Glucocorticoid Receptor Induced Down Regulation of Metalloproteinase-9 (MMP-9) by Ginseng Components, Panaxadiol (PD) and Panaxatriol (PT), Contributes to Inhibition of the Invasive Capacity of HT1080 Human Fibrosarcoma Cells

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ABSTRACT

This study showed the anti-invasive activity of ginseng components, panaxadiol (PD) and panaxatriol (PT) on the highly metastatic HT1080 human fibrosarcoma cell line. PD and PT reduced tumor cell invasion through a reconstitute basement membrane in the transwell chamber. A significant down regulation of MMP-9 by PD and PT was detected by Northern blot analysis. However, MMP-2 was constantly expressed. Quantitative gelatin based zymography confirmed a marked reduced expression of MMP-9 but not MMP-2 in the treatment of PD and PT. Since the chemical structures of PD and PT are very similar to that of dexamethasone, a synthetic glucocorticoid, it was investigated whether PD and PT act through GR. Western blot analysis and immunocytochemistry showed that PD and PT increased the GR fraction in the nucleus. These results suggest that ursolic acid may induce repression of MMP-9 by stimulating the nuclear translocation of GR and hence inhibiting the activity of AP-1 to TPA-responsible element of MMP-9 promoter region. In conclusion, we suggest that GR-induced down-regulation of MMP-9 by PD and PT contributes to reduce the invasive capacity of HT1080 cells.

Introduction

Ginseng, the root of *Panax ginseng* C.A. Meyer, is a medicinal plant used world-wide and has been reported to have various biological effects [1-3]. Ginseng extracts have been reported to inhibit the incidence and proliferation of tumors induced by some carcinogens [4], and the anticarcinogenic effect of ginseng was shown to be related to the modulation of immune response [5]. Recently, the ginsenosides Rh_1 and Rh_2 purified from the crude ginseng extracts were reported to affect the growth of B16 melanoma cells and the expression of their melanotic phenotype [6, 7]. Ginsenoside Rb_2 has been shown to possess various biological activities such as anti-diabetes, anti-hyperlidemia, and anti-angiogenic effect in the tumor angiogenesis and metastasis [8]. In addition, ginsenoside Rh_2 was recently reported to induce the apoptosis of human hepatoma SK-HEP-1 cells [9].

We previously reported that ursolic acid (UA), a pentacyclic triterpene acid has the anti-invasive activity in the HT1080 human fibrosarcoma cell by reducing the expression of MMP-9 [10]. The structural formula of the several ginseng components are characterized to be the pentacyclic triterpenoids and very similar to that of UA. Among many kinds of ginseng components, the structure of the aglycone, PD and PT is the most analogous to that of UA and may down-regulate the MMP-9 expression through the similar manner to UA.

The promoter region of MMP-9 was characterized as having three important *cis*-element. These elements are putative binding sites for AP-1, Sp-1, and NF-κB, or related proteins. The AP-1 binding site on MMP-9 gene was shown to play a master role in the induction of MMP-9, cooperating synergistically with the element of the Sp-1-like and the NF-κB-like factors [11]. The signal to the AP-1 sites is common for the genes of several matrix metalloproteinases such as MMP-1 and MMP-3 [12]. Glucocorticoid hormones such as dexamethasone have been reported to be potent inhibitors of MMP-1 and MMP-3 of which expression can be induced by AP-1 [13, 14]. It has been also reported that the repression of AP-1-responsive genes is mediated by glucocorticoid receptor (GR) through a direct interaction with c-Jun [14-18] or c-Fos [19]. In fact, the down-modulation of the trans-activating function of AP-1 by GR was suggested to be mediated through the interaction with either the preexisting unbound or DNA-bound AP-1. This suggestion was supported by coprecipitation experiments with AP-1 and GR [12]. The structures of plant triterpenoids are much similar to that of Dexa. Thus, PD and PT may reduce the expression of MMP-9 through the similar manner to Dexa.

In this study, we investigated whether PD and PT reduced the expression of MMP-9 and invasive capacity of HT1080 cells. Furthermore, we analysed the reduction of the expression of MMP-9 by these ginseng components is mediated by the nuclear translocation of GR.

Materials and Methods

Cell culture and treatment of PD and PT

HT1080 human fibrosarcoma cells were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin and were incubated at 37℃ in a humidified atmosphere containing 5% CO_2 in air. The cells were treated with 10 μM of PD and 10, 20, 30, 40 μM of PT. After 3- and 6-day incubation, the cells were harvested or conditioned media were collected for Northern blot analysis and gelatin based zymogram assay.

MTT assay

The cytotoxicity of PD and PT was measured by the MTT assay [20]. 0.1 mg /ml MTT was added to each well and incubated at 37℃ for 4 h and cellular reduction of MTT by the mitochondrial dehy-

drogenase of viable cells forms a blue formazan product which can be measured spectrophotometrically at 540 nm.

