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AQUEOUS EXTRACT FROM *RHIZOMA NOTOPTERYGII* REDUCES CONTACT SENSITIVITY BY INHIBITING LYMPHOCYTE MIGRATION VIA DOWN-REGULATING METALLOPROTEINASE ACTIVITY

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In the present paper we examined the effects of the aqueous extract from *Rhizoma notopterygii* (RN-ext) on picryl chloride-induced contact sensitivity (PCl-CS). The extract, administered during either induction or effector phase showed a significant inhibition on the ear swelling in mice with PCl-CS. By using the isolated spleen cells from the mice with PCl-CS, we demonstrated the inhibitory effects of RN-ext on matrix metalloproteinase-2 (MMP-2) and MMP-9 activities. However, such inhibition was not found in those from normal mice. The inhibitory effects on MMP-2 and MMP-9 of RN-ext were also observed when it was administered *in vivo*. In addition, the extract significantly inhibited the migration of spleen cells from PCl-CS mice in transwell system without affecting the cell adhesion to fibronectin. These results suggest that RN-ext exerts its inhibitory activity on the contact sensitivity through decreasing the localization to the inflammation site via down-regulating MMP activities.

KEY WORDS: *Notopterygium incisum*, contact sensitivity, matrix metalloproteinases, migration, lymphocytes.

INTRODUCTION

Metalloproteinase (MMP) is a family of endopeptidases that are characterized by their ability to digest very stable components of extracellular matrix (ECM) [1–3]. They are involved in various physiological and pathological processes, such as trophoblast implantation [4], angiogenesis [5], bone development [6], and cancer metastasis [7]. As common pathological substrates, the MMPs also play an important role in many inflammatory disorders and autoimmune diseases, such as rheumatism arthritis [8], multiple sclerosis [9], and experimental allergic encephalomyelitis [10]. Furthermore, the proteinases are required in the process of inflammatory infiltration, such as T lymphocytes into target tissues. This process includes two consequence steps. Firstly, lymphocytes encounter endothelial cells through a complex array of surface receptors [11–13] and transmigrate through the endothelial cell junctions. Secondly, the cells interact with the underlying basement membrane and interstitial matrix [14–16]. The basement membrane is synthesized by epithelial and endothelial cells that is composed essentially of collagen type IV, laminin, and perlecan. Vectorial motility across the basement membrane and intestinal matrix requires coordinated series of adhesion-release steps and focal matrix degradation [17, 18]. Recent contributions suggested an important role for matrix MMP in basement membrane disruption by T lymphocytes [19]. The 92 kDa gelatinase (MMP-9) and 72 kDa gelatinase (MMP-2) efficiently degrade native collagen types IV and V, fibronectin, entactin, and elastin. Therefore, these proteases are believed to be of crucial importance in the processes requiring basement membrane disruption, such as tumor invasion and metastasis [20, 21] and presumably, tissue infiltration by leukocytes, the pathological substrate of inflammatory diseases. Clinical and laboratory researches have given convincing evidences that inhibiting MMPs will ameliorate various inflammation disorders, such as rheumatism arthritis [22] and asthma [23].

Rhizoma notopterygii (RN) is widely used in traditional Chinese medicine for treating various inflammatory diseases including rheumatoid arthritis. The extract from RN has been shown to have an analgesic activity. Its analgesic component, notopterol also showed an

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anti-inflammatory activity by inhibiting vascular permeability [24]. In addition, the *n*-hexane extract from RN inhibited 5-lipoxygenase and cyclooxygenase activities [25]. In the present paper, we first examined the effects of the aqueous extract from RN (RN-ext) on picryl chloride-induced contact dermatitis. Then, the mechanisms were elucidated in the aspect of lymphocytes functions, especially the expression of MMPs.

MATERIALS AND METHODS

Animals

Female Kunming strains of mice, 6–8-week-old and 20 ± 2 g, were obtained from the Experimental Animal House of China Pharmaceutical University (Nanjing, China). They were kept in plastic cages at $21 \pm 2\,^{\circ}$C with free access to pellet food and water and were on a 12-h light/dark cycle. This study complied with the current ethical regulations on animal research of the university and the mice used were treated humanely.

