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Induction of cyclooxygenase-2 in human synovial cells by β2-microglobulin

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Background. Prostaglandins (PGs) are important mediators of inflammation in arthritis. We evaluated the role of the cyclooxygenase-2 (COX-2) enzyme, which regulates PG biosynthesis, in osteoarthropathy associated with hemodialysis-associated amyloidosis (HAA) by characterizing COX-2 expression in β2-microglobulin–treated human synovial cells.

Methods. We examined the effects of β2-microglobulin (β_2m), a major constituent protein of amyloid fibrils in HAA, on the COX-2 protein and mRNA expression in human synovial cells using Western blot and reverse transcriptase-polymerase chain reaction.

Results. β_2m selectively increased the biosynthesis of COX-2 protein and induction of COX-2 mRNA in a dose-dependent manner. Immunoabsorption of β_2m–containing media by anti-β_2m–specific antibody abrogated β_2m–mediated COX-2 expression on synovial cells. On the other hand, dexamethasone markedly suppressed the induction of COX-2 protein and mRNA in β_2m–stimulated synovial cells.

Conclusions. Our results suggest that induction of COX-2 expression by β_2m may be an important component of the inflammatory process in hemodialysis-associated osteoarthropathy.

Amyloid arthritis has been described previously in patients with systemic amyloidosis such as immunoglobulin light-chain–derived amyloidosis [1, 2]. Also, it is well known that amyloid protein induces the production of proteinases from synovial cells, which may contribute to articular destruction [3]. β2-microglobulin (β_2m) is the major constituent protein of amyloid fibrils in hemodialysis-associated amyloidosis (HAA) [4]. The main clinical manifestations of this disease include a group of rheumatological disorders, including carpal tunnel syndrome, chronic synovitis, and progressive bone destruction [5–8]. Although β_2m plays an important role in the development of HAA, the precise molecular pathogenic mechanisms of these complications remain unknown.

Excessive production of prostaglandins (PGs) is associated with the development of major pathological processes and plays a critical role in eliciting inflammation [9]. Cyclooxygenase (COX) is a key regulatory enzyme in the biosynthesis of PGs [10, 11]. COX exists in two forms [12, 13]. While COX-1 is present in most tissues and is involved in the physiological production of PGs [14], COX-2 is almost undetectable under physiological conditions in most tissues. Its expression is highly induced by proinflammatory agents such as cytokines and growth factors [15–17]. Therefore, up-regulation of COX-2 is considered the mechanism through which cellular PG levels are elevated in inflammatory processes. Experimental evidence suggests the involvement of elevated biosynthesis of PGs in the inflammatory process affecting the synovial cells and articular destruction in inflammatory joint diseases [18, 19]. For example, COX-2 is expressed in human rheumatoid synovial tissues, and inflammatory cytokines induce the synthesis of COX-2 mRNA and protein in cultured synovial cells [20].

Interestingly, the clinical manifestations of HAA are similar to those of inflammatory joint diseases. Recent studies suggest that β_2m has biological functions in a variety of cells, including synovial cells and osteoblasts [21–23]. This study was designed to assess the effects of β_2m on the expression of COX-2 in human synovial cells.

Key words: amyloidosis, prostaglandin, dexamethasone, osteoarthropathy, hemodialysis.

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METHODS

Reagents

Human β_2m was purchased from Sigma Chemical Co. (St. Louis, MO, USA). It was dissolved in endotoxin-free distilled water (Seikagaku Co., Tokyo, Japan) and

was subsequently diluted in experimental culture media. β_2m was analyzed by 14% sodium dodecyl sulfate-polyacrylamide gel under reducing conditions and migrated as a single band (data not shown). No endotoxin was detected (<0.18 EU/ml) under our experimental culture conditions, as confirmed by using an endotoxin-specific detection kit (Toxicolar system; Seikagaku Co.). Human serum amyloid A protein (SAA) was kindly provided by Dr. N. Kubota (Eiken Chