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Role of four major components in the effect of Si-Ni-San, a traditional Chinese prescription, against contact sensitivity in mice

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Abstract

Previously, we demonstrated the inhibitory effects of Si-Ni-San, a traditional Chinese prescription, on picryl chloride-induced ear contact sensitivity (PCl-CS). This study aimed to evaluate the role of the four major constituents contained in the prescription (saikosaponins, paeoniflorin, naringin and glycyrrhizin) in the inhibitory effect. When administered during the induction phase, saikosaponin a and glycyrrhizin showed significant inhibitory effects, while paeoniflorin and naringin did not. These components in Si-Ni-San also inhibited the activation and proliferation of T lymphocytes as well as the production of cytokines such as tumour necrosis factor-α and interferon-γ to different extents. Saikosaponin a and paeoniflorin dose-dependently reduced the splenocyte adhesion to type I collagen, while glycyrrhizin only showed a slight tendency. Furthermore, treatment with glycyrrhizin or saikosaponin a, rather than paeoniflorin or naringin, moderately inhibited the matrix metalloproteinase (MMP)-2 activity of the splenocytes from PCl-CS mice, and the combination of all four components showed a strong inhibition against MMP-2. Moreover, the components markedly decreased the serum level of nitric oxide in PCl-sensitized mice. The results indicated that saikosaponin a and glycyrrhizin may be the major contributors in the alleviation effect of Si-Ni-San on contact sensitivity, and paeoniflorin and naringin may exhibit a co-operative effect.

Introduction

Delayed-type hypersensitivity (DTH) reaction, a typical T cell-mediated immune response, has been known to be involved in the pathogenesis of various immunological related diseases, such as hepatitis, contact dermatitis, experimental allergic encephalomyelitis, rheumatoid arthritis and multiple sclerosis (Napoli et al 1996; Askenase 2001; Matarese et al 2001; Grom & Hirsch 2000; Morgan et al 2001). The key process of the DTH reaction is the activation of T lymphocytes and their migration and infiltration to the inflammation locus, which always accompanies the development and exacerbation of these T cell-mediated diseases (Kobayashi et al 2001). Increasing evidence has demonstrated that the inhibition of the activation and functions of T cells, such as adhesion, migration, and proteinase production, may represent a useful approach to the treatment of T cell-mediated immune diseases (Haworth et al 1999; Mattei et al 2002).

In an important attempt to explore such approaches, in addition to finding potential immunosuppressant and anti-inflammatory agents, our research has been focused on various traditional Chinese medicines. Among the traditional prescriptions, Si-Ni-San is believed to be effective in curing some inflammatory diseases and has been widely used as a mediation recipe to treat hepatitis, gastritis, neuralgia, and appendagitis (Guo et al 1999; Cai & Liu 2004; Zhang & Zhang 2000). The formula comprises an equal ratio of four traditional Chinese medicines: Chaihu (*Radix Bupleuri Chinensis*), Shaoyao (*Radix Paeoniae Alba*), Zhishi (*Fructus Citri Aurantii*) and Gancao (*Radix Glycyrrhizae Uralensis*). As the major components contained in these herbal drugs, saikosaponins are well known to have excellent anti-inflammatory activity (Bermejo Benito et al 1998); paeoniflorin showed a potent analgesic action (Kobayashi et al 1990); naringin had an antioxidant effect in cholesterol-fed rabbits (Jeon et al 2002); and glycyrrhizin possessed various pharmacological effects

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including anti-inflammation, anti-ulcer, anti-allergy, anti-carcinogenesis and immune modulation (Matsui et al 2004). Although Si-Ni-San has been extensively applied in clinics and its mechanisms have been partially elucidated (Jiang et al 2003; Sun et al 2003), the active constituents and their pharmacological properties have not been sufficiently clarified. As a famous prescription in traditional Chinese medicine, Si-Ni-San is composed of herbal drugs with different effects. These effects are believed to be complementary according to the theory of the Yin-Yang balance. It is reasonable to suggest that the immunosuppressive activity of Si-Ni-San is exerted by these different herbal drugs containing different components as described above. This study, therefore, was undertaken to clarify the role of the four main constituents of Si-Ni-San in its immunosuppressive activity against picryl chloride-induced contact hypersensitivity, a typical DTH reaction.

