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Aqueous extract from aerial parts of *Artemisia vestita*, a traditional Tibetan medicine, reduces contact sensitivity in mice by down-regulating the activation, adhesion and metalloproteinase production of T lymphocytes

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Abstract

In the present paper, the effect of the aqueous extract from aerial parts of *Artemisia vestita* (AV-ext), a traditional Tibetan medicine, on ear contact sensitivity was examined. AV-ext significantly reduced the ear swelling when administered during the induction phase of picryl-chloride (PCl)-induced ear contact sensitivity in mice. The extract also showed a dose-dependent inhibition on lymphocyte proliferation and IL-2 production in Con A-activated spleen cells. The proliferation inhibition was confirmed in the mixed lymphocytes reaction. Furthermore, the adhesion of the isolated spleen cells from PCl-sensitized mice to type IV collagen was significantly decreased in a dose-dependent manner by AV-ext. Such decrease was also seen in AV-ext-treated Jurkat T cells and the T cells purified from above spleen cells. The purified spleen T cells from PCl-sensitized mice produced more matrix metalloproteinase-9 (MMP-9) than naive T cells, and AV-ext remarkably reduced MMP-9 production both in vivo and in vitro. These results suggest that AV-ext may alleviate contact sensitivity through blocking the activation of T lymphocytes and decreasing their localization to the inflammatory sites via down-regulating the potential of cell adhesion and metalloproteinase production.

Keywords: *Artemisia vestita*; Delayed-type hypersensitivity; Picryl chloride; Contact sensitivity; Matrix metalloproteinase; Adhesion

1. Introduction

Delayed-type hypersensitivity (DTH) is a T cell-mediated immune reaction and plays a major role in the pathogenesis and chronicity of various inflammatory disorders. The cellular and molecular mechanisms

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have been intensively studied using mainly rodent models [1,2]. One of the most characteristic DTH phenomena is contact sensitivity [3]. During the lymphocytes localization and infiltration to inflammation locus in the DTH reaction, lymphocytes should interact with endothelial cells, the underlying basement membrane and extracellular matrix. Such interaction has been generally accepted as a crucial step in the DTH reaction [4,5]. In this step, matrix metalloproteinases (MMPs) are known to degrade basement membranes and extracellular matrices such as type IV collagen, laminin and fibronectin, and to enhance the inflammatory infiltration of T lymphocytes into target tissues [6]. Furthermore, there are increasing evidences showing that inhibition of MMPs may lead to an alleviation of inflammations [7,8], and some monoclonal antibodies and synthetic peptides can ameliorate inflammation by inhibiting lymphocytes adhesion to extracellular matrix [5,9,10]. These findings suggest that inhibition of T cell adhesion and interference with the activities of proteinases including MMPs may represent a useful approach to the treatment of T cell-mediated immune diseases.

On the other hand, *Artemisia vestita* (AV) has been widely used in traditional Tibetan and Chinese medicine for treating various inflammatory diseases including rheumatoid arthritis, but it is still poorly understood for its chemical components and how it works on those inflammatory disorders. Although it was reported that some compounds including sesquiterpens, flavone and coumarin existed in *Artemisia vestita* [11] and that some components isolated from the same genus plant could alleviate the inflammatory diseases [12–14], there is still lack of evidence to treat these diseases using the traditional medicines. In the present paper, therefore, we first demonstrated the inhibitory effect of AV-ext on picryl-chloride-induced contact dermatitis. Then, its preliminary mechanisms were elucidated in the aspect of T lymphocytes especially in their potential of proliferation, adhesion and MMP production.

2. Materials and methods

2.1. Animals

Female BALB/c and C57BL/6 mice (SPF), aged 6–8 weeks (18–22 g) were obtained from the Laboratory Animal Center of Shanghai (Shanghai, China). They were maintained with free access to pellet food (Jiangsu Cooperation Medical and Pharmaceutical, Nanjing, China) and water in plastic cages at 21 ± 2 °C and kept on a 12-h light/dark cycle. Animal welfare and experimental procedures were carried out strictly in accordance with the guide for the care and use of laboratory animals (National Research Council of USA, 1996) and the related ethical regulations of our university. All efforts were made to minimize animal's suffering and to reduce the number of animals used.

