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Inhibitory Effect of Paeoniflorin on Fos-Jun-DNA Complex Formation and Stimulation of Apoptosis in HL-60 Cells

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The Fos-Jun heterodimers are part of the regulatory network of gene expression and nuclear proteins encoded by proto-oncogenes. The activation of Fos-Jun is important in the transmission of the tumor-promoting signal from the extracellular environment to the nuclear transcription mechanism. To search for the inhibitors of the Fos-Jun DNA complex formation, several natural products were screened and water-soluble paeoniflorin reduced the binding activity of the Fos-Jun heterodimer. This active compound was purified by silica gel column chromatography and HPLC. The electrophoresis mobility shift assay and reverse-phase HPLC test showed that paeoniflorin reduced the AP-1 function. The cytotoxic effect of paeoniflorin was observed in HL-60. These results indicate that paeoniflorin blocks the Fos-Jun heterodimer-binding site of the AP-1 DNA and it also has cytotoxic effects on human leukemia cell lines.

Keywords: Paeoniflorin, AP-1 site, Fos-Jun heterodimer, HL-60

Introduction

Transcription is controlled by two types of DNA sequences, namely, promoters and enhancers. The control is exerted through specific binding proteins, called nuclear transcription factors that recognize short sequence motifs within the promoters and enhancers. One such factor activator protein 1, (AP-1), is a heterodimer formed by the products of the *fos* and *jun* protooncogene families. The fact that these transforming proteins play a role in transcriptional regulation became apparent as a consequence of two discoveries. First, Jun is related to a set of sequence-specific DNA-binding proteins collectively termed AP-1. Second, the Fos and Jun families of eukaryotic transcription factors heterodimerize to form complexes capable of binding 5'-TGAGTCA-3' DNA elements (Curran and Franza, 1988; Rauscher *et al.*, 1988; Angel and Karin, 1991). This sequence is also referred to as the TPA-response element (TRE), which binds the nuclear factor AP-1 (Lee *et al.*, 1987; Sassone-Corsi *et al.*, 1988). The dimerization occurs via interaction within leucine zipper domains at their carboxyl terminals. The actual contact with the DNA occurs via the basic region, which is located immediately upstream of the leucine zipper. Disruption of the fos-jun dimerization has been shown to impair the transcriptional activation and cell transformation regulated by these proteins (Baichwal and Tjian, 1990).

Paeoniae Radix, is one of the most important natural medicines and is prescribed in various Chinese medicinal preparations as an anodyne, sedative, antispasmodic and astringent (Murakami *et al.*, 1996). Various chemical studies reveal that the constituents of this natural medicine are paeoniflorin, albiflorin, and paeoniflorigenone (Aimi *et al.*, 1969). Pharmacological studies on the anti-inflammatory effect, as well as the antiallergic and anti-platelet aggregation effects of paeoniflorin and benzoylpaeoniflorin, have been reported. However, there has been no report that is related to the jun-fos transcription factor. Through various natural product extracts, Paeoniflorin was selected as a target for the AP-1 inhibitor. The active compound extracts were purified by column chromatography and HPLC. The electorphoresis mobility shift assay and reverse-phase HPLC test were performed to show the effect of paeoniflorin on the AP-1 function. The cytotoxic effect of paeoniflorin was observed in HL-60. With these results, it may be suggested that paeoniflorin inhibits the Fos-Jun heterodimer-binding site of the AP-1 DNA. It also has cytotoxic effects on human leukemia cell lines.

Materials and Methods

Fos and Jun Protein Expression The expressing vectors (pLM1) containing the c-fos and c-jun gene were provided by Dr. J. N. Mark Glover at the Harvard Medical School. Overnight

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cultures of *E. coli* BL21, transformed with pLM1-c-fos and pLM1-c-jun expressing plasmid, were diluted in 500 ml of LB with ampicillin (50 μg/ml). After shaking it until OD_{600} reaches 0.5-0.7, at 37°C, protein expression was induced with 0.5 mM IPTG for 4 h. The cells were pelleted and resuspended in 1 × PBS containing 1 mM EDTA, 2 μg/ml aprotinin, 0.1 mM PMSF, 0.1 mM $Na_2S_2O_5$ and 0.1 mM leupeptin, and then lysed by sonication. Each supernatant of the lysed cells (containing Fos and Jun proteins) was mixed at an appropriate proportion and kept at room temperature for 30 min to dimerize (Halazonetis *et al.*, 1988; Lee *et al.*, 1998). We used only a Fos-Jun heterodimer, checking by gel electrophoresis.

