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Suppressive effects of PG201, an ethanol extract from herbs, on collagen-induced arthritis in mice

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Objective. **PG201 has been formulated using 12 herbs known to have anti-inflammatory and protective effects on damaged tissue and bone among other functions. The present study was done in order to assess the therapeutic effects of PG201 in collagen-induced arthritis (CIA) in mice.**

Methods. **DBA/1 mice were immunized with bovine type II collagen. After a second collagen immunization, mice were treated with PG201 orally at 10 mg/kg once a day for 18 days. Paws were evaluated macroscopically for redness, swelling and deformities. The levels of TNF-α and IL-1β in the ankle were examined. The severity of arthritis within the knee joints was evaluated by histological assessment of cartilage destruction and pannus formation. Molecular indicators related to CIA pathology were analysed by measuring the serum levels of matrix metalloproteinase 2 (MMP-2), tissue inhibitor of matrix metalloproteinase 2 (TIMP-2) and the anti-inflammatory cytokines interleukin (IL)-4 and IL-10.**

Results. **Administration of PG201 significantly suppressed the progression of CIA and inhibited the production of TNF-α and IL-1β in the paws. The erosion of cartilage was dramatically reduced in mouse knees after treatment with PG201. In the serum of PG201-treated mice, the level of TIMP-2 and the ratio of TIMP-2 to MMP-2 were significantly elevated, and the level of IL-4, but not of IL-10, was increased.**

Conclusion. **Administration of PG201 has therapeutic effects on CIA. Protection of cartilage was particularly prominent. PG201 is a potential therapy for rheumatoid arthritis.**

KEY WORDS: PG201, Collagen-induced arthritis, Cytokines, MMP-2, TIMP-2.

Rheumatoid arthritis (RA) is a chronic inflammatory disease involving multiple joints. The main pathology of the affected synovial tissue consists of hyperplasia and subintimal infiltration of T and B lymphocytes. Synovial tissue hyperplasia forms the pannus tissue that irreversibly destroys the cartilage and bone in the affected joint. RA progression is associated with elevated levels of tumour necrosis factor α (TNF-α) and interleukin (IL)-1β produced by macrophages and dendritic cells, an imbalance of Th1/Th2 and overproduction of antigen-specific immunoglobulins [1–4]. TNF-α and IL-1β directly induce synthesis of proteolytic enzyme such as matrix metalloproteinases (MMPs), which can break down the extracellular matrix macromolecules. Under

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normal conditions, tissue inhibitors of metalloproteinases (TIMPs) normally bind to MMPs in the ratio 1:1. Imbalance of the ratio of TIMPs to MMPs, which is generally caused by up-regulation of MMPs, results in continued matrix destruction in RA [5, 6].

Most of the current treatments are directed to correction of the immune aberration that supposedly drives synovial cell proliferation and cartilage erosion. Glucocorticoids and non-steroidal anti-inflammatory drugs (NSAIDs) are relatively old and conservative treatments for RA. Disease-modifying anti-rheumatic drugs (DMARDs) have been developed. For example, methotrexate became a benchmark agent with efficacy and tolerability in the early phase of RA [7]. The recently approved drug leflunomide, an inhibitor of dihydroorotate dehydrogenase, affects lymphocyte function *in vivo* and *in vitro*, although its specific mechanism of action in RA is not known [8–10]. The first group of biological response modifiers (BRMs) approved for treatment of RA were the antagonists of TNF-α. They work by binding to the TNF-α receptor or binding directly to the TNF-α protein [11]. The use of DMARDs has been impeded by their potential for long-term side-effects and toxicity [12], while BRM therapy, despite substantial efficacy and clinical improvement, entails high cost and hypersensitivity to medications and infections due to TNF-α blockage [13, 14]. The action of all these drugs is based on suppression of the inflammatory reaction; to our knowledge no drugs have been developed for cartilage protection.

