,000 × *g* for 7 min, and the resulting nuclear extract supernatant was collected in a chilled Eppendorf tube. Consensus oligonucleotides were end-labeled using T4 polynucleotide kinase and [γ-^{32}P] ATP for 10 min at 37°C. Gel shift reactions were assembled and allowed to incubate at room temperature for 10 min followed by the addition of 1 μl (50,000 – 200,000 cpm) of ^{32}P-labeled oligonucleotide and another 20 min of incubation at room temperature. Subsequently, 1 μl of gel loading buffer was added to each reaction and the mixture was then loaded onto a 4% nondenaturing gel and electrophoresis performed until the dye was three-fourths of the way down the gel. The gel was dried at 80°C for 1 h and exposed to film overnight at −70°C. The relative density of the protein bands was scanned by densitometry using MyImage (SLB, Seoul, Korea) and quantified by Labworks 4.0 software (UVP, Inc., Upland, CA, USA).

Transfection and AP-1 luciferase activity assay

SW620 human colon cells were transfected with AP-1-Luc plasmid (AP-1; Stratagene, La Jolla, CA, USA) using a mixture of plasmid and lipofectAMINE PLUS in OPTI-MEN according to manufacturer's specifications (Invitrogen, Carlsbad, CA, USA). After 24-h transfection, the cells were treated with cinnamaldehyde derivatives in the presence of TPA (100 ng/ml) for 6 h since AP-1 was maximumly increased after 6-h treatment with TPA. Luciferase activity was measured by using the luciferase assay kit (Promega) according to the manufacturer's instructions (WinGlow, Bad Wildbad, Germany).

Western blot analysis

Cells were treated for 24 h with HCA. Then the cultured cells were washed twice with 1× PBS, followed by the addition of 1 ml of PBS, and the cells were scraped into a cold Eppendorf tube. Cells were homogenized with lysis buffer [50 mM Tris pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.2% SDS, 1 mM PMFS, 10 μl/ml aprotinin, 1% igapel 630 (Sigma Chem. Co., St. Louis, MO, USA), 10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA, and 0.5% sodium deoxycholate] and centrifuged at 23,000 × *g* for 1 h. The protein concentration was measured by the Bradford method (Bio-Rad Protein Assay; Bio-Rad Laboratories Inc., Hercules, CA, USA), and equal amount of proteins (20 μg) were separated on a SDS/12%-polyacrylamide gel and then transferred to a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA). Blots were blocked for 2 h at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline [10 mM Tris (pH 8.0) and 150 mM NaCl] solution containing 0.05% Tween-20. The membrane was incubated for 5 h at room temperature with specific antibodies: rabbit polyclonal antibodies against caspase-3 (1:500; Cell Signaling Technology Inc., Beverly, MA, USA), Bax (1:500), caspase-9 (1:500), and mouse monoclonal Bcl-2 antibody (1:500) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The blot was then incubated with the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology, Inc.). Immunoreactive proteins were detected with the ECL Western blotting detection system. The relative density of the protein bands was scanned by densitometry using MyImage and quantified by Labworks 4.0 software.

Statistical analysis

Data were analyzed using one-way analysis of variance followed by the Tukey test as a post hoc test. Differences were considered significant at $P<0.05$.

Results

Effect of cinnamaldehyde derivatives on AP-1 transcriptional activity in SW620 human colon cancer cells

Since activation of AP-1 is critical in cancer cell growth, we first determined the effect of cinnamaldehyde derivatives on AP-1 luciferase activity in SW620

Table 1. Effect of cinnamaldehyde derivative on AP-1 luciferase activity and cell growth inhibition

Compound	Name	AP-1-Luc, IC_{50} (μg/ml)	Cell cytotoxicity, IC_{50} (μg/ml)
1	2'-hydroxycinnamaldehyde (HCA)	9	12.5
2	3-(2'-methylcarboxyphenyl)-2-propenate	>100	>100
3	3-(2'-hydroxyphenyl)-2-propenol	37.5	36
4	3-(2'-methoxyphenyl)-2-propenol	35	32.5
5	3-(2'-*O*-acetylphenyl)-2-propenol	16	17.5
6	3-(2'-*O*-benzylphenyl)-2-propenol	40	38
7	3-[2'-*O*-(4-methoxybenzyl)-phenyl]-2-propenol	80	30
8	3-[2'-*O*-(4-methylbenzyl)-phenyl]-2-propenol	70	22.5

