2'-Hydroxycinnamaldehyde Shows Antitumor Activity against Oral Cancer In Vitro and In Vivo in a Rat Tumor Model

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Abstract. Background: 2'-Hydroxycinnamaldehyde (HCA) exerts antitumor activity against several human cancer cell lines. However, its antitumor activity in oral cancer has not been demonstrated. Materials and Methods: The antitumor activity of HCA was assessed in oral cancer cell lines and in a rat oral tumor model. Results: Cell cycle analysis confirmed that HCA showed anti-proliferative activity via cell cycle arrest at the G2/M-phase and increased the number of cells in the sub-G1 (apoptotic cells) phase in SCC-15 and HEp-2 oral cancer cells. Additionally, direct injection of HCA into an RK3E-ras-Fluc-induced tumor significantly inhibited growth of the tumor mass. Histological analysis showed that HCA decreased tumor cell proliferation and induced apoptosis in a rat tumor model. Conclusion: Taken together, these observations suggest the potential value of HCA as a candidate for the treatment of oral cancer.

Cinnamon, one of the oldest and most versatile spices in the world, is a good source of polyphenol. Recent studies have suggested that cinnamon extract exerts chemopreventive properties such as anti-proliferative and anti-oxidative effects (1, 2).

We purified 2'-hydroxycinnamaldehyde (HCA) from the stem bark of Cinnamomum cassia and synthesized a novel HCA derivative, 2-benzoyl-oxycinnamaldehyde (BCA). HCA and BCA exerted anti-angiogenic, anti-inflammatory and anti-proliferative activities against human cancer cell lines such as breast, leukemia, ovarian, lung and colon cancer cells (3-7).

Oral carcinomas are the world’s eleventh most common form of human neoplasm and account for 3% of all newly diagnosed cancer cases (8-10). Despite efforts to improve overall outcomes, survival rates have not changed during the last 20 years. In fact, the prognosis is very poor and approximately 50-70% of patients die within 5 years (9). Late presentation, lack of suitable markers for early detection and failure of advanced lesions to respond to chemotherapy contribute to the poor outcome of oral carcinomas.

Biologically and clinically relevant animal models are essential to investigate the progression of disease and the elaboration of diagnostic or therapeutic protocols. Recently, we developed a tumor animal model using k-ras-transformed RK3E cells in Sprague-Dawley rats (11). This model has the advantage of allowing short-term screening of antitumor agents (12).

Although the antitumor activity of HCA has been widely studied, previous studies have primarily been conducted in vitro. Therefore, in the present study, the antitumor activity of HCA was assessed in vitro and in vivo using a rat oral tumor model.

Materials and Methods

Cell cultures. Two human oral squamous cell carcinoma cell lines were used. SCC-15 was obtained from the Korean Cell Line Bank (Seoul, South Korea) and HEp-2 was obtained from the American Type Culture Collection (Manassas, VA, USA). Luciferase and green fluorescent protein-transformed RK3E-ras-Fluc cells (RK3E-ras-Fluc) were described previously (12). The cells were grown in DMEM (GibcoBRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin (GibcoBRL). The cells were maintained at 37°C in a 5% CO2 humidified atmosphere.
Cell proliferation assays. The cells (5,000 cells/well) were seeded into 96-well plates, which were subsequently incubated for 24 h. The cells were then replenished with fresh complete medium containing HCA or BCA (dissolved in 0.1% DMSO), after which they were incubated for an additional 24 h. The cell proliferation was then evaluated by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays as described previously (13).

Cell cycle analysis. The cells were harvested and fixed with ice cold 70% ethanol overnight. The fixed cells were washed with PBS containing 1% fetal bovine serum and then incubated with 100 μg/mL RNase A at 37˚C for 30 min. Propidium iodide was added to a final concentration of 50 μg/mL for DNA staining, after which the fixed cells were analyzed by flow cytometry using a FACScalibur (BD Biosciences, San Jose, CA, USA).

