Mycoplasma fermentans glycolipid-antigen as a pathogen of rheumatoid arthritis

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Abstract

Mycoplasma fermentans has been suspected as one of the causative pathogenic microorganisms of rheumatoid arthritis (RA) however, the pathogenic mechanism is still unclear. We, previously, reported that glycolipid-antigens (GGPL-I and III) are the major antigens of M. fermentans. Monoclonal antibody against the GGPL-III could detect the existence of the GGPL-III antigens in synovial tissues from RA patients. GGPL-III antigens were detected in 38.1% (32/84) of RA patient’s tissues, but not in osteoarthritis (OA) and normal synovial tissues. Immunoelectron microscopy revealed that a part of GGPL-III antigens are located at endoplasmic reticulum. GGPL-III significantly induced TNF-α and IL-6 production from peripheral blood mononuclear cells, and also proliferation of synovial fibroblasts. Further study is necessary to prove that M. fermentans is a causative microorganism of RA; however, the new mechanisms of disease pathogenesis provides hope for the development of effective and safe immunotherapeutic strategies based on the lipid-antigen, GGPL-III, in the near future.

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synovitis is also characterized by the infiltration of variable numbers of B cells and antibody-producing plasma cells. Finally, the synovial fibroblasts in RA may be activated, and may degrade components of the articular matrix. These activated fibroblasts are particularly prominent in the lining layer, and at the interface with bone and cartilage.

The rheumatoid synovium is characterized by the presence of a number of secreted products of activated lymphocytes, macrophages, and fibroblasts. The local production of these cytokines and chemokines appears to account for many of the pathologic and clinical manifestations of RA.

The cause of RA remains unknown. It has been suggested that RA might be a manifestation of the response to an infectious agent in a genetically susceptible host. A number of possible causative agents have been suggested, including Mycoplasma, Epstein-Barr virus (EBV), cytomegalovirus, parvovirus, and rubella virus, but convincing evidence that these or other infectious agents cause RA has not emerged. One possibility is that there is a persistent infection of articular structures or retention of microbial products in the synovial tissues that generated a chronic inflammatory response.

*Mycoplasma fermentans* has been reported as a pathogenic microorganism of chronic inflammatory diseases, such as RA, chronic fatigue syndrome, fibromyalgia, and neurological diseases [1–8]. However, this association has been difficult to prove. Previously, we reported that phosphocholine-containing glycerolipid (GGPLs: GGPL-I and GGPL-III) are the lipid-antigens of *M. fermentans* [9]. The lipid-antigens are expressed in *M. fermentans* specifically, and these lipid-antigens are the major lipid-antigens of *M. fermentans* [9]. Matsuda et al. found and determined the complete structures of GGPL-I and GGPL-III as 6-O-phosphocholine-α-glucopyranosyl(1’-3)-1, 2-diacyl-sn-glycerol, and 1’-phosphocholine 2’-amino dihydroxypropane-3’-phospho-6’-α-glucopyranosyl(1’-3)-1,2-diacyl-glycerol, respectively [10–13]. Based on the structures and bioactivities of GGPLs, we have proposed the hypothetical role of *M. fermentans* in the pathogenesis [14–16]. Because GGPLs have strong immunogenicity, they may play roles as immunodisturbing agents in cell functions such as inflammation and cell differentiation [17]. We established monoclonal antibody which specifically recognizes the GGPL-III structure [18].

In this study, we demonstrated a direct evidence which suggested that *M. fermentans* is regarded as the pathogen of RA.

**Materials and methods**

**Collection of tissues.** Synovial tissues were obtained from the knee, elbow, and hip joint during replacement surgery from patients with RA (*n* = 84, 71 women and 13 men, mean age is 62.1 ± 11.0 years), osteoarthritis (OA) (*n* = 20, 18 women and 2 men, mean age is 60.4 ± 7.9 years), and traumatic injury (*n* = 10, 7 women and 3 men, mean age is 73.3 ± 13.4 years) according to the criteria of the American College of Rheumatology.