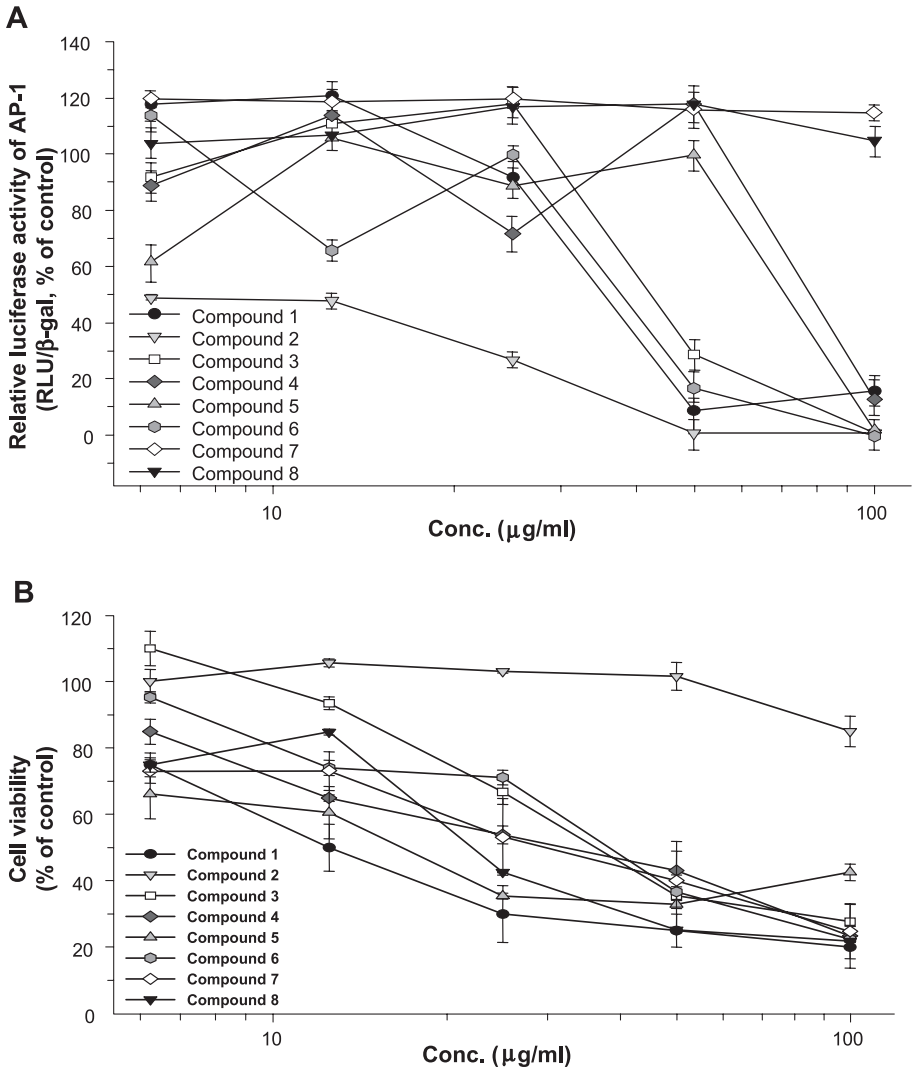


Fig. 2. Effect of cinnamaldehyde derivatives on TPA-induced AP-1-dependent luciferase activity in SW620 cells and cell viability. A: SW620 cells were transfected with AP-1-luciferase, treated with TPA (100 ng/ml) alone or TPA plus different concentrations of cinnamaldehyde derivatives at 37°C, and then the luciferase activity was determined. All values represent means ± S.D. of three independent experiments performed in triplicate. RLU is relative to the luciferase activity in transfected unstimulated cells. B: Cell viability was determined by MTT assay after 72-h treatment of chemicals as described in Materials and Methods. Values are each the mean ± S.D. of three experiments, with each experiment performed in triplicate.

colon cancer cells. Because AP-1 luciferase activity in the untreated cells was limited for detection, TPA (100 ng/ml)-induced AP-1 luciferase activity in SW620 colon cancer cells was determined to investigate the structure-activity relationship. After co-treatment with TPA and cinnamaldehyde derivatives for 24 h, TPA-induced AP-1 luciferase activities were decreased remarkably in a concentration-dependent manner by cinnamaldehyde derivatives with the exceptions of 3-[2'-*O*-(4-methoxybenzyl)-phenyl]-2-propenal and 3-[2'-*O*-(4-methylbenzyl)-phenyl]-2-propenal (Table 1). Among the derivatives, 2'-hydroxycinnamaldehyde (HCA) has the most potent inhibitory effect on TPA-induced AP-1 activity. The IC_{50} value of HCA in the inhibition of TPA-induced AP-1 transcriptional activity was 9 μg/ml (Fig. 2A and Table 1). Cinnamaldehyde (compounds 2 and 3) containing methylcarboxylphenyl or hydroxylphenyl showed no significant effect (Table 1). Compound 4 containing a methoxy group inhibited the TPA-induced AP-1 luciferase activity slightly, and compound 5 containing an acetyl group also showed marginal effect (IC_{50}: 16 μg/ml). Compound 6 containing a benzyl group also slightly inhibited AP-1 luciferase activity. Changing the alkyl substitute R_2 from methyl to a polar group such as methoxy was examined with the hope of inhibiting activity in comparison to the parent compound 6. As shown in Table 1, a polar group such as methoxy did not inhibit AP-1 activity (compounds 7 and 8). Therefore, these results suggested that the aldehyde group of the side chain (R1), and no derivatives in the R2 position seems to play a critical role in inhibition of AP-1 activity.

Effect of cinnamaldehyde derivatives on SW 620 human colon cancer cell growths

To investigate the inhibitory effect of cinnamaldehyde derivatives on cell growth of SW620 human colon cancer cells and compare the cell growth inhibitory effect with AP-1 luciferase activity, we analyzed cell viability using MTT. Among the tested compounds (Fig. 2B, Table 1), HCA had the strongest inhibitory effect on cell growth. This effect paralleled the effect on the AP-1 luciferase activity. The IC_{50} value of HCA in cell growth inhibition was 12.5 μg/ml, which is to the similar dose that inhibits AP-1 luciferase activity (9 μg/ml). Similar to the effect on AP-1 transcriptional activity, cinnamic acids (compound 2), containing methylcarboxylphenyl or hydroxylphenyl showed no significant effect on cancer cell growth (Table 1). Compounds 3 and 4 containing an alcohol or methoxy group inhibited the cell growth at the IC_{50} dose of about 30 μg/ml. However, compound 5 containing an acetyl group showed a significant effect (IC_{50} value: 17.5 μg/ml). Compound 6 (IC_{50} value: 38 μg/ml) and 7 (IC_{50}: 30 μg/ml) containing a benzyl group have slight inhibitory effects. The IC_{50} value of compound 8 was 22.5 μg/ml. Therefore, these results suggested that the aldehyde group of the side chain of cinnamic acid and hydroxyl or acetyl groups seem to play a critical role in inhibition of SW620 human colon cancer cell growth and suggested that the effect of cinnamaldehyde derivatives on cell growth inhibition agreed with the effect on AP-1 transcriptional activity. To further demonstrate the effect of HCA, the most potent cinnamaldehyde derivative on SW620 human colon cancer cell growth, dose- and time-dependent effects were next determined. HCA (0 – 20 μg/ml) treatment for up to 72 h significantly inhibited SW620 human colon cancer cell growth in dose- and time-dependent manners (Fig. 3).

Effect of HCA on AP-1 DNA binding activity and c-Jun and c-Fos expression in SW620 human colon cancer cells

To further investigate the possible cell growth inhibitory effect of cinnamaldehyde derivatives on colon cancer cell growth, we used HCA as a representative compound. We first determined whether HCA can also inhibit AP-1 DNA binding activity. SW620 colon cells were treated with HCA for 60 min, which is sufficient time to activate AP-1 maximally (data not shown). Nuclear extracts from treated cells were prepared and assayed for AP-1 DNA binding by EMSA. SW620 colon cells have a strong AP-1 DNA binding activity, which was attenuated by treatment with HCA in a dose-dependent manner (Fig. 4A). This effect was consistent with the inhibition of TPA-induced AP-1 luciferase activity by treatment with HCA, but other cinnamic acids, even compound 4 that had the lowest IC_{50} (16 μg/ml) and AP-1 luciferase activity (17.5 μg/ml), did not show any significant effect (data not shown). These data indicate that only HCA inhibits both the DNA binding activity of AP-1 and AP-1 luciferase activity, consistent with the inhibitory effect on cell growth. Consistent with the inhibitory effect on AP-1 activity, the expressions c-Jun and c-Fos, components of AP-1, were also inhibited (Fig. 4B). The inhibitory pattern by increasing the dose of HCA in c-Jun was very similar to the inhibition of AP-1 activity, suggesting that inhibition of c-Jun expression may be more related to AP-1 activity inhibition.