2.2. Cell line

Human leukemia Jurkat cell line was maintained in RPMI 1640 medium supplemented with 100 U mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin and 10% fetal calf serum under a humidified 5% (v/v) CO_2 atmosphere at 37 °C.

2.3. Extracts, drugs and reagents

The aerial parts of *Artemisia vestita* used in this study were purchased from Tibet pharmaceutical factory of Tibet university (Lhasa, China) and identified as *Artemisia vestita* Wall. by Dr. Ciren Dunzhu (Tibet Tibetan medicine college). The material was used for making aqueous extract as reported [15] with small modification. Briefly, the material (100 g) was extracted twice with fivefold volumes of water (500 mL) at 100 °C for 1 h each time. The supernatants from each extraction were mixed and centrifuged at $1870 \times g$, then the precipitation was discarded and the supernatant was lyophilized to make a powder with 18.1% of yields. The dosage of the extract was indicated as the powders. The powder was dissolved in water for in vivo assay by gavage administration to mice and in RPMI 1640 medium for in vitro assay. Other reagents used in this study were as follows: Injection Dexamethasone Sodium Phosphate (Dex, Nanjing 3rd pharmaceutical factory, Nanjing, China), picryl chloride (PCl, Nacalai tesque, Kyoto, Japan), concanavalin A (Con A, Sigma), mitomycin C (Sigma), M-MLV Reverse Transcriptase (Promega, USA), bovine serum albumin (BSA, Sigma), rat tail type IV collagen (Sigma), phorbol myristate acetate (PMA, Sigma), acrylamide and bis-acrylamide (Shanghai Sangon Biotechnical, Shanghai,

China), gelatin and Coomassie brilliant blue R-250 (Sigma), crystal violet (Shanghai Yuanhang reagent factory, Shanghai, China), mouse T cell Enrichment columns (R&D Systems, USA), 96-well culture plates (Nunclon), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Sigma), ELISA kit for Murine IL-2 (Jingmei Biotech, Shengzhen, China).

2.4. PCl-induced ear contact sensitivity

Female BALB/c mice were sensitized by painting 0.1 mL of 1% PCl in ethanol on the shaved skin of their abdomens. Five days later, they were challenged by painting 30 μL of 1% PCl in olive oil on right ear lobe [3]. Eighteen hours later, ear thickness of right against left was measured with a digimatic micrometer (0.001 mm, Mitutoyo, Tokyo, Japan). The control animals were run parallel with other groups except for gavage p.o. the same volume of water.

2.5. Preparation of splenocyte suspensions and purification of T cells

Spleen was aseptically taken from BALB/c mice, crushed gently and separated into single cells by squeezing in 5 mL D-Hank's solution (GIBCO BRL). The cells obtained were passed though the gauze of eight layers and centrifuged at 200×*g* for 5 min at 4 °C. Pellet was added into 10 mL sterile 0.17 M Tris (hydroxymethyl aminomethane)–0.75% NH_4Cl (pH 7.5) followed by centrifugation to remove erythrocytes. After washing twice with RPMI 1640 medium (GIBCO BRL), they were re-suspended in the medium and used for culture. In some cases, the prepared mouse spleen cell suspensions were loaded onto T cell Enrichment Columns [16]. Briefly, the column was equilibrated with column wash buffer (8 mL). After the wash buffer had drained down to the level of the white filter, 2 mL cell suspension (5×10^7 cells mL^{-1}) was applied to the top of the column and incubated at room temperature for 10 min. Then cells were eluted from the column with 4 aliquots of 2 mL of column wash buffer. The collected cells were centrifuged at 250×*g* for 5 min at 4 °C and re-suspended in the RPMI 1640 medium. Total T cell recovery from these columns was about 55% and the purity ($CD3^+$ cells) of recovered cells reached about 88% determined by fluorescence-activated cell sorter (FACS).

