Original Article

Inhibitory Effect of Rosmarinic acid Extrcted from Euonymus Alatus on Cyclooxygenase-2

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Objectives and methods : Previous mechanistic studies suggest the cyclooxygenase-2 (COX-2) inhibitors represent the good candidates against tumor progression. MeOH extract of the stem barks of Euonymus alatus induced the strong inhibition of COX-2. A phenolic compound responsible for the anti- COX-2 known to involve in tumor adhesion and invasion has been studied through the methanol extracts. The compound, rosmarinic acid (ROS-A) was an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. ROS-A showed a strong inhibitory effect of COX-2 activity in a concentration-dependent manner. Then we have measured the IL-1β, IL-6 and TNF-α production related the immune regulation, induction of inflammatory related genes.

Results and Conclusions : Hep3B cells produce proinflammatory cytokines of IL-1β, IL-6 and TNF-α while ROS A inhibited the cytokines production. Since IL-1β, IL-6 and TNF-α need the transcription factors such as nuclear factor-κB (NF-κB) and activated protein-1 (AP-1), we measured the transcription factors. ROS-A inhibited the activation of p65, p50, c-Rel subunits of NF-κB and AP-1 transcription factors. These findings indicate that ROS A from the stem bark of E. alatus inhibits proliferation in metastatic cancer cells. It was suggested that stem barks of E. alatus could be suitable for anti-cancer drugs.

Key Words : Euonymus alatus, cyclooxygenase-2, Rosmarinic acid, transcription factor, cytokines

Introduction

An understanding of the mechanisms of tumor metastasis may be important for chemopreventive therapies aimed for preventing tumor cell growth. Cyclooxygenase-2 (COX-2) activity is elevated in tumor cells[1]. Previous studies on euonymus have resulted in the isolation of cardenolides[2]. The anti-cancer and cytotoxic activities of the crude extract however, have not yet been demonstrated. Euonymus alatus (Thunb.) Siebold has been used in traditional medicine for cancer treatment[3]. In a previous paper[4], methanol extracts prepared from E. alatus showed a strong inhibitory effect of MMP-9 activity involved in tumor cell metastasis in a concentration-dependent manner. Assay guided fractionation led to the isolation of a caffeic acid (CA) as the compound responsible for the anti-MMP-9 activity[6,7]. The result was related to the total phenolic content (TPH) determined by the Folin-Ciocalteu method, and were compared with two reference substances, quercetin and (-)-epigallo-catechin gallate (EGCG). During cancner progression, inflammatory cytokines may influence survival, growth, proliferation and migration of tumor cells. In addition, COX-2, an enzyme in the cancer progression, affects carcinogenesis via the inflammation

and immunological reaction[1].

Transcription factors of nuclear factor-κB (NF-κB) and activated protein (AP-1) up-regulate the production of cytokines such as IL-1, IL-6, and TNF-α as well as MMP-9[5,6]. NF-κB group is comprised of five different subunits of p50, p52, p65 (Rel A), Rel B and c-Rel. AP-1 consists of homodimers and heterodimers of Jun, Fos or activating transcription factor (ATF)[8]. AP-1 activation plays an important role on cell proliferation[9]. Immune control, induction of different inflammatory and growth related genes such as IL-1β, IL-6 and TNF-α need transcription factors such as NF-κB and AP-1.

In this study, we have studied ROS-A as a COX-2 inhibitor. ROS-A is a phenolic acid and has many different activities, mainly one being anti-inflammatory like other phenolic compounds, to the antioxidative activity of plants used in the medicinal[11]. For cancer cell proliferation, we have focused on the activation and nuclear translocation of NF-κB and AP-1. Hep3B cells produced cytokines such as IL-1β, IL-6 and TNF-α while ROS-A inhibits the levels of the cytokines. ROS-A significantly inhibited the nuclear translocation of p65, p50, NF-κB and AP-1. Therefore, the present study has aimed on the new COX-2-inhibitory activity of the ROS-A, suggesting that the ROS-A can be used for the cancer chemotherapy.

Materials and Methods

1. Materials

Rosmarinic acid (536954-5G) was also purchased from Aldrich-Sigma to compare with the purified ROS-A. Other chemicals were of analytical grade.

2. Cell culture

Human hepatocellular carcinoma cell line (Hep3B) was obtained from the Korean cell line bank and cultured in Dulbecco's modified eagle's medium (DMEM, Gibco-BRL, USA) containing 10% heat inactivated fetal bovine serum (FBS, Gibco-BRL, USA) supplemented with penicillin (100 units/mℓ), streptomycin (100 μg/mℓ) and sodium bicarbonate (2.2 g/L) at 37℃ in 5% CO_2-air. Cells were grown and were washed with phosphate-buffered saline (PBS) and then incubated in serum-free medium for 24 h.

