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Si-Ni-San, a traditional Chinese prescription, and its drug-pairs suppress contact sensitivity in mice via inhibition of the activity of metalloproteinases and adhesion of T lymphocytes

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Abstract

In this paper, the effect of Si-Ni-San, a famous traditional Chinese prescription, on ear contact sensitivity was examined. Si-Ni-San significantly inhibited the ear swelling when administered during the induction phase of picryl-chloride-induced ear contact sensitivity in mice. The adhesion to type I collagen of isolated spleen cells was significantly decreased in the Si-Ni-San group, especially in the presence of protein kinase C activator, phorbol 12,13-dibutyrate. The inhibition of adhesion was seen in purified T cells from the spleen, as well as in Jurkat cells. Furthermore, the adhesion to collagen involves the production of matrix metalloproteinase-2 and -9 in spleen cells, and the oral administration of Si-Ni-San remarkably reduced the matrix metalloproteinase production. Three drug-pairs composed in Si-Ni-San were used for comparison. The combination of Chaihu and Shaoyao showed a similar effect to Si-Ni-San, while Chaihu and Zhishi, and Shaoyao and Gancao only showed a tendency in most situations. These results suggest that Si-Ni-San may contribute to the treatment of immunologically related diseases by down-regulating the activation and function of T lymphocytes, especially the cell adhesion and matrix metalloproteinase production. Its effect is mainly displayed by the combination of Chaihu and Shaoyao.

Introduction

Delayed-type hypersensitivity, a typical T cell-mediated immune response, has been known to be involved in the pathogenesis of various immunologically related diseases, such as hepatitis, contact dermatitis, experimental allergic encephalomyelitis, rheumatoid arthritis and multiple sclerosis (Napoli et al 1996; Grom & Hirsch 2000; Askenase 2001; Matarese et al 2001; Morgan et al 2001). The cellular and molecular mechanisms of delayed-type hypersensitivity reactions have been extensively studied. One of the key points is the activation of lymphocytes and their migration and infiltration to inflammation loci (Kobayashi et al 2001). For the inflammatory infiltration, lymphocytes should interact with endothelial cells, the underlying basement membrane and interstitial matrix. Such interactions play a crucial role in the occurrence and progress of delayed-type hypersensitivity reactions (De Fougerolles et al 2000). During the progress, matrix metalloproteinases (MMPs) are important for the degradation of basement membrane and extracellular matrix. The activated T lymphocytes that migrate to areas of inflammation may produce MMPs in the presence of numerous inflammatory factors (Goetzl et al 1996). Some monoclonal antibodies and synthetic peptides could ameliorate inflammation via inhibiting lymphocytes adhesion to extracellular matrix (Haworth et al 1999; De Fougerolles et al 2000). Clinical and experimental research has demonstrated that inhibition of MMP leads to an alleviation of various inflammation disorders (Kumagai et al 1999; Di Sebastiano et al 2001; Elliott & Cawston 2001). These findings suggest that inhibition of T cell adhesion and interference with the activity of proteinases, including MMPs, may represent a useful approach to the treatment of T-cell-mediated immune diseases.

On the other hand, traditional Chinese medicine has a long history of treating various diseases by using various prescriptions that were formulated according to the