PG201 is an ethanol extract from 12 herbs and its safety is recognized. The formulation has been developed on the basis of the known functions of each herb, as described in the literature of traditional Chinese and Korean medicine [15–17]. *Chaenomelis Fructus, Achyranthis Radix*, *Carthami Flos*, *Cnidii Rhizoma* and *Cinnamomi Cortex* are known for their functions in maintaining or assisting blood circulation. *Angelica Radix* and *Gastrodiae Rhizoma* were chosen for their activities in 'tonifying' the blood. *Ledebouriellae Radix* is reported to catalyse the function of other reagents. *Gentianae Macrophylla Radix* and *Acanthopanacis Cortex* are reported to assist in the functions of both muscle and bone, while *Clematidis Radix* and *Phlomidis Radix* have been used for protection against degeneration of cartilage and regeneration of damaged tissue [15–17]. All raw materials used in the preparation of PG201 are listed in the Korean Pharmacopoeia or the Korean Herbal Pharmacopoeia, and PG201 has been used by a selective group of oriental medicine physicians in Korea. Here, we show that PG201 (i) inhibited the development of CIA in mice, (ii) lowered the level of proinflammatory cytokines, such as TNF-α and IL-1β, in the paws of mice with CIA, (iii) increased the concentration of the anti-inflammatory cytokine IL-4 in the serum, and (iv) had dramatic protective effects against cartilage destruction in the affected knee joint, presumably by affecting the levels of matrix metalloproteinase (MMP) and tissue inhibitor of matrix metalloproteinase (TIMP). PG201 is an outstanding candidate for use in general therapeutics and for use as a cartilage-protective medicine for use in RA.

Materials and methods

Preparation of PG201

All herbs used were purchased from a market specializing in herbs (Kyungdong herb market, Seoul, Korea). The herbs had a moisture content of $<10\%$ by weight, and were air-dried. The herbs used were as follows: *Chaenomelis speciosa* Nakai (*Chaenomelis Fructus*, 8 g), *Achyranthes bibentata* Blume (*Achyranthis Radix*, 8 g), *Angelica sinensis* Oliv. (*Angelica Radix*, 5 g), *Cnidium officinale* Makino (*Cnidii Rhizoma*, 5 g), *Gastrodia elata* Blume (*Gastrodiae Rhizoma*, 5 g), *Acanthopanax senticosus* Maxim. (*Acanthopanacis Cortex*, 8 g), *Carthamus tinctorius* L. (*Carthami Flos*, 5 g), *Cinnamomum aromaticum* Nees (*Cinnamomi Cortex*, 8 g), *Gentiana macrophylla* Pall. (*Gentianae Macrophyllae Radix*, 5 g), *Ledebouriella seseloides* Wolff (*Ledebouriellae Radix*, 4 g), *Clematis chinensis* Retz. (*Clematidis Radix*, 5 g), and *Phlomis umbrosa* Turczaninow (*Phlomidis Radix*, 4 g) [18, 19]. The herbs (totalling 70 g dry weight) were mixed, minced with a grinder (Rong Tong Iron Works, Taichung, Taiwan), and extracted by storing in 1 litre of 25% ethanol in water for 3 months at 4°C. The supernatant was filtered with 10 μm cartridge paper and ethanol was removed by rotary evaporation (Eyela, Tokyo, Japan), and concentrated extracts were freeze-dried. This process generally produced 10 g of brown powder.

MTT assays

HT1080 and U937 cells were seeded at 3000 and 5000 cells per well respectively in a 96-well plate. Cells were exposed to PG201 at concentrations in the range 5–5000 μg/ml at 37°C under an atmosphere containing 5% CO_2. After 96 h of incubation with PG201, viable cells were stained with MTT (5 mg/ml; Sigma, St Louis, MO, USA) for 30 min. The medium was then removed and the formazan crystals produced were dissolved by the addition of 200 μl dimethylsulphoxide. Absorbance was determined at 540 nm using an ELISA (enzyme-linked immunosorbent assay) microplate reader. The median inhibitory concentration (IC_{50}) was defined as the drug concentration that resulted in a 50% reduction in cell number compared with untreated controls. These values were derived from semi-log plots percent viability (% absorbance of treated sample/absorbance of untreated control $\times$ 100) *vs* drug concentration.