2.6. Proliferation of spleen cells

Spleen cells were cultured in 96-well plates at a density of 5×10^5 cells/well in RPMI 1640 medium (0.2 mL) and stimulated with 5 μg/mL of concanavalin A (Con A) for 72 h at 37 °C in 5% CO_2/air. Then cell growth was evaluated with modified MTT assay [17]. Briefly, 20 μL of 5 mg/mL MTT in RPMI 1640 medium were added for a further 4-h incubation. After removing the supernatant, 200 μL of DMSO (dimethyl sulfoxide) were added to dissolve the formazan crystals. The absorbance was read on an ELISA reader (Sunrise Remote/Touch Screen, TECAN, Austria) at 540 nm.

2.7. IL-2 production

Spleen cells isolated from normal BALB/c mice were suspended in RPMI 1640 medium at a density of 5×10^6/mL. Portions (0.1 mL) were seeded onto 96-well flat-bottom microplates and co-cultured with 5 μg/mL Con A in the presence or absence of AV-ext at 37 °C in 5% CO_2. After 24 h, the supernatants were aspirated and stored at −70 °C until the ELISA assay. The threshold of detection was 10 pg mL^{-1} for IL-2 and the standard curve's range was from 0 to 1000 pg mL^{-1}.

2.8. Reverse transcriptase-polymerase chain reaction (RT-PCR) [18]

Total RNA was extracted from Con A-activated splenocytes or naive splenocytes using Tripure reagent (Roche) as described by the manufacturer. Single-stranded cDNA was synthesized from 2 μg of total RNA by reverse transcription using 0.5 μg primer of $oligo(dT)_{18}$. Following cDNA synthesis, amplification was performed using the following primers (Genebase, Shanghai, China): beta-actin 5′-ACATCTGCTGGAAGGTGGAC and 3′-GGACCCATGTACCACCATGG, IL-2 5′-TGCTCCTTGTCAACAGCG and 3′-CTCACGGTTAAGCTACTACT. PCR cycle conditions were as follows: 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min for 30 cycles. After amplification, PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide dying. The relative expressions were quantified densitometrically using the LabWorks

4.0 software, and calculated according to the reference bands of beta-actin.

2.9. Single mixed lymphocytes reaction [19]

The lymphocytes (5×10^5) from C57BL/6 mice, which had been treated with mitomycin C (500 μg/mL) for 1 h, were co-cultured with the lymphocytes (5×10^5) from BALB/c mice in the presence or absence of the various concentrations of AV-ext at 37 °C in 5% CO_2 for 72 h. The proliferation of the lymphocytes from BALB/c mice was measured by MTT method. The OD_{540} was determined by an ELISA reader. Stimulation index was counted as following formula: stimulation index=$(OD_{sample}-OD_{C57BL/6\ alone})/OD_{BALB/c\ alone}$. The experiment was repeated three times.

2.10. Cell adhesion to type IV collagen

Adhesion assay was performed according to the report [20] with some modifications. Briefly, a 96-well flat-bottomed microplate was coated with 50 μL solution containing type IV collagen (50 μg mL^{-1}) and left at 4 °C overnight. Nonspecific binding sites were blocked with 0.2% bovine serum albumin (BSA) for 2 h at room temperature followed by washing three times with phosphate-buffered solution. The cells were suspended in RPMI 1640 medium and spleen cells (5×10^5) or spleen T cells (5×10^5), or Jurkat cells (1×10^5) were added to each well. The cells were incubated at 37 °C for 1 h with or without PMA (100 nM) and the non-adherent cells were removed by washing three times with RPMI 1640 medium. Then cells were fixed with methanol/acetone (1:1), and stained with 0.5% crystal violet in 20% methanol. Unbound dye was removed in tap water and the plate was dried in air. Bound dye was extracted with 1% sodium dodecyl sulfate (SDS). The absorbance of the samples was measured at 592 nm. The wells, which were fixed and stained without previous washing, were regarded as the absorbance of total cells. The results were expressed as the mean percentage of total cells from triplicate wells and the experiments were repeated three times. Spleen cells from control animals were subjected to the same assay procedures in parallel. Specificity of cell adhesion assays was corroborated using BSA as substratum.

