Evaluation of the effect of glucosamine administration on biomarkers for cartilage and bone metabolism in soccer players

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Abstract. In the present study, to investigate the effect of glucosamine, a component of glycosaminoglycans with a chondroprotective action, on articular cartilage in athletes, we looked at soccer players, who expose their joints to excessive motion and loading, and compared the levels of biomarkers for type II collagen degradation (CTX-II) and type II collagen synthesis (CPII) between soccer players and non-athlete controls, and in soccer players before and after glucosamine-administration. CTX-II (P<0.01) and CPII (P=0.08) levels were substantially elevated in soccer players compared with those in controls, indicating that cartilage metabolism (type II collagen degradation and synthesis) is increased in soccer players. Of note, glucosamine administration (1.5 g and 3 g/day for 3 months) significantly decreased the CTX-II level (P<0.05); however, the effect disappeared after withdrawal of administration. In contrast, glucosamine administration did not essentially affect the increased level of CPII. Furthermore, cartilage damage was evaluated by using the ratio of type II collagen breakdown to synthesis (CTX-II/CPII). The ratio in soccer players was significantly higher than that in controls (P<0.05), suggesting that type II collagen degradation is relatively enhanced compared with type II collagen synthesis in soccer players than in control students. Of importance, the ratio was reduced by glucosamine administration but returned to the pre-administration level after withdrawal of administration. Together these observations suggest that glucosamine is expected to exert a chondroprotective action in athletes (soccer players) by preventing type II collagen degradation but maintaining type II collagen synthesis, although the effect is transient and disappears after withdrawal of administration.

Introduction

The frequency and severity of joint loading are important determinants for the development of joint destruction characterized by the damage to articular cartilage. In fact, excessive motion and exposure of load on the joint are known to clinically and experimentally cause damage of the articular cartilage (1-7). Thus, sports that subject the joints to repetitive impact and torsional loading increase the risk of articular cartilage degeneration, which results in the clinical syndrome of osteoarthritis (OA) (6,7). The disease process of OA is related to the degradation and functional loss of joint cartilage. Importantly, studies with experimental OA models have shown that the early changes in the metabolic and chemical properties of cartilage matrix can be detected before the appearance of radiological changes (2). Thus, various molecular markers have been developed as indicators of cartilage and bone metabolism in patients with joint and bone disorders (8-11). In this context, it is interesting to note that sports-related mechanical loading on the joints affects the turnover rate of cartilage as well as bone in humans and experimental animal models, which can be detected by systemic biomarker assays (1-5).

Type II collagen is one of the major constituents of cartilage and represents 90-95% of the total cartilage collagen (10). Thus, fragments of type II collagen have been targeted as biomarkers for cartilage breakdown. A C-terminal crosslinking peptide (CTX-II) is cleaved during degradation of type II collagen (12), whereas a neoepitope (C2C) is generated by intrahelical cleavage at the C terminus of the 3/4 piece of degraded type II collagen (13); both CTX-II and C2C are used as markers for type II collagen degradation. In contrast, a C-terminal type II procollagen peptide (CPII),

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which is localized in newly formed type II collagen and cleaved during processing of synthesized type II procollagen, can be used as a marker for type II collagen synthesis (14). In addition, cross-linked N-terminal telopeptides of type I collagen (NTx) and deoxypyridinoline (Dpyr), a crosslink product of type I collagen are used as markers for bone resorption (degradation of type I collagen in bone) (11).

Nutritional supplements (such as glucosamine, chondroitin and collagen) are sometimes used for ‘joint health’ to treat or prevent sports-related cartilage injuries (including OA) in athletes (15-17). Among these, glucosamine, a naturally-occurring amino monosaccharide, has been widely used to treat OA in humans (18-20). Glucosamine is present in the connective and cartilage tissues as a component of glycosaminoglycans, and contributes to maintaining the strength, flexibility and elasticity of these tissues. Several short- and long-term clinical trials in osteoarthritis have shown the significant symptom-relieving and structure-modifying effects of glucosamine (18-20). Importantly, it has been revealed in vitro that glucosamine can inhibit the degradation and stimulate the synthesis of glycosaminoglycans (proteoglycans), thereby possibly exhibiting chondroprotective action (21,22). Moreover, glucosamine has been shown to inhibit the expression of collagen-degrading enzymes (matrix metalloproteinases, MMPs) but augment the expression of type II collagen in chondrocytes in vitro (23,24). Based on these observations, glucosamine is estimated to exert a chondroprotective action on cartilage injuries by retaining not only proteoglycans but also type II collagen in the articular cartilage.

