Osteoarthritis patients with high haemoglobin A1c have increased Toll-like receptor 4 and matrix metalloprotease-13 expression in the synovium

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Purpose: While research has identified diabetes mellitus (DM) as a risk factor for knee osteoarthritis (KOA), the underlying mechanisms are not fully understood. Studies suggest that Toll-like receptor 4 (TLR4) expression is elevated in osteoarthritic lesions of OA patients and in target tissues of insulin resistance such as adipose tissue and skeletal muscle in patients with DM. TLR4 is associated with inflammation and catabolic response via regulation of matrix metalloproteases (MMPs). We hypothesized that TLR4 and MMP expression may be increased in the synovial tissue (SYN) of KOA patients with diabetic pathology. We therefore investigated TLR and MMP expression in the SYN of KOA patients with and without high haemoglobin A1c concentrations.

Patients and methods: A total of 171 patients radiographically diagnosed with KOA were grouped based on their HbA1c concentration (HbA1c ≥6.5 and HbA1c <6.5). We used real-time polymerase chain reaction to compare the expression of TLRs (TLR2, TLR4) and MMPs (MMP2, MMP3, MMP9 and MMP13) in patients’ SYN between the two groups. MMP13 regulation by the TLR4 ligand, lipopolysaccharide (LPS), in SYN cells was examined in culture by stimulating SYN cells with LPS or vehicle (culture medium) for 24 h.

Results: The expression of TLR4 and MMP13 in the HbA1c ≥6.5 group was significantly elevated compared to that in the HbA1c <6.5 group. In contrast, TLR2, MMP2, MMP3 and MMP9 expression levels were similar between the groups. MMP13 mRNA and MMP13 protein levels in SYN cells were significantly higher following stimulation with LPS compared to vehicle.

Conclusions: TLR4 and MMP13 expression were elevated in the synovium of osteoarthritis patients with high HbA1c concentrations. Our results may provide insight into the pathology of OA patients with DM.

Keywords: osteoarthritis, diabetes mellitus, TLR4, MMP13

Introduction
Several studies have reported an association between over-loading due to obesity and the development of knee osteoarthritis (KOA).1–4 However, based on the epidemiological correlation between hand OA and overweight or obesity, systemic factors identified in the association with KOA may also be involved in general OA pathology.5–8 Some epidemiological studies have also suggested an association of diabetes mellitus (DM) and hyperglycemia with OA.9–13 However, the mechanism underlying such an association is not fully understood.

Toll-like receptors (TLRs) are transmembrane proteins with roles in DM pathology. Among the TLR subtypes, TLR2 and TLR4 play an important role in DM
The study protocol received approval by the Ethics Review Board of Kitasato University (reference number: KMEO B13-113). Written informed consent was obtained from all participants regarding participation in the study and the removal and use of their SYN one day prior to surgery. This study was conducted in accordance with the Declaration of Helsinki.

**Quantitative polymerase chain reaction (qPCR) analysis**

To evaluate the effect of the diabetic state on TLR and MMP expression, OA patients were grouped based on their HbA1c concentration (HbA1c ≥ 6.5 and HbA1c < 6.5). Patients’ clinical characteristics are summarized in Table 1 by group. Methods for total RNA extraction, cDNA synthesis and qPCR using SYBR Green are described elsewhere. The following PCR primers were used for qPCR: TLR2-sense 5'-CCT GTG TGA CTC TCC ATC CC-3', TLR2-antisense 5'-GAC ATT CCG ACA CCG AGA GG-3 (product size: 71 bp); TLR4-sense 5'-CGA CAA CCT CCC CTT CTC AAC-3', TLR4-antisense 5'-AGA GTG GCC TTA GGC TCT GAT-3 (product size: 167 bp); MMP2-sense 5'-GTG AAG TAT GGG AAC CGA TGG AGT CAC CTC TTC CCA GA-3', MMP2-antisense 5'-AGA GTG GCC TTA GGC TCT GAT-3 (product size: 154 bp); MMP3-sense 5'-GTG GAG TCT CTC AGC TGG GTG TCT GAT-3', MMP3-antisense 5'-TGG AGT CAC CTC TCC TTC CCA GA-3' (product size: 164 bp); MMP9-sense 5'-TTT GAG TCC GGT GGA CGA TG-3', MMP9-antisense 5'-GCT CCT CAA AGA GCA TG-3' (product size: 197 bp); MMP13-sense 5'-TGA CTC AGA GGC TCC GAG AA-3', MMP13-antisense 5'-CAT CAG GAA CCC CCC ATC TT-3 (product size: 111 bp); GAPDH-sense 5'-TGT TGC CAT CAA TGA CCC CTT-3' and GAPDH-antisense 5'-CTC CAG GAC GTA CTC AGC G-3' (product size: 202 bp). TLR and MMP mRNA expression levels were normalized to GAPDH levels using the delta-delta Ct method.