2.11. Gelatin zymography

Analysis by zymography on gelatin gel allows detection of enzymatic activity of the secreted collagenases MMP-9 [21]. Briefly, purified spleen T cells were suspended in serum-free RPMI 1640 medium at a density of 5×10^5/well and incubated at 37 °C in 5% CO_2 for 24 h. Spleen T cells from control animals were subjected to the same assay procedures in parallel. Twenty microliters of the supernatants was mixed with 10 μL sample buffer (62.5 mM Tris–HCl containing 10% glycerol, 0.00125% bromophenol blue and 12% SDS) without reducing agent, and they were subjected to SDS-PAGE in 5% polyacrylamide gels that were copolymerized with 2 mg mL^{-1} of gelatin at 4 °C for 1 h. After electrophoresis, the gels were washed twice in the rinsing buffer (50 mM Tris–HCl containing 2.5% Triton X-100, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.05% NaN_3) for 1 h at room temperature to remove SDS. Then, they were incubated for 36 h at 37 °C in the incubation buffer (50 mM Tris–HCl containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.05% NaN_3). The gels were stained with 0.1% Coomassie brilliant blue R250 for 30 min, and destained for 8 h in a solution of 10% acetic acid and 10% isopropanol. The proteolytic activity was evidenced as clear bands (zones of gelatin degradation) against the blue background of stained gelatin.

2.12. Statistical analysis

Results were expressed as mean±S.D. of three independent experiments and each experiment includes triplicate sets in vitro and of eight animals of each group in vivo. Statistically evaluated by Student's *t*-test when only two value sets were compared, and one-way ANOVA followed by Dunnett's test when the data involved three or more groups. $P<0.05$ was considered to be significant.

3. Results

3.1. Effect of AV-ext and dexamethasone on PCl-induced ear contact sensitivity in mice

When given for 6 days from the sensitization, AV-ext significantly inhibited contact sensitivity in mice

Table 1
Effects of AV-ext and dexamethasone on PCl-induced ear contact sensitivity in mice

Group	Number of mice	Dose (mg kg^{-1})	Induction phase		Effector phase	
			Ear swelling (10^{-3} mm)	Inhibition (%)	Ear swelling (10^{-3} mm)	Inhibition (%)
Control	8	0	93.4±49.3	0	79.4±27.2	0
AV-ext	8	150	45.0±23.2*	51.8	58.1±15.9	26.8
	8	300	41.3±24.7*	55.8	56.1±27.1	29.3
Dex	8	10	18.1±13.2**	80.6	16.1±15.5**	79.7

Mice were sensitized by painting 0.1 mL of 1% PCl in ethanol on the shaved skin of their abdomens. Five days later, they were challenged by painting 30 μL of 1% PCl in olive oil on the right ear lobe. Eighteen hours after the challenge, the thickness of right and left ears were measured and the swelling was evaluated by the increase in ear thickness. In the induction phase, AV-ext and Dex were given p.o. and i.m. for 6 days from the sensitization, respectively. In the effector phase, the drugs were given for three times at 0, 5 and 10 h after the challenge. Dex: dexamethasone.

* $P<0.05$ vs. Control (Dunnett's test).

** $P<0.01$ vs. Control (Dunnett's test).

in a dose-dependent manner (Table 1). When given for three times (0, 5 and 10 h) after the PCl challenge, AV-ext showed a tendency to inhibit the ear swelling. Dexamethasone strongly inhibited the contact sensitivity in both administration schedules.

3.2. Effect of AV-ext on Con A-induced splenocytes proliferation and IL-2 production

Spleen cells from naive mice were isolated and stimulated with or without 5 μg/mL of Con A at 37 °C

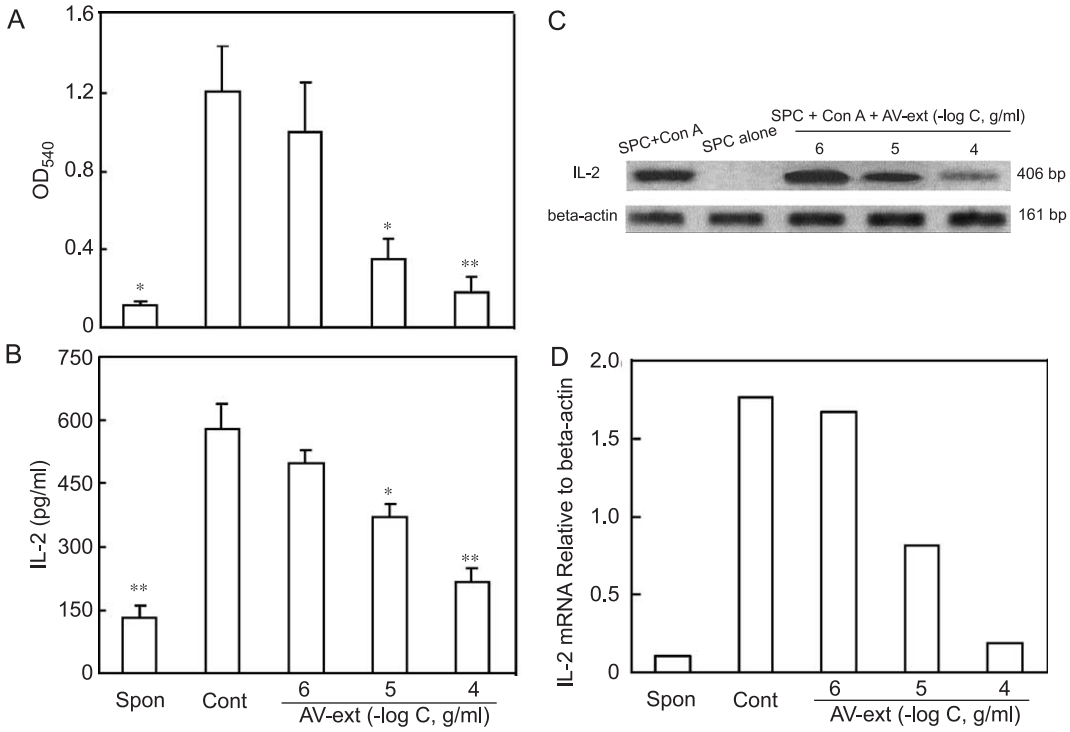

Fig. 1. Effect of AV-ext on Con A-induced splenocytes proliferation and IL-2 production. Spleen cells from naive BALB/c mice were isolated and stimulated with or without 5 μg/mL of Con A at 37 °C in 5% CO_2/air for 72 h. (A) The lymphocyte proliferation was measured by MTT method. (B) The IL-2 production in the supernatant was measured by ELISA. (C) IL-2 mRNA expression in the Con A-activated splenocytes was examined by RT-PCR. (D) The semi-quantification result of IL-2 expression of activated splenocytes by LabWorks 4.0 software. SPC, spleen cells. *$P<0.05$, **$P<0.01$ vs. Cont (Dunnett's test).

for 72 h. As shown in Fig. 1A, AV-ext showed a dose-dependent inhibition on Con A-induced lymphocyte proliferation. Also, the inhibition of Con A-induced IL-2 production by AV-ext was seen in both protein and RNA levels (Fig. 1B–D). However, the extract in the concentrations of 10^{-6}–10^{-4} g/ml did not show any cytotoxicity to spleen cells when incubated at 37 °C for 72 h (data not shown).