**Immunohistochemical staining.** Immunohistochemical staining was performed as previously described [19]. Synovial tissue specimens were preserved in 10% formalin, embedded in paraffin, serially sectioned onto microscope slides at a thickness of 4 μm. Deparaffinization, blocking of endogenous peroxidase activity is treated with alcohol quickly. Nonspecific binding sites were saturated by exposure to 0.2% bovine serum albumin and normal rabbit serum diluted 1:66.7 in PBS for 20 min. Immunohistochemical staining was done with the Vectastain avidin-biotin peroxidase complex kit (Vector Laboratories, Burlingame, California, USA). Anti-GGPL-III monoclonal antibody (50 μg/ml) [18] or control normal mouse IgM (50 μg/ml) was used. Counterstaining was done with Hematoxylin staining.

**TLC immunostaining.** GGPLs were extracted and isolated according to the purification method, which we previously reported [10]. The developing solvent was a mixture of chloroform: methanol: 0.2% aqueous CaCl2 (50:45:10, by volume). Lipids were separated on a high performance TLC (HPTLC)-plate (Merck, Darmstadt, Germany). Horseradish peroxidase (HRPO)-conjugated goat anti-mouse IgG + IgM conjugated to 10-nm gold particles, diluted 1:10 in PBS. After three rinsings in diluted water, these were stained with uracyl acetate and examined using a HITACHI H-7100 electron microscope (Hitachi, Hitachinaka, Japan).

**IL-6 and TNF-α production assay from peripheral blood monocytes.** The mononuclear cell pellet from healthy volunteers was seeded in 24-well flat bottom microtiter plates (Nunc, Roskilde, Denmark) at a density of 5 × 10⁵ cells/well in a total volume of 400 μl of RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (Biowhittaker, Walkersville, MD, USA), 100 U/ml penicillin, and 100 g/ml streptomycin (Gibco BRL/Life Technologies Inc., Rockville, MD, USA). Duplicate cultures were then prepared by adding the medium containing GGPL-III at concentrations of 0, 10, 20, and 50 μg/ml. After incubation at 37 °C in a humidified 5% CO₂ atmosphere at 37 °C, the culture supernatants were recovered and stored at 80 °C until cytokine measurements. The concentrations of TNF-α and IL-6 were measured using the respective commercial ELISA kits (BioSource International Inc., Camarillo, CA, USA). All manipulations were carried out under endotoxin-free conditions.

**Synovial cell proliferative studies.** Synoviocytes were cultured as previously described [20]. Tissues from RA (*n* = 5, 4 women and 1 man, mean age is 61.89 ± 8 years) were minced and stirred with 1 mg/ml collagenase (Gibco BRL/Life Technologies Inc., Rockville, MD, USA). Dupli cate cultures were then prepared by adding the medium containing GGPL-III at concentrations of 0, 10, 20, and 50 μg/ml. After incubation at 37 °C in a humidified atmosphere of 5% CO₂/95% air for 24 h, the culture supernatants were recovered and stored at 80 °C until cytokine measurements. The concentrations of TNF-α and IL-6 were measured using the respective commercial ELISA kits (BioSource International Inc., Camarillo, CA, USA). All manipulations were carried out under endotoxin-free conditions.

**Statistical analysis.** The results of bioassy and synovial cell proliferative studies are analyzed by paired t-test. Differences were considered significant when *P* < 0.05.
Results

Detection of *M. fermentans* lipid-antigens in synovial tissue cells in patients with RA

Rheumatoid arthritis (RA) is a chronic inflammatory polyarticular disease characterized by massive synovial proliferation and subintimal infiltration of inflammatory cells that leads to the destruction of cartilage and bone. To see whether *M. fermentans* lipid-antigens (GGPL-III) exist in the pathological lesions of RA, we applied the monoclonal antibody specific for the GGPL-III for immunohistochemical study. Fig. 1 shows the distribution pattern of these lipid-antigens in synovial tissue sections from patients with RA. Glycolipid-antigens (GGPL-III) specific for *M. fermentans* species are seen as fine brownish particles. The existence of GGPL-III could be visualized in the cytoplasm of mononuclear cells (Fig. 1A) and synovial lining cells (Fig. 1B). There are distinctly positive cells and also clearly negative cells, therefore, the anti-GGPL-III monoclonal antibody specifically recognizes the lipid-antigens in cytoplasm. Synovial tissue specimens from 84 RA patients were obtained at the time of synovectomy or joint replacement surgery, and these specimens were used for analyses. The percentage of lipid-antigen positive specimens was 38.1% (32/84) of RA tissues, while none was positive in synovial tissues from patients with OA (n = 20) or normal (n = 10).