Acute oral toxicity study

To evaluate the acute toxicity of PG201 after a single oral dose, 10 male and 10 female Sprague–Dawley rats were assigned randomly to two experimental groups (five rats of each sex in each group) and were treated by gavage at doses of 0 and 8.0 g/kg body weight. Mortality, clinical signs, body weight changes and gross findings were monitored during the 14 days after treatment. This study was carried out in compliance with the Testing Guidelines for Safety Evaluation of Drugs (Notification No. 1999-61) issued by the Korea Food and Drug Administration on 22 December 1999, the Good Laboratory Practice Regulations for Non-clinical Laboratory Studies (Notification No. 2000-63) issued by the Korea Food and Drug Administration on 11 December 2000, and the Principles

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of Good Laboratory Practice issued by the Organization for Economic Cooperation and Development (1997).

Induction of CIA and PG201 treatment

Inbred male DBA/1 mice (Jackson Laboratory, Bar Harbor, ME, USA), aged 9–10 weeks at the start of the experiments, were immunized intradermally at the base of the tail with 100 μg bovine type II collagen (Chondrex, Redmond, WA, USA) emulsified in Freund's complete adjuvant (Gibco BRL, Grand Island, NY, USA). On day 21, all mice were boosted with an intradermal injection of 100 μg type II collagen. The next day, mice that had no macroscopic signs of arthritis were selected and divided into two groups, which each contained 10 mice. The control group were treated orally with 100 μl distilled water and the PG201-treated group were treated orally with 100 μl PG201 at the concentration of 2 mg/ml for 18 days. The gradual onset of arthritis normally starts approximately 4 weeks after initial immunization. The progression of CIA was evaluated by macroscopic scoring of the paws every 3 days and histological analysis of the knees on day 18.

Macroscopic scoring of CIA

Erythema and swelling of the paws were scored on a scale of 0–4, with a maximum score of 4 for each paw, as described previously [20]. Arthritis was considered to be present if the score was >2. Two independent observers, without prior knowledge of the experimental groups, performed the scoring.

Histological processing and analysis of knee joints

Mice were killed by cervical dislocation. Thereafter, knee joints were dissected, fixed in 10% phosphate-buffered formalin for 2 days, decalcified in 10% EDTA (ethylene diamine tetraacetate) for 7 days, then embedded in paraffin. Standard frontal sections of 7 μm were prepared and stained with either haematoxylin–eosin or safranin O–fast green. Histopathological changes were scored using the following method, as described previously [21]. Cartilage depletion was indicated visually by diminished safranin O staining of proteoglycan matrix, and was scored arbitrarily as 0 when normal or 1–3 according to the degree of depletion (loss of staining). A characteristic feature of CIA is the progressive loss of articular cartilage. The destruction was graded separately on a scale of 0–3, ranging from fully stained cartilage to destained cartilage or complete loss of articular cartilage. Pannus formation was scored arbitrarily as 0 when no pannus formed in the joint space or as 1–2 according to the degree of pannus formation. All these histological evaluation procedures were performed blind.

Measurement of cytokine levels in mouse ankles and serum

TNF-α, IL-1β, IL-4, MMP and TIMP were measured using commercially available ELISAs for TNF-α, IL-1β, IL-4 (R & D Systems, Minneapolis, MN, USA), MMP-2 and TIMP-2 (Biotrak, Buckinghamshire UK) according to the manufacturer's recommendations. Briefly, for TNF-α and IL-1β, mice tarsi were snap-frozen in liquid nitrogen and ground into powder with a pestle, then lysed with lysis buffer (25 mM Tris–HCl, 50 mM NaCl, 0.5% sodium deoxycholate, 2% NP-40, 0.2% sodium dodecyl sulphate, 1 mM phenyl methyl sulphonyl fluoride). The tissue lysates were used to measure the level of cytokines. For MMP-2, TIMP-2 and IL-4, mice were killed on the final day of experimentation and serum then was drawn to measure their levels. The levels of all these proteins were normalized to the total amount of cellular protein in prepared tissue lysates as measured by the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Data are expressed as mean $\pm$ S.E.M. Differences in protein levels were compared by means of Student's *t*-test. Data on the incidence of arthritis were evaluated with the χ^2 test. Data on the progression of arthritis in the paws and the test of severity of pannus formation and cartilage erosion were evaluated with Student's *t*-test or the Wilcoxon rank sum test. *P* values <0.05 were considered significant.