3.3. Effect of AV-ext on single mixed lymphocytes reaction

As shown in Fig. 2, AV-ext significantly inhibited mixed lymphocytes reaction in a concentration-dependent manner.

3.4. Effect of AV-ext on the adhesion of splenocytes and spleen T cells from PCl -sensitized mice, and Jurkat T cells to type IV collagen

Mice were sensitized by PCl as described in Materials and methods. Five days after sensitization, the spleen cells were isolated for adhesion assay. As shown in Fig. 3A, the PCl sensitization significantly increased the adhesion potential of spleen cells. Compared with this, AV-ext dose-dependently inhibited the adhesion. Furthermore, when using spleen T cells purified from the isolated spleen cells, AV-ext showed a stronger inhibition on the adhesion than that of total splenocytes (Fig. 3B). In the case of Jurkat T cells, PMA was used to activate the cells and a significant increase of the adhesion to collagen was observed (Fig. 3C). Pretreatment with AV-ext for 2 h caused a dose-dependent decrease of the cell adhesion.

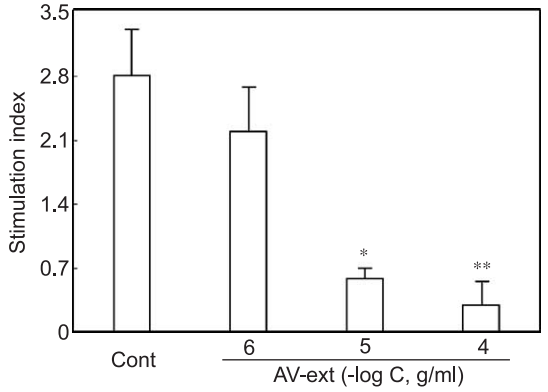

Fig. 2. Effect of AV-ext on single mixed lymphocytes reaction (sMLR). The lymphocytes (5×10^5) from C57BL/6, which had treated by mitomycin C (500 μg/mL) at 37 °C for 1 h, were co-cultured with the lymphocytes (5×10^5) from BALB/c mice in the presence or absence of the various concentrations of AV-ext at 37 °C in 5% CO_2 for 72 h. The proliferation of lymphocytes in sMLR was measured by MTT method. *P<0.05, **P<0.01 vs. Cont (Dunnett's test).