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characteristic theory. Si-Ni-San is one representative of the prescriptions derived from *Treatise on febrile diseases*, a medical classic written by Zhongjing Zhang in East Han Dynasty. The prescription comprises an equal ratio of four drugs: Chaihu (Radix Bupleuri Chinensis), Shaoyao (Radix Paeoniae Alba), Zhishi (Fructus Citri Aurantii) and Gancao (Radix Glycyrrhizae Uralensi). Si-Ni-San has been believed to be effective in curing some inflammatory diseases and is widely used as a medication recipe to treat hepatitis, gastritis, neuralgia, appendagitis, etc., in clinics (Guo et al 1999; Xue 2001; Zhang & Zhang 2000). Among the herbal drugs composed in the prescription, the extract from Radix Bupleuri has been reported to show a strong anti-inflammatory activity, and saikosaponins were identified as the effective components (Bermejo et al 1998). The main component of Radix Paeoniae, paeoniflorin, showed a potent analgesic action (Kobayashi et al 1990), while naringin from Fructus Citri Aurantii had antioxidant effects in cholesterol-fed rabbits (Jeon et al 2002). Glycyrrhizin from Radix Glycyrrhizae possesses various actions, such as anti-inflammatory, anti-allergic and hepatoprotection activity (Sato et al 1996). However, as seen with most other traditional prescriptions, Si-Ni-San is lacking scientific evidence of how it was formulated and how it takes effect. This is a unique prescription since it contains several drug-pairs that are also used as a single prescription or formulated in other prescriptions. The pairs are: Chaihu and Shaoyao (C-S); Chaihu and Zhishi (C-Z); and Shaoyao and Gancao (S-G). These drug-pairs have also been used for treating various inflammatory diseases such as cosalgia (C-S), dyspepsia (C-Z) and trigeminal neuralgia (S-G). Although the scientific reason why these drug-pairs are used in the same prescription is unclear, the efficacy of Si-Ni-San and these drug-pairs has been confirmed by clinical experience (Huang 1983; Guo et al 1999; Zhang & Zhang 2000; Xue 2001). Therefore, it is worth trying to find scientific evidence for the effect of this traditional prescription. Previously, we have demonstrated that Si-Ni-San significantly ameliorated liver injury in mice induced by carbon tetrachloride (Jiang et al 2000) as well as by delayed-type hypersensitivity to picryl chloride (Zhou et al 2000). However, the detailed mechanisms of Si-Ni-San against delayed-type hypersensitivity are still unknown. In this study, therefore, we examined the mechanisms of Si-Ni-San in inhibiting ear contact sensitivity, a typical delayed-type hypersensitivity reaction, with regard to adhesion and MMPs and compared the effect of the prescription with its drug-pairs.

Materials and Methods

Animals

Female Kunming strains of mice, aged 6–8 weeks (18–22 g) were obtained from the Experimental Animal Center of China Pharmaceutical University (Nanjing, China). They were maintained with free access to pellet food and water in plastic cages at $21 \pm 2\,^{\circ}C$ 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 animals' suffering and to reduce the number of animals used.

Cell line

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

Drugs and reagents

The crude drugs used in this study were purchased from Nanjing Medicinal Material Co. (Nanjing, China) and identified as *Bupleurum chinense* DC (Radix Bupleuri Chinensis, Chaihu), *Paeonia albiflora* Pall. (Radix Paeoniae Alba, Shaoyao), *Citrus aurantium* L. (Fructus Citri Aurantii, Zhishi) and *Glycyrrhiza uralensis* Fisch. (Radix Glycyrrhizae Uralensis, Gancao) by Dr Boyang Yu (Department of Chinese Medicinal Prescription, China Pharmaceutical University). They were mixed in an equal ratio (25 g of each drug in total 100 g) to make up Si-Ni-San, a mixed powder of material crude drugs. Furthermore, Chaihu and Shaoyao (C-S), Chaihu and Zhishi (C-Z) and Shaoyao and Gancao (S-G) were mixed in a ratio of 1:1 (50 g of each drug in 100 g of drug-pair) to make up traditional drug-pairs as C-S, C-Z and S-G, respectively. These materials were used for making 70% ethanol extracts as reported (Kobayashi et al 1993) with small modification. Briefly, the materials (100 g) were extracted twice with 5-fold volumes of 70% ethanol (500 mL) at $70\,^{\circ}C$ for 1 h each time. Then the supernatant, after centrifuging at 1870 *g*, was pooled and lyophilized to make a powder with 13.6, 26.3, 18.1 and 23.3% yields for C-S, C-Z, S-G and Si-Ni-San, respectively. The dosages of these extracts were indicated as the powders. The contents of main components in the 70% ethanol extract of Si-Ni-San were determined by HPLC as 0.25% of saikosaponin a, 2.49% of paeoniflorin, 1.89% of naringin and 4.24% of glycyrrhizin. The powders were dissolved in water for in-vivo assay by gavage oral administration to mice and in RPMI 1640 medium for in-vitro assay. Other drugs and reagents used in this study were as follows: dexamethasone sodium phosphate injection (Dex, Nanjing 3rd pharmaceutical factory, Nanjing, China), picryl chloride (Nacalai tesque Inc, Kyoto, Japan), bovine serum albumin (BSA, Sigma), rat tail type I collagen (Collaborative Biomedical Products, MA), phorbol 12,13-dibutyrate (PDBu, Wako Pure Chemical Industry Ltd., Japan), acrylamide and bis-acrylamide (Shanghai Sangon Biotechnical Ltd. Co., 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).

Picryl-chloride-induced ear contact sensitivity

Mice were sensitized by painting 0.1 mL of 1% picryl chloride in ethanol on the shaved skin of their abdomens. Five days later, they were challenged by painting 30 μL of 1% picryl chloride in olive oil on the right ear lobe (Liu et al 2002). Eighteen hours later, the thickness of the right ear was measured against the left with a digimatic micrometer (0.001 mm; Mitutoyo Co., Tokyo, Japan). The control mice were run parallel with other groups except for being given, orally, the same volume of water. At the same time, the spleen cells were isolated and used for adhesion or zymography assay.

Preparation of splenocyte suspensions and purification of T cells

Spleen was aseptically taken from 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 eight-layers of gauze and centrifuged at 200 *g* for 5 min at 4 °C. The 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 (Binz & Wigzell 1975). 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 4×2 mL 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 ranged between 45% and 65% and the purity ($CD3^+$ cells) of recovered cells reached about 88%.

Gelatin zymography assay

Analysis by zymography on gelatin gel allows detection of enzymatic activity of the secreted collagenases MMP-2 and MMP-9 (Torimura et al 2001). Briefly, spleen cells isolated from various treated mice were suspended in serum-free RPMI 1640 medium at a density of 2×10^6/well and incubated at 37° in 5% CO_2 for 36 h. Spleen cells from control mice were subjected to the same assay procedures in parallel. The supernatants (20 μL) were mixed with 10 μL sample buffer (62.5 mM Tris-HCl containing 10% glycerol, 0.00125% bromophenol blue and 12% sodium dodecyl sulfate (SDS)) without reducing agent, and they were subjected to SDS-PAGE (SDS-polyacrylamide gel electrophoresis) 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 shown as clear bands (zones of gelatin degradation) against the blue background of stained gelatin. Note that the zymography technique classically shows two bands for MMP-9, corresponding to pro MMP-9 (released as inactive proenzyme) and active MMP-9 (after cleavage of the regulation domain).

Adhesion assay

Adhesion assay was performed according to the report (Franitza et al 2000) with some modifications. Briefly, a flat-bottom 96-well microplate was coated with 50 μL solution containing type I collagen (50 μg mL^{-1}) and left at 4 °C overnight. Nonspecific binding sites were blocked with 0.2% BSA for 2 h at room temperature followed by washing three times with phosphate buffer solution. The cells were suspended in RPMI 1640 and spleen cells (1×10^6), spleen T cells (1×10^6) or Jurkat cells (1×10^5) were added to each well. The cells were incubated at 37 °C for 1 h with or without PDBu (100 ng mL^{-1}) and the non-adherent cells were removed by washing three times with RPMI 1640. 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% SDS. The absorbance of the samples was measured at 592 nm. The wells that 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 mice were subjected to the same assay procedures in parallel. The specificity of cell adhesion assays was corroborated using BSA as substratum.

Statistical analysis

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

Results

Effect of Si-Ni-San, its drug-pairs and dexamethasone on picryl-chloride-induced ear contact sensitivity in mice

When given in the induction phase, Si-Ni-San and its drug-pairs, C-S and S-G, significantly inhibited ear contact sensitivity in mice (Table 1). C-Z also inhibited the contact sensitivity, although not significantly. When given

Table 1 Effects of Si-Ni-San, its drug-pairs, and dexamethasone on the induction and effector phase of ear contact sensitivity in mice.

