Inhibitory Effects of Caffeic Acid Phenethyl Ester Analogues on Experimental Lung Metastasis of Murine Colon 26-L5 Carcinoma Cells

Takema NAGAOKA, Arjun H. BANSKOTA, Yasuhiro TEZUKA, Yuko HARIMAYA, Keiichi KOIZUMI, Ikuo SAIKI, and Shigetoshi KADOTA*

Institute of Natural Medicine, Toyama Medical and Pharmaceutical University; 2630 Sugitani, Toyama 930–0194, Japan.
Received November 22, 2002; accepted January 16, 2003

We have previously examined the antiproliferative activity of caffeic acid phenethyl ester (CAPE) and its 20 analogues against six tumor cell lines, and found that CAPE analogues possess selective antiproliferative activity toward the murine colon 26-L5 carcinoma cell line. To extend our study, the effects of CAPE analogues on the metastatic development of murine colon 26-L5 carcinoma cells in the lung were examined. The oral administration of CAPE (5 mg/mice/d) for 7 d after tumor inoculation decreased the tumor weight and the number of tumor nodules in the lung by 50% and 50%, respectively, compared to the control, while CAPE (5 mg/mice/d) administered for 7 d before tumor inoculation showed no significant effect. Besides CAPE, 4-phenylbutyl caffeate, 8-phenyl-7-octenyl caffeate, 2-cyclohexylethyl caffeate and n-octyl caffeate at an oral dose of 2 mg/mice/d caused a 55%, 43%, 55% and 35% reduction of the tumor nodules in their lung metastasis formation, respectively. These results further elaborate the possibility of CAPE and its analogues to become a new class of chemopreventive agents for the treatment of colon cancer metastasis.

Materials and Methods

Materials CDDP (cis-diamine-dichloroplatinum; Randa®, 0.5 mg/ml) was purchased from Nippon Kayaku Co., Ltd. (Tokyo, Japan). RPMI-1640 was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Heat-inactivated fetal calf serum (FCS) and trypsin were from Gibco BRL Products (Gaitherburg, MD, U.S.A.). A cell culture flask (150, 75 cm²) coated with polystyrene (Corning Incorporated, Corning, NY, U.S.A.) was used to culture the cells. EDTA and Tween-20 were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), glutamine, sodium bicarbonate and corn oil were from Wako Pure Chemical Industries (Osaka, Japan). Caffeic acid esters (1—5) were synthesized by esterification of caffeic acid chloride with corresponding alcohol, as describe in our previous report, and were pure in TLC and 1H-NMR spectral observation.

Cell Lines and Cell Culture The murine colon 26-L5 carcinoma cell line was established by one of the authors (I. Saiki). Murine colon 26-L5 carcinoma cells were cultured in RPMI-1640 medium, supplemented with 0.1% sodium bicarbonate and 2 mM glutamine and 10% fetal calf serum. The cells were propagated at 37°C in a humidified atmosphere containing 5% CO₂.

Animals Inbred 6-week-old female Balb/c mice were purchased from Shizuoka Laboratory Animal Center, Hama-matsu, Japan. All mice were housed in a controlled environment with a 12 h light/dark cycle, a temperature of 24±2°C and a humidity of 55±10%, and they were given a laboratory pellet chow (Labo MR, Nosan, Kanagawa, Japan) and tap water ad libitum. This study was conducted in accordance

Fig. 1. Structure of CAPE Analogues

To whom correspondence should be addressed. e-mail: kadota@ms.toyama-mpu.ac.jp © 2003 Pharmaceutical Society of Japan
with the standards outlined in the Guidelines for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

**Experimental Lung Metastasis Assay** Log-phase cell cultures of colon 26-L5 cells were harvested with 1 mM EDTA in phosphate-buffered saline (PBS), washed three times with serum-free RPMI-1640, and resuspended at 2×10⁶ cells/200 μl concentration in serum-free medium, then 200 μl of the cell suspension was injected intravenously into the mice. The mice were then divided randomly into different groups of 6 mice per group. The CAPE analogues 1—5 were suspended in corn oil/Tween 20/water (6/1/13, w/w) and administered orally by gastric tube to mice at the appropriate dose for 7 d before or after tumor inoculation. CDDP was injected intravenously at the dose of 80 μg/mice on the 1st and 8th days after tumor inoculation. At the 15th day after tumor inoculation, the mice were sacrificed under an anesthetic condition and their lungs were removed. The weight of the lung was taken immediately after washing with distilled water and drying on tissue paper. The tumor nodules in the lungs were counted under a dissection microscope. The tumor weight was calculated by subtracting the lung weight of the normal mice group (200 μl serum free medium inoculated group) from that of colon 26-L5 cell treated group.

**Statistical Analysis** All data are expressed as the mean±S.D. Student’s t-test for unpaired observations between control and tested samples was carried out to identify statistical differences; p values of less than 0.05 were considered significantly different.