Immunochemical identification of *M. fermentans* lipid-antigens in synovial tissue in patients with RA

To exclude the possibility that the lipid-antigens detected by immunohistochemical study are non-specific immune reaction, we extracted lipids and isolated GGPL-III from tissues, and stained positively by immunohistochemical analysis (Fig. 2). Six specimens were analyzed, and GGPL-III-specific bands were seen in three of six specimens as shown. This result shows that the GGPL-III antigens exist in the RA synovial tissue which are positive for immunohistochemical staining using the anti-GGPL-III monoclonal antibody (Fig. 2).

Detection of *M. fermentans* lipid-antigens on endoplasmic reticulum in synovial tissue cells in patients with RA

To investigate the mechanism of GGPL-III pathogenesis in RA, immunoelectron microscopic studies with monoclonal antibodies against GGPL-III were carried out to determine the pattern of distribution of these molecules at the rheumatoid synovial tissues (Fig. 3). The ultrastructural analysis of GGPL-III localization revealed that there were no *M. fermentans* cell bodies. A part of GGPL-III antigens existed on endoplasmic reticulum in plasmacyte-like cells. Gold particles are approximately 10 nm in diameter.

It was suggested that *M. fermentans* cells were already degraded and GGPL-III antigens remained in the RA tissues.

Pathological activities of *M. fermentans* lipid-antigens: cytokine induction from peripheral blood mononuclear cells

The immunological dysfunction, especially abnormal helper T-cell balance, has been thought to be important for the pathogenesis of autoimmune diseases including RA. Microbial infection has been suspected as a causative

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Fig. 1. Glycolipid-antigens (GGPL-III) specific for *M. fermentans* species are seen as fine brownish particles in synovial tissue sections from patients with RA. Applying the monoclonal antibody specific for the GGPL-III, the existence of GGPL-III could be visualized in the cytoplasm of mononuclear cells (A) and synovocytes (B). For first antibodies, anti-GGPL-III monoclonal antibody (A,B) or normal mouse IgM (C,D) was used. Counterstaining was done with hematoxylin staining. Original magnification 200×.
agent of the abnormal Th1/Th2 balance. Th1 helper T cells produce inflammatory cytokines (TNF-α, IFN-γ, and IL-6). Th2 helper T cells produce regulatory cytokines (IL-4 and IL-10). In order to determine whether the GGPLs might induce these cytokines, we performed a cytokine production assay. After 24-h incubation, natural GGPL-III induces significantly TNF-α (Fig. 4A) and IL-6 (Fig. 4B) production in peripheral blood monocytes from healthy volunteers at 10, 20, 50, and 100 ng/ml (P < 0.05, paired t-test), dose-dependently.

Pathological activities of M. fermentans lipid-antigens: synoviocyte proliferation

An increase in the number of synovial lining cells appears to be the earliest lesions in rheumatoid synovitis. To investigate the effect of GGPL-III on RA synoviocyte proliferation, we analyzed cell viability in vitro by a modified MTT assay (Fig. 4C).

Equal numbers (5 × 10⁵ in media, n = 5) of these cells were seeded into replicate 96-well plates. As shown, RA synoviocyte at concentration of 10, 20, and 50 μg/ml of natural GGPL-III significantly increased viability compared with control after 24 h of cell culture, in a dose-dependent manner (P < 0.05, paired t-test). These findings suggest that GGPLs from M. fermentans, as a persistent pathogen, play a role in initiating and perpetuating synovitis of RA.