Drugs and reagents

RN was purchased from Nanjing Medicinal Material Co. (Nanjing, China) and identified as *Notopterygium incisum* ex H. T. Chang by Miss Huijuan Liu (Department of Pharmacognacy, China Pharmaceutical University). The RN-ext was made by a common method. Briefly, RN material was extracted twice with distilled water (10:1) at 100 °C for each 1 h. Then, the supernatant after centrifugation at 1700 ***g*** was pooled and lyophilized to obtain a powder with 20% of yield. Reagents used in this study were as follows: picryl chloride (PCl; Nacalai Tesque, Inc., Kyoto, Japan), 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Amresco, USA), acrylamide and bis-acrylamide (Shanghai Sangon Biotechnical Limited Co., Ltd, Shanghai, China), gelatin and Coomassie brilliant blue R-250 (Sigma), crystal violet (Yuanhang Reagent Factory, Shanghai, China).

Picryl chloride-induced contact sensitivity (PCl-CS)

Mice were sensitized by painting 0.1 ml of 1% picryl chloride in ethanol on the skin of their abdomens where the hair had been shaved. Five days later, they were challenged by painting 30 μl of 1% PCl in olive oil on the right ear. The ear swelling was evaluated by the difference of the thickness between the two ears measured with an engineer's micrometer (0.001 mm, Mitutoyo Corporation, Japan) 24 h after the challenge.

Preparation of splenocyte suspensions

Spleen was aseptically taken from mice, crushed gently and separated into single cells by squeezing in Hank's solution containing 5 U ml^{-1} of heparin. The cells obtained were passed though a gauze of eight-layers and centrifuged at 1000 rpm for 10 min at 4 °C. Pellet was added into 10 ml sterile Tris–NH_4Cl, pH 7.5 followed by centrifugation to remove erythrocytes. After washing twice with RPMI 1640 (GIBCO BRL) medium supplied with 100 U ml^{-1} of penicillin, 100 U ml^{-1} of streptomycin and 10% FCS, they were resuspended in the medium and used for culture.

Gelatin zymography assay

Analysis by zymography on gelatin gel allows detection of enzymatic activity of the secreted collagenases MMP-2 and MMP-9. This was performed as described by Hauzenberger *et al.* [26] with modification. Briefly, spleen cells isolated from mice were suspended in serum-free RPMI 1640 medium at a density of 2×10^7 ml^{-1} and incubated with various concentrations of RN-ext at 37 °C in 5% CO_2 for 36 h. The supernatants (20 μl) were collected and mixed with 10 μl Tris buffer (62.5 mM Tris containing 10% glycerol, 0.00125% BPB, 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 (2.5% Triton X-100, 1 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 50 mM Tris buffer containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 0.05% NaN_3. The gels were stained with 0.25% Coomassie brilliant blue R250 for 30 min, and destained for 8 h in a solution of 10% acetic acid and 30% methanol. The proteolytic activity was evidenced as clear bands (zones of gelatin degradation) against the blue background of stained gelatin. Note that zymography technique classically evidences two bands for MMP-9, corresponding to pro-MMP-9 (released as inactive proenzyme) and active MMP-9 (after cleavage of regulation domain).

Adhesion assay

Cell adhesion assay was conducted as described [27] with modification. Briefly, flat-bottom 96-well microplate was coated with 50 μl solution of fibronectin (50 μg ml^{-1}) at 4 °C overnight, and nonspecific binding sites were blocked with 0.2% BSA for 2 h at room temperature. Then the plate was washed three times with PBS. Spleen cells (5×10^5/0.2 ml) suspended in RPMI 1640 were added to each well. The cells were incubated at 37 °C for 60 min after which nonadherent cells were removed by washing three times with RPMI 1640. The cells were fixed with methanol:acetone (1:1) and stained with 0.5% crystal violet in 20% ethanol. Unbound dye was removed in tap water and the plate was dried in air. Bound dye was extracted with 1% SDS. The absorbance of 5×10^5 cells, which were fixed and stained without previous washing, was considered as 100% cell adhesion. Specificity of cell adhesion assay was corroborated using BSA as substratum. All assays were run in triplicate.