Group	No. 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	85.4 ± 28.8	0	109.0 ± 26.6	0
Si-Ni-San	8	200	47.4 ± 24.6*	40	84.0 ± 18.6	23
C-S	8	200	43.6 ± 20.2**	49	93.7 ± 9.5	14
C-Z	8	200	63.1 ± 41.4	26	104.6 ± 8.3	4
S-G	8	200	53.0 ± 19.7*	38	84.9 ± 29.2	22
Dexamethasone	8	10	12.0 ± 5.1**	86	22.9 ± 9.8**	79

Ear contact sensitivity was induced in mice. Eighteen hours after the challenge, the thickness of right and left ears was measured and the swelling was evaluated by the increase in ear thickness. In the induction phase, the extracts were given orally and the dexamethasone given intramuscularly for 6 days after the sensitization. In the effector phase, the drugs were given 3 times in an interval of 5 h from the challenge. Each figure indicates the mean ± s.d. of data from 8 mice. C-S, C-Z and S-G: combinations of Chaihu and Shaoyao, Chaihu and Zhishi, Shaoyao and Gancao, respectively. *$P < 0.05$, **$P < 0.01$ vs control (Dunnett's test).

in the effector phase, Si-Ni-San, C-S and S-G tended to inhibit the contact sensitivity, although not significantly. Dexamethasone strongly inhibited contact sensitivity in both phases.

Effect of Si-Ni-San and its drug-pairs on the adhesion of T lymphocytes from mice with ear contact sensitivity to type I collagen

Drugs were given orally (200 mg kg^{-1})for 6 days following the picryl chloride sensitization. In the absence of PDBu, the isolated spleen cells at 18 h after picryl chloride challenge showed a remarkably increased adhesion to collagen as compared with normal cells. All the extracts inhibited this adhesion, although not significantly. PDBu remarkably increased the cell adhesion. Against this, Si-Ni-San and C-S significantly inhibited the adhesion; S-G and C-Z also inhibited the adhesion, although not significantly (Figure 1). Furthermore, the isolated spleen cells were used for purifying T lymphocytes. The latter also relatively strongly adhered to collagen in the absence of PDBu (Spon, Figure 2). Stimulation with PDBu significantly increased the adhesion (Cont, Figure 2). Compared with the control, Si-Ni-San and C-S dose-dependently inhibited the adhesion.

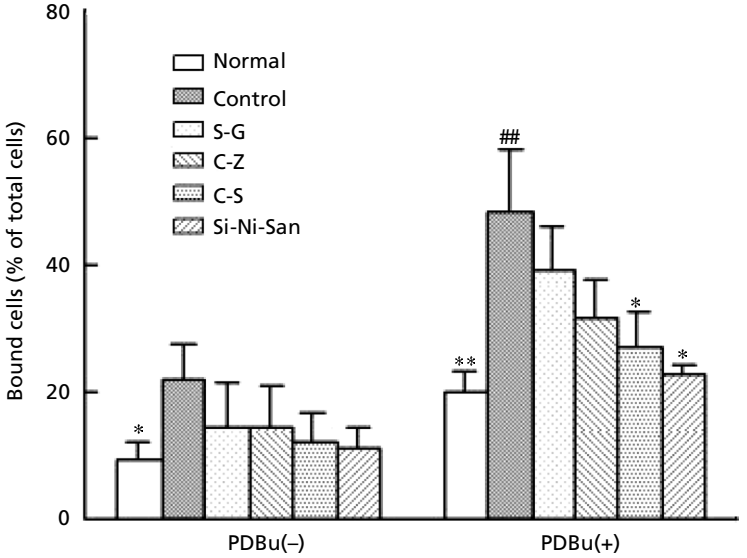

Figure 1 Effect of Si-Ni-San and its drug-pairs on the adhesion activity of spleen cells from mice with ear contact sensitivity to type I collagen. Drugs (200 mg kg^{-1}) were given orally for six days after the sensitization. At 18 h after the challenge, spleen cells were isolated and 1×10^6 cells were incubated in the collagen-coated 96-well microplate in the absence or presence of PDBu (100 ng mL^{-1}) for 1 h. After incubation, the cell adhesion assay was performed. Data were expressed as the mean ± s.d. of three independent experiments and each experiment included triplicate sets. C-S, C-Z and S-G: combinations of Chaihu and Shaoyao, Chaihu and Zhishi, Shaoyao and Gancao, respectively. *$P < 0.05$, **$P < 0.01$ vs control (Dunnett's test); ##$P < 0.01$ vs control of PDBu(−) (Student's t-test).

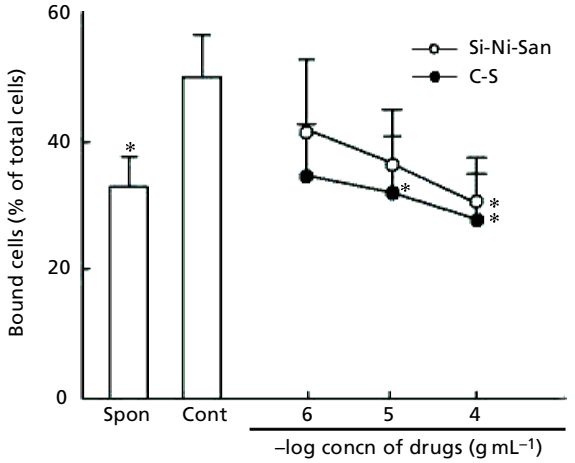

Figure 2 Effect of Si-Ni-San and C-S on the adhesion activity of T cells purified from spleen cells in mice with ear contact sensitivity to type I collagen activated by PDBu. Ear contact sensitivity was induced in mice. Eighteen hours after the picryl chloride challenge, spleen cells were isolated for purifying T cells. After being treated with various concentrations (10^{-6}, 10^{-5}, 10^{-4} g mL^{-1}) of the extracts for 2 h at 37 °C and then washed twice with RPMI 1640 medium, the cells (1×10^6 cells/well) were used for the adhesion assay in the presence of PDBu (100 ng mL^{-1}) for 1 h. Data were expressed as mean ± s.d. of three independent experiments and each experiment included triplicate sets. Spon: T cells alone; Cont: T cells + PDBu; C-S: Chaihu and Shaoyao. *$P < 0.05$ vs control (Dunnett's test).

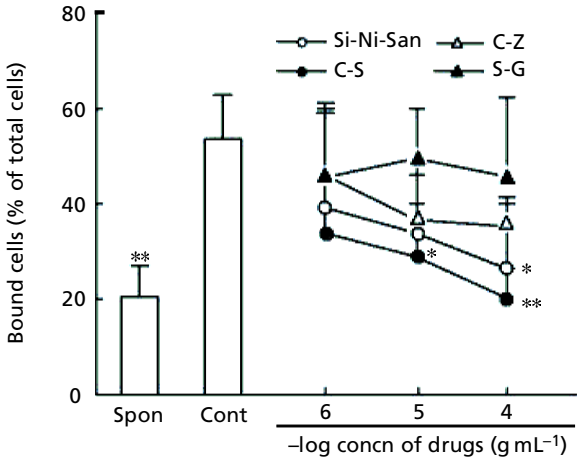

Figure 3 Effect of Si-Ni-San and its drug-pairs on the adhesion activity of Jurkat cells to type I collagen activated by PDBu. Jurkat cells (1×10^5/well) were incubated with the extracts for 2 h at 37 °C. Then the cells were washed twice with RPMI 1640 medium and subjected to the adhesion assay. Data were expressed as mean ± s.d. of three independent experiments and each experiment included triplicate sets. Spon: Jurkat alone; Cont: Jurkat + PDBu; C-S, C-Z and S-G: Chaihu and Shaoyao, Chaihu and Zhishi, Shaoyao and Gancao, respectively. *$P < 0.05$, **$P < 0.01$ vs control (Dunnett's test).

Effect of Si-Ni-San and its drug-pairs on the adhesion of Jurkat T cells to type I collagen

PDBu significantly induced the adhesion of Jurkat cells to collagen (Figure 3). Pre-treatment with Si-Ni-San, C-S and C-Z for 2 h produced a dose-dependent decrease in the cell adhesion. S-G did not influence the cell adhesion.

Increase in MMP activity in the process of cell adhesion to type I collagen

Spleen cells isolated from mice with ear contact sensitivity at 18 h after challenge were applied to the adhesion assay in the presence or absence of PDBu. As shown in Figure 4, PDBu or collagen alone did not influence MMP production in 24 h of incubation. In the presence of both PDBu and collagen, spleen cells remarkably increased the production of MMP-2 and MMP-9.