Induction of apoptotic cell death of SW620 human colon cancer cells by HCA

Next, we investigated the effect of HCA on cell death to see whether the inhibitory effect of cinnamaldehyde derivatives on SW620 colon cancer cell growth was due

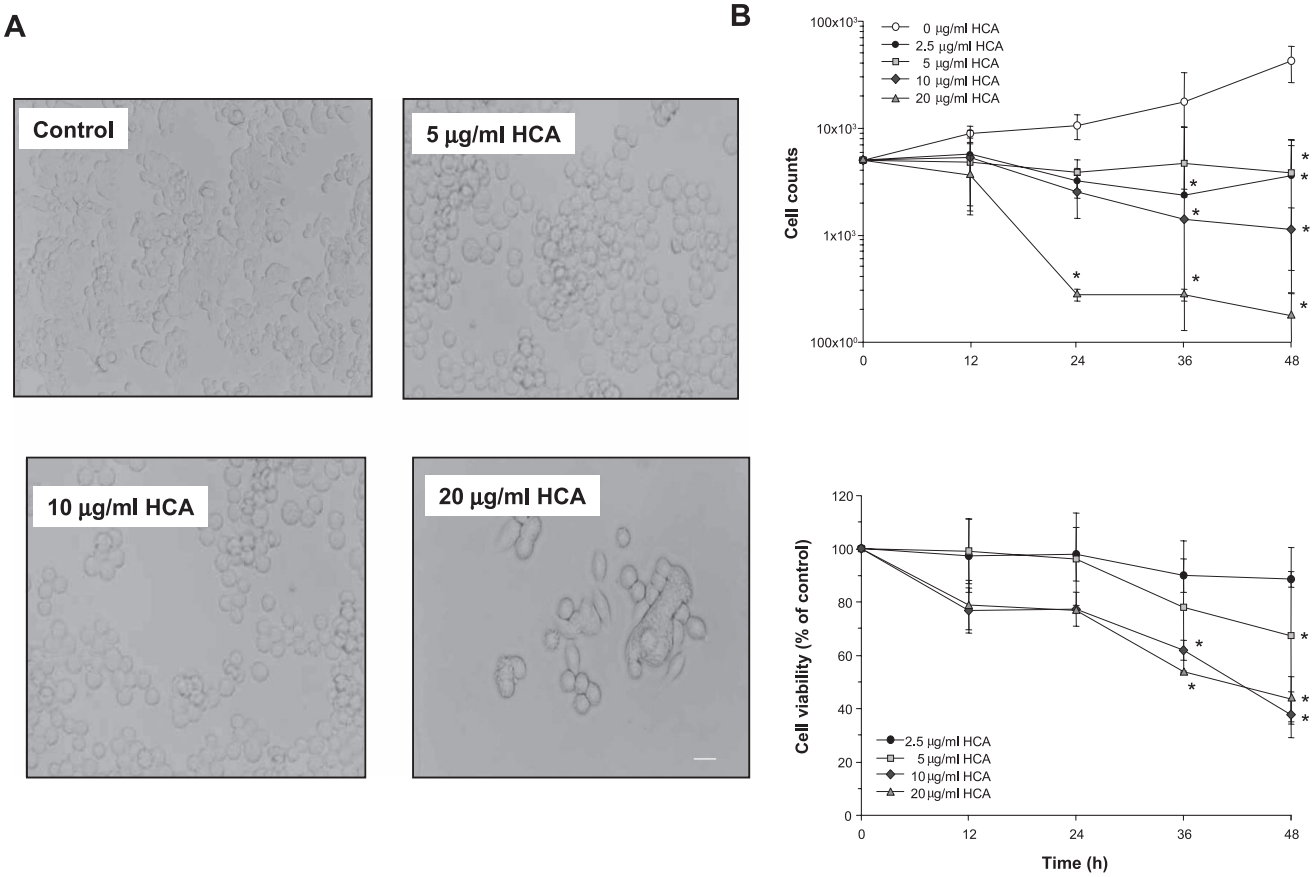


Fig. 3. Morphological changes and cell viability of SW620 cells by HCA. A: Morphorogical changes were observed under a microscope (magnification, 200×) (scale bar = 50 μm). B: Cell viability was determined by directly counting (upper panel) or by MTT assay (lower panel, percentage of control: 100%) after 72-h treatment by different doses of HCA as described in Materials and Methods. Values are each the mean ± S.D. of three experiments, with each experiment performed in triplicate. *P<0.05 indicates statistically significant differences from the untreated control group.