3. Extraction and COX-2-inhibiting fractionation

Two ㎏ (dry weight) of the stems of E. alatus (Thunb.) Siebold was extracted with methanol (3 × 10 L) by maceration for 24 h. The combined methanolic extracts were filtered and concentrated under vacuum to dryness affording 525.3 g of crude extract (CME) (extraction yield 27.8% (w/w) dry weight). The crude extract was then redissolved in water (1 L), filtered and then partitioned with hexane until the organic solvent was colorless, thus obtaining a hexane fraction (HXF), which was dried under vacuum (115.4 g, extraction yield 24% (w/w) of CME) and a "defatted crude extract (DCE). An aliquot of 10 mℓ of DCE was freeze-dried for extraction yield calculation. The DCE was partitioned with ethyl acetate obtaining an ethyl acetate fraction (EAF) which was dried under vacuum (83.2 g, extraction yield 17% (w/w) of DCE) and an aqueous fraction (EA-AF) which was freeze-dried (335.6 g, extraction yield 65%(w/w)) of DCE.

An aliquot (10 g) of the dried EAF was dissolved in MeOH and fractionated by gel filtration using Sephadex LH-10 (Pharmacia, 5 ㎝ × 50 ㎝, MeOH, 1 mℓ/min) to afford 50 fractions (10 mℓ each). The fractions were monitored by TLC (Whatman silicagel plates, EtOAc:AcOH:H20, 10:2:3). After drying, the plates were sprayed with 1% diphenylboric acid in MeOH for UV detection of phenolic compounds, and then visualized under UV light at 254 nm and 365 nm. The TLC plates were sprayed with 2,2-diphenyl-1-picrylhydrazyl (DPPH) methanolic solution (20 g/L) and examined 20 min after spraying as yellow spots. Fractions were combined to obtain three active fractions (from A to C).
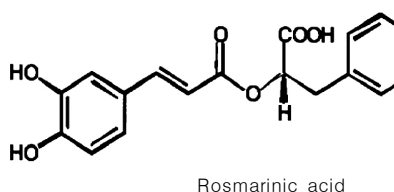
Fig. 1. Chemical structure of ROS-A

Then fraction C was subjected to gel chromatography using Sephadex LH-20 (Pharmacia, 50 ㎝ × 10 ㎝, MeOH, 0.5 ㎖/min). One ㎖ fraction was collected on the TLC. The active fractions were collected as the COX-2-inhibitory compound (Fig. 1).

4. Total phenolic content

The total phenolic content was decided by the Folin-Ciocalteu agent[10]. The content level was expressed as equivalent gallic acid (GAE)/mg of dry extract[10].

5. XTT proliferation assay

Cell growth was examined using a proliferation kit II (XTT, Boehringer Mannheim, Germany). Hep3B cells were cultured into 96-well plates at a density of 10,000 cells per well in 150 µl of DMEM medium and incubated for 24 h at 37°C. Following incubation, the cells were replenished with 100 µl of new medium and were treated with various concentrations (0 to 200 µg/㎖) of CA. The plates were incubated in a 37°C humidified incubator at 5% CO_2 for 24 h. At the end of the incubation, the cells were washed with PBS. Fifty µl of XTT test solution (sodium 3'-[1-(phenyl-aminocarbonyl) -3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate and N-methyl dibenzopyrazine methyl sulfate; mixed in proportion 50:1) prepared by XTT reagent and electron coupling reagent, was added. After 4 h of incubation in a 37°C and 5% CO_2 incubator, the absorbance was measured on an ELISA reader at 490 nm. All determinations were confirmed at least three experiments.

6. Enzyme assay for artificial substrate

For enzyme inhibition assay, the COX-2 enzyme was preincubated with ROS-A for 60 min. COX-2 activity from the culture of Hep3B cells was assayed[1].

7. Measurement of IL−1β, IL−6 and TNF−α in Hep3B cells

Hep3B hepatoma cells ($2×10^5$ cells/well) in 96-well plate were incubated at 37°C in 5% CO_2 in DMEM with 10% FBS. After 24 h, the cells were incubated for 24 h in serum-free medium in the presence and absence of ROS-A (2-10 µg/㎖). The total volume in the wells was 300 µl. The culture supernatant was centrifuged and IL-1β, IL-6 and TNF-α were determined by ELISA.