**Materials and methods**

**Patients**

Power analysis using an alpha of 0.05, power of 0.80, and N2 (number of patients with HbA1c ≥ 6.5)/N1 (number of patients with HbA1c < 6.5) ratio of 0.155 was conducted in G*POWER3 to determine the optimal sample size. Power analysis revealed that 23 patients with HbA1c ≥ 6.5 and 148 patients with HbA1c < 6.5 were needed to detect a difference in TLR4 between HbA1c ≥ 6.5 and HbA1c < 6.5 groups.

We examined SYN from patients (38 men and 133 women) with KOA diagnosed by radiography who underwent total knee arthroplasty at our institution. KOA patients with a cancer diagnosis, trauma, rheumatoid arthritis or other collagen diseases were excluded from this study. SYN samples were harvested from the operated knee during the operation. A piece of each of the 171 SYN specimens was snap frozen in liquid nitrogen at −80 °C prior to RNA extraction. Eight SYN specimens were used for cell culture.
compared the expression of TLR2, TLR4, MMP2, MMP3, MMP9 and MMP13 in the SYN between the two HbA1c groups. Additionally, we grouped the patients based on the World Health Organization Body Mass Index (BMI) classification (Table 2) (normal, overweight, obese) and compared TLR2, TLR4, MMP2, MMP3, MMP9 and MMP13 expression among these groups. Relative expression was calculated using the mean of all control samples (samples from SYN from the HbA1c <6.5 group or α-MEM-treated SYN cells in vitro). To investigate potential gender effects, female and male KOA patients were grouped based on their HbA1c level and BMI and TLR4 and MMP13 levels were analyzed.

Cell culture
SYN cells derived from 8 patients were used to examine the effect of the TLR4 agonist, LPS, on MMP13 mRNA expression and MMP13 protein production. Synovial cells were obtained following collagenase digestion of SYN as reported previously. Isolated synovial cells were cultured in 6-well plates in α-minimal essential media (α-MEM) with 10% fetal bovine serum. One week later, the cell population of cultured SYN cells was determined using flow cytometric analysis as reported previously. The cultured SYN cells were incubated with FITC-conjugated anti-CD45 (Biolegend, CA, USA) and allophycocyanin (APC)-conjugated anti-CD90 (Biolegend) antibodies for 45 min at 4 °C. After washing twice in PBS, 50,000 total events were acquired using FACSVerseTM (BD Biosciences, San Jose, CA, USA), and the data were analyzed using Flow Jo 10.0 (Tree Star, Ashland, OR). SYN cells were exposed to 1000 ng/mL LPS (Sigma, St. Louis, MO, USA) for 24 h. Subsequently, MMP13 mRNA expression was examined using qPCR with the primers described above. MMP13 protein concentration in the cell supernatant was analyzed using a commercial ELISA kit (Human Pro-MMP-13 Quantikine ELISA Kit, R&D Systems, Inc., Minneapolis, MN, USA).

Statistical analysis
The SPSS 25.0 statistical package was used for statistical analysis. Continuous variables were analyzed using the Mann-Whitney U test and Tukey multiple comparisons test, and categorical variables were analyzed using Fisher’s exact test. Statistical significance was defined by P<0.05.

Results
Clinical characteristics of patients with HbA1c ≥6.5 and HbA1c <6.5
BMI among KOA patients with HbA1c ≥6.5 was significantly higher than that among KOA patients with HbA1c <6.5 (Table 1). Age, male/female ratio and Kellgren/Lawrence grade 2/3/4 ratio were similar between the groups (Table 1).

Expression of TLRs and MMPs among patients with HbA1c ≥6.5 and HbA1c <6.5
To determine whether TLR and MMP expression levels are increased in diabetic KOA patients, we examined TLRs and MMPs in the SYN of KOA patients with HbA1c ≥6.5 and HbA1c <6.5. TLR2 expression was similar between patients with HbA1c ≥6.5 and HbA1c <6.5 (P=0.078; Figure 1A), while TLR4 expression in the HbA1c ≥6.5 group was significantly higher than that in the HbA1c <6.5 group (P=0.040; Figure 1B). There were no differences in MMP2, MMP3, or MMP9 expression between the groups (MMP2, P=0.626; MMP3, P=0.876; MMP9, P=0.912; Figure 1C–E). MMP13 expression in the HbA1c ≥6.5 group was significantly higher than that in the HbA1c <6.5 group (P<0.001; Figure 1F).