**RESULTS**

**Effect of CAPE Before or After Cell Inoculation on Lung Metastasis Formation** Initially, we investigated the effect of CAPE (1, 5 mg/mice/d), by oral administration before or after inoculation of colon 26-L5 carcinoma cells, on the experimental lung metastasis model. Mice administered CAPE (1) before cell inoculation did not show any suppression of tumor formation. In contrast, mice administered CAPE (1) after cell inoculation showed a decrease in their lung tumor formation; the tumor weight was reduced by 50% and the number of nodules by 50% (Fig. 2). Thus, the antimitastatic effect of CAPE (1) might be due to cytotoxicity or growth inhibition against tumor cells, or due to blocking of the invasion process, an initial step of lung metastasis.

**Effect of CAPE at Various Doses** We further examined the effect of CAPE (1) on the same experimental model at different doses, or of CDDP, a well-known anticancer drug having strong anti-metastatic properties toward lung metastasis.29 Oral administration of CAPE (1) at 5 and 2 mg/mice/d did not produce any significant difference in suppressive effect on the formation of lung tumor (Figs. 3a,b). The tumor weight, as well as the number of nodules, was reduced by 30—40% at both doses, while CDDP suppressed tumor weight by 89% and the number of nodules by 72%. However, CDDP injection caused a significant reduction in body weight, i.e., a toxic effect, but CAPE (1) at 5 mg/mice/d had little effect on body weight (Fig. 3c). These results suggest that CAPE (1) could suppress tumor metastasis without side effects. On the other hand, there was no significant anti-metastatic effect of CAPE (1, 5 mg/mice/d) on lung metastasis by murine B16-BL6 melanoma cells20 (data not shown), which led to the conclusion that CAPE (1) selectively inhibits the metastasis of colon 26-L5 carcinoma cells.

**Effect of CAPE Analogues** In our previous in vitro study, we observed that 4-phenylbutyl caffeate (2), 8-phenyl-7-octenyl caffeate (3), 2-cyclohexylethyl caffeate (4) and n-octyl caffeate (5) (Fig. 1) had potent antiproliferative activity toward the murine colon 26-L5 carcinoma cell line.17 Thus, these compounds 2—5 were subjected to an in vivo experiment at a dose of 2 mg/mice/d. Ester 2 showed the highest degree of suppressive effect on the reduction of the number of nodules by 55% (Fig. 4), but there was no significant reduction in tumor weight, even it was reduced by 58%. Similarly, ester 4 possessed stronger inhibitory activity than CAPE (1) at 2 mg/mice/d. On the other hand, ester 3 and 5 possessed inhibitory activities a little weak but identical with CAPE (1).

**DISCUSSION**

The results indicate that CAPE and its analogues possess anti-metastatic properties. CAPE was previously reported to be a strong antioxidant, to inhibit soybean 15-lipoxygenase, and to completely block the production of reactive oxygen species (ROS) in human neutrophils and in the cell-free xanthine/xanthine oxidase (XOD) system.21 These antioxidative properties of CAPE are considered important factors in the protection of cancer formation in mice bearing an Apc gene.80 CAPE at a dietary level of 0.15% decreased tumor formation in C57BL/6-Min/+ mice bearing germline mutation in the Apc gene.81 The author concluded that the tumor
prevention by CAPE was associated with increased enterocyte apoptosis and proliferation closely associated with ROS. In our previous report, we also observed that CAPE inhibits the proliferation of the colon 26-L5 carcinoma cell line via apoptosis. Moreover, it has been well documented that ROS are associated with tumor promotion. ROS are thought to act as second messengers for signal transduction pathways that regulate cell proliferation, and by reducing intracellular peroxides, antioxidants may expect to inhibit carcinogenesis. We further tested the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of CAPE and its analogues. All these compounds possessed an equal strength of DPPH free radical scavenging activity (EC50, 5.20—6.99 μM) to that of caffeic acid (EC50, 4.83 μM). Thus, it may be partly due to its antioxidative property that CAPE and its analogues show anti-metastatic activity.

Fig. 3. Effect of CAPE (1) at Various Doses
CAPE (1) was administered orally daily for 7 d after tumor inoculation into mice. CDDP was injected intravenously into mice on day 1 and day 8. At the 15th day after tumor inoculation, the number of nodules on the surface of the lungs was counted (a), tumor weight calculated (each group average lung weight—normal group average lung weight) (b), and mean body weight of the mice measured during the experiment (c). The data are expressed as mean±S.D. of 6 mice. *p<0.05, **p<0.01.

In conclusion, oral administration of CAPE (5 mg/mice) for 1 week after tumor inoculation significantly decreased the tumor weight and the number of nodules in the lung. Besides CAPE, 4-phenylbutyl caffeate, 8-phenyl-7-octenyl caffeate, 2-cyclohexylethyl caffeate and n-octyl caffeate also significantly reduced the tumor nodules in their lung metastasis formation. These results further elaborate the possibility of CAPE and its analogues as a new class of chemopreventive agents for the treatment of colon cancer metastasis.

REFERENCES