Materials and Methods

Drugs and reagents

The crude drugs used in this study were purchased from Nanjing Medicinal Material Co. (Nangjing, China) and identified as the roots of *Bupleurum chinensis* DC. (Chaihu), *Paeonia albiflora* Pall. (Shaoyao), *Glycyrrhiza uralensis* Fisch. (Gancao), and the fruit of *Citrus aurantium* L. (Zhishi) 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 a mixed powder of material crude drugs which were used for making 70% ethanol extracts and lyophilized to make a powder with a 23.2% yield for Si-Ni-San. The powders were dissolved in water for the in-vivo assay for administration by oral gavage to mice and in RPMI 1640 medium for the in-vitro assay. Other drugs and reagents were as follows: paeoniflorin and naringin were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), glycyrrhizin was purchased from Wako Pure Chemical Industries (Japan). Saikosaponin a was isolated from Radix Chinensis in our laboratory; dexamethasone sodium phosphate (Nanjing 3rd pharmaceutical factory, Nanjing, China), picryl chloride (PCl, Nacalai Tesque Inc, Kyoto, Japan), concanavalin A (Con A, Sigma), mitomycin (Sigma), M-MLV Reverse Transcriptase (Promega, USA), bovine serum albumin (BSA, Sigma), 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), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma).

Animals

Female ICR, BALB/c and C57BL/6 strains of mice (6–8-weeks old, 18–22 g) were obtained from the Experimental Animal Center of China Pharmaceutical University (Nanjing, China). 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) and the related ethical regulations of our university. All efforts were made to minimize the animals' suffering and to reduce the number of animals used.

Quantitative analysis of the major compounds in Si-Ni-San

Analytical HPLC was performed (Okamura et al 2000) to quantify the contents of the marked compounds in Si-Ni-San, which contained 1.2% (w/w) saikosaponin a, 1.4% (w/w) paeoniflorin, 7.9% (w/w) naringin and 2.1% (w/w) glycyrrhizin.

Picryl chloride-induced ear contact sensitivity

Groups of eight mice (Strain ICR) were each sensitized by painting 0.1 mL 1% PCl in ethanol on the shaved skin of their abdomens. In the induction phase, Si-Ni-San and its major components were given orally by gavage and dexamethasone was given intramuscularly for six days from the sensitization. The control animals were run parallel with the other groups except that water was administered orally by gavage (same volume). Six days later, mice were challenged by painting 40 μL 1% PCl in olive oil on the right ear lobes (Sun et al 2003). After 18 h, the mice were killed by ether anaesthesia and the ear thickness of the right against the left was measured with a digimatic micrometer (0.001 mm, Mitutoyo Co., Tokyo, Japan). Meanwhile, cells from the mice were used in the in-vitro assays. Among them, pre-sensitized mice meant those that were sensitized for six days without the challenge and they were used for adhesion and gelatin zymography assays. PCl-CS mice indicated that mice suffered from a PCl-induced contact sensitivity with both sensitization and challenge. In some assays, we used the cells from naive mice without any pretreatment.

Preparation of splenocytes suspension

The spleen was aseptically taken from naive or PCl-sensitized mice, crushed gently and separated into single cells by squeezing in 5 mL RPMI 1640 medium (GIBCO BRL). The cells obtained were passed through a gauze of eight-layers and centrifuged. After removing erythrocytes and washing twice with RPMI 1640 medium, cells were re-suspended in the medium and used for culture.

Proliferation of spleen cells

Spleen cells from naive BALB/c mice 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^{-1} (final concentration) of concanavalin A (Con A) at 37°C in 5% CO_2 for 72 h. The cell growth was evaluated with modified MTT assay (Sargent & Taylor 1989). Briefly, 20 μL 5 mg mL^{-1} MTT in RPMI 1640 medium were added for a further 4-h incubation. After removing the supernatant, 200 μL DMSO (dimethylsulfoxide) was added to dissolve the formazan crystals. The absorbance was read on an ELISA reader (Sunrise Remote/Touch Screen, TECAN, Austria) at 540 nm.

Reverse transcriptase-polymerase chain reaction (RT-PCR) (Mitsui et al 2004)

Total RNA was extracted from spleen cells with or without concanavalin A-activation, from naive mice using Tripure reagent (Roche) as described by the manufacturer. Single-stranded cDNA was synthesized from 2 μg 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): β-actin 5′-ACATCTGCTGGAAGGTGGAC and 3′-GGACCCATGTACCACCATG, tumour necrosis factor-α (TNF-α) 5′-CATCTTCTCAAAATTCGAGTGACAA and 3′-CCCAACATGGAACAGATGAGGGT, interferon-γ (IFN-γ) 5′-CTTCTTCAGCAACAGCAAGGCGAAAA and 3′-ACTAACGCCCCAACATAGACCCCC. PCR cycle conditions were: 94°C for 30 s, 60°C for 1.5 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 dyeing. The relative expressions of the cytokine genes were quantified densitometrically using the BandScan 5.0 software, and calculated according to the reference bands of β-actin.