In vitro invasion assay

In vitro invasion assay was carried out by the method of Seiki *et al.,* [21]. Invasion was measured by use of 24-well transwell units with 8 μm porosity polycarbonate filters. The cells treated with PD and PT were incubated on the traswell plate coated with matrigel for 16 h in a humidified atmosphere of 5% CO_2 at 37℃. Cells were stained with hematoxyline and eosin and invasion was determined by counting the cells migrated to the lower side of the filter with optical microscopy at X400 magnification. Thirteen fields were counted for each assay. Each sample was assayed in triplicate, and each assay was repeated twice.

RNA extraction and Northern blot analysis

Total cellular RNA was prepared from HT1080 cells according to the acid/guanidinium thiocyanate/phenol/chloroform extraction method [22]. The RNA samples were resolved on 1 % agarose/formaldehyde gels under denaturing conditions and transferred to nylon membranes (Zeta-Probe membrane, Bio-Rad). The RNA was hybridized to [^{32}P]dCTP-labeled cDNA probes of MMP-2 and MMP-9. Then the hybridized filters were exposed to X-ray film for 1 day.

Gelatin zymography

The amount of gelatinase (MMP-2 and MMP-9) in the conditioned media was quantified by cell number. The conditioned media of 10^4 cells were analyzed by the gelatin-based zymography, using slightly modified procedure from that of Herron *et al.,* [23].

Immunocytochemistry

Immunocytochemistry was conducted as the method of Ylikomi *et al.* [24]. HT1080 cells grown on the coverslip were incubation with specific GR antibodies stained AEC chromogen as a substrate (DAKO LSAB kit). The immunoperoxidase- stained samples were photographed using a phase-contrast microscope (Olympus BX 40).

Western blot analysis

Proteins were separated on 15 % nonreducing PAGE [25]. After transfer, nitrocellulose membrane were washed in TBS-T solution (20 mM Tris-HCl buffer, pH 7.6, containing 137 mM NaCl, 1% Tween 20). They were incubated in blocking buffer (5-10 % skim milk in TBS-T) for 12 h at 4℃. The filter was incubated with first antibody for 12 h at 4℃, and then washed three times with TBS-T. After washing, the filter was incubated with the second antibody. The band was detected with

enhanced chemiluminescence (ECL) reagents according to the supplier's protocol.

Results and Discussion

Effects of PD and PT on the cell viability

We first tested the effects of PD and PT on the cell viability using MTT assay. The cell viability was impaired about 60 - 70 % of control for 3 or 6 days at 20 μ M of PD and 50 μ M of PT. Thus, we determined the doses of treatment as 10 μ M for PD and 10 to 40 μ M for PT.
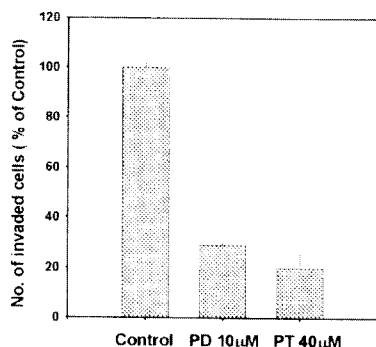
Anti-invasive effects of PD and PT

Fig 1. Anti-invasive activity of PD and PT. The effects of PD and PT on the invasive capacity of HT 1080 cells were measured by *in vitro* invasion assay. The assay was performed after treatment with 10μM of PD or 40 μM of PT for 6 days. HT1080 cells treated with PD and PT were incubated on the transwell chamber for 16 h. The number of invaded cells was counted and mean values were determined under X400 light microscopy.

We examined whether PD and PT affects the invasive capacity of HT1080 cells by *in vitro* invasion assay. The invasion of HT1080 cells through a reconstituted basement membrane (Matrigel) to the collagen-coated lower surface of the filters was inhibited by PD and PT (Figure 1). HT1080 cells treated with 10 μ M of PD or 40 μ M of PT for 6 days, comparing with a control, inhibited the invasion into Matrigel by about 70 and 80%, respectively. This result suggested that the PD and PT sufficiently inhibit the invasive capacity of the HT1080 cells.

Down regulation of the expression of MMP-9 gene by PD and PT

In order to elucidate the mechanism of anti-invasive action of PD and PT, we investigated the effect of PD and PT on the expression of MMPs by Northern blot analysis. As shown in Figure 2, treatment with 10 μ M of PD for 3 or 6 days significantly reduced the expression of MMP-9. Treatment with 10 to 40 μ M of PT for 3 or 6 days also down-regulated the mRNA level of MMP-9 drastically. In contrast, the expression of MMP-2 was not significantly changed by treatment with PT or PD. The effects of PD and PT on the repression of the MMP-9 expression were confirmed at the protein level by the gelatin-based zymography. As expected, treatment with PD and PT reduced the enzyme activity of MMP-9. As demonstrated in Figure 3, treatment with 10 μ M of PD for 6 days showed meaningful decrease of MMP-9 expression. However, treatment for 3 days did not change explicitly. Treatment with 40 μ M of PT for 3 days and 20 to 40 μ M for 6 days significantly reduced the activi-