Results

Toxicity of PG201

To examine the cytotoxicity of PG201 in the *in vitro* cell culture system, the MTT assay was performed. The results demonstrated that the concentration of PG201 required to inhibit growth by 50% (IC_{50}) after 96 h was 332.01 and 664.05 μg/ml for HT1080 and U937 cells, respectively.

To evaluate the acute oral toxicity of PG201, we determined its single-dose toxicity in both sexes of rats at the dose of 8.0 g/kg body weight. This dose had no effect on mortality, clinical signs, body weight changes and gross findings in either sex. The results suggest that the lethal dose of PG201 is higher than 8.0 g/kg in rats of both sexes.

Inhibition of the progression of arthritis by PG201

CIA in mice is an autoimmune type of arthritis which displays many characteristics in common with human RA [22, 23]. The onset of arthritis in DBA/1 mice occurs approximately 4 weeks after initial immunization with type II collagen. We first determined a therapeutically optimal concentration of PG201 by measuring the incidence of disease and the arthritis index by macroscopic examination of joint swelling and erythema at intervals of 3 days. Mice were treated orally with four concentrations of PG201, ranging from 5 to 300 mg/kg body weight. The incidence of arthritis was significantly less in PG201-treated animals, and was lowest in animals receiving 10 mg/kg PG201 (Fig. 1A). Consistent with this result, the arthritis index was also lowest at this dose (Fig. 1B). On the basis of this result, all other experiments were performed using PG201 at 10 mg/kg.

The time course of the disease status of animals is shown in Fig. 2A. When animals were treated with 10 mg/kg of PG201 daily, the progression of arthritis was dramatically inhibited in mice treated with PG201 compared with control mice treated with vehicle (distilled water). The increase in paw thickness was significantly less in mice treated with PG201 than in control mice (Fig. 2B). These data showed that administration of PG201 could suppress the course of collagen-induced arthritis in mice.

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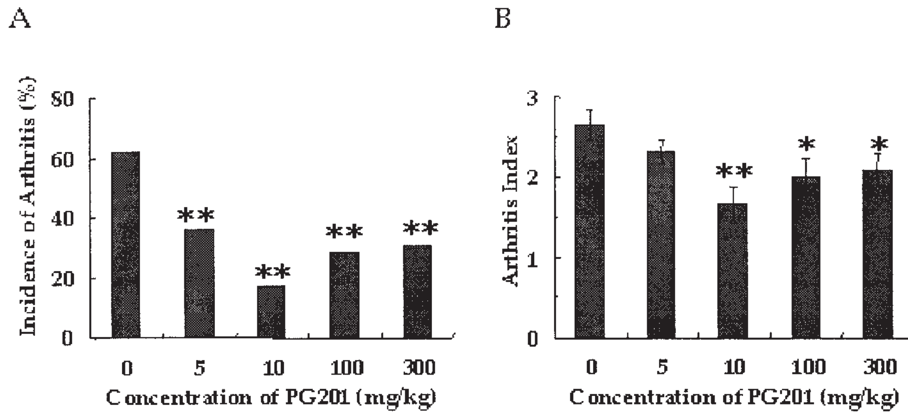

FIG. 1. Dose–response experiment on the treatment of arthritis with PG201 in CIA mice. From the day after the booster immunization, PG201 was given orally once a day. Inhibition of arthritis (A) and the arthritis index (B) were measured on day 21 after PG201 treatment. Data are mean ± S.E.M. (10 mice in each group). $^*P < 0.05$; $^{**}P < 0.01$ *vs* control (dose = 0 mg/kg) (χ^2 test in A and Wilcoxon rank sum test in B).