Single mixed lymphocytes reaction (sMLR) (Tahara et al 2004)

The lymphocytes (5×10^5) from naive C57BL/6 mice were pretreated with mitomycin (final concentration was 500 μg mL^{-1}) in RPMI 1640 medium for 1 h. These cells (5×10^5/well) were co-cultured with the lymphocytes (5×10^5/well) from naive BALB/c mice in a 96-well plate in the presence or absence of the indicated concentrations of Si-Ni-San and its major components at 37°C for 72 h. The proliferation of the lymphocytes from BALB/c mice was measured by the MTT method. The stimulation index was calculated as following: stimulation index = $(OD_{sample}-OD_{C57BL/6\ alone})/OD_{BALB/c\ alone}$.

Cell adhesion to type I collagen

The adhesion assay was performed according to Franitza et al (2000) with some modifications. Briefly, flat-bottom 96-well microplates were coated with type I 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 buffer solution. Then spleen cells from PCl-sensitized mice, which were pre-incubated with or without the drugs at 37°C for 3 h and then washed three-times to remove the drugs, were added 1×10^6/well and incubated at 37°C for 1 h with or without PDBu (final concentration: 100 ng mL^{-1}). The non-adherent cells were removed by washing with RPMI 1640 medium. The cells were then 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 air-dried. Bound dye was extracted with 1% sodium dodecyl sulfate (SDS) and the absorbance of the samples was measured at 592 nm. The absorbance of the control wells, which were fixed and stained without previous washing, was regarded as the absorbance of total cells. All assays were run in triplicate and the results were expressed as percentage of bound cells.

Gelatin zymography assay

Analysis by zymography on gelatin gel allows the detection of enzymatic activity of the secreted collagenases MMP-2 (Torimura et al 2001). Briefly, spleen cells isolated from PCl-CS mice were suspended in serum-free RPMI 1640 medium at a density of 1×10^6/well and were incubated with the various drugs (10^{-5} or 10^{-4} g mL^{-1}) at 37°C for 36 h. The activity of MMP-2 in the supernatant was determined by gelatin zymography assay. Cell supernatants were subjected to SDS-PAGE. After electrophoresis, the gels were washed twice in the raising buffer for 1 h at room temperature and then incubated at 37°C for 36 h in the incubation buffer. After staining with 0.1% Coomassie brilliant blue, evidence of proteolytic activity was observed as clear bands (zones of gelatin degradation) against the blue background of stained gelatin.

Determination of NO

NO production was assessed according to Rockett et al (1994) using nitrate reductase kit (Nanjing JianCheng Bioengineering Institute, Nanjing, China). Briefly, Si-Ni-San and its major constituents were given orally and dexamethasone was given intramuscularly for six days from the PCl sensitization. Eighteen hours after the challenge, the serum was separated and the NO_2^- level was determined. Serum samples (50 μL) were incubated at 37°C with 50 μL reduction solution (NADPH 5 mg mL^{-1}; FAD 41.5 mg mL^{-1}, KH_2PO_4 0.5 M, pH 7.5 plus 0.5 U nitric oxide reductase). After 60-min incubation, 100 μL Griess reagent (0.1% naphthylenediamine hydrochloride, 1% sulphonylamide, 3% H_3PO_4) was added and the samples were incubated for 10 min. NO_2^- concentrations were determined from a standard curve prepared with $NaNO_2$.

Statistical analysis

Results were expressed as mean ± s.d. of three independent experiments and each experiment included triplicate sets in-vitro and eight animals of each group in-vivo. Data were statistically evaluated by Kruskal–Wallis test followed by Dunnett's test between control group and multiple dose groups, with the level of significance chosen as $P<0.05$.

Results

Effect of the major constituents in Si-Ni-San on PCl-induced ear contact sensitivity in mice

As shown in Table 1, when orally administered for six days from the sensitization, Si-Ni-San 200 mg kg^{-1} significantly inhibited ear swelling in mice. Two of its major constituents, saikosaponin a and glycyrrhizin, as well as their combinations, saikosaponin a plus paeoniflorin, saikosaponin a plus naringin, paeoniflorin plus glycyrrhizin, and all four components,

Table 1 Effect of the major constituents in Si-Ni-San and their combinations on PCl-induced ear contact sensitivity in mice