Isolation and Purification of Paeoniflorin Paoeniflorin was isolated and purified from Korean white peony by modification of the method used by Ishida *et al.* (1987). Grounded Paeoniae radix (100 g) was purchased from a herb clinic and extracted with H_2O (500 ml) under reflux for 0.5 h. The mixture was centrifuged at 2,500 rpm for 20 min and the supernatant was lyophilized to produce the crude extract. The H_2O extract was dissolved in water (210 ml) and extracted with ethylene acetate (210 ml) four times. Concentration of the ethyl acetate layer under reduced pressure afforded the active fraction as a brown gum. This was subjected to column chromatography on a silica gel (2.4 × 28 cm) with the lower layer of $CHCl_3$-MeOH-H_2O (7 : 3 : 1) to produce three fractions; namely Fractions I, II, and III. For this experiment, we used significantly active Faction II. The Fraction II was loaded on silica gel (1.1 × 27 cm) and eluted using $CHCl_3$-MeOH (10 : 1). Significant activity was found only in Fraction IV_2, the fourth fraction. This fraction was applied to a reverse-phase high-performance liquid chromatography (HPLC) to obtain the inhibitory element, paeoniflorin.

Gel-Electrophoresis Mobility Shift Assay The DNA-binding reaction was carried out by mixing the Fos-Jun heterodimer with the DNA probe in a gel shift buffer (20% glycerol, 5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl [pH 7.5], 0.25 mg/ml poly(dI-dC). The ^{32}P-labeled oligonucleotide was prepared by the standard radioactive 5-terminal labeling method. Briefly, 1.75 pmol of AP-1 consensus oligonucleotide (Santa Cruz) was added to 10 μ Ci-[^{32}P] dATP (Amersham-Pharmacia, Arlington., USA), T4 polynucleotide kinase (Takara Biomedicals). The mixture was incubated at 37°C for 30 min and the reaction was quenched with 0.5 M EDTA. The total volume was adjusted to 50 μl with water. After 1 h incubation at ambient temperature, samples were resolved on a 6% nondenaturing gel according to the standard technique. A gel-retardation assay was quantified by cutting the corresponding bands and measuring the radioactivity with liquid-scintillation counting (Halazonetis *et al.*, 1988).

Cell Culture and Cytotoxic Assays K562 (human erythroleukemia), HL-60 (human promyelocytic leukemia), A549 (human lung cancer), and Colo205 (human colon cancer) were maintained in RPMI 1640 (Gibco, BRL) supplemented with heat-inactivated 10% (v/v) fetal-bovine serum and 100 × antibiotic-antimicotic (Gibco BRL, Gaithersburg, USA) at 37°C in a fully humidified atmosphere of 5% CO_2. The paeoniflorin (0, 25, 50, 100, 200, 400, and 800 μM) were directly added to the suspension and grown at 37°C in a fully humidified atmosphere of 5% CO_2. Each of the human tumor culture mediums (10^4 cells/100 μl) was plated on a 96-well microtiter and incubated for 24 h. The incubated microtiter were centrifuged at 1,000 rpm for 10 min and the supernatants were removed by flicking. The formazan formed by the mitochondria succinate dehydrogenase for MTT was dissolved in 100 μl of DMSO. The absorbance of each well was measured at 540 nm by a micro ELISA-reader. The IC_{50} (50% Inhibition Concentration) value was calculated by the linear regression method (Carmichael *et al.*, 1987). The cell number and viability were estimated using a hematocytometer and trypan blue dye exclusion.

Reverse-Phase HPLC Analysis The AP-1 consensus nucleotide (HPLC grade, 1.75 pmol) was purchased from Promega. HPLC analysis was performed on Waters (150 × 2-mm column) with a flow rate of 1.0 ml/min; a 20 μl injection loop was utilized for these analyses. The Beckman-HPLC gold system was used with a detector channel setting at 254 nm. The buffer system was prepared using reagent-grade mono- and dibasic potassium phosphate, deionized water, and HPLC- grade methanol and acetonitrile. A linear gradient elution system was utilized using Buffer A and B. Buffer A was made up of 10 mM KPO_4, pH 7 and Buffer B, acetonitrile/Buffer A/methanol, 7 : 2 : 1 pH 7.2. The HPLC analysis was performed using the reverse-phase C_{18} column at a flow rate of 1 ml/min employing the Buffer A and B system with an elution gradient of 0-10 min, 0-10% B; 10-15 min, 10% B; 15-40 min 10-30% B; 40-60 min, 30-100% B; and 60-65 min, 100-0% B (Arghavani and Romano, 1995; Chuang *et al.*, 1996)