Among various sports with different intensity, frequency and rate of joint injury, soccer is classified as a representative that subjects joints to repetitive high levels of impact and torsional loading (6,7). Thus, in the present study, to investigate the effect of glucosamine on articular cartilage in athletes, we looked at soccer players and determined the levels of biomarkers for type II collagen degradation (CTX-II and C2C) and type II collagen synthesis (CPII) before and after the glucosamine-administration.

Materials and methods

Subjects. The research project was undertaken following approval from the Human Experimentation Ethics Committees of Juntendo University. Subjects gave written informed consent prior to participation in the study. As a soccer group, 21 soccer players belonging to the soccer team of Juntendo University School of Health and Sports Science (all males, ranging in age from 19 to 22, 20.3±0.9 years old, mean ± SD) were recruited. All players were actively training for soccer during the study period: they performed the training session five times a week (from Tuesday to Saturday) for ~2 h/day and played the official match almost every Sunday (running distance reached 8-10 km in the training and 10-12 km in the match). As a control group, 10 college students (all males, age from 20 to 27, 23.5±2.8 years old) were recruited; none of the controls participated in collegiate athletics, nor had experienced moderate and hard exercise for over one year. There was no essential difference in age between soccer players and control students. None of the participants exhibited the symptoms of joint injuries (such as pain, stiffness and disability) and were taking any medication likely to influence cartilage and bone metabolism during the study period (with the exception of glucosamine administration, as described below).

Glucosamine administration. To evaluate the effect of glucosamine administration on the biomarkers for cartilage as well as bone metabolism, soccer players were orally administered with glucosamine hydrochloride (GlcN) (500 mg/capsule, supplied by Koyo Chemical Co., Ltd., Tokyo, Japan) at doses of 1.5 g/day (1.5 g after supper) or 3 g/day (1.5 g after breakfast and 1.5 g after supper) for 3 months. Urine samples were collected before (at 0 month) and after the glucosamine administration (at 3 months), and further 3 months after the withdrawal of glucosamine administration (at 6 months).

Measurement of biomarkers by enzyme linked immunoassay (ELISA). To measure the biomarkers for cartilage and bone metabolism, second morning void urine specimens were used. Urine samples were collected after a overnight fast, and stored in aliquots at -80˚C until use.

Assay for CTX-II was performed with a Urine CartiLaps® ELISA kit (Nordic Bioscience Diagnostic A/S, Herlev, Denmark), which detects a C-terminal telopeptide (CTX-II) of type II collagen (12). CTX-II is cleaved by collagenases during degradation of type II collagen. Furthermore, C2C was assayed with a Collagen type II Cleavage ELISA kit (IBEX Pharmaceuticals Inc., Montreal, Canada), which detects a neoepitope created by the collagenase cleavage of type II collagen (13). The neoepitope is located at the C terminus of the 3/4 length type II collagen cleavage product. Both CTX-II and C2C can be used as type II collagen-degradation markers.

Assay for CPII (14) was performed with a Procollagen type II C-propeptide ELISA kit (IBEX Pharmaceuticals), which detects a C-terminal propeptide of newly formed type II collagen (C-propeptide, also referred as CPII). CPII is cleaved from type II procollagen during processing of synthesized procollagen, and thus can be used as a type II collagen-synthesis marker.

CTX-II, C2C and CPII were measured in duplicates on the same microtiter plate, and their detection limits were <0.6 ng/ml, <10 ng/ml and <50 ng/ml, respectively. Concentrations of CTX-II, C2C and CPII were corrected by urinary creatinine (Cr), and expressed as ng/mmol Cr.

Cross-linked N-terminal telopeptides of type I collagen (NTx) and deoxypyridinoline (Dpyr), a crosslink product of type I collagen are excreted in urine during bone degradation, and can be used as markers of bone turnover (resorption) (11). Urinary NTx, Dpyr and creatinine were measured by Mitsubishi Chemical Medience Corporation (Tokyo, Japan) based on the ELISA (NTx and Dpyr) and enzyme assay (creatinine), and the concentrations of NTx and Dpyr were expressed as nmol BCE (bone collagen equivalent)/mmol Cr and nmol/mmol Cr, respectively, after correction with urinary creatinine.

Statistical analysis. Data are expressed as mean ± SD, unless otherwise noted, and analyzed for significant difference by
a one-way analysis of variance (ANOVA) with multiple comparison test or Student’s t-test (Prism 4, GraphPad Software, San Diego, CA). Correlation analysis was also performed with Prism 4. Differences were considered statistically significant at $P<0.05$.