to the induction of apoptotic cell death. We determined DAPI and TUNEL-positive cell number to evaluate cell death. As seen in Fig. 5, HCA dose-dependently induced cell death, and maximum induction of apoptotic cell death was about 50% of the cell population, which corresponds to the cell growth inhibition (50% – 60% inhibition by the highest dose of HCA), suggesting that most of the cell growth inhibition was due to the apoptotic cell death. However, other cinnamaldehyde derivatives did not show any significant effect (data not shown).

Effect of HCA on the expression of genes relating apoptotic cell death

To figure out the relationship between the induction of apoptosis by HCA and expression of apoptotic gene expression, expressions of apoptosis-related proteins were investigated. Expressions of pro-apoptotic proteins, active form of caspases 3, were increased but the expression of anti-apoptotic protein Bcl-2 was decreased. However, other genes (p53, caspase 9, and Bax) were not changed (Fig. 6).

Discussion

Cinnamaldehyde derivatives have been shown to exert a potent anti-tumor effect in a number of different cancer cells (4). In the present study, treatments with some cinnamaldehyde derivatives resulted in inhibition of AP-1 transcriptional activity in SW620 human colon cancer cells. This effect was consistent with the cell growth inhibition by these compounds. Among them, HCA showed the most potent inhibitory effect on cell growth as well as AP-1 transcriptional activity. HCA also significantly inhibited AP-1 DNA binding activity with down regulation of c-Jun and c-Fos. This cell growth inhibitory effect is associated with induction of cell death and modification of the cell death regulatory proteins. The aldehyde group of the side chain and free hydroxy-substituted groups seem to play a critical