Migration assay

Spleen cell migration along a gradient of substratum-bound gelatin was assayed in a Transwell cell culture chamber according to the methods previously reported [28]. The filters with an 8.0 μm pore size were precoated

with 2.5 μg fibronectin in a volume of 50 μl in the lower surface as described above. One hundred thousands of the cells in 100 μl RPMI 1640 medium were added to the upper compartment of Transwell cell chamber and incubated at 37 °C for 6 h. After incubation, filters were harvested and fixed with methanol and stained with hematoxylin and eosin. The spleen cells on the upper surface of filters were removed by wiping with cotton swabs. The cells, which had migrated to various areas of the lower surface, were counted under a microscope in five predetermined fields. At the same time, the cell number was also determined by MTT method. Briefly, 20 μl of MTT solution (5 mg ml^{-1}) was added to the cells, and incubated at 37 °C for 10 h. After centrifuged at 1000 rpm for 5 min, the supernatants were discarded and 200 μl of DMSO was added. After thorough dissolution, the optic density was read by an ELISA reader. Each assay was performed in triplicate.

Statistical analysis

One-way analysis of variance (ANOVA) for multiple comparisons was used to detect whether there was any significant differences among the different treatment. Once significant differences were detected ($P < 0.05$) Student two-tailed *t*-test was used to evaluate the difference between two groups. All experimental results were shown as the mean ± SD.

RESULTS

Effect of RN-ext on PCl-CS

Mice were administrated p.o. with RN-ext and i.m. with dexamethasone for 6 days from the PCl sensitization. Compared with the control, 200 mg kg^{-1} of RN-ext significantly inhibited the ear swelling. Dexamethasone (20 mg kg^{-1}) also showed a strong inhibition (Table I, upper panel). When the drugs were given for four times at 0 (immediately), 5, 10, and 15 h after the PCl challenge, the extract dose-dependently inhibited the ear swelling. Dexamethasone also showed a strong inhibition (Table I, the lower panel).

Table I
Effect of RN-ext and dexamethasone on PCl-CS

Groups	*Dose (mg kg^{-1})*	*Number of animals*	*Ear swelling (10^{-3} cm)*	*Inhibition (%)*
Induction phase				
Control	–	7	86.6 ± 20.1	
RN-ext	200	7	49.6 ± 11.8*	42.8
Dex	20	7	39.6 ± 10.6**	54.3
Effector phase				
Control	–	8	10.4 ± 14.3	
RN-ext	100	8	94.9 ± 15.5	8.9
	200	8	68.9 ± 14.0**	33.8
	400	8	59.4 ± 15.9**	43.0
Dex	20	8	41.5 ± 15.9**	60.1

Mice were sensitized by painting 0.1 ml of 1% PCl in ethanol on the skin of their abdomens. Five days later they were challenged by painting 30 μl of 1% PCl in olive oil on the right ear. The ear swelling was evaluated by the difference of the thickness between the two ears 24 h after the challenge. RN-ext and dexamethasone were given p.o. and i.m., respectively for 5 days from the PCl sensitization (induction phase), or for four times at 0, 5 10, and 15 h after PCl challenge (effector phase). * $P < 0.05$. ** $P < 0.01$ versus control.

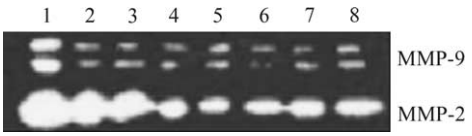

Fig. 1. Effects of RN-ext on the activities of MMP-2 and MMP-9 in spleen cells isolated from mice with PCl-CS *in vitro*. Spleen cells were isolated 6 h after the PCl challenge and normal mice. These cells were cultured *in vitro* at a density of 2×10^6/0.2 ml well^{-1} for 36 h in the presence or absence of various concentrations of RN-ext. After incubation, the supernatants were collected and subjected to the zymography assay. Lanes 1–4: 0, 10^{-6}, 10^{-5}, and 10^{-4} g ml^{-1} of RN-ext-treated spleen cells from PCl-CS mice; lanes 5–8: 0, 10^{-6}, 10^{-5}, 10^{-4} g ml^{-1} of RN-ext-treated spleen cells from normal mice.