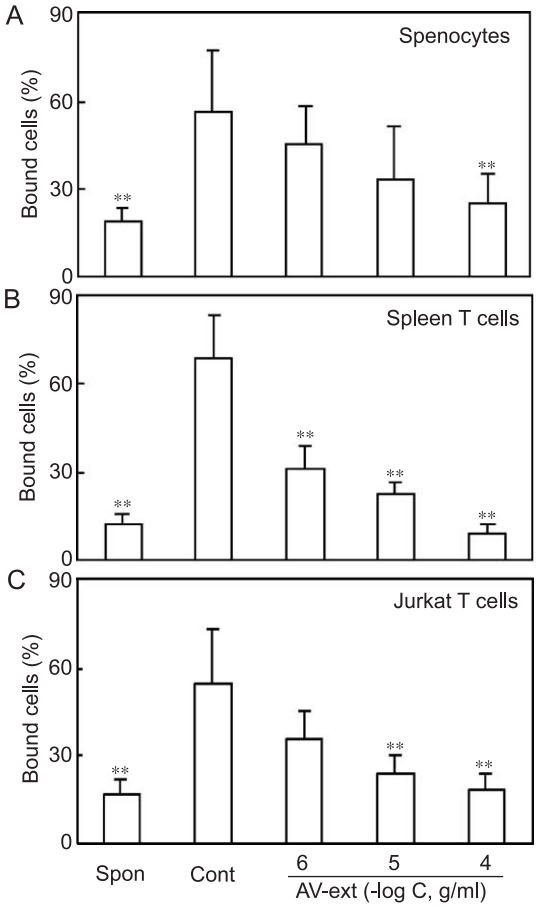

Fig. 3. Effect of AV-ext on the adhesion of splenocytes, spleen T cells from PCl-sensitized mice and Jurkat T cells to type IV collagen. Mice were sensitized by PCl as described in Materials and methods. Five days after the sensitization, the spleen cells (A) were isolated and spleen T cells (B) were purified from these spleen cells. They were incubated with various concentrations of AV-ext at 37 °C in 5% CO_2 for 2 h. Then the cells were washed and used for adhesion assay. In the case of Jurkat cells (C), the cells were incubated with AV-ext for 2 h at 37 °C. After washing twice with RPMI 1640 medium, they were subjected to the adhesion assay. Each column represents the mean±S.D. of three independent experiments and each experiment includes triplicate sets. Spon: cell alone; Cont: cells+PMA; **P<0.01 vs. Cont (Dunnett's test).

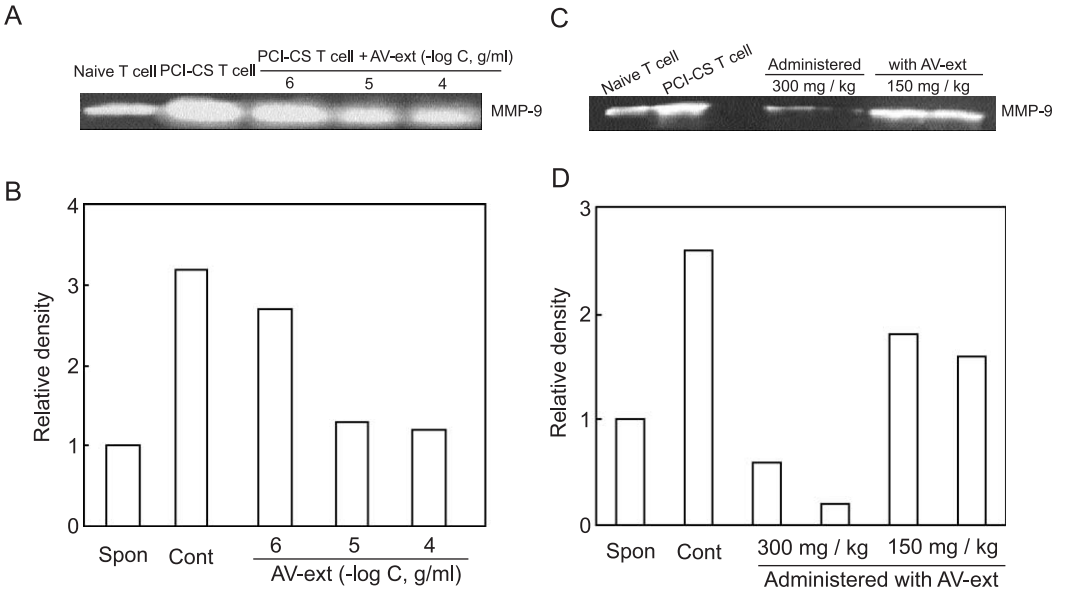

Fig. 4. Effect of AV-ext on MMP-9 production by spleen T cells from PCl-sensitized mice in vivo and in vitro. (A, B) In vitro effect of AV-ext on MMP-9 production. The spleen T cells purified from PCl-sensitized mice were incubated with different concentrations of AV-ext at 37 °C for 24 h. After the incubation, the supernatants were aspirated and used for zymography assay. (C, D) In vivo effect of AV-ext on MMP-9 production. Mice were given p.o. 150 and 300 mg kg^{-1} of AV-ext for 5 days from the PCl sensitization. At the fifth day, the spleen T cells were purified from different treated groups and incubated at 37 °C for 24 h. The supernatants were aspirated and used for zymography assay. The figure shown here is the representative of three different experiments.