Results and Discussion

Paeoniflorin (PF) was isolated and purified from paeoniae radix with repeated silica-gel column chromatography and reverse-phase HPLC. Its structure was identified with instrumental analysis, which included ^1H-NMR, ^{13}C-NMR, and FAB-MS (Fig. 1). The ^{13}C-NMR spectrum data of paeoniflorin are shown in Table 1. The weight of the

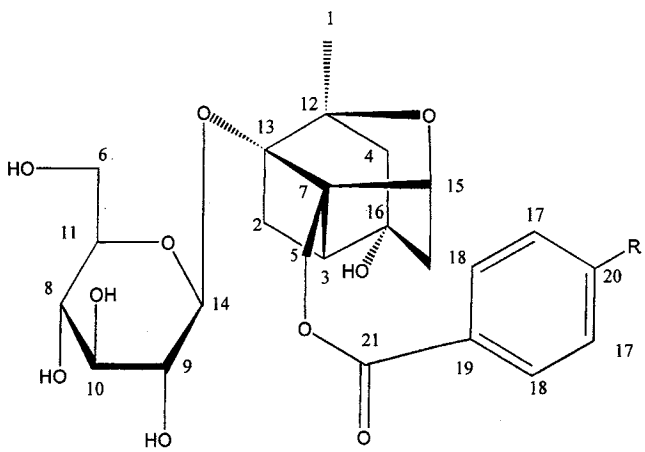

Fig. 1. The structure of paeoniflorin. R = H, paeoniflorin (PF); R = OH, oxypaeoniflorin (PF-1).

Table 1. ^{13}C-NMR Data of Paeoniflorin

Carbon (DEPT)		δ	Carbon (DEPT)		δ
1	CH_3	19.08	12	C	84.93
2	CH_2	21.96	13	C	87.45
3	CH	42.26	14	CH	98.59
4	CH_2	43.56	15	CH	100.01
5	CH_2	60.41	16	C	104.71
6	CH_2	61.17	17	CH	128.75
7	C	69.92	18	CH	129.22
8	CH	70.18	19	C	129.63
9	CH	73.41	20	CH	133.42
10	CH	76.87	21	C	165.73
11	CH	76.89	Dimethyl-d_6 sulphoxide (solvent)		

molecular ion was determined by mass-spectrum analysis and found to be 480. The FAB-MS spectrum showed that a molecular ion peaks at m/z = 503.5 $[M+Na]^+$ and 519.5 $[M+K]^+$.

The inhibitory effect of paeoniflorin on the Fos-Jun-DNA complex formation was measured by the gel-retardation assay method. Fig. 2(a), an autoradiograph demonstrates the inhibitory effect of paeoniflorin on the Fos-Jun-DNA complex formation. PF was preincubated with DNA prior to binding of the Fos-Jun heterodimer complex to the AP-1 site. The binding activity of the Fos-Jun-DNA complex was reduced by the paeoniflorin with 1.17 mmol/ml of an IC_{50} value (Fig. 2 b). We also detected the interaction between AP-1 DNA and paeoniflorin by a HPLC assay. As shown in Fig. 3 (c), the interaction of paeoniflorin (PF) or oxypaeoniflorin (PF-1) with 5'-d(TGAGTCAG) gave rise to several peaks when analyzed by the reverse-phase HPLC assay method. When the DNA and paeoniflorin were analyzed, respectively, they showed their own peaks (Fig. 3 (a), (b)). We conclude that the additional minor peaks correspond to the PFs-DNA complex involving PF, PF-1, and the AP-1 site. At this stage, the elution order of AP-1 DNA and PFs-DNA complex is not known with certainty. However, based on HPLC analysis of PFs and oligonucleotide (Ishida *et al.*, 1987; Arghavani and Romano, 1995), this result indicates that paeoniflorin interacts with AP-1: The peak of the nucleotides interacted with PF and PF-1 and was expected to split into three peaks and to elute prior to PF and PF-1. These PF and PF-1 peaks were collected, freed of solvent by lyophilization, and identified with instrumental analyses including ^{1}H- NMR, ^{13}C-NMR, and mass spectrum. To test the specificity of the interaction between paeoniflorin and the AP-1 binding site, both a competition assay and HPLC analysis were performed. Electrophoresis was performed with an increasing amount of either Fos-Jun heterodimer or DNA in the presence of paeoniflorin. As shown in Fig. 4, the change rate of radioactivities with the increasing dimer was equivalent to that of the control radioactivities. On the other hand, the change