Effect of RN-ext on matrix MMP activity in spleen cells isolated from mice with PCl-CS in vitro

Spleen cells isolated from normal mice or the mice with PCl-CS 6 h after challenge were incubated with various concentrations of RN-ext for 36 h. The supernatants were collected and subjected to the zymograph assay. As shown in Figure 1, a remarkable increase in both MMP-2 and MMP-9 were observed in spleen cells from PCl-CS mice, compared with those from normal mice. Against the increase (lane 1), RN-ext dose-dependently inhibited both MMP-2 and MMP-9 activities in the cells from PCl-CS mice (lanes 2–4), while did not influence those from normal mice (lanes 6–8).

Effect of RN-ext on the migration of spleen cells from mice with PCl-CS in vitro

Spleen cells were isolated 6 h after PCl challenge. Compared with the control, the number of cells that migrated to the lower surfaces of the filters and in the lower compartment of the transwell system were significantly reduced in a dose-dependent manner due to RN-ext exposure (Fig. 2).

Effect of RN-ext on the adhesion of spleen cells to fibronectin

As shown in Figure 3, the treatment of spleen cells isolated at 6 h with 10^{-6} to 10^{-4} g ml^{-1} of RN-ext did not influence the cell adhesion to fibronectin.

Effect of RN-ext, administered in vivo, on matrix MMP activities produced by spleen cells from mice with PCl-CS

Mice were administered p.o. 200 mg kg^{-1} of RN-ext for 6 days from the PCl sensitization. Six hours after the PCl challenge, the splenocytes were isolated and incubated at 37 °C for indicated time. Then, the supernatants were collected and subjected to zymograph assay. Compared with the control, a remarkable reduction in the activities of MMP-2 and MMP-9 was observed in the spleen cells from RN-ext-administered mice (Fig. 4).

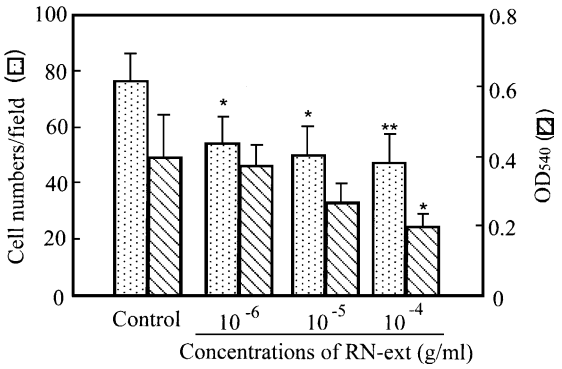

Fig. 2. Effect of RN-ext on the migration activities of spleen cells from mice with PCl-CS. Spleen cells were isolated 6 h after the PCl challenge. The filters with an 8.0 μm pore size in the transwell system were precoated with 2.5 μg fibronectin in a volume of 50 μl on the lower surface. Then, one hundred thousands of the spleen cells were added in the upper compartment and cultured at 37 °C for 6 h in the presence or absence of RN-ext. Then, the number of cells, which migrated to various areas of the lower surface of the filters and the lower compartment was determined as described in Materials and Methods. Each column represents the mean ± SD of four independent experiments and each experiment includes triplicate sets.

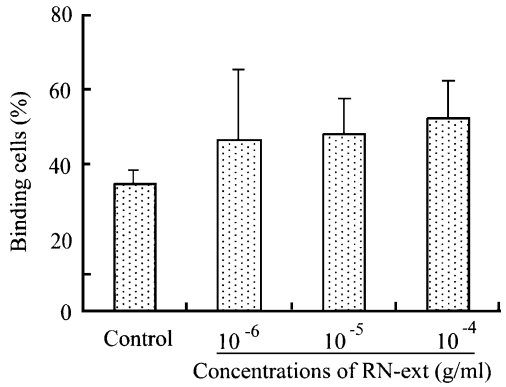

Fig. 3. Effect of RN-ext on the adhesion of spleen cells from PCl-CS mice to fibronectin. Spleen cells were isolated 6 h after the PCl challenge. The 96-well microplate were coated with 2.5 μg fibronectin overnight, and the nonspecific binding sites were blocked by 0.2% BSA. Cells (5×10^5) that had been pretreated with or without RN-ext at 37 °C for 30 min were added into the culture well. After incubation for 60 min at 37 °C, the percentage of hinding cells was determined as described in Materials and Methods. Each column represents the mean ± SD of four independent experiments and each experiment includes triplicate sets.