Group	Number of mice	Dose (mg kg^{-1})	Swellings (10^{-3} mm)	Inhibition (%)
Control	8	0	89.5 ± 14.7	0
Si-Ni-San	8	200	55.3 ± 23.1**	37.7
Saikosaponin a	8	2.5	58.1 ± 15.8**	35.1
Paeoniflorin	8	3	83.9 ± 18.3	6.3
Naringin	8	15 7	68.1 ± 17.5*	23.9
Glycyrrhizin	8	4	62.6 ± 15.6**	30.1
S + P	8	2.5 + 3	43.6 ± 11.2**	51.3
S + N	8	2.5 + 15	57.8 ± 18.8**	35.4
P + G	8	3 + 4	51.8 ± 12.4**	42.1
S + P + N + G	8	2.5 + 3 + 15 + 4	37.6 ± 9.8**	58.0
Dexamethasone	8	10	11.8 ± 3.5**	86.8

PCl-CS was induced in ICR mice. Each result indicates the mean ± s.d. of eight animals. S + P, S + N, P + G and S + P + N + G: combinations of saikosaponin a and paeoniflorin, saikosaponin a and naringin, paeoniflorin and glycyrrhizin, the four components, respectively. *$P < 0.05$, **$P < 0.01$ vs control (Dunnett's test).

at the doses that corresponded to their proportion contained in Si-Ni-San, showed strong inhibition to the contact sensitivity. However, naringin only exhibited a slight reduction and paeoniflorin hardly exerted any inhibitory effect. Dexamethasone as a positive drug also exhibited a strong inhibition. Moreover, the mixture of these four compounds (S + P + N + G) exerted a stronger influence as compared with Si-Ni-San and with each compound alone on the resulting inhibitory effect. In terms of the drug pairs, despite the ineffectiveness of paeoniflorin alone, it raised the inhibition rate of saikosaponin a and of glycyrrhizin to some extent.

Effects of the major constituents in Si-Ni-San on concanavalin A-induced splenocyte proliferation and single mixed lymphocytes reaction

Si-Ni-San and its major constituents, except naringin, significantly inhibited concanavalin A-induced lymphocyte proliferation (Figure 1A) and the mixed lymphocytes reaction (Figure 1B), both in a dose-dependent manner. However, the drugs at concentrations of 10^{-6}–10^{-4} g mL^{-1} did not have any cytotoxicity to normal spleen cells when incubated at 37°C for 24 h (data not shown).

Effects of the major constituents in Si-Ni-San on TNF-α and IFN-γ mRNA expressions in concanavalin A-activated murine spleen cells

Total mRNA was extracted from naive spleen cells incubated with 5 μg mL^{-1} concanavalin A alone or with indicated drugs. As shown in Figure 2 (A and B), concanavalin A activation markedly increased the mRNA expressions of TNF-α and IFN-γ. Against this, Si-Ni-San almost completely down-regulated the IFN-γ expression rather than TNF-α. Meanwhile, its four major constituents showed the inhibitory effect to different degrees. TNF-α expression was markedly reduced by

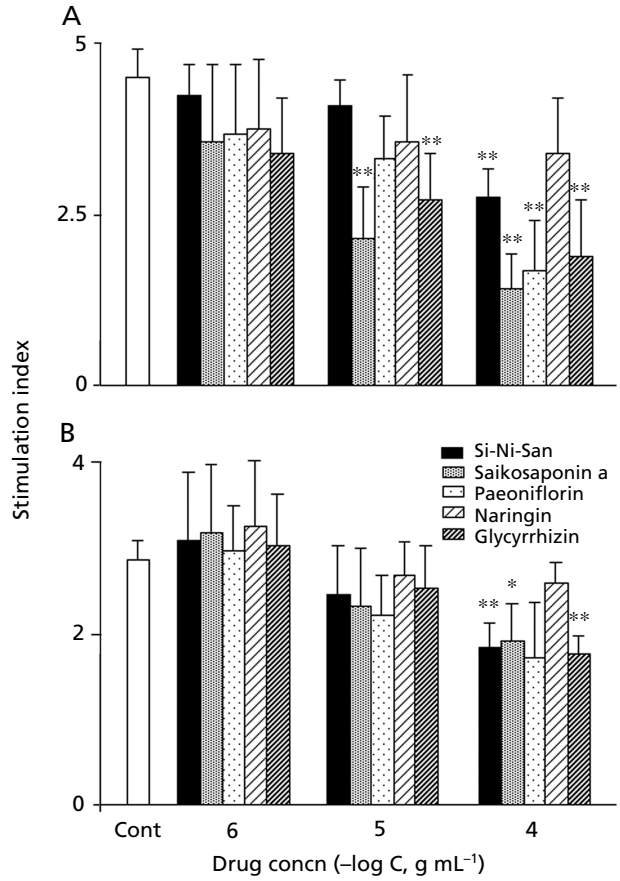

Figure 1 Effect of the major constituents in Si-Ni-San on concanavalin A-induced splenocyte proliferation (A) and single mixed lymphocyte reaction (sMLR; B). A. Spleen cells from naive BALB/c mice were isolated and stimulated in-vitro with 5 μg mL^{-1} concanavalin A and cultured in the presence or absence of the indicated concentrations of Si-Ni-San and its major constituents for 72 h. B. sMLR was performed in the presence or absence of the indicated drugs for 72 h. Cell proliferation in A and B was measured at 540 nm by MTT uptake. The stimulation index was calculated as the ratio of the absorbance between stimulated and non-stimulated cells. Each datum indicated mean ± s.d. of three independent experiments. *$P < 0.05$, **$P < 0.01$ vs control (Dunnett's test).

saikosaponin a and paeoniflorin, while the expression of IFN-γ was noticeably inhibited by paeoniflorin, naringin and especially glycyrrhizin. Naringin or glycyrrhizin did not inhibit the TNF-α expression, and saikosaponin a did not lower the IFN-γ expression.

Effect of the major constituents in Si-Ni-San on the adhesion of splenocytes from PCl-sensitized mice to type I collagen

The spleen cells for the adhesion assay were obtained and treated as described in Materials and Methods. As shown in Figure 3, the adhesion of splenocytes from PCl-sensitized mice was strongly increased as compared with the cells without PDBu activation. Against this, co-culture with saikosaponin a or paeoniflorin dose-dependently decreased the

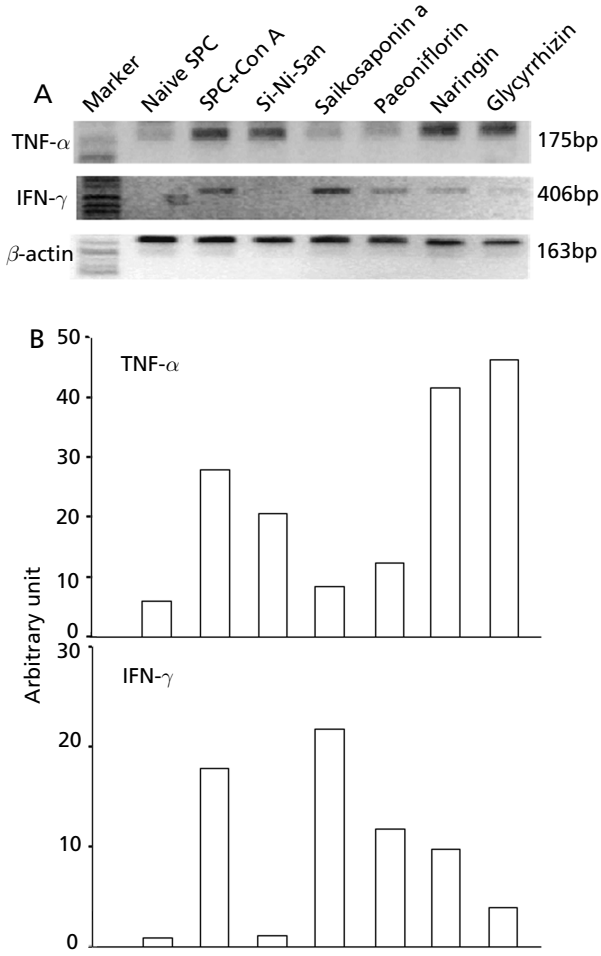

Figure 2 Effects of the major constituents in Si-Ni-San on the TNF-α and IFN-γ mRNA expressions. Spleen cells (SPC, 1×10^7) were incubated with 5 μg mL^{-1} concanavalin A and various concentrations of the drugs for 14 h. The dose of Si-Ni-San was 10^{-4} g mL^{-1} and the other drugs were used at 10^{-5} g mL^{-1}. A. The TNF-α, IFN-γ and β-actin mRNA expressions were examined by RT-PCR. B. The semi-quantification for the results shown in A. Data were representative of three independent experiments.

splenocyte adhesion to type I collagen, while glycyrrhizin only showed a slight inhibition, and naringin did not influence the adhesion.