Results

Urinary levels of the biomarkers for type II collagen degradation, type II collagen synthesis and bone resorption in soccer player. We examined the urinary levels of CTX-II, NTx and Dpyr in soccer players, and compared with those in control students. The levels of a type II collagen degradation marker (CTX-II) and bone resorption markers (NTx and Dpyr) were significantly elevated in soccer players than those in controls ($P<0.01$) (Fig. 1A-C), as previously reported in endurance athletes such as cross-country runners and rowers (4).

Next, we evaluated another type II collagen degradation marker (C2C) and a type II collagen synthesis marker (CPII) in soccer players. In contrast to CTX-II, C2C was only marginally increased in soccer players ($P=0.28$) compared with that in controls (Fig. 2A). Of note, the level of CPII, a type II collagen synthesis marker, was substantially increased than that in controls ($P=0.08$) (Fig. 2B), suggesting that cartilage metabolism including type II collagen synthesis is also increased in soccer players.

Correlation analysis of the biomarkers for type II collagen degradation, type II collagen synthesis and bone resorption in controls and soccer players. As mentioned above, CTX-II level was significantly elevated but C2C level was only slightly increased in soccer players than in control students (Figs. 1A and 2A). This observation suggests that the levels of CTX-II and C2C are unlikely to be changed in parallel within the body, although both are known as markers for type II collagen degradation. To test this, we analyzed the correlation between

**Figure 1.** Urinary levels of CTX-II, NTx and deoxypyridinoline in soccer players and control students. CTX-II (A), NTx (B) and deoxypyridinoline (C) were measured by ELISA using urine specimens. CTX-II, NTx and deoxypyridinoline levels were corrected by urinary creatinine (Cr), and expressed as ng/mmol Cr, nmol BCE/mmol Cr and nmol/mmol Cr, respectively. Data are the mean ± SD of 10 control students and 18 soccer players. Values were compared between control students and soccer players. **$P<0.01$.**

**Figure 2.** Urinary levels of C2C and CPII in soccer players and control students. C2C (A) and CPII (B) were measured by ELISA using urine specimens. C2C and CPII levels were corrected by urinary creatinine (Cr), and expressed as nmol/mmol Cr. Data are the mean ± SD of 10 control students and 18 soccer players. Values were compared between control students and soccer players. $P=0.28$ and $P=0.08$ for C2C and CPII, respectively.
CTX-II and C2C levels using urine samples from controls and soccer players. As expected, there was no significant correlation between CTX-II and C2C levels (P=0.31) (Fig. 3A).

In contrast, the levels of NTx and Dpyr, both of which are known as bone resorption markers, were significantly correlated (P<0.001) (Fig. 3B).

Figure 3. Correlation analysis of the biomarkers for type II collagen degradation, type II collagen synthesis and bone resorption in controls and soccer players. Correlation analysis was performed between CTX-II and C2C (A), NTx and deoxypyridinoline (Dpyr) (B), CTX-II and CPII (C), C2C and CPII (D), CTX-II and NTx (E), CTX-II and Dpyr (F), C2C and NTx (G), or C2C and Dpyr (H), based on the data from 28 urine specimens (10 control students and 18 soccer players). P-values are shown in the panels.
Furthermore, we analyzed the correlations between the markers for type II collagen degradation (CTX-II and C2C) and type II collagen synthesis (CPII) or bone resorption (NTx and Dpyr). Of importance, not only CTX-II but also C2C was significantly correlated with CPII (P<0.05) (Fig. 3C and D), suggesting that type II collagen degradation is correlated with type II collagen synthesis (CPII), even when type II collagen degradation was assessed by both CTX-II and C2C.

In contrast, CTX-II (P<0.01) (Fig. 3E and F) but not C2C (P>0.44) (Fig. 3G and H) was significantly correlated with NTx and Dpyr, indicating that type II collagen degradation is correlated with bone resorption (NTx and Dpyr), only when type II collagen degradation was assessed by CTX-II but not C2C.

These observations support our hypothesis that although both CTX-II and C2C are regarded as type II collagen degradation markers, their levels are not necessarily changed in parallel within the body.
Effect of glucosamine administration on the biomarkers for type II collagen degradation and bone resorption in soccer players. Finally, we examined the effect of glucosamine, a chondroprotective agent for OA, on the biomarkers for type II collagen degradation, type II collagen synthesis, and bone resorption in soccer players. Before glucosamine administration, CTX-II levels (ng/mmol Cr) were 1074.2±689.6 in 1.5 g GlcN/day-group (n=9) and 1232.2±872.2 in 3 g GlcN/day-group (n=10); C2C levels (ng/mmol Cr) were 7387.7±2104.0 and 7734.4±3702.7; CPII levels (ng/mmol Cr) were 9204.5±5274.9 and 9268.6±4076.3; NTx levels (nmol BCE/mmol Cr) were 76.4±46.4 and 98.4±45.5; Dpyr levels (nmol/mmol Cr) were 5.8±2.4 and 4.8±1.9 for 1.5 g and 3 g GlcN/day-groups, respectively. Thus, there were no significant differences in the CTX-II, C2C, CPII, NTx, and Dpyr levels between 1.5 g and 3 g GlcN/day-groups before glucosamine administration, although C2C levels were somewhat decreased in 3 g GlcN/day-group (Fig. 4B).

