Cell Wall β-Glucan Derived from Candida albicans Acts as a Trigger for Autoimmune Arthritis in SKG Mice

Shunsuke Hida, Noriko N. Miura, Yoshiyuki Adachi, and Naohito Ohno∗

Laboratory for Immunopharmacology of Microbial Products, School of Pharmacy, Tokyo University of Pharmacy and Life Science; 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan.

Received January 12, 2007; accepted June 4, 2007; published online June 5, 2007

SKG mice are a recently established experimental model for rheumatoid arthritis (RA). Although they spontaneously develop chronic autoimmune arthritis under conventional conditions, SKG mice failed to develop chronic arthritis in a strictly controlled specific pathogen-free (SPF) environment. Beta-glucan (BG) from Lammaricia digitata, laminarin (LAM), induced arthritis under SPF conditions, thus BG would be a pathogenic factor for arthritis in SKG mice. Therefore, we prepared BG from Candida albicans, a pathogenic fungus and investigated whether BG from C. albicans induced arthritis in SKG mice under SPF conditions. SKG mice were injected intraperitoneally with particulate BG (oxidative-Candida albicans (OX-CA)), soluble BG (Candida soluble beta-glucan (CSBG)) from C. albicans and LAM as a positive control. In addition, schizophyllan (SPG) from Schizophyllum commune or Mycobacterium whole cells were injected into SKG mice to induce arthritis. Mice injected with OX-CA, CSBG and SPG had more severe arthritis than with LAM, and whole Mycobacterium cells. IL-6 concentration in sera from SKG mice injected with OX-CA or CSBG was high, whereas not detected in sera from mice treated with LAM. In histological analysis, infiltration of inflammatory cells was observed in SKG mice injected with BG. These results suggest that fungal infection may be a factor to induce and exacerbate autoimmune diseases such as RA.

Key words β-glucan; arthritis; SKG mice; Candida albicans; rheumatoid arthritis

Rheumatoid arthritis (RA) is one of the most common systemic autoimmune diseases. It is characterized by chronic joint inflammation, the formation of a rheumatoid pannus and eventually, tissue degradation and joint destruction.1,2 Although there are newer and effective therapeutic approaches, the etiology of RA is unknown.

Animal models of RA are used to investigate the pathogenesis and to develop therapeutic drugs. For example, collagen-induced arthritis (CIA) is an animal model of RA.2,3 CIA is induced by inoculation with type II collagen (CII) emulsified with Freund’s complete adjuvant (FCA) consisting of mineral oil (Freund’s incomplete adjuvant) and heat-killed Mycobacterium, followed by a booster injection.4 In the classical CIA model, FCA is essential for the induction of arthritis in mice. These results suggested that the activation of immune systems by Mycobacterium components is necessary for induction of autoimmune diseases. Recently, we reported that particles of β-glucan (BG) derived from Candida albicans acted as an adjuvant for CIA.5 We used a BG prepared from C. albicans by oxidation with sodium hypochlorite (NaClO), oxidative-Candida albicans (OX-CA).5 The results suggested that not only bacterial components in FCA but also BG derived from C. albicans as an adjuvant for the induction of CIA.

SKG mice also spontaneously develop chronic autoimmune arthritis as a consequence of a mutation of the gene encoding an SH2 domain of ZAP-70, a key signal transduction molecule for T-cells.6 SKG mice develop arthritis at about 2 months of age under conventional conditions. The symptoms of SKG such as bilateral swelling of finger, wrist and ankle joints, and the production of various autoantibodies, resemble human RA; however, in a strictly controlled specific pathogen-free (SPF) environment, SKG mice failed to develop chronic arthritis.7,8 These results suggested that infection from environmental agents, especially fungal infections, is a trigger for the induction of arthritis in SKG. In fact, SKG mice injected with BG such as laminarin (LAM) derived from seaweed Laminaria digitata and curdlan derived from the bacterium Alcaligenes faecalis, developed arthritis under SPF conditions, but not LPS or CpG derived from bacteria or viruses; therefore, a combination of genetic and environmental factors such as fungi may evoke autoimmune arthritis in SKG mice.