Discussion

In this study, we demonstrated that M. fermentans-species-specific lipid-antigen (GGPL-III) existed in 38.1% of RA synovial tissues from patients with RA of replacement surgery, but not in osteoarthritis (OA) and normal synovial tissues. It has been reported that M. fermentans was isolated in synovial fluid samples from arthritic patients with inflammatory diseases including RA [2–6]. M. fermentans was detected in the joints and peripheral blood in patients with RA, it is not yet clear as to how the bacteria enter the body and reach to the joints [4]. On the basis of the animal model of M. fermentans-infection it has been reported that M. fermentans can induce experimental arthritis in rabbits following inoculation of the bacteria in the trachea and knee joints, and it could induce arthritis regardless of the inoculation route [21]. Considering from these reports, M. fermentans could be a pathogen of reactive arthritis. Because it is said that 10% of reactive arthritis shifts to rheumatoid arthritis, M. fermentans reactive arthritis might shift to RA.

Usually, it is difficult for such a patient to gather the synovial fluid to show the mycoplasma infection by PCR method, because the joints are severely destructed we could not get the synovial fluid from our patients. In these cases, we could prove the existence of M. fermentans lipid-antigen, GGPL-III, in infiltrated mononuclear cells and synovial lining cells in RA synovial tissues. The monoclonal antibody is useful for the histochemical or immunoelectron microscopic analysis of pathological tissues, and may be an important tool in helping to determine the mechanisms underlying these processes.

Biofunctional study of M. fermentans lipid-antigen showed that it significantly induced inflammatory cytokines, TNF-α and IL-6, production from peripheral blood monocytes and proliferation of synovial fibroblasts. The immunological dysfunction, especially abnormal helper T-cell balance, has been thought to be important for the pathogenesis of autoimmune diseases including RA. Since therapies targeted against TNF-α and IL-6 are very effective for controlling systemic and joint inflammation of RA [22], these cytokines play a role in initiating and perpetuating synovitis of RA. Microbial infection has been suspected as a causative agent of the abnormal Th1/Th2 balance, but there was not enough evidence that
demonstrated confirmative evidence and mechanism. To support the idea, we presented the data which showed that the lipid-antigen including GGPL-III induced an inflammatory cytokine from the patient blood mononuclear cells, and the proliferation of synoviocytes. We have proposed that GGPLs (GGPL-I and GGPL-III) might be a pathogen of RA based on their structure, because the GGPLs have residues which are important for the biofunctions and molecular similarity to second messengers of cell signal transduction, for example, TNF-α sphingomyelin pathway [14–17]. Recently, it has been reported that proinflammatory IL-17 and IFN-γ production and increase of anti-inflammatory IL-10, IL-13 in splenocytes and production of IL-1beta, IL-6, and IL-23 in local joint tissue are important in the pathogenesis of RA [23]. To confirm these speculations, further study is necessary.

Specific T cell responses to a variety of self and microbial lipids depend on proper assembly and intracellular trafficking of CD1 molecules that intersect with and load processed lipid-antigens [24,25]. These pathways involve unique membrane trafficking and chaperones that are distinct from those utilized for major histocompatibility complex (MHC)-mediated presentation of peptide-antigens, and thus define unique lipid-antigen presentation pathways. Furthermore, recent studies have identified components of lipid metabolism that participate in lipid delivery, uptake, processing and loading onto CD1 molecules [25]. Defects in these pathways may result in impaired T cell development and function, underscoring their critical role in the lipid-specific T cell immune responses [26]. *M. fermentans* lipid-antigens were detected on endoplasmic reticulum in synovial tissue cells in patients with RA. Whether *M. fermentans* lipid-antigen, GGPL-III, certainly has pathogenetic activities specifically is not known at present. Immunoelectron microscopic studies with monoclonal antibodies against GGPL-III, revealed that a part of GGPL-III antigens existed at endoplasmic reticulum in plasmacyte-like cells. Because it has been reported that glycolipid-antigens, which bind to CD1 molecule such as a peptide antigen binding to MHC class I or II, are presented to NKT cells recently. GGPLs, may play pathogenic roles through NKT cells [27–29].

We have prepared the chemical synthesis of GGPLs and its purification system for biofunctional study to analyze the pathological functions of GGPLs in early to chronic stage of RA or animal models *M. fermentans* infection [12,13,30,31]. Further study, including genetical background [32,33], will be necessary to prove that *M. fermentans* lipid-antigen as a cause of autoimmune arthritis, newly emerging mechanisms of disease pathogenesis, provides hope for the development of effective and safe immunotherapeutic strategies in the near future.

References