3.5. Effect of AV-ext on MMP-9 production by spleen T cells from PCl-sensitized mice in vivo and in vitro

As shown in Fig. 4A and B, the spleen T cells isolated from PCl-sensitized mice secreted a higher level of MMP-9 than that from naive mice, while AV-ext treatment for 24 h dose-dependently decreased MMP-9 production. On the other hand, p.o. administration of AV-ext to the isolated spleen T cells sensitized by PCl during the induction phase of DTH also markedly reduced MMP-9 level (Fig. 4C and D). However, AV-ext did not affect MMP-9 production from normal spleen T cells both in vivo and in vitro (data not shown).

4. Discussion

In the present study, we first examined the effect of AV-ext, an extract from a traditional Tibetan medicine, on PCl-induced ear contact sensitivity, a typical DTH reaction. As the result, AV-ext showed a significant inhibition on the ear swelling when given in the induction phase of the DTH reaction. However, the extract only showed a slight tendency of inhibition when given in the effector phase (Table 1). These results suggest that AV-ext may mainly inhibit the activation rather than the function of the effector T cells involved in DTH reaction.

Lymphocyte proliferation and IL-2 production are considered indispensable in the activation of the effector T lymphocytes [22]. In the next examinations, we found that AV-ext dose-dependently inhibited Con A-induced lymphocyte proliferation, as well as the IL-2 production in both protein and RNA levels (Fig. 1). The inhibitive activity on lymphocyte proliferation of AV-ext was further confirmed by mixed lymphocytes reaction assay (Fig. 2). These findings indicate that the inhibition of lymphocyte proliferation and IL-2 production by AV-ext may block the activation of T lymphocytes and finally contribute to the alleviation of the ear swelling when given in the induction phase as seen in Table 1.

Considering the crucial role of the adhesion of lymphocytes to extracellular matrix in the progress of DTH reaction [5], we next examined the effect of AV-ext on the adhesion of spleen cells from PCl-sensitized mice to type IV collagen in vitro. The adhesion of the spleen cells was significantly enhanced when the cells were stimulated in vitro by PMA, a protein kinase C (PKC) activator. Against the increase, the pretreatment with AV-ext showed an inhibitory tendency (Fig. 3A). Furthermore, the effect of AV-ext was confirmed in T cells purified from above spleen cells (Fig. 3B) as well as Jurkat T cells (Fig. 3C) with a more significant inhibition than in total spleen cells. These findings indicate that the inhibition of spleen cells adhesion by AV-ext was mainly directed to T lymphocytes.

Lymphocyte localization to the inflammatory sites requires cooperation between adhesion molecules and MMPs. During the lymphocyte migration, the degradation of extracellular matrix needs the help of MMPs and excretion of MMP-9 conspicuously increases when the integrins on the surface of T cells bind to their ligands in extracellular matrix [23]. In Fig. 4, we also observed the MMP-9 production by spleen T lymphocytes from antigen-sensitized mice. Such production of MMP-9 was dose-dependently inhibited when pretreated with AV-ext in vitro and in vivo. The inhibitive effect of AV-ext was also confirmed in the MMP-9 mRNA level by RT-PCR (data not shown). MMP-9 has been reported to be involved in the migration of T cells through extracellular matrix and inhibition on MMPs may lead to an alleviation of various inflammation disorders including colitis, rheumatoid arthritis and contact sensitivity [7,8,24]. These results indicated that the inhibition of MMP-9 production by AV-ext might contribute to the recovery of mice from contact sensitivity.

In summary, AV-ext may inhibit DTH reaction mainly through blocking the activation of antigen-primed T lymphocytes. The mechanisms may involve the inhibition of lymphocyte proliferation, down-regulation of MMP production and the cell adhesion to extracellular matrix. These findings, together with the previous data on the improvement against rheumatoid arthritis in clinic [25], suggest that this traditional Tibetan medicine is worthy of being investigated as a novel remedy for the treatment of DTH-related diseases.

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