Although LAM and curdlan were used to induce arthritis in SKG mice, they were not derived from fungi; furthermore, LAM is polydispersed with a degree of structural heterogeneity.8 It was reported that TNF-alpha production was induced from human monocytes with BG-oligomer prepared from LAM but not original LAM.9 As the structure and biological activity of LAM remain unclear, it is not suitable to use LAM as a pure BG associated with fungal infection.

C. albicans is the most frequently isolated opportunistic fungal pathogen and is reported to have a role in the pathogenesis of arthritis.4,10,11 In the present study, we examined whether BGs derived from C. albicans induced arthritis in SKG mice under SPF conditions.

MATERIALS AND METHODS

Animals SKG mice (5—7 weeks old) were purchased from Clea Japan (Tokyo, Japan), and housed in a specific pathogen-free facility.

Materials Candida albicans IFO 1385 purchased from the Institute of Fermentation (Osaka, Japan), was maintained on potato dextrose agar (Difco, Michigan, U.S.A.) at 27 °C and transferred once every three months. 10% Formaldehyde neutral buffer was purchased from Nacalai Tesque (Kyoto, Japan). Laminarin (LAM) from Sigma Chemical Co. (Tokyo, Japan). LAM was dissolved in PBS at 50 mg/ml before injection. Schizophyllan (SPG) from Kaken Pharm. Co. (Tokyo,

* To whom correspondence should be addressed.  e-mail: ohnonao@ps.toyaku.ac.jp  © 2007 Pharmaceutical Society of Japan

**Preparation of *C. albicans* Beta-Glucan**  
Particles of BG, OX-CA were prepared from *C. albicans* as described previously.1,2 Briefly, OX-CA (5 mg) was suspended in 1 ml of saline and sonicated for 30 s. The supernatant was removed and OX-CA was resuspended in saline. The suspension was kept in a refrigerator prior to use. To prepare of Candida soluble BG (CSBG), OX-CA suspended in DMSO was ultrasonically disrupted and the resulting supernatant was designated CSBG.12 CSBG was resolublized in 0.2 N NaOH and neutralized with 0.2 N HCl. A solution of CSBG was prepared at 5 mg/ml prior to use.

**Induction of Arthritis by Administration of BG to SKG Mice**  
Male or female SKG mice were injected with OX-CA, CSBG and SPG at 3 mg/mouse intraperitoneally. LAM was injected as a positive control at 30 mg/mouse.7 PBS was used as a negative control. Cells of *Mycobacterium tuberculosis* H37 Ra were suspended in PBS and injected into female SKG mice as material derived from gram-positive bacterium. Joint swelling was monitored by inspection and scored as mentioned below.

**Evaluation of Arthritis**  
To evaluate the severity of arthritis, animals were assessed for redness and swelling of the limbs and allocated a clinical score weekly, for up to 10 weeks. The scoring system was based on that described previously1 as follows: 0 = no joint swelling, 1 = swelling of one finger joint, 0.5 = mild swelling of wrist or ankle, and 1 = more severe swelling of wrist or ankle. Scores for all fingers and toes, wrist, and ankles were totaled for each mouse.

**Histology**  
Mice were killed at 12 weeks after the first administration of BG. Control mice injected with PBS were killed similarly. Their paws were amputated, fixed in 10% neutral formalin and decalcified. The tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

**Measurement of IL-6 in Serum from SKG Mice**  
Mice were killed at 12 weeks after the first administration of BG and sera were collected for measurement of IL-6. IL-6 concentration was measured immunochemically using following reagents: capture antibody anti-mouse IL-6 monoclonal antibody (BD Pharmingen, New Jersey, U.S.A.), recombinant mouse IL-6 (BD Pharmingen), biotinylated rat anti-mouse IL-6 monoclonal antibody (BD Pharmingen), avidin-horse-radish peroxidase conjugate (BD Pharmingen), and a TMB (Tetramethyl benzidine) substrate system.

**Statistics**  
Two-way repeated measures ANOVA was used to evaluate comparisons of the arthritis scores. The Mann–Whitney *U* test was applied to evaluate comparisons of IL-6 concentration. *p*<0.05, *p*<0.01 and *p*<0.001 was considered significant in all analyses.