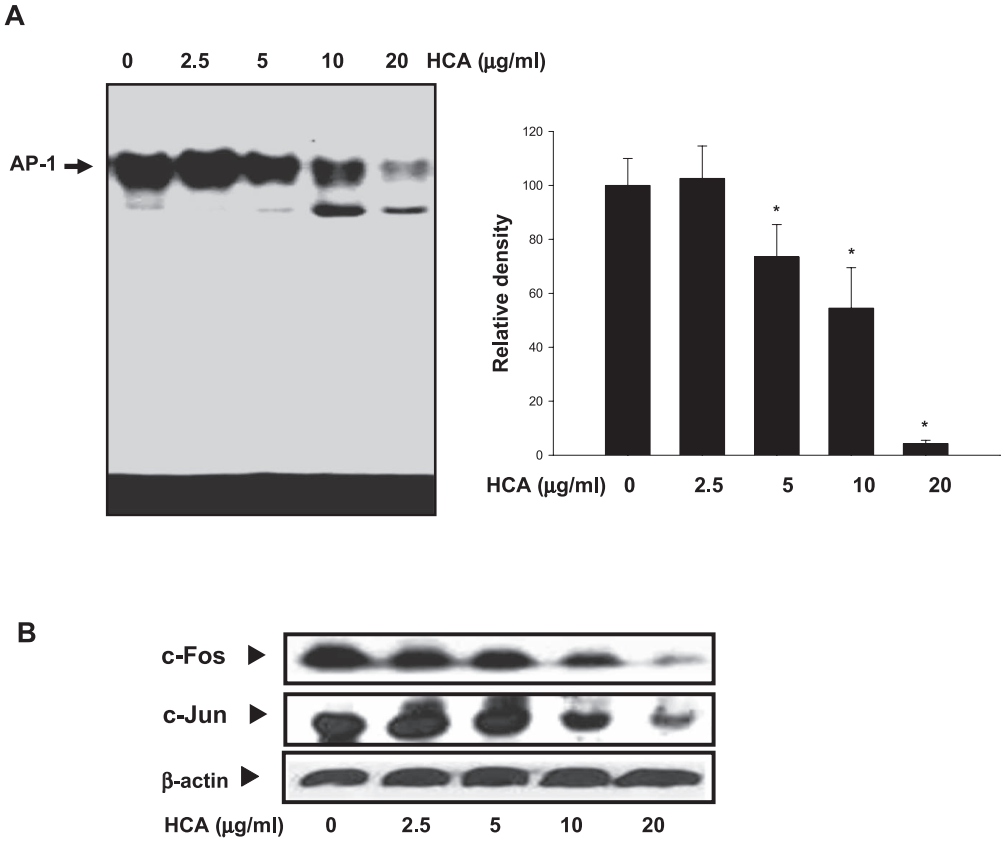


Fig. 4. Effect of HCA on AP-1 activation in SW620 cells. A: The activation of AP-1 was investigated using EMSA as described in Materials and Methods. Nuclear extract from SW620 cells treated with the combination with HCA (2.5, 5, 10, 20 μg/ml) was incubated in binding reactions of ^{32}P-end-labeled oligonucleotide containing the AP-1 sequence. AP-1 DNA binding activity was determined by EMSA. The density ratios of AP-1 DNA binding were calculated, and the mean value in the untreated control group was set to 100. All values represent the means ± S.E.M. of three independent experiments performed in triplicate. **P*<0.05 indicates statistically significant differences from the untreated group. B: Effect of HCA on expression of c-Fos and c-Jun proteins. The cells were treated with different concentrations (2.5, 5, 10, 20 μg/ml) of HCA at 37°C for 24 h. Equal amounts of total proteins (50 μg/lane) were subjected to 12% SDS-PAGE. Expressions of c-Jun, c-Fos, and β-actin were detected by Western blotting using specific antibodies. β-Actin protein was used as an internal control.

role in the anti-tumor activity of the cinnamaldehydes (12). These results suggested that HCA has the most inhibitory activity against human colon cancer cell growth, and DNA binding and transcriptional activities of AP-1 may be an important mechanism of the inhibitory effect of HCA on SW620 human colon cancer cell growth.

The mechanisms for how HCA can interfere AP-1 activation are not clear. However, it is known that HCA is an α,β-unsaturated carbonyl compound that can react with nucleophiles, especially with cysteine sulfhydryl groups of target molecules, in a Michael-type addition reaction. Transcription factors are subject to transcriptional and posttranslational regulation. AP-1 is eukaryotic transcriptional activator, which is regulated by redox (reductant/oxidant) molecules at posttranslational levels. The redox (reduction/oxidation) regulation of transcriptional activators occurs through highly conserved cysteine residues in the DNA binding domains of these proteins. In vitro studies have shown that reducing environments increase, while oxidizing conditions inhibit sequence-specific DNA binding of these transcriptional activators. Interestingly, AP-1 inhibitors have been recently shown to target the cysteine residue of c-Jun and c-Fos (13 – 16). For example, cyclopentenone prostaglandins (cyPG) are reactive compounds that possess an α,β-unsaturated carbonyl group in the cyclopentenone ring. This group reacts with sulfhydryl groups of cysteine residues of proteins by a Michael's addition reaction, resulting in an alteration of protein function (13). The DNA binding domain of human c-Jun possesses two functionally important cysteine residues. Cys^{269} is located in close contact with DNA, and it is involved in the redox and nitric oxide-dependent regulation of DNA binding by means of specifically targeted *S*-glutathionylation (14 –