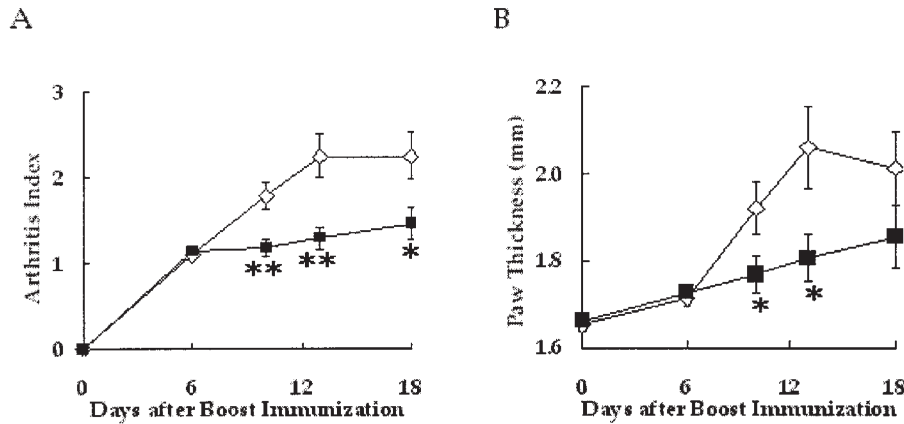

FIG. 2. Time course of the suppressive effect of PG201 on arthritis in CIA mice. From the day after the booster immunization, PG201(filled squares) and control vehicle (distilled water, open diamonds) were given orally once a day. The arthritis index (A) and paw thickness (B) were measured at 3-day intervals. Data are mean ± S.E.M. (10 mice in each group). $^*P < 0.05$; $^{**}P < 0.01$ *vs* control (Wilcoxon rank sum test in A and Student's *t*-test test in B).

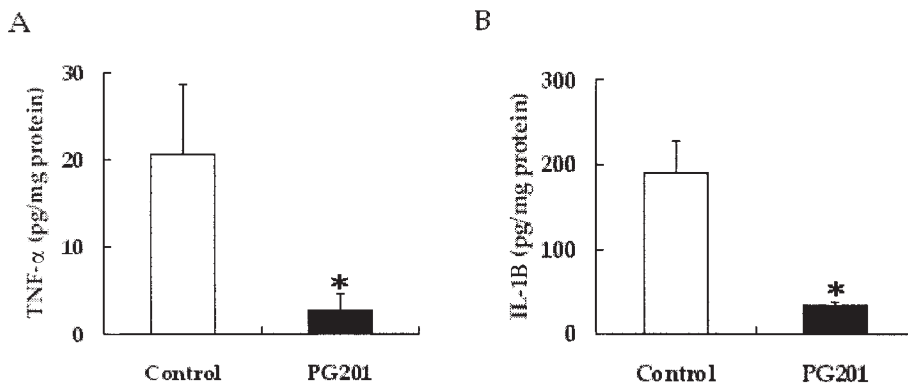

FIG. 3. Effects of PG201 on levels of inflammatory cytokines in ankle joints. Ankle joint extracts were prepared from joint tissues and analysed for TNF-α (A) and IL-1β (B). Data for TNF-α and IL-1β are mean ± S.E.M. of 20 joints. $^*P < 0.05$ (Students' *t*-test).

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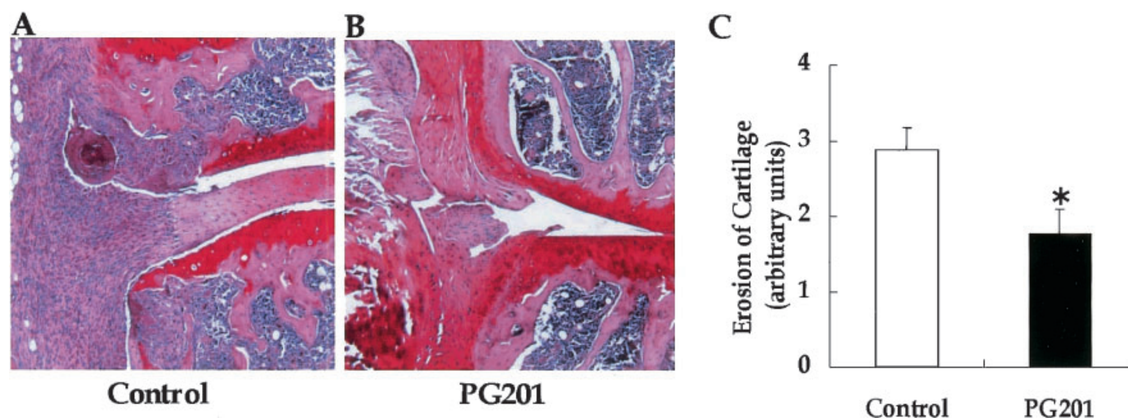