**RESULTS AND DISCUSSION**

**β-Glucan Derived form *Candida albicans*, Induced Severe Arthritis in SKG Mice**  
It is known that the administration of β-glucan (BG) such as laminarin (LAM) and curdlan trigger chronic arthritis in SKG mice.3 Although BG is found in various species, it is mainly a component of the cell wall of fungi. *Candida albicans* is a fungus distributed through out the environment and provokes opportunistic mycosis in a compromised host. It seems that humans, mice and so on are exposed to *C. albicans* in daily life and their immune systems may be stimulated with components derived from *C. albicans*. Therefore, to assess whether BG derived from pathogenic fungi such as *C. albicans* induces arthritis in SKG mice, we administered particulate BG (OX-CA) or soluble BG (CSBG) prepared from *C. albicans*.2,5 Male or female SKG mice were administered intraperitoneally with OX-CA (3 mg), CSBG (3 mg) and LAM (30 mg), and then the arthritis score was followed for 10 weeks after administration (Figs. 1A, B). SKG mice injected with PBS as a control did not develop arthritis in this experimental condition.

The administration of LAM, OX-CA or CSBG induced chronic arthritis in both genders of SKG mice at 4—5 weeks after administration (Figs. 1A, B). SKG mice injected with BG derived from *C. albicans* developed more severe arthritis and earlier than with LAM in both genders. Some mice did not develop arthritis with LAM treatment in spite of high dose-injection. Arthritis in female SKG injected with OXCA or CSBG was more serious than that in male mice, for example, not only wrists but also all fingers were swollen. We previously studied the relationship between the structure and activity of various BG.15 OX-CA and CSBG showed various biological activities, such as IL-6 synthesis of macrophages *in vitro*, anti-tumor effect, TNF-alpha production from RAW264.7 cells and adjuvant effect for the induction of CIA.4,12,16 whereas LAM hardly showed those activities. The difference in biological activities induced with BG might be related to the development of arthritis in SKG.

**Infection with *C. albicans* induces various immune reactions such as cytokine production.**14 There was a report that inoculation of *C. albicans* exacerbated inflammation with cytokine production such as TNF-alpha and IFN-gamma in CIA.11 Recently, we have demonstrated that BG from the cell wall of *C. albicans* acts as an adjuvant to induce CIA.4 These results suggested that *C. albicans* is a candidate for the
induction of inflammation in RA patients.

Next, we examined the ability of BG other than that derived from *C. albicans* to induce arthritis. Schizophyllan (SPG) from *Schizophyllum commune* is a fungal BG used clinically in Japan for cervical cancer patients in combination with irradiation to enhance the immunological surveillance system; thus, we injected SPG intraperitoneally into SKG mice. As a result, SPG also induced arthritis in SKG mice, and the arthritis induced by SPG was more severe and high incidence than that by LAM (Figs. 2A, B). A previous report recommended the use of LAM for the induction of arthritis in SKG mice. According to that report, we injected LAM at 30 mg into SKG mice in this study. However, some mice did not induce arthritis (Fig. 1). On the other hand, BG prepared from *C. albicans* induced arthritis with high incidence and more severe symptoms. Furthermore, although SPG is derived from *Schizophyllum commune*, not from pathogenic fungi, the injection of SPG induced arthritis as well as BG from *C. albicans* (Fig. 2). It seems that a high incidence, reproducibility, convenient procedure and period to onset of disease are important for the development of animal models. Therefore, OX-CA and CSBG used in this study are useful tools as BG for the induction of arthritis in SKG mice.

Macroscopically, joint swelling was observed at the wrist and ankle joints and then progressed to other joints, such as the fingers, and so on. In SKG mice injected with BG from *C. albicans*, swelling of wrist joints was more serious (Figs. 1E, F) than with LAM (Fig. 1D). Some SKG mice developed severe swelling of all paws by injection with OX-CA and CSBG.

Swollen joints in SKG mice treated with BG were investigated by histological analysis (Fig. 3). There was destruction of the articular cartilage and bone in mice injected with OX-CA, CSBG and LAM (Figs. 3B—D). Infiltration of neutrophils was observed in SKG mice treated with BG from *C. albicans* (Fig. 3E).