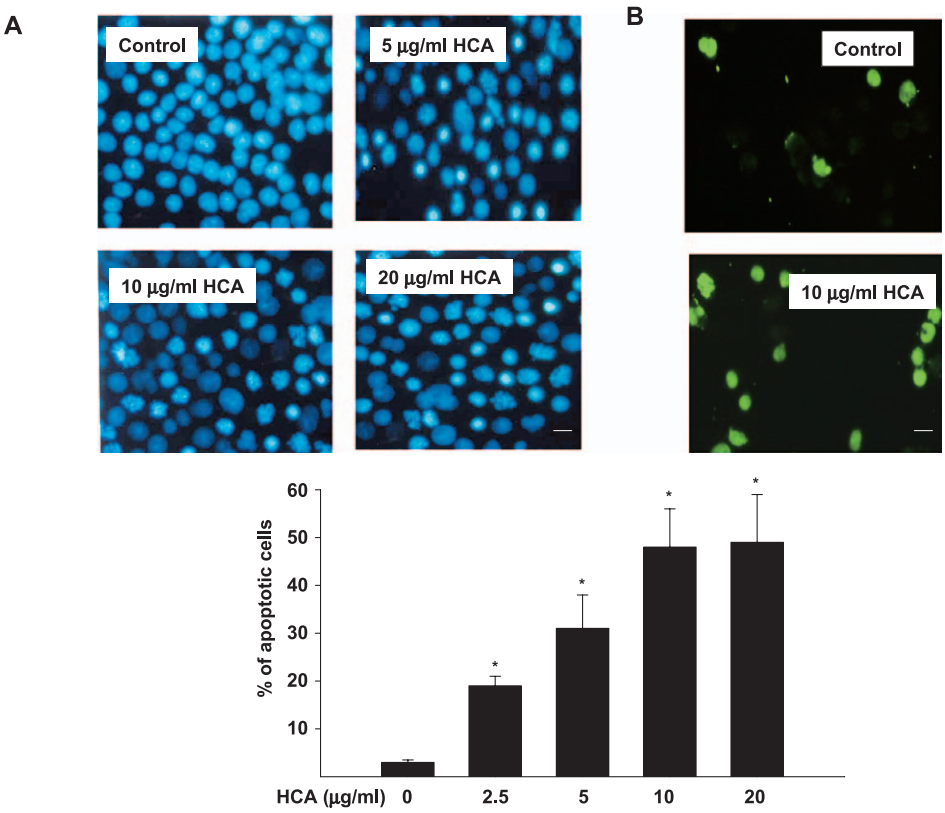


Fig. 5. Effects of HCA on the induction of apoptosis of SW620 cells. A: The apoptotic cells were examined by fluorescence microscopy. Treatment of HCA for 24 h caused apoptosis characterized by marked chromatin condensations, small membrane-bound bodies (apoptotic bodies), cytoplasmic condensations, and cellular shrinkage. The cell indicated by arrows is an example of the apoptotic cells (magnification, 200×) (scale bar = 50 μm). B: TUNEL assays were performed by the in situ Cell Death Detection Kit (Roche Diagonostics GmbH, Mannheim, Germany) according to manufacturer's instructions. The TUNEL-positive cells were confirmed as apoptotic cells by examination under a microscope (magnification, 200×) (scale bar = 50 μm). Apoptotic cells were estimated by direct counting of fragmented nuclei after DAPI staining. The value are means ± S.D. of three experiments, with each experiment performed in triplicate. *P<0.05 indicates statistically significant differences from the untreated group.

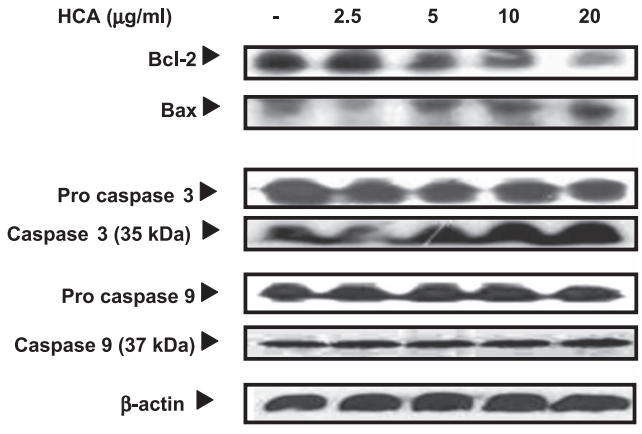


Fig. 6. Effect of HCA on expression of apoptosis regulatory proteins. The cells were treated with different concentrations (2.5, 5, 10, 20 μg/ml) of HCA at 37°C for 24 h. Equal amounts of total proteins (50 μg/lane) were subjected to 12% SDS-PAGE. Expression of Bcl-2, Bax, Caspase-3, Caspase-9, and β-actin were detected by Western blotting using specific antibodies. β-Actin protein was used as an internal control.

16). Cys^{320} is located in the leucine zipper region and participates in c-Jun homo- or heterodimerization with proteins of the Fos family through the formation of an intermolecular disulfide bridge (17). Thus, the α,β unsaturated carbonyl group of HCA has a possibility to react directly with the cysteine sulfhydryl groups of AP-1 protein in a manner similar to that described above, which results in a decrease in the DNA binding activation of AP-1. Other covalent modifications of AP-1 by HCA may be possible. The α,β unsaturated carbonyl group of prostaglandin A_1 was shown to be essential for the covalent modification of IKKβ, putatively via interaction with cysteine 179 of IKKβ (15, 18). However, it is noteworthy that not only the α,β-unsaturated carbonyl group in the R1 position, but group in the R2 position of cinamaldehyde compound can influence the AP-1 activity. Thus, hindrance of the derivatives of R2 position of the compound (4, 5, and 6) could reduce AP-1 activity. It was also noted that

covalent modification of cysteine 179 of IKKβ has been proposed to mediate the pathological effects of arsenite and parthenolide (19). Alternatively, the aldehyde of HCA may possibly react with the catalytic hydroxyl or thiol groups in the active sites of this protein to form a reversible hemi (thio) acetal as a peptide aldehyde, thus modify the function (affinity) of protein (20).