Effect of obesity on expression of TLRs and MMPs
Patients in the HbA1c ≥6.5 group had higher BMI than those in the HbA1c <6.5 group. We therefore investigated the effect of obesity on the expression of TLRs and MMPs. Patients in the obese group were significantly younger than those in the normal and overweight groups (Table 2). HbA1c levels in the obese group were significantly higher than those in the normal and overweight groups (Table 2). The Kellgren/Lawrence grade 2/3/4 ratio was similar among the groups (Table 2). Expression levels of TLR2,
TLR4, MMP2, MMP3, MMP9, and MMP13 were also similar among the normal, overweight, and obese groups (TLR2, P=0.821; TLR4, P=0.075; MMP2, P=0.294; MMP3, P=0.636; MMP9, P=0.659; MMP13, P=0.853; Figure 2).

Gender differences in expression of TLR4 and MMP13

Previous studies have suggested that there may be gender differences in pathological conditions in obese and diabetes patients. Therefore, we analyzed TLR4 and MMP13 expression between HbA1c ≥ 6.5 and HbA1c < 6.5 and among normal, overweight, and obese groups separately in male and female patients. Female patients but not male patients had higher BMI in the HbA1c ≥ 6.5 group than in the HbA1c < 6.5 group (Table 3). Among male OA patients, there was no difference in TLR4 or MMP13 between the HbA1c ≥ 6.5 and HbA1c < 6.5 group (P=0.216 and P=0.341, respectively; Figure 3A and B). TLR4 and MMP13 expression in female KOA patients with HbA1c ≥ 6.5 was significantly higher than that in female KOA patients with HbA1c < 6.5 (P=0.021 and P<0.001, respectively; Figure 3C and D). Both male and female patients in the obese group were significantly younger than those in the normal group (Table 4). HbA1c levels in female patients but not male patients were significantly higher in the obese group than in the normal and overweight groups (Table 4). There were no differences in TLR4 or MMP13 among the normal, overweight, and obese groups in male (TLR4, P=0.888; MMP13, P=0.320; Figure 4A and B) or female (TLR4, P=0.096, MMP13, P=0.812; Figure 4C and D) KOA patients.

Effect of TLR4 ligand, LPS, on MMP13 expression and MMP13 production

Flow cytometric analysis showed that almost all cells (94.5±0.9%) among cultured SYN cells were CD45-CD90+ (synovial fibroblasts) (Figure 5A). TLR4 and MMP13 expression levels were elevated in the synovium of OA patients with high HbA1c concentrations. Therefore, we investigated whether a TLR4 agonist regulates MMP13 in synovial cells. Synovial cells stimulated with 100 or 1000 ng/mL LPS exhibited significant increases in MMP13 mRNA expression compared to vehicle-treated cells (P=0.006 and P=0.007, respectively; Figure 5B). MMP13 protein levels in the supernatant of synovial cells treated with 100 or 1000 ng/mL LPS were also
significantly higher than those of cells treated with vehicle \((P=0.021\) and \(P=0.022,\) respectively; Figure 5C).

**Discussion**

Studies have shown that \(TLR2\) and \(TLR4\) expression are raised in traditional target tissues of insulin resistance such as adipose tissue and skeletal muscle in DM patients.\(^{33,34}\) Consistent with these reports, we observed higher \(TLR4\) expression in the SYN of KOA patients with high HbA1c concentrations. \(TLR4\) is associated with inflammatory and catabolic responses, and is up-regulated in inflamed SYN\(^{35}\) and osteoarthritic lesions in OA patients.\(^{36}\) Elevated \(TLR4\) expression in the SYN of KOA patients with high HbA1c concentrations may therefore be associated with OA pathology.

Clinical studies have reported elevated \(MMP13\) expression in patients with articular cartilage destruction,\(^{37,38}\) indicating that raised \(MMP13\) levels may contribute to cartilage degradation. A previous study showed that transgenic mice overexpressing \(Mmp13\) exhibit OA phenotype, including cartilage degradation. \(TLR4\) signaling is associated with catabolic response via \(MMP13.\)^{39} \(TLR4\) ligands stimulate \(MMP13\) in human chondrocytes\(^{40,41}\) and synovial fibroblasts.\(^{42}\) Here, \(MMP13\) expression was increased in the SYN of KOA patients with high HbA1c concentrations. In addition, the \(TLR4\) agonist, LPS, stimulated \(MMP13\) expression and \(MMP13\) production. Several endogenous \(TLR4\) ligands that may exacerbate diabetic conditions have been reported, including advanced glycation end products,\(^{41}\) macroglobulin,\(^{43}\) and amyloid-\(\beta.\)^{44} However, it is unclear which endogenous ligands contribute to the elevation of \(MMP13\) in KOA patients with high HbA1c concentrations. Nevertheless, elevation of \(TLR4\) and \(MMP13\) expression in the SYN of KOA patients