Effect of the major constituents in Si-Ni-San on the activity of MMP-2 produced by spleen cells from mice with PCl-CS

As shown in Figure 4 (A and B), the spleen cells from PCl-CS mice secreted a higher level of MMP-2 than those from naive mice. In comparison with this, treatment with saikosaponin a or glycyrrhizin in-vitro, rather than paeoniflorin or naringin, moderately inhibited the MMP-2 release. In terms of the combinations of these four major constituents shown in Figure 4 (C and D), the combination of saikosaponin a and naringin, or that of paeoniflorin and glycyrrhizin decreased

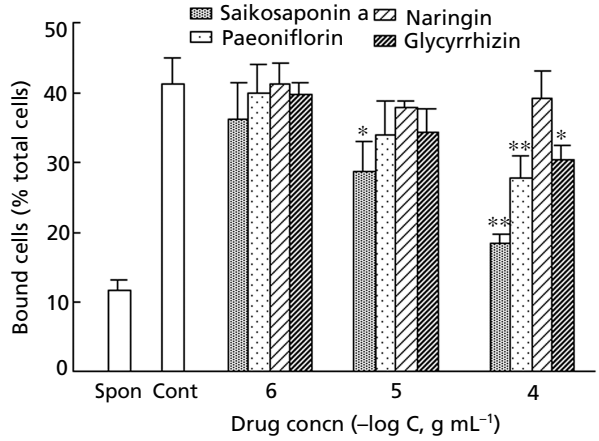

Figure 3 Effect of the major constituents in Si-Ni-San on the adhesion of spleen cells from PCl-sensitized mice to type I collagen. All cells came from PCl-sensitized mice for the adhesion assay. Cont indicates the cells that were only activated with PDBu without any drug pretreatment. Spon indicates cells alone. Data were expressed as mean ± s.d. of three experiments and each included triplicate sets. $^*P < 0.05$, $^{**}P < 0.01$ vs control (Dunnett's test).

the MMP-2 level as significantly as the combination of all the four constituents.

Effect of the major constituents in Si-Ni-San on the serum NO_2^- level of mice with PCl-CS

As shown in Figure 5, mice with PCl-CS secreted an obviously high level of NO_2^- in serum compared with the naive mice. Against this, oral administration with Si-Ni-San and either of its four constituents as well as dexamethasone for six days from the sensitization significantly reduced the production of NO in the serum of the mice undergoing PCl-induced contact sensitivity. Among them, Si-Ni-San as well as saikosaponin a, glycyrrhizin, naringin, and dexamethasone all exhibited an almost complete inhibition.

Discussion

In this study, we first elucidated the effects of the active constituents in Si-Ni-San in ameliorating picryl chloride-induced ear contact sensitivity. As a result, four major constituents in Si-Ni-San (saikosaponin a, paeoniflorin, naringin, glycyrrhizin) and their combinations attenuated the ear sensitivity to different degrees when administered in the induction phase of the DTH reaction. Among the four constituents, saikosaponin a and glycyrrhizin exhibited a much stronger inhibition on the ear swelling than naringin and paeoniflorin. When the constituents were combined in pairs, and especially when all four were combined, the inhibition shown was much stronger compared with the compounds alone (Table 1). Besides, a similar tendency of the inhibitory effect was observed in the model of SRBC-induced DTH reaction in mice (data not shown). Meanwhile, these compounds did not have any obvious toxicity on the bodyweight of the mice and immune