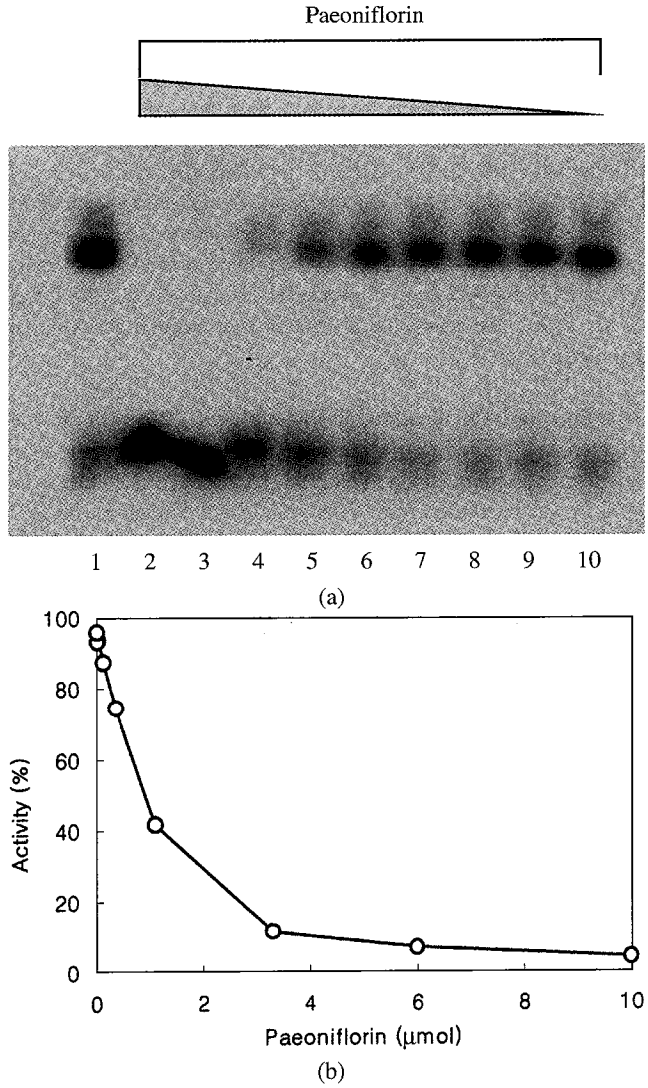

Fig. 2. Inhibitory effect of paeoniflorin on Fos-Jun- DNA complex formation. (a) Electrophoresis was performed with increasing amounts of paeoniflorin. Lane 1 control (probe and dimer); Lane 2~10, 10 μmol~1.4 *n*mol of paeoniflorin. The Fos-Jun dimer was prepared using the gene expression in *E. coli* (BL21) as described under Materials and Methods. The entire reaction mixture with paeoniflorin dissolved in water was incubated at room temperature for 30 min and then electrophoresis was performed. (b) Fos-Jun-DNA binding activities were measured by liquid-scintillation counting of the corresponding bands ($y = 86.17e^{-0.47x}$, $R^2 = 0.95$).

rate of radioactivities with increasing DNA did not correspond to its control (data not shown). This result shows that paeoniflorin interacts more with AP-1 DNA than with a Fos-Jun heterodimer.

This drug showed cytotoxicity against human leukemia cell lines (HL-60, K562); two of the four human cancer cell lines that were tested. No cytotoxicity was observed in any of the other cell lines (Colo205, A549), even after the exposure of an excess amount of paeoniflorin. The IC_{50} was determined using