Similarly, the levels of CPII, NTx and Dpyr were not essentially changed even after the glucosamine administration and withdrawal of glucosamine administration, although C2C levels were still reduced in 3 g GlcN/day-group (P<0.05). In contrast, C2C levels were not substantially affected by glucosamine administration and withdrawal of glucosamine administration, although C2C levels were somewhat decreased in 3 g GlcN/day-group (Fig. 4B).

The decrease in the ratio is based on the finding that type II collagen degradation (CTX-II) was reduced but type II collagen synthesis (CPII) was maintained after glucosamine administration, whereas the increase in the ratio is due to that type II collagen degradation is relatively enhanced compared with type II collagen synthesis in soccer players than control students. Of importance, the ratio was reduced by glucosamine administration especially at a high dose (3 g GlcN/day), and returned to the pre-administration level after withdrawal of glucosamine in soccer players (Fig. 5B). The decrease in the ratio is based on the finding that type II collagen degradation (CTX-II) was reduced but type II collagen synthesis (CPII) was maintained after glucosamine administration, whereas the increase in the ratio is due to that type II collagen degradation (CTX-II) was increased but type II collagen synthesis (CPII) was maintained after withdrawal of glucosamine (Fig. 4A and C). These observations suggest that glucosamine may exert a chondroprotective action in soccer players (athletes) by preventing type II collagen degradation but maintaining type II collagen synthesis; however, its effect on type II collagen degradation is transient and disappears after withdrawal of administration.

Discussion

Glucosamine is currently used as a health supplement to relieve the pain of OA (18-20). Several clinical trials have shown the significant symptom-modifying effect of glucosamine in OA (18-20). In this context, biochemical and pharmacological studies have suggested in vitro that glucosamine possibly exhibit chondroprotective action by inhibiting the degradation and stimulating the synthesis of proteoglycans (21,22).

Currently, many researchers are trying to evaluate joint disorders using biomarkers. Markers under study are basically...
the constituents of cartilage, such as aggrecan, chondroitin sulfate and collagens (8-11). Type II collagen is a major constituent of articular cartilage, representing 90-95% of a total collagen content and forming the fibrillar structure that give cartilage its tensile strength (10). Among several biomarkers reported (11), components of type II collagen are recognized as the most important biomarkers for joint disorders (such as OA) (9), since type II collagen is specifically localized in cartilage, and essentially catabolism and anabolism of articular type II collagen are involved in joint disorders.

In this study, we evaluated the effect of glucosamine on the cartilage metabolism by using biomarkers for type II collagen-degradation (CTX-II and C2C) and -synthesis (CPII) (12-14) in athletes (soccer players), who expose their joints to repetitive impact and torsional loading, with the risk of articular cartilage degeneration and development of OA (7). Furthermore, we assessed the bone metabolism using degradation makers (NTx and Dpyr) of type I collagen, a major component of bone collagen (11), since subchondral bone remodeling is developed during the progression of joint disorders such as OA (27,28). Thus, this is the first study to investigate the effects of glucosamine on athletes by evaluating cartilage and bone metabolism using biochemical markers such as CTX-II, C2C, CPII, NTx and Dpyr.