Conversely related with the effect of HCA on the cell growth inhibition, HCA treatment in SW620 human colon cancer cells resulted in a dose-dependent increase of apoptotic cell death. It was further found that consistent with the increase of apoptotic cell death, the expression of apoptotic proteins (active caspase-3) was dose dependently increased but that of anti-apoptotic protein Bcl-2 was decreased. Apoptotic cell death is an important mechanism for eliminating unwanted cells in a wide variety of physical processes, and deregulation of this process is implicated in pathogenesis of many chronic diseases, including cancer development (21). Some proteins within this family, including Bcl-2, inhibit apoptosis, while others such as caspase and Bax promote apoptosis (22). Hence, an alteration in the levels of anti- apoptotic Bcl-2 is likely to influence HCA-induced apoptosis. Similar to our findings, Wu demonstrated that cinnamaldehyde causes apoptotic cell death of human PLC/PRF/5 cells through activation of the proapoptotic Bcl-2 family proteins (23). Caspases are proteases that also play critical role in the execution of apoptotic cell death (24). Activation of caspase-3 can be significant in cancer agent-induced cell death (24). In this study, active caspase-3 expressions were increased in HCA-treated cells, suggesting a caspase 3-dependent mechanism may be an important mechanism in SW620 human colon cancer cell death by HCA. We also previously found that HCA inhibited TNF-α sensitized SW620 colon cancer cell growth via induction of apoptotic cell death (25). In the TNF-α-sensitized SW620 colon cancer, HCA induced (enhanced) other apoptotic cell death-related proteins such as caspase-9. Interestingly, in untreated cells, HCA showed no effect on caspase-9 and p53 expression. This difference can not be explained properly with the present data. However, we assumed that HCA-induced colon cancer cell death may not be related with a p53-dependent mechanism because p53 was not changed by HCA in both untreated and TNF-α-treated SW620 colon cancer. There are several reports showing p53-independent apoptotic cell death (26 – 28). HCA did not alter caspase-9 expression either. Even though HCA induced the expression of active caspase-3, HCA may affect another apoptotic cell death pathway that converges into the caspase-3 pathway instead of the caspase-9/caspase-3 one. Direct activation of caspase-3 by MAP kinase activation could be possible. In this point, it is noteworthy that 2'-bezoyloxyxinnamaldehyde induces apoptosis of human breast and colon cancer cells via activation of caspase-3 in a p53-independent manner (29). Possible mechanisms are proposed in Fig. 7. In fact, we found that HCA inhibited activation of MAP kinase (ERK as well as p38 MAP kinase, data not shown), but MAP kinase was inhibited in TNF-α-treated SW620 colon cancer cells (25). Further study to clarify this issue is required.

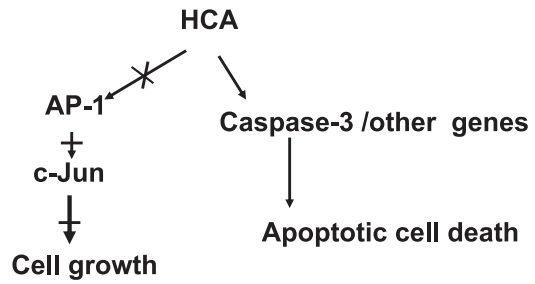


Fig. 7. Possible molecular mechanisms of the anti-cancer property of 2'-hydroxycinnamaldehye (HCA) in colon cancer. HCA can inhibit AP-1 activity through down regulation of c-Jun and c-Fos, which resulted in the inhibition of cell growth. HCA also induced apoptotic cell death via activation of the apoptotic cell death pathway through up regulation of the expression of caspases and other genes.

In conclusion, the current study showed that HCA exerts its cell growth inhibition by reaction with AP-1 in SW620 human colon cancer cells and suggested that HCA can be a useful agent for prevention of colon cancer cell growth.

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