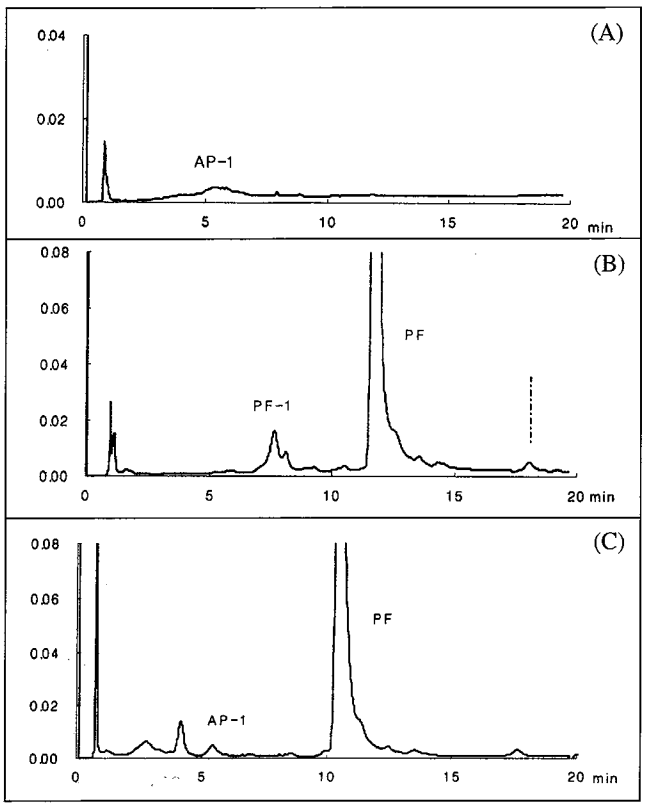

Fig. 3. Effect of AP-1 nucleotide on the retention time of paeoniflorin. (a) Elution of AP-1 nucleotide (b) Elution of PF and PF-1 complex (c) Elution of PF and PF-1 complex mixed with AP-1 nucleotide prior to injection.

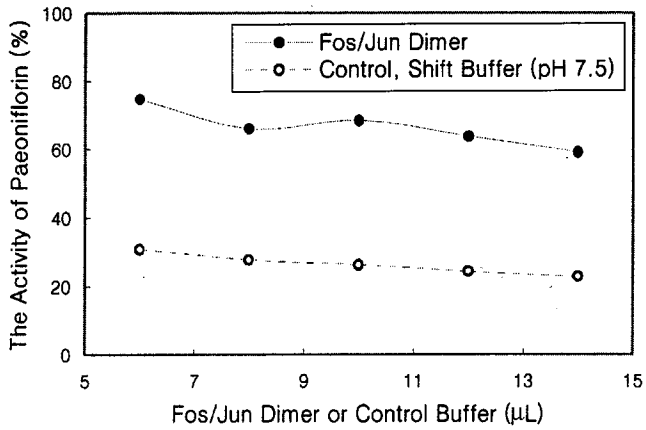

Fig. 4. Radioactivity with increasing Fos-Jun dimer. Electrophoresis was performed with increasing amounts of Fos-Jun heterodimer in the presence of 1.17 μmol paeoniflorin (IC_{50}). The change rate of radioactivities with an increasing Fos-Jun dimer is similar to that of control radioactivities.

the MTT assay. IC_{50} for HL-60 cells were 72 μg/ml, while it was 98 μg/ml for K562 cells.

The activation of Fos and Jun plays a key role in transmitting a signal that promotes the growth of a tumor. The AP-1 activity is present in most cell types prior to their stimulation, but is further induced in response to external stimuli, which results in the induction of AP-1 target genes. The c-Jun oncoprotein plays an important role in the regulation of gene expression and the signal transduction process. The c-Jun is also active in the differentiation of B-chronic lymphocytic leukemia cells (B-CLL) and in the malignant transformation of melanocytes (Murphy *et al.*, 1990; Yamanishi *et al.*, 1991). Thus, the inhibition of the binding of the human transcription factor Fos-Jun binding to a conserved nucleotide sequence (TRE) is likely to be related to the reduced transcription of different cellular genes in response to tumor promoting signals in cancer cell lines.

Paeoniflorin has several pharmacological effects, including CNS (central nervous system)-depressant, immunostimulant, myorelaxant, and anticoagulative and anti-inflammatory actions (Ishida *et al.*, 1987; Williamson and Evans, 1988). Paeoniflorin from peony radix also shows no clear toxic effect on the liver of rats. The absorption and excretion of paeoniflorin after intravenous and oral administration was studied in rats to evaluate the significance of paeoniflorin in the pharmacological action (Yoshimi *et al.*, 1992; Takeda *et al.*, 1995). However, these pharmacological effects of paeoniflorin are mainly studied in mice and rats. Although most oriental herbal medicines containing paeoniflorin are administered orally to patients in clinical use, its mode of action related to oncoprotein is unknown. We propose that paeoniflorin has an inhibitory effect on the action of the Fos-Jun-AP-1 complex and stimulates apoptosis in HL-60. Further studies are required to determine the precise molecular mechanisms of inhibition by paeoniflorin.

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