8. RNA isolation and RT−PCR

To examine the effect of ROS-A on the gene expression level of IL-1β, IL-6 and TNF-α, total RNA was subjected to PCR. Hep3B cells ($2×10^5$ cells/well) were grown in the presence and absence of ROS-A (2-10 µg/㎖). Cells were treated with ROS-A for 6 h at 37°C in 5% CO_2 in serum-free medium. Total RNA was extracted from Hep3B cells and cDNA was synthesized. PCR was performed with inflammatory cytokine PCR kit for GAPDH, IL-1β, IL-6 and TNF-α genes. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

9. Preparation of nuclear extracts

Nuclear extracts were prepared by slightly modified previously published methods[6]. The cells were grown in 25㎠ culture flask. When the cells were getting subconfluent, cells were treated with ROS-A (2-10 µg/㎖) for 2 h at 37°C in 5% CO_2 in serum-free medium. The cells were washed with PBS twice and incubated with TNF-α (100 pg/㎖) for 30 min at 37 °C in 5% CO_2. Cells were washed with PBS, dislodged with a cell scraper and collected by centrifugation at 450×g. The cell pellet was resusp-

ended in cell lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 mM PMSF, 1 mM DTT, 0.5% Nonidet P40, 0.1 mM EGTA and 0.1 mM EDTA) and allowed to swell on ice for 15 min, followed by centrifugation at 400×g for 10 min. The cell pellet was resuspended in lysis buffer twice. Disrupted cells were incubated for 15 min on ice, and the disrupted cell suspension was centrifuged at 10,000×g for 30 min at 4°C. Nuclear pellet was resuspended in extraction buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl2, 420 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF and 1 mM DTT) and incubated on ice for 30 min. The nuclear suspension was centrifuged at 20,000×g for 20 min at 4°C and the supernatant was collected as nuclear extract.

10. Transcription factor profiling

Subunits of NF-κB such as p65, p50, c-Rel and AP-1 were analyzed in the nuclear extract. Using an ELISA, the transcription factors was targeted. Each transcription factors were detected by specific primary antibody towards NF-κBp65, NF-κBp50, NF-κB c-Rel and AP-1. A horseradish peroxidase-conjugated secondary antibody was used to detect the bound primary antibody. Percentage inhibition was calculated by the formula: 100-([OD of piperine-treated/OD of control]×100).

11. Densitometric and statistical analysis

The intensity of the bands obtained from zymogram studies was estimated with Gel-Print System (Core Bio Corp., Seoul, KOREA). The values are expressed as means ± SE.

12. HPLC analysis

HPLC analyzes were performed on a liquid chromatograph system consisting of a quaternary pump, an autosampler Waters 700 and UV-vis detector, a C18 reverse column; solvent, MeOH/H2O/AcOH (25:70:5), linear gradient of MeOH/H2/AcOH (25:70:5)-MeOH; flow rate.

13. Determination of ROS-A

ROS-A was determined by HPLC after extraction. ROS-A was quantitatively determined by an external standard method. The detection limits for ROS-A was 0.05 μM with linear detector response up to 20 μM. When necessary, samples were diluted with mobile phase before HPLC analysis. For the bioassay test, samples were dissolved in dimethyl sulfoxide (DMSO) and further diluted in incubation buffer.

Results

1. ROS-A from E. alatus

In a preliminary study, MeOH extract from E. alatus showed the strong COX-2 inhibitory activity in a dose-dependent manner. Therefore, to show the compound for this inhibitory activity, an active phenolic compound was obtained from the ethyl acetate fraction, as described below: the total phenolic content of the different obtained extracts and fractions are shown in Table 1. The EAF fraction contained the phenolics (613 GAE/mg extract), which was correlated with the highest COX-2 activity (71.2%). After fractionation of the EAF fraction, the COX-2-inhibitory activity was distributed among the different fraction. COX-2-inhibitory activity was mainly found in fraction C (82.3%).

Fraction C was fractionated as described to isolate the compound. It was identified as ROS-A on the basis of its UV. In addition, its identity was compared with those of an authentic sample (Fig. 1). The spectrum was in agreement with that reported for ROS-A.

2. Effect of ROS-A on the cell viability, cytokine production by Hep3B cells

Hep3B cells were treated with various concentrations of ROS-A and cell viability was measured using the XTT assay. The survival curve showed that ROS-A has no cytotoxicity on the cells. The addition of 100 μg/㎖ of ROS-A did not change the viability, while treatment with 200 μg/㎖ of ROS-A

Table 1. COX-2-inhibitory activity of different extracts and fractions of E. alatus compared to that of some reference compounds

Extract/fraction	Dry weight (g)	Total phenolic content	COX-2-inhibitory activity (%)
CME	525.3	356.55± 21.64	58.3±5.2
HXF	115.4	9.23±1.43	4.6±0.2
DCE	312.5	387.35±19.52	66.31±7.6
EAF	83.2	613.23± 87.45	72.6±3.1
EA-AF	335.6	289.55±23.24	52.4±4.3
EAF fraction			
A	0.22	-	12.2±2.2
B	0.21	-	18.3±1.2
C	0.76	-	87.3±1.2

Phenolic content was expressed as GAE/㎎ extract. Values of COX-2 inhibitory activity were expressed as % of inhibition (10 μg/㎖).

to the cells reduced 12% of the cell viability. Inflammatory cytokines in the culture supernatant of the Hep3B cells were determined by ELISA using antibodies. Although the Hep3B cells produced IL-1β, IL-6 and TNF-α, ROS-A (2-10 μg/㎖) significantly (P<0.01) reduced the cytokine production (Table 2).