FIG. 4. Effects of PG201 on cartilage erosion in CIA. Safranin O staining of knee joint tissues from control mice (A) and PG201-treated mice (B). Data represent 20 samples for each group. Erosion of cartilage was markedly inhibited in the knees of mice treated with PG201 (C). Original magnification, ×100. $*P < 0.05$ *vs* control (Wilcoxon rank sum test).

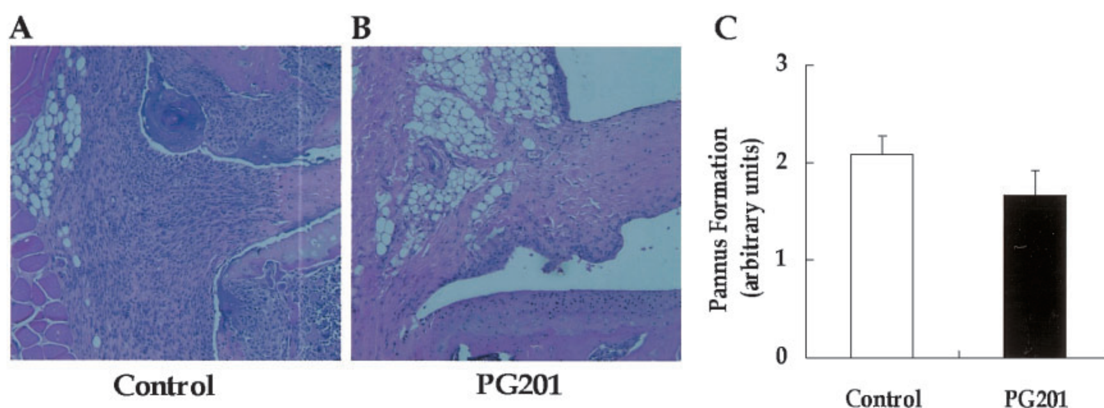

FIG. 5. Effects of PG201 on pannus formation in CIA. Haematoxylin–eosin staining of knee joint tissues from control mice (A) and mice treated with PG201 (B). Data represent 20 samples for each group. The grade of pannus formation was markedly lower in the knees of mice treated with PG201 (C). Original magnification, ×100.

Effects of PG201 on levels of TNF-α and IL-1β in the paws

TNF-α and IL-1β are potent proinflammatory proteins and are important in the pathogenesis of RA [24, 25]. TNF-α and IL-1β are known to be present in large quantities in affected synovial fluid. Effects of PG201 on the levels of TNF-α and IL-1β in the paws were examined. The paws were excised and total cellular proteins were extracted. The levels of the two cytokines were measured after standardization of the protein concentration. Consistent with the joint swelling result, marked decreases in the levels of TNF-α and IL-1β were observed in the paws of mice treated with PG201 compared with the control mice ($P < 0.05$) (Fig. 3A and B), whereas the levels of TNF-α and IL-1β were very low in normal synovial fluid (data not shown). These results suggest that PG201 inhibits the production of proinflammatory cytokines in the affected paws of CIA mice.

Inhibition of cartilage destruction

Effects of PG201 were also examined by histological examination in synovial tissues. Safranin O staining of proteoglycan in the cartilage showed that the proteoglycan was well preserved in joints treated with PG201 but not in joints treated with control vehicle (Fig. 4A and B). A statistically significant difference (46% inhibition) in the severity of cartilage erosion was found between the PG201-treated group and the control group (Fig. 4C). Sections stained with haematoxylin and eosin showed that pannus in the knee joint was decreased in mice treated with PG201 compared with those treated with control vehicle. Thinning and hyalinization of the cartilage were also inhibited (Fig. 5A and B). Comparison of the histological grades of pannus formation between the experimental group and the control group showed that the difference was about 20%, representing mild inhibition of pannus formation (Fig. 5C). These results indicate that PG201 might have suppressive

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effects on CIA through cartilage protection rather than inhibition of pannus formation.

Effects on regulation of MMP and TIMP by PG201

Matrix metalloproteinases (MMPs) degrade the tissue component in the pathological stage of RA, whereas tissue inhibitors of MMPs (TIMPs) regulate the activity of MMPs by forming complexes with MMPs [26, 27]. It has been reported that an imbalance in the ratio of MMPs to TIMPs in favour of MMPs promotes proteolysis. To evaluate whether PG201 administration affects the activities of the proteolytic enzymes related to cartilage erosion, we determined the serum levels of MMP-2 and TIMP-2, and calculated the ratio of TIMP-2 to MMP-2. The serum level of MMP-2 in PG201-treated mice was not significantly different from that in control mice, whereas the level of TIMP-2 was considerably higher in the experimental group than in the control mice (Fig. 6A). The TIMP-2/MMP-2 ratio was also compared. Consistent with the results on cartilage protection, a marked increase in the ratio of TIMP-2 to MMP-2 was observed in the serum of PG201-treated mice (Fig. 6B). These data suggest that administration of PG201 might protect against cartilage erosion by regulating the proteolytic enzymes.

Effects of PG201 on IL-4 and IL-10

It has been reported that systematic treatment with IL-4 ameliorates disease progression and protects against cartilage destruction [28]. IL-10 has also been reported to exert a protective effect in CIA at high doses, although its precise role remains controversial [29–31]. Levels of IL-4 and IL-10 in serum were compared between the experimental and control groups. The serum level of IL-4 in PG201-treated mice was significantly higher than that in the control group (Fig. 7A), whereas there was no significant difference in the level of IL-10 (Fig. 7B). This suggests that PG201 might have cartilage protection effects in the CIA model by regulating anti-inflammatory cytokines such as IL-4.

Discussion

PG201 is an ethanol extract developed to have therapeutic effects in inflammatory diseases involving cartilage destruction, such as RA. According to published

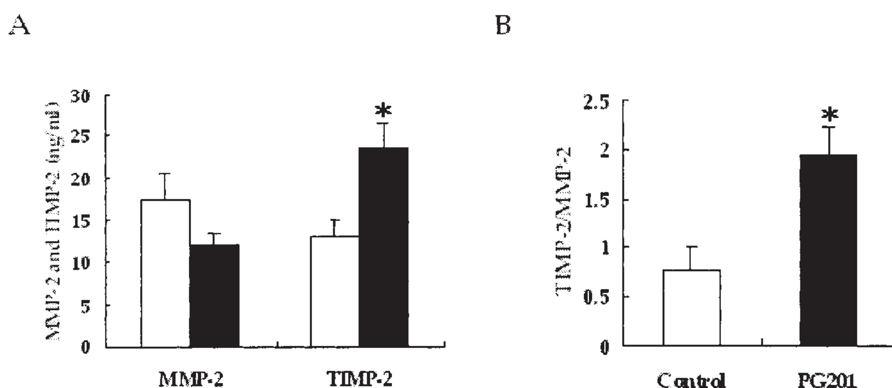

FIG. 6. Effects of PG201 on serum concentrations of MMP-2 and TIMP-2. Concentrations (A) were determined by ELISA in the control (open columns) and PG201-treated (filled columns) groups. The ratio of TIMP-2 to MMP-2 was calculated for each mouse and the average ratio for each group was calculated (B). Data are mean ± S.E.M. of 10 mice. *$P < 0.05$ *vs* control (Students' *t*-test).