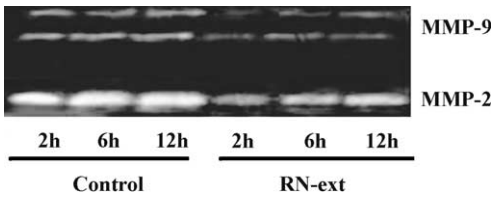

Fig. 4. Effect of RN-ext, administered *in vivo*, on the activity of matrix MMP produced by spleen cells isolated from mice with PCl-CS. Mice were administered p.o. 200 mg kg^{-1} RN-ext for 6 days from the PCl sensitization. Six hours after the PCl challenge, the splenocytes were isolated and incubated at 37 °C for 2, 6, and 12 h, respectively. Then, the supernatants were collected and subjected to zymography assay. The figure shown here is the representative of three experiments.

DISCUSSION

The present study first examined the effect of RN, used for treating rheumatoid arthritis in traditional Chinese medicine, on the cellular immune response, PCl-CS. As the result, the aqueous extract from the plant significantly inhibited the ear swelling whenever given during induction or effector phase (Table I). This result suggests that the extract could interfere with the activation and functions of T lymphocytes involved in the ear inflammation.

To confirm the effect of the extract on the lymphocytes, we next examined the activities of MMPs produced by the spleen cells from mice with PCl-CS. As compared with those from normal mice, the cells from PCl-CS mice produced much more increased MMP-2 and MMP-9 levels, suggesting the involvement of MMPs in the process of the contact sensitivity. Against the increased release, the *in vitro* exposure to RN-ext of the cells from PCl-CS mice but not naïve mice dose-dependently inhibited the production of both MMP-2 and MMP-9 (Fig. 1). When the extract was given p.o. to mice after the PCl sensitization, a reduced MMP production was also found in the spleen cells isolated 6 h after the PCl challenge (Fig. 4). These results indicated that the inhibition of MMP production by RN-ext might contribute to the recovery of mice from the contract sensitivity. Although the detailed mechanisms of anti-MMPs by RN-ext needs to be investigated, an inhibition on the synthesis, secretion and activity of MMPs might be considered since the extract did not show any influence on the activities by the cells from naïve mice.

MMP-2 and MMP-9 have been reported to be involved in transmigration of T cells through basement membrane both *in vitro* and *in vivo*, and this process was specifically blocked by the inhibitors of matrix MMPs [23, 29]. Next we examined the effect of RN-ext on the ability of spleen cells from mice with PCl-CS to transmigrate through basement membrane. By using a transwell culture system, a significant inhibition of the cell transmigration was observed (Fig. 2). However, RN-ext did not show any inhibitory effects on the cell adhesion to fibronectin (Fig. 3). As two aspects in the tissue infiltration of the lymphocytes, the migration of lymphocytes needs to get a moving potential and a direction to the inflammatory sites, while the adhesion process includes the interaction between the lymphocytes and endothelial cells as well as between lymphocytes and ECMs. The migration is usually mediated by various humoral factors including MMPs. Above results suggest that in the effect of RN-ext on the lymphocyte transmigration, MMP might be crucial as its main target.

On the other hand, previous investigators reported the inhibitory activities of the plant on 5-lipoxygenase and cyclooxygenase [25]. COX-1 and COX-2 overexpression by stable transfection has recently been shown to induce membrane type MMP-1, which activates MMP-2 [30]. These findings may give a further explanation for the anti-inflammatory activity of RN-ext in addition to MMP-inhibiting activity. The inhibition of COX by

RN-ext may also result in the down-regulation of MMP activities.

In summary, RN-ext exhibited a significant inhibition on the PCl-induced contact sensitivity mediated by a cellular immune response. Its preliminary mechanism has been given in this study as a reduction in the migration potential of lymphocytes due to the down-regulation of MMP production. These findings may be helpful for the explanation on its traditional use in treating inflammatory disorders including rheumatoid diseases.

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