3. Effect of ROS-A on the transcription factors

Transcription factors were detected by the trans-fector-specific primary antibodies. A horseradish peroxidase-conjugated secondary antibody was used to detect the primary antibody by spectrophotometrically, as described in Methods. The results have clearly shown that ROS-A (2-10 μg/㎖) inhibits the translocation of NF-κB subunits such as p65, p50 and c-Rel, and also AP-1 (Table 3). The high dose of ROS-A (10 μg/㎖) showed significant inhibitory activities of the above transcription factors (2 μg/㎖).

Table 2. Effect of ROS-A on the IL-1β, IL-6 and TNF-α production by Hep3B cells

Cytokine	Untreated control	ROS-A (2 μg/㎖)	ROS-A (10 μg/㎖)
IL-1β	145±9.3 pg/㎖	104.2±8.4 pg/㎖[*]	67.7±4.5 pg/㎖[*]
IL-6	176±15.3 pg/㎖	134.5±9.5 pg/㎖[*]	87.3±5.7 pg/㎖[*]
TNF-α	156.3±14.4 pg/㎖	112.8±8.2 pg/㎖[*]	68.3±7.8 pg/㎖[*]

Hep3B cells were incubated for 24 h in the presence or absence of ROS-A (2-10 μg/㎖). IL-1β, IL-6 and TNF-α were determined by ELISA. Values are the mean±S.D. * Statistically significant from the untreated control: P<0.01.

Table 3. Effect of ROS-A on the translocation of transcription factors in Hep3B cells

Transcription factor	Control	ROS-A (2 μg/㎖)	ROS-A (10 μg/㎖)
NF-κB p65	0.41±0.01	0.32±0.01	0.14±0.01[*]
NF-κB p50	0.52±0.01	0.42±0.05	0.21±0.02[*]
NF-κB c-Rel	0.62±0.03	0.25±0.05[*]	0.14±0.01[*]
AP-1	0.32±0.05	0.21±0.02	0.14±0.02[*]

Hep3B cells were grown and treated with ROS-A (2-10 μg/㎖) for 2 h at 37°C in 5% CO2 cells and incubated with TNF-α (100 pg/㎖) for 30 min at 37°C in 5% CO2. Optical density was measured. Values are the mean±S.D. * Statistically significant from the untreated control: P<0.015

Discussion

A multitude of biological activities ROS-A are antioxidative and antiinflammatory[12]. The antiinflammatory properties are based on the inhibition of cyclooxygenases[12]. Phenolic compounds can provide protection against cancer and ROS-A contributes to the anti-oxidant activity in Rosmarinus officinalis and Sanicula europaea[13]. Tumor invasion is an important step in the sequential process of metastasis, and is subdivided into three steps including tumor cell adhesion, migration and enzymatic degradation of the ECM and basement membrane. Therefore, many attempts have been made to discover promising agents with anti-metastatic activity. Anticancer properties have been associated with the components of various natural products including green tea polyphenols, resveratrol, limonene and organosulfur compounds from garlic. Especially, EGCG inhibited the COX-2 activity.

In the present study, we have studied COX-2-inhibitory compound ROS-A and confirmed the inhibitory effect of ROS-A on COX-2. ROS-A dramatically inhibits COX-2 enzyme activity in a dose-dependent manner. These results suggest that ROS-A could be used as a potential anti-tumor agent having anti- COX-2 activity. For the COX-2 gene expression, transcription factors such as NF-κB and AP-1 proteins have been implicated as a role in cancer. In addition, these factors have also been regarded as a cellular factor by growth stimuli mediated by cytokine pathways[14]. Translocated NF-κBs are rich in cancers such as colorectal cancer and breast cancer[8]. The NF-κB is a heterodimer with p50 and p65 subunits and these are the most frequent components of active NF-κB. Subunit p65 containing complexes bind with high binding affinity to the consensus DNA sequences, leading to the transcription initiation of target genes[8,15]. AP-1 consists of homodimers and heterodimers of Jun, Fos or activating transcription proteins[5,6,8]. AP-1 gene expression plays an important role in cell proliferation, cell cycle regulation and tumor promotion[5,6,8]. In the present study, ROS-A could inhibit the activation or nuclear translocation of NF-κB and AP-1. From the above results, the findings suggested for the therapeutic potential of ROS-A reducing the inflammatory cytokine production.

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