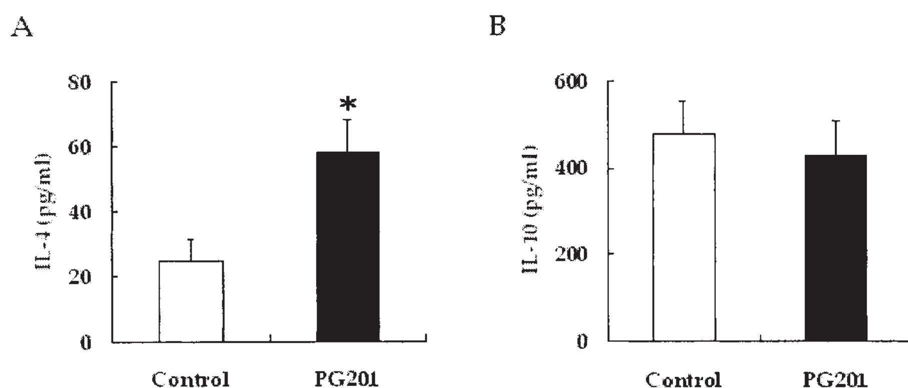

FIG. 7. Effects of PG201 on serum levels of IL-4 and IL-10. Sera were obtained on the last day of the experiments and diluted appropriately; levels of IL-4 (A) and IL-10 (B) were determined with ELISAs. Data are mean ± S.E.M. of 10 mice. *$P < 0.05$ *vs* control (Students' *t*-test).

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work that is well accepted by the traditional oriental medicine community, PG201 was formulated to facilitate blood circulation as well as to reduce anti-inflammatory activity. The herbs used in the preparation of PG201 have been used for hundreds of years in this oriental region, and their safety and efficacy are well established through a long history of human use, but their use still lacks scientific support [17, 32].

We demonstrated the marked effectiveness of oral administration of PG201 in protecting CIA mice against joint destruction. Histological analysis revealed pronounced protection against cartilage and bone erosion. This protective effect of PG201 appears to result from its control of key components in RA pathogenesis, including the down-regulation of TNF-α and IL-1β and the up-regulation of TIMPs and IL-4. TNF-α plays a major role in the inflammatory process, while IL-1β is involved in the destruction of cartilage matrix [33]. MMPs are critical in late, irreversible cartilage damage, which is usually associated with bone erosion [25, 34]. Our data suggest that PG201 reduces collagenase activity, either directly or indirectly by up-regulation of TIMPs, resulting in a ratio of MMPs to TIMPs that favours the rebuilding of cartilage. Therefore, PG201 is an important negative regulator of both inflammatory and destructive proteins related to RA.

According to our results, PG201 also appears to act as positive regulator of IL-4. IL-4 and IL-10 have been thought to be upstream regulators that control the progression of RA negatively [35]. IL-4 was not detected in the synovium of patients with RA [36], while IL-10 was reported to be produced in substantial amounts in the RA joint [30–36]. This lack of IL-4 may contribute to the uneven balance between destructive and regulatory mediators in the synovium of RA. IL-4 appears to be involved in the CIA model, especially in the protection of cartilage and bones [37]. Consistent with these findings, IL-4 has been shown to enhance the synthesis of type I procollagen and the production of TIMP in human mononuclear phagocytes and cartilage explants, but to suppress metalloproteinase production [27]. In the serum of mice treated with PG201, the level of IL-4 was maintained consistently at a level two-fold higher than that in control mice, which may lead to the protection of cartilage. Therefore, our data suggest that the cartilage-protective effects of PG201 might result from the up-regulation of IL-4.

It is well known that drugs used currently to treat RA have limitations in that they have many undesired effects, and there is therefore a need for improved treatments. The major concerns with glucocorticoids, NSAIDs and DMARDs are poor efficacy, delayed onset of action and toxic effects. Most importantly, many RA patients, despite the use of these agents, continue to show progression of irreversible joint destruction. In the case of the recently developed recombinant proteins that control TNF-α, which can be given only via the parenteral route, frequent and costly injections are needed because of their relatively short half-lives [14]. Our results indicate that PG201 has great potential as an alternative to these treatments, and has no adverse effects. PG201 can be given orally, and it inhibits disease progression by both controlling inflammatory proteins and protecting cartilage. Its cost is also estimated to be substantially lower than that of recombinant proteins. The data presented in this study show that PG201 warrants further investigation, including preclinical and clinical studies.

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