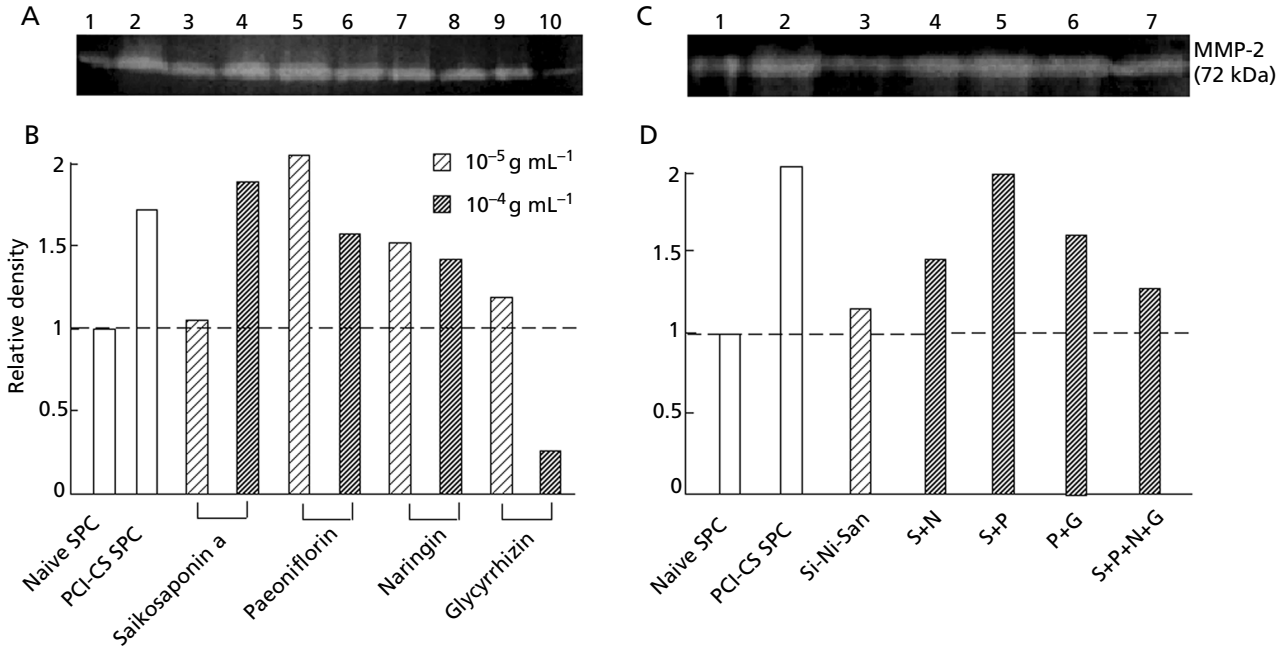

Figure 4 Effect of the major constituents in Si-Ni-San and their combinations on the activity of MMP-2 produced by spleen cells from PCl-CS mice. A and B; lane 1, normal spleen cells; lane 2, spleen cells from PCl-CS mice; lane 3–10, spleen cells from PCl-CS mice treated with indicated drugs. C and D; lane 1, normal spleen cell; lane 2, spleen cells from PCl-CS mice; lane 3–7, spleen cells from PCl-CS mice treated with indicated drugs. S + P, S + N, P + G and S + P + N + G: combinations of saikosaponin a and paeoniflorin, saikosaponin a and naringin, paeoniflorin and glycyrrhizin, the four components, respectively. Each value shown here is the representative of three independent experiments.

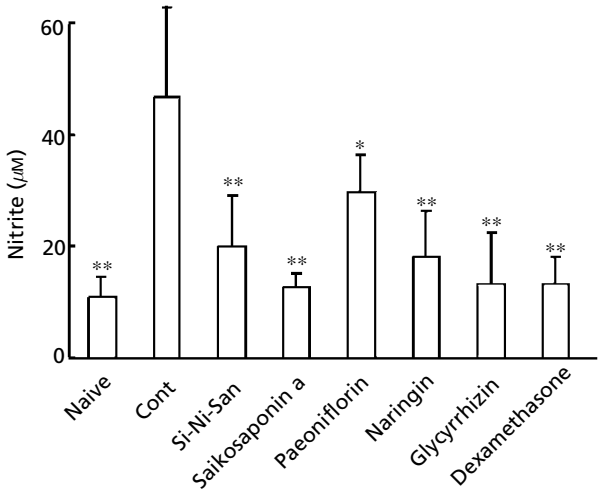

Figure 5 Effect of the major constituents in Si-Ni-San on the serum NO_2^- level of mice with PCl-CS. The serum samples were obtained simultaneously from the model in Table 1. Each datum indicates the mean ± s.d. of eight animals. $^*P < 0.05$, $^{**}P < 0.01$ vs control (Dunnett's test). Cont: spleen cells from mice with PCl-CS.

organs (spleen and thymus), whereas dexamethasone put an intensively harmful effect on them. The results suggested that the four major constituents might be involved in the therapeutic effects of the whole prescription with their own characteristics by influencing different stages during the progress of the DTH reaction. The co-operation activity of the constituents shown above might have also reflected the integrated effect of the whole prescription based on the theory of traditional Chinese medicine. Subsequently, we examined the role of these constituents in the effect of the whole prescription.

Lymphocyte proliferation and cytokines secretion are considered very crucial to the exertion of effector lymphocytes (Jeon et al 2001). To check the role of each of the four constituents in the full prescription, we examined their effects on concanavalin A-induced splenocyte proliferation and sMLR. Saikosaponin a and glycyrrhizin were the most efficient on both concanavalin A-induced splenocyte proliferation and mixed lymphocyte reaction (Figure 1). However, paeoniflorin showed an efficacy to block the lymphocyte proliferation in-vitro in spite of no influence on the PCl-CS model in-vivo. This was perhaps due to the metabolism of paeoniflorin by intestinal bacteria (Hsiu et al 2003; He et al 2004). The pharmacokinetics of paeoniflorin requires research.

During the activation of the effector T lymphocytes, some inflammatory cytokines, such as TNF-α and IFN-γ, are released. We examined the cytokines production of concanavalin A-induced spleen T cells. As our results showed in Figure 2, the reduction varied from different components to different cytokines. The TNF-α expression in mRNA level was significantly reduced by saikosaponin a, while the expression of IFN-γ was markedly decreased by paeoniflorin, naringin and especially glycyrrhizin. A reduced TNF-α gene expression may hint at a diminished activation of endothelial cells by up-regulating markers for extravasations of immune cells to the site of infection (Boldrini et al 2006). However, glycyrrhizin and naringin did not inhibit the TNF-α gene

expression but showed an enhancement. Another observation indicated that saikosaponin a did not influence IFN-γ gene expression while it showed inhibition on TNF-α gene expression and excellent in-vivo activity. Although these results on gene expression should be confirmed in the protein levels, such different patterns obtained here are really quite typical for complex plant extracts containing many pharmacologically active ingredients with overlapping and balanced activities. The findings indicated that each of the constituents in the full prescription influenced the production of different cytokines and finally contributed to the alleviation of the inflammation.

In the progress of DTH reaction, lymphocytes adhere to extracellular matrix, such as collagen, laminin and fibronectin, and then localize to the inflammatory sites in co-operation with adhesion molecules and MMPs (Tarlton et al 2000). As many reports have indicated, MMPs that can degrade extracellular matrix are indispensable for the adhesion of lymphocytes to extracellular matrix (de Fougerolles et al 2000; Yakubenko et al 2000). We subsequently checked the influences of the major constituents on the adhesion of splenocytes to type I collagen as well as on the activity of MMP-2 production. In the adhesion experiment, saikosaponin a, paeoniflorin and glycyrrhizin all reduced the adhesion of splenocytes from PCl-sensitized mice to the pre-coated type I collagen in a dose-dependent fashion (Figure 3). As to the secretion of MMPs, saikosaponin a or glycyrrhizin exhibited a marked reduction in MMP-2 activity (Figure 4). These findings implied that saikosaponin a and glycyrrhizin most likely worked through the inhibition of cellular-matrix adhesion and MMP-2 secretion so as to block the lymphocyte mobilization and localization to the inflammation sites. Here may also exist a modulation between the compounds when combined, with the pairs of P+G or S+P being less inhibitory compared with the single compound of saikosaponin a or glycyrrhizin. This finding suggested that paeoniflorin might have some modulation for the effect of saikosaponin a or glycyrrhizin since paeoniflorin itself did not exert much inhibition on the MMP secretion.

On the other hand, NO as an inflammatory molecule has been well reported to show a modulating role in contact hypersensitivity reaction (Morita et al 1996; Petricevich et al 2000; Verma & Goldin 2003). In this study, pretreatment with the four components significantly reduced the serum NO level of PCl-sensitized mice, whose effects approached those of dexamethasone (Figure 5). This suggested that the reduction in NO production by the constituents might have contributed to the alleviation of the inflammation.

In summary, the anti-inflammatory effect of Si-Ni-San might have been due mostly to saikosaponin a and glycyrrhizin, which mainly inhibited the T lymphocyte activation and proliferation, reduced the release of cytokines and down-regulated the adhesion ability as well as the production of MMP-2 and NO. Our previous findings had clearly clarified that glycyrrhizin was the main contributor to this action of Si-Ni-San by selective depletion (Zhang et al 2005). Since traditional Chinese prescription is the mixture of several medicinal herbs, there is a strong possibility that the chemicals interact to modify the individual pharmacological activity. It was found that other components such as paeoniflorin and naringin showed some activity against the lymphocyte proliferation, adhesion, and cytokine production in the process of the DTH reaction. All these components showed an integrated and synergic effect to the whole prescription. The co-operative effect of the components was confirmed by the reduction of the MMP-2 level after treatment with a combination of the four constituents. Our group has also found the strong effect of Si-Ni-San in treating immunological liver injury and contact sensitivity (Jiang et al 2003; Sun et al 2003). Meanwhile, many extracts from Chinese herbs and their principles have been shown to improve T cell-mediated diseases, such as hepatitis and arthritis, via inhibiting the T cell function, which could strongly underline the medically important characteristics of immunomodulatory drugs (Wu et al 2005; Asano et al 1998; Wang et al 2004). Our results have revealed the potential benefit in elucidating the underlying pharmacological mechanisms of the traditional combination therapy of herbal constituents in Si-Ni-San. Further study on the detailed mechanisms of its therapeutic effect is necessary.

Conclusions

The alleviation effect of Si-Ni-San on contact sensitivity might have been due mostly to saikosaponin a and glycyrrhizin, and co-operatively due to paeoniflorin and naringin. Its underlying mechanisms included the inhibition of T lymphocyte activation and proliferation, the reduction of cytokines release and down-regulation of the adhesion ability as well as the production of MMP-2 and NO.

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