PD   -   +   +   (10 μM)
         3   6   days

←MMP-9

←MMP-2

←28S
←18S

PT   -   10 20 30 40 10 20 30 40 μM
              3   6   days
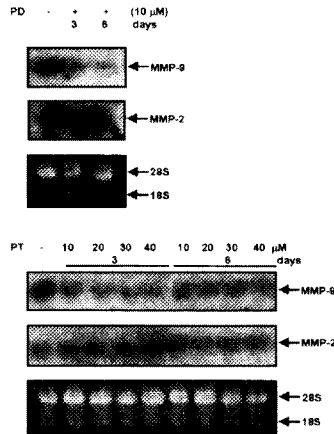
←MMP-9

←MMP-2

←28S
←18S

Figure 2. Effects of PD and PT on the expression of MMP-9 and MMP-2. HT1080 cells were treated with 10 μM of PD or 10 to 40 μM of PT and cultured for 3 or 6 days. Expressions of MMP-9 and MMP-2 were assayed by Northern blot analysis. Filters were hybridized with 32P-labeled cDNA probes of MMP-9 and MMP-2. Molecular sizes of the transcripts are 3.1 and 2.8 kb for MMP-2 and MMP-9, respectively
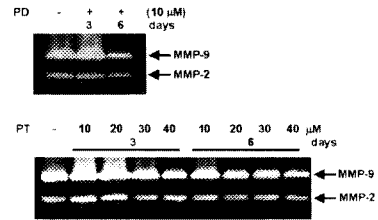

PD   -   +   +   (10 μM)
         3   6   days

←MMP-9
←MMP-2

PT   -   10 20 30 40 10 20 30 40 μM
              3   6   days

←MMP-9
←MMP-2

Figure 3. Gelatin based zymography of the culture medium of HT1080 cells treated with PD and PT. After treating with 10 to 40 μM of PT or 10 μM of P for 3 or 6 days, the culture media were used in gelatin based electrophoresis and stained with Coomassie brilliant blue. Arrows indicate the MMP-9 and MMP-2 respectively
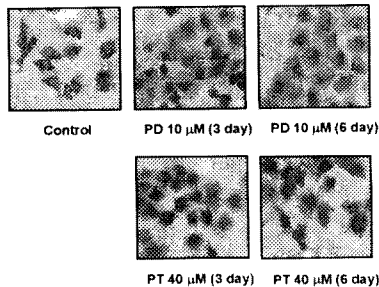

Control   PD 10 μM (3 day)   PD 10 μM (6 day)

PT 40 μM (3 day)   PT 40 μM (6 day)

Figure 4. PD and PT induce the nuclear translocation of GR in the immunocytochemistry. HT 1080 cell treated with 10 μM of PD and 40 μM of PT for 3 or 6 days and immunocytochemistry were carried out as described in Material and Methods

Control   PD   PT
                                    ←GR

Days   3   6   3   6

Figure 5. Effects of PD and PT on the nuclear translocation of GR in the Western blot analysis. After treatment with 10 μM of PD and 40 μM of PT for 3 o 6 days, the cell were harvested and nuclear protein (40 μg) were purified and transferred to Hybond-ECL. The filters were blotted with anti-GR antibody and detected by ECL.

ty of MMP-9, whereas, treatment with 10 to 30 μM for 3 days and 10 μM for 6 days did not change the level of MMP-9 drastically. These results suggest that the inhibitory effect of PD and PT on tumor cell invasion can be partially attributable to the down-regulation of the expression of MMP-9.

PD and PT- induced nuclear translocation of GR

In order to determine whether PD and PT induce the translocation of GR from cytosol to nucleus like the action of Dexa, HT1080 cells were stained with anti-GR antibody after treatments with 10 μM of PD and 40 μM of PT for 3 or 6 days. Figure 4 shows that the immunoactivity of GR in untreated cells was mainly localized in the cytosol whereas the treatments with 10 μM of PD and 40 μM of PT for 3 or 6 days drastically induced the translocation of GR from cytosol to nucleus, respectively.

To confirm whether PD and PT promote the

translocation of GR, we carried out Western blot analysis and gel retardation assay using nuclear extracts of HT1080 cells treated with PD and PT. Figure 5 shows that the treatments with 10μM of PD and 40 μM of PT for 3 or 6 days increased the amount of nuclear GR, respectively on the protein level. Furthermore, in the treatment with 40 μM of PT for 3 or 6 days, the amount of translocated GR was more increased than that in the treatment with 10 μM of PD for 3 or 6 days. These results suggested that the effect of PT on the nuclear translocation of GR is more stronger than that of PD. These results suggest that the nuclear translocation of GR induced by PT is more effective than that by PD.

In conclusion, PD and PT inhibit tumor cell invasion *in vitro* by inhibiting the transcription of MMP-9 gene required for the degradation of basement membrane and these down regulation of MMP-9 by PD and PT might be mediated through translocation of GR from cytosol to nucleus.

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