Western blot analysis. The cells were treated with HCA or BCA for 24 h. The cells were then washed twice with ice-cold PBS and lysed in RIPA buffer (0.01 M Tris-HCl [pH 7.4], 0.15 M NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). Next, the protein contents of the cell extracts were determined by a Bradford assay (Bio-Rad, Hercules, CA, USA). The proteins were then resolved by SDS-polyacrylamide gel electrophoresis, electrotransferred onto a polyvinylidene fluoride (PVDF) membrane and then immunoblotted with anti-poly (ADP-ribose) polymerase (PARP) antibody, anti-caspase-3 antibody (Cell Signaling Technology, Danver, MA, USA), or anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Animals and tumor growth. Three-week-old male Sprague-Dawley rats (Samtaco, Osan, South Korea) were kept under standard housing conditions. RK3E-ras-Fluc cells were harvested by trypsinization, centrifuged and then resuspended in DMEM without serum at a density of 2.5×10^5/mL. Two hundred µL of cell suspension (total 5×10^6 cells) were then injected into the oral mucosa of control and treated rat groups; each group consisted of five rats. HCA treatment began on the 5th day after oral injection of RK3E-ras-Fluc cells. HCA (50 mg/kg) or an equal volume of the vehicle, DMSO, was directly injected into the tumor every other day for five times. On the next day after the final treatment, the animals were sacrificed and the solid tumors were excised for further analysis. The tumor growth was evaluated using a caliper and the tumor volume (V) was calculated using the following formula described by Carlsson: \( V = \frac{ab^2}{2} \), where ‘a’ is the longest diameter and ‘b’ is the shortest diameter of the tumor (14). The excised solid tumors were immediately fixed in 10% buffered formalin and then embedded in paraffin. Body weight was regularly checked to determine cinnamaldehyde toxicity. All the experiments were conducted following protocols approved by the Animal Care and Use Committee at Chosun University College of Dentistry (Gwangju, South Korea).

**Bioluminescence imaging.** Luciferin ( Molecular Probes, Palo Alto, CA, USA) was injected i.p. at a dose of 80 mg/kg of body weight with xylazine/ketamine anesthesia. The bioluminescent signals emitted from the tumor of the rats were then evaluated using a Xenogen IVIS-100 Imaging System (Xenogen Co., Alameda, CA, USA) equipped with a CCD camera system for emitted light acquisition. The Living Image software (Xenogen) was used for data analysis.

**Histopathology and immunohistochemistry.** For light microscopic examination, 4-μm sectioned tissues were stained with hematoxylin-eosin (H-E). Immunohistochemical staining was performed on similar sections by the avidin-biotin peroxidase complex (ABC) method using anti-proliferating cell nuclear antigen (PCNA) antibody (Dako, Glostrup, Denmark). Immune reactions were visualized with 3,3'-diaminobenzidine (DAB) and counterstained with Mayer's hematoxylin.

**TUNEL assay.** A TUNEL assay was performed using an ApopTag Plus Peroxidase In Situ Apoptosis Detection kit (Intergen, Purchase, NY, USA) according to the manufacturer’s instructions. Briefly, the tumor sections, mounted on slides were deparaffinized and incubated with 20 μg/mL proteinase K at 37˚C for 15 min, then immersed in 3% hydrogen peroxide and incubated with terminal
deoxynucleotidyl transferase containing reaction buffer at 37˚C for 1 h. Finally, the sections were incubated with peroxidase-conjugated anti-digoxigenin antibody for 30 min, after which the reaction products were visualized with 0.03% DAB solution containing 2 mM/L hydrogen peroxide. Counterstaining was performed using 0.5% methyl green.

Statistical analysis. The differences in mean values among groups were evaluated and the values were expressed as the means±SD. All the statistical calculations were conducted using Microsoft Excel.

Results

Effect of HCA on cell proliferation and the number of cells in the sub-G1-phase. As shown in Figure 1, HCA strongly inhibited the growth of SCC-15 and HEp-2 cells in a dose-dependent manner with IC50 values of 20.2 and 40.5 μmol/L, respectively. BCA, one of the HCA derivatives, also showed similar growth inhibitory activity against these two cell lines. Because the growth-inhibitory effects of BCA did not differ from those of HCA, the following experiments were...
performed using HCA. The cell cycle distribution was analyzed under the HCA-induced growth inhibitory conditions using flow cytometry. When treated with HCA for 48 h, the SCC-15 cells were arrested in the G2/M-phase depending on the HCA concentrations (Figures 2A and 2C). Specifically, the proportion of cells arrested in the G2/M-phase increased from 27.97% in the untreated control cells to 40.88% in the 30 μM HCA-treated SCC-15 cells. More importantly, the number of cells in the sub-G1 phase (apoptotic cells) also increased markedly, from 5.41% in the untreated control cells to 21.97% in the 30 μM HCA-treated SCC-15 cells (Figure 2A and 2C). The number of HEp-2 cells arrested in the Sub-G1 phase also increased from 3.64% to 22.32% on HCA treatment (Figure 2B and 2C).