The present results revealed that CTX-II, NTx and Dpyr levels were significantly elevated in soccer players compared to those in controls (Fig. 1), indicating that cartilage and bone metabolism (type II collagen degradation and bone resorption) is increased in soccer players as reported in other sports players and experimental animal models with extremes of skeletal exercise (1-5). Moreover, CPII level was substantially increased in soccer players compared with that in controls (Fig. 2B), suggesting that cartilage metabolism as evaluated by type II collagen synthesis is also increased in endurance athletes. Of note, glucosamine administration significantly decreased CTX-II level at both 1.5 g and 3 g GlcN/day; however, the effect was transient and disappeared after withdrawal of administration especially at a low dose (1.5 g GlcN/day). In this context, glucosamine has been shown to inhibit the production of MMP-13 from chondrocytes and synoviocytes in vitro (23,24) and decrease the serum level of MMP-3 in sera of patients with rheumatoid arthritis (29). Based on these findings, it is interesting to speculate that glucosamine suppresses MMP production, thereby inhibiting type II collagen degradation (as evaluated by CTX-II) in vivo. In contrast, glucosamine administration did not essentially affect the levels of CPII as well as NTx and Dpyr, indicating no effect of glucosamine on the increased levels of type II collagen synthesis (CPII) and bone resorption (NTx and Dpyr) in soccer players. Of importance, glucosamine has been shown to augment the expression of type II collagen in chondrocytes in vitro (24). Probably, type II collagen synthesis (as evaluated by CPII) was already increased in soccer players (Fig. 2B), and consequently the level could not be further elevated by glucosamine administration.

In contrast to CTX-II (Figs. 1A and 4A), C2C, another marker for type II collagen degradation, was only marginally increased in soccer players compared with that in controls (Fig. 2A), and no significant effect of glucosamine administration was detected on C2C (Fig. 4B). This may be due to that urinary level of C2C (~6000 ng/mmol Cr) is much higher than that of CTX-II (~1400 ng/mmol Cr) and thus can not sensitively detect the changes in type II collagen degradation observed between soccer players and control students, and after glucosamine administration. Furthermore, the discrepancy could be explained by the results of correlation analysis that the levels of CTX-II and CPII are not necessarily changed similarly within the body (Fig. 3).

As mentioned above, components of type II collagen are recognized as valuable biomarkers for joint disorders (9). Moreover, the ratio of type II collagen breakdown to synthesis can be used for predicting the progression of joint damage (25,26). In this study, CTX-II levels (ng/mmol Cr, mean ± SEM) were 1348±213 for soccer players (n=18) and 510±88 for controls (n=10) (soccer players vs. controls, P<0.01), CPII levels (ng/mmol Cr) were 11000±1179 for soccer players and 7691±1340 for controls (P=0.08); and the ratio of CTX-II/ CPII was significantly higher in soccer players (0.135±0.020) than in controls (0.078±0.013) (P<0.05) (Fig. 5). Recently, we analyzed the urinary levels of CTX-II and CPII in patients with knee OA (74.3±7.8 years old, 6 males and 10 females, n=16) and healthy controls (70.5±5.2 years old, 5 males and 12 females, n=17) (30). OA patients radiographically exhibited obvious cartilage damage with Kellgren and Lawrence grades of 2-4 (31), whereas healthy controls showed no essential cartilage damage (Kellgren and Lawrence grades of 0-1). CTX-II levels (ng/mmol Cr, mean ± SEM) were 434±77 for OA patients and 196±28 for controls (OA vs. controls, P<0.01), CPII levels (ng/mmol Cr) were 6983±3409 for OA patients and 5378±1218 for controls (P=0.65), and the ratio of CTX-II/ CPII was significantly higher in OA patients (0.136±0.028) than in controls (0.067±0.019) (P<0.05). Based on the levels of CTX-II and CPII, the cartilage tissue of soccer players seem to be metabolically active than that of OA patients. However, the similar ratios of CTX-II/CPII likely suggest that both in soccer players and OA patients, type II collagen degradation is relatively enhanced compared with type II collagen synthesis. Since early changes in the chemical properties of cartilage can be detected with biomarkers (2), these observations indicate that the articular cartilage may be metabolically damaged in soccer players, although they exhibited no symptoms of joint injuries. Of note, glucosamine administration prevented the increase of type II collagen degradation (CTX-II) but maintained that of type II collagen synthesis (CPII), which results in the reduction of CTX-II/CPII ratio, in soccer players with metabolically active cartilage tissue (Figs. 4 and 5). In this context, it is important to note that glucosamine administration exhibited a chondroprotective action against OA patients with high cartilage turnover (as evidenced by high level of urinary CTX-II) by suppressing the joint space narrowing and reducing CTX-II level (32).

In summary, the present study revealed that cartilage metabolism (type II collagen degradation and synthesis) as well as bone metabolism (bone resorption) is significantly elevated in soccer players, when evaluated with biomarkers such as CTX-II, CPII, NTx and Dpyr. Moreover, glucosamine administration suppressed the increase of type II collagen degradation but did not affect the increased level of type II collagen synthesis in soccer players. These observations suggest that glucosamine is expected to exert a chondroprotective...
action in athletes (soccer players) by preventing type II collagen degradation but maintaining type II collagen synthesis. However, the detailed action of glucosamine on the articular cartilage of athletes remains to be elucidated in the future.

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