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**Table 3** Patients’ clinical characteristics by HbA1c group and gender

| Gender | HbA1c <6.5 (male, n=34; female, n=114) | HbA1c ≥6.5 (male, n=4; female, n=19) | \(P\) |
|--------|------------------------------------|---------------------------------|-----|
| Age (years) | Male 73.4±1.8 Female 74.1±0.7 | Male 70.0±2.5 Female 74.9±1.5 | \(P=0.535\) \(=0.636\) |
| KL (2/3/4), n | Male 0/2/2 Female 2/38/74 | Male 1/13/20 Female 0/3/16 | \(P=1.000\) \(=0.296\) |
| BMI | Male 26.6±3.2 Female 25.5±0.4 | Male 28.3±2.6 Female 30.4±1.0 | \(P=0.309\) \(=0.001\) |

Notes: Data represent mean ± standard error or n. *\(P<0.05\) compared with gender-matched HbA1c <6.5 group.

Abbreviations: KL, Kellgren/Lawrence grade; BMI, body mass index.
with high HbA1c concentrations may explain some of the epidemiological findings showing that DM is associated with OA. Previous epidemiological studies have reported that DM patients have high prevalence of overweight/obesity. Fatani et al reported that 41% of a DM patient population was obese. Hedley et al reported that 70% of DM patients were obese. In our study, patients with HbA1c ≥6.5 had higher BMI than those with HbA1c <6.5, and 56% of OA patients with HbA1c ≥6.5 were obese. However, TLR4 and MMP13 expression were comparable among normal, overweight, and obese patients. Therefore, our results suggest that higher TLR4 and MMP13 expression may be reflective of diabetic conditions but not obesity.

Several studies have reported gender differences in type 2 DM and obese patients. For example, the effect of type 2 DM on the risk of coronary heart disease is greater in females than males. Fat distribution with menopause is the main contributor to obesity in females. In our study, there were no significant differences in TLR4 or MMP13

Table 4 Patients’ clinical characteristics by body mass index group and gender

| Gender | Normal male, n=10 | Overweight male, n=24 | Obese male, n=4 | P |
|--------|------------------|-----------------------|----------------|---|
| Age (years) | Male 74.2±2.9 | Female 76.7±0.9 | Male 74.9±1.7 | Female 73.0±1.3 | 59.0±7.0 | 70.8±1.3 | 0.011 | <0.001 |
| KL (2/3/4), n | Male 0/2/8 | Female 2/16/40 | Male 1/12/11 | Female 0/17/32 | 0/1/3 | 0/8/18 | 0.352 | 0.038 |
| HbA1c ≥6.5 | Male 5.8±0.1 | Female 5.8±0.0 | Male 5.9±0.1 | Female 6.0±0.1 | 6.0±0.3 | 6.3±1.1 | 0.820 | <0.001 |

Notes: *P<0.05 compared with gender-matched normal group. **P<0.05 compared with gender-matched overweight group.
expression among the normal, overweight, and obese groups in either males or females. In contrast, TLR4 and MMP13 expression in female KOA patients with HbA1c ≥6.5 was significantly higher than that in female KOA patients with HbA1c <6.5. No such difference was observed between male KOA patients with HbA1c ≥6.5 and HbA1c <6.5. Our results may indicate gender differences in KOA patients with diabetes. However, our study lacked sufficient numbers of male patients for statistical analysis. Further investigations are needed to reveal the potential gender differences in KOA pathology with diabetes.

Several limitations of the present study warrant mention. First, the present study lacked oral glucose tolerance test (OGTT) data. Further characterization with OGTT data may more accurately reflect TLR4 and MMP13 expression in diabetic conditions. Second, the inclusion
of a non-KOA population is needed to confirm whether TLR4 and MMP13 expression are increased in diabetic individuals and if this directly contributes to KOA progression. Measurement of MMP levels in the serum of diabetic patients with and without OA and non-diabetic controls with and without OA is needed to support our conclusion. Third, elucidation of the mechanism by which TLR4 and MMP13 contributes to KOA pathology is needed. Fourth, we assessed the mRNA expression of TLRs and MMPs in SYN. Further investigation, such as a protein profiling study, is needed to tie the gene expression profile results together. Fifth, a positive control using a human synovial fibroblast or chondrocyte cell line was lacking in the in vitro study. Finally, whether there is also an increase in MMP/TLR expression in cartilage remains to be determined.

In summary, TLR4 and MMP13 were elevated in the synovium of osteoarthritis patients with high HbA1c concentrations. Our results may provide insights into the pathology of KOA patients with DM.

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Disclosure
The authors report no conflicts of interest in this work.

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