**Effect of HCA on apoptosis.** Because HCA treatment increased the population of apoptotic cells, the mechanism of apoptosis induction was investigated. The SCC-15 or HEp-2 cells were treated with various concentrations of HCA or BCA for various times. The levels of cleaved PARP and cleaved caspase-3 in the total cell lysates were determined by western blot analysis. As shown in Figure 3, treatment with HCA led to increased levels of cleaved PARP and caspase-3 in a time- and dose-dependent manner. Treatment with BCA also led to increased levels of PARP and caspase-3 cleavage in the oral carcinoma cells (Figure 3).

**Effect of HCA on the growth of oral tumors in vivo.** Both the gross appearance and bioluminescence imaging showed significant inhibition of tumor growth after 10 days of treatment with HCA (Figures 4A, 4B and 4C). Tumor masses were reduced to 23.9±8.8% upon HCA treatment compared to the controls (Figure 4D) (p<0.05). Furthermore, no difference in the body weight loss between groups treated with HCA and the vehicle controls was evident for 14 days (data not shown).

Histologically, the oral tumors were anaplastic undifferentiated carcinoma with characteristic mitotic figures, multifocal necrosis and hemorrhage. However, HCA treatment led to extensive tumor cell death surrounded by granulomatous

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**Figure 3. Effect of HCA on induction of apoptosis.** SCC-15 or HEp-2 cells were treated with 30 μM HCA for the indicated time periods (A), or the indicated concentrations of HCA or BCA for 24 h (B). Cleaved PARP and cleaved caspase-3 in total cell lysates were determined by Western blot analysis. Actin was used as loading control.
inflammation with calcification (Figure 5, top panel). In addition, the immunohistochemistry of the tumor cells revealed that nearly all the cells in the control tumors were PCNA positive, which suggested a high proliferation rate. However, treatment of the tumor with HCA resulted in a notably decreased number of PCNA-positive cells (Figure 5, middle panel). Conversely, the number of TUNEL-positive cells increased greatly in response to HCA treatment, which suggested that HCA inhibited tumor growth by inhibiting cell proliferation and activating apoptosis (Figure 5, bottom panel).

Discussion

Previous study showed that HCA induced apoptosis in human colon and breast cancer cells through the elevation of reactive oxygen species (ROS), caspase-3 activation, and degradation of PARP (15). It was also reported that HCA-induced apoptosis was associated with proteasome inhibition that led to the increase of ER stress and mitochondrial perturbation, which are representative events associated with apoptosis induced by proteasome inhibitors (16). In this study, HCA showed strong anti-proliferative activity toward the human oral squamous cell carcinoma cell lines, SCC-15 and HEP-2 by inducing cell cycle arrest at the G2/M phase and apoptosis (Figures 2 and 3). Furthermore, direct intratumoral injection of HCA successfully inhibited growth of tumor mass via inhibition of cell proliferation and induction of apoptosis (Figures 4 and 5).

As a part of preclinical evaluations, plasma pharmacokinetics and metabolism of BCA have been characterized recently (17). BCA was not detected in the plasma because of its rapid conversion to HCA. In addition, HCA was converted to its inactive metabolite o-coumaric acid (t1/2=2.1±0.1) in a quantitative manner. The biotransformation of HCA to o-coumaric acid is probably mediated by aldehyde oxidase. Therefore, the biotransformation process of HCA could be partially inhibited in the hypoxic conditions of a solid tumor (18). Solid tumors are inherently resistant to some types of chemotherapy due to hypoxia and to target this resistant population, bioreductive drugs that are preferentially toxic to the tumor cells in a hypoxic environment are being evaluated in clinical trials (19). HCA, from this point of view, would therefore be an effective and valuable antitumor agent for oral cancer treatment, since oral cancer is easily accessible for intratumoral injection. Furthermore, the hypoxic environment would become more pronounced upon HCA treatment, because of its anti-angiogenic effect (4).

In general, proteasome inhibitors induce apoptosis in cancer cells that are resistant to apoptosis due to mutations in some components of the apoptotic machinery, such as p53 (20, 21). The SCC-15 and HEP-2 cells used in this study carried p53 mutations, which suggest that HCA induces p53-independent apoptosis in cancer cells. According to our
unpublished data, HCA induces potent apoptosis in cancer cells, while normal cells show much less sensitivity to HCA. The mechanisms for the different responses of HCA in normal cells and tumor cells are now under investigation.

HCA shows antitumor activity in SCC-15 and HEp-2 oral carcinoma cells and inhibits cell proliferation by inducing cell cycle arrest and apoptosis. More importantly, HCA effectively arrests tumor growth by inhibiting cell proliferation and inducing apoptosis in a rat oral tumor model. These results suggest the potential value of HCA as a candidate for oral cancer therapies.

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References


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