Effect of Si-Ni-San and its drug-pairs on MMP production by spleen cells

Mice were given 200 mg kg^{-1} of the extracts orally for 6 days from the picryl chloride sensitization. The isolated spleen cells at 18 h after picryl chloride challenge secreted a higher level of MMP-2 and MMP-9 than did normal spleen cells. Compared with the control, Si-Ni-San and its drug-pairs produced an inhibition, especially in the group administered with Si-Ni-San and C-S (Figure 5).

However, Si-Ni-San and its drug-pairs, 200 mg kg^{-1} given orally for 6 days to normal mice, did not affect MMP activity of spleen cells (data not shown).

Discussion

It is well known that the induction phase of the delayed-type hypersensitivity reaction represents the formation of antigen-specific T lymphocytes and the effector phase includes the release of cytokines from effector T cells against the antigen challenge and the consequent inflammatory reaction with an infiltration of monocytes and lymphocytes. In this study, we first examined the effect of Si-Ni-San and its drug-pairs on picryl-chloride-induced ear contact sensitivity in mice. As a result, Si-Ni-San, C-S and S-G significantly inhibited the ear swelling when given in the induction phase of the delayed-type hypersensitivity reaction. However, the extracts only slightly inhibited the ear swelling when given in the effector phase. Another drug-pair, C-Z, did not show a significant inhibition (Table 1). These results suggest that Si-Ni-San may inhibit the delayed-type sensitivity reaction through influencing the formation and activation rather than the function of the effector cells. The effect of Si-Ni-San is mainly displayed by the combination of C-S and S-G.

Next, we examined the effect of the extracts on the adhesion of spleen cells from mice with ear contact sensitivity to type I collagen when administered in the induction phase. A remarkable increase in adhesion activity of spleen cells from the mice was observed as compared with the activity in spleen cells from normal mice. The oral

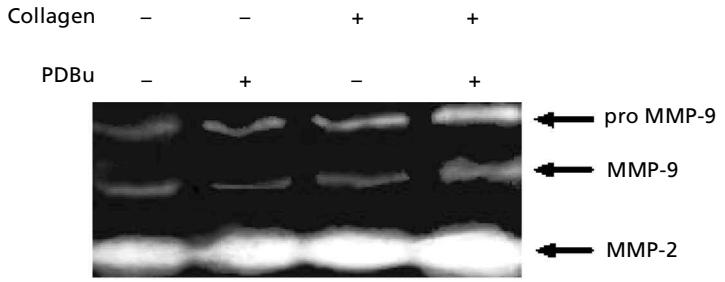

Figure 4 Effect of type I collagen-induced adhesion on the activity of MMP-2 and MMP-9 in spleen cells from mice with ear contact sensitivity. The 96-well microplate was coated overnight with 2.5 μg type I collagen in a volume of 50 μL. After washing with phosphate-buffered saline, spleen cells isolated from the contact sensitivity mice at 18 h after the picryl chloride challenge were added and incubated at a density of 1×10^6/0.2 mL/well with or without PDBu (100 ng mL^{-1}) for 24 h. Then the supernatants were collected and subjected to gelatin zymography analysis. The figure is a representative of three experiments.

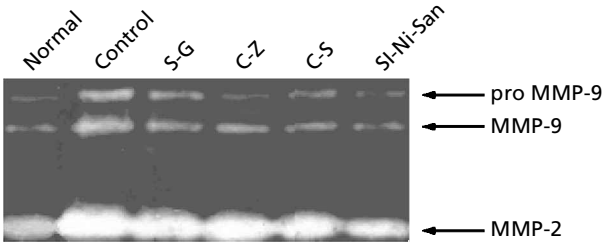

Figure 5 Effect of Si-Ni-San and its drug-pairs, administered in the induction phase, on the activity of MMP-2 and MMP-9 in spleen cells isolated from mice with ear contact sensitivity. Mice were administered orally 200 mg kg^{-1} of drugs for 6 days after the picryl chloride sensitization. Eighteen hours after the challenge, spleen cells were isolated and incubated (1×10^6/well) at 37 °C for 36 h, the supernatants were

administration of Si-Ni-San and its drug-pairs tended to inhibit this increase. The adhesion activity of the spleen cells was significantly enhanced when the cells were stimulated by protein kinase C (PKC) activator, PDBu, in-vitro. An almost complete blockage of this enhancement was seen in groups treated with Si-Ni-San or C-S, while C-Z and S-G non-significantly inhibited the enhancement. These results are in accordance with those represented in ear swelling and suggest that the inhibitory effect of Si-Ni-San and its drug-pairs on ear swelling is related to the reduction in adhesive potential of lymphocytes to extracellular matrix. It should be noticed that the spleen cells isolated from Si-Ni-San-treated mice almost lost their responsiveness to PDBu activation. Furthermore, T cells were purified from the spleen cells and their adhesion ability was examined. As shown in Figure 2, adhesion of spleen T cells to type I collagen activated by PDBu was inhibited by Si-Ni-San and C-S in a dose-dependent manner. This finding indicates that the inhibition of spleen cells by Si-Ni-San and its drug-pair was mainly directed to T lymphocytes.

The adhesion of T cells to extracellular matrix plays a crucial role in the progress of delayed-type hypersensitivity as T cells act as the major inducer and effector in the delayed-type hypersensitivity reaction (Black 1999). To confirm the effect of Si-Ni-San on T lymphocytes, we examined the adhesion activity of human T lymphoma Jurkat cells in-vitro. As shown in Figure 3, PDBu significantly enhanced the adhesion of Jurkat cells to collagen. Si-Ni-San and C-S dose-dependently inhibited the cell adhesion to collagen, while C-Z only inhibited the adhesion non-significantly. However, S-G did not influence the adhesion. Upon the activation of T cells, the stimulation through CD3/TCR complex usually leads to the up-regulation of adhesive function of the cells. This process involves more than one intracellular signal transduction pathways (Tsuchida et al 2000). Among these, the PKC pathway seems to be essential for the up-regulation of adhesion. Since Si-Ni-San and its drug-pairs could block the PDBu-induced cell adhesion increase, the involvement of PKC pathway may be considered in the inhibition of T cell adhesion by Si-Ni-San.

Cell invasion requires cooperation between adhesion receptors and MMPs. As many reports indicated, the adhesion of lymphocytes to extracellular matrix is considered to be indispensable in the process of lymphocyte migration. During this process, the degradation of extracellular matrix needs the help of MMPs. Excretion of MMP-2 and MMP-9 conspicuously increases when the integrins on the T cell surface bind to their ligands in extracellular matrix (Esparza et al 1999; Yakubenko et al

2000). In Figure 4 of the present paper, we also observed that the activity of MMP-2 and MMP-9 is markedly enhanced in the adhesion to collagen when activated by PDBu in spleen cells from mice with ear contact sensitivity. In fact, a high level of MMP was observed in the spleen cells isolated from the contact sensitivity mice at 18 h after picryl chloride challenge when the inflammation reached a peak (Figure 5). These findings suggest an involvement of MMPs in the adhesion process. The oral administration of Si-Ni-San and its drug-pairs in the induction phase inhibited MMP production in the spleen cells. Such inhibition was especially seen in the Si-Ni-San and C-S-treated groups (Figure 5). This result is quite consistent with that of the spleen cells' adhesion to collagen in the contact sensitivity mice. The above findings make a linkage between the inhibition of cell adhesion and the reduction in MMP activity. However, Si-Ni-San and its drug-pairs did not inhibit the MMPs in the spleen cells isolated from normal mice (data not shown), suggesting that the effect of Si-Ni-San might be displayed only on the over-expression of MMP-2 and MMP-9 in pathological conditions, which reveals a selectivity to some degree.

Overall, Si-Ni-San may inhibit delayed-type hypersensitivity reactions mainly through influencing the formation and activation of effector T lymphocytes. The mechanisms may involve the down-regulation of MMP activity and inhibition of the cell adhesion to extracellular matrix. These findings, together with our previous data on the improvement against the liver injury by a delayed-type hypersensitivity reaction to picryl chloride (Zhou et al 2000), suggest that Si-Ni-San may be used for the treatment of delayed-type-hypersensitivity-related diseases. By analysing the composition of Si-Ni-San, we found that the combination of Chaihu and Shaoyao (C-S), as a major composition, showed a comparatively coincident effect with Si-Ni-San, while C-Z and S-G may contribute to the efficacy in a minor way. These findings are also quite in accordance with the theory of traditional Chinese medicine by which Chaihu and Shaoyao were two of the key drugs in Si-Ni-San.

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