Next, we examined IL-6 concentration in serum from mice with arthritis. IL-6 is known as a major inflammatory cytokine and plays an important role for pathogenesis of RA and in RA model animals. It is reported that genetic deficiency in IL-6 completely suppressed the development of arthritis in SKG mice. Sera were collected from SKG mice after 12 weeks at injection of BG and then IL-6 concentration in serum was measured by enzyme linked immunosorbent assay (ELISA). In both genders, IL-6 was high levels in sera from SKG mice with severe arthritis (Fig. 4). For example, in sera from normal mice and treated with LAM, IL-6 was not detected, but in serum from mice treated with OX-CA or CSBG, IL-6 at high levels was detected. This result suggested that SKG mice treated with BG derived form *C. albicans* induced excess inflammatory response.

These results suggested that OX-CA and CSBG derived from *C. albicans* act as inducers of more severe arthritis in SKG mice. *C. albicans* is widely distributed in the natural environment, thus the environmental factors are suggested to be associated with the development of arthritis in SKG mice.

**Mycobacterium tuberculosis** Induced Milder Arthritis in SKG Mice

It is known that arthritis in SKG mice was induced by the administration of zymosan (ZYM), but not...
CpG and LPS, which are known as ligands of toll-like receptors (TLRs).\textsuperscript{23} These results suggested that bacterial infection was not a trigger for the induction of arthritis; therefore, we examined whether whole cells of \textit{Mycobacterium tuberculosis} can trigger arthritis. Whole cells of \textit{M. tuberculosis}, dosage of 3 mg, were administered as above and monitored incidence as well as score of arthritis. As shown in Fig. 2B, viewed from the incidence, half of the mice induced arthritis within 10 weeks. However, viewed from the arthritis score, it is milder than that induced by BG (Fig. 2A). \textit{Mycobacterium} was contained in Freund’s complete adjuvant (FCA), which is one of the most commonly used adjuvant in research. The components of \textit{Mycobacterium}, for example, liparabinomannan, CpG DNA, mycolic acids and peptidoglycan,\textsuperscript{22–24} effectively activates antigen-presenting cells such as macrophages and dendritic cells (DC) in a toll-like receptors (TLRs)-dependent manner. Although the components of \textit{Mycobacterium} significantly activate innate immunity via TLRs, they may not be suitable for the activation of arthrogenic T-cells in SKG. Not only the components of \textit{Mycobacterium} but also the components of gram-negative bacteria, lipopolysaccharide (LPS), could not induce arthritis in SKG mice.\textsuperscript{7} There are hundreds of pathogen-associated molecular patterns (PAMPs) stimulating various types of immune systems in animals and human. Further examination is needed to fully demonstrate efficacy of bacterial and fungal PAMPs on SKG model.

It was reported that \textit{C. albicans} associated with the differentiation of IL-17-producing helper T cells (Th17), which mediated an autoimmune arthritis,\textsuperscript{21,29} and deficiency of IL-17 completely inhibits arthritis development in SKG mouse.\textsuperscript{30} Therefore, it cannot be said that the pathogenesis of arthritis in SKG mice was the simple activation of innate immunity. Dectin-1 was shown to be the major receptor for BG on macrophages and to mediate cellular responses to particulate BG, including the production of proinflammatory cytokines.\textsuperscript{25,26} In SKG mice, blockade of Dectin-1 reduced the incidence and severity of arthritis.\textsuperscript{7} These results suggested that cell signaling via Dectin-1, not TLRs, is very important for the activation of arthrogenic T-cells in SKG. Furthermore, cell signaling of Dectin-1 has been actively researched,\textsuperscript{27,28} which were shown the signaling dectin-1 is required for the development of Th17,\textsuperscript{31} which was considered as pathogenic cell population for SKG mice.\textsuperscript{30} These reports show that Dectin-1 mediated the novel innate immune pathway for C-type lectin, not TLR signaling. The comparison of signaling on Dectin-1 and TLRs may indicate the role of BG in the pathogenesis of arthritis.

In this study, we showed that BG derived from \textit{C. albicans} acts as an inducer of arthritis in SKG mice. The severity and incidence of arthritis induced by BG from \textit{C. albicans} were greater and higher than LAM, a positive control in a previous study, and than \textit{M. tuberculosis}, the most potent adjuvant for animal experiments. These results suggest that fungal infection may, at least in part, induce and exacerbate autoimmune diseases such as RA.

Acknowledgments The authors thank Mr. Masashi Yoshihara for his technical assistance. This work was partly supported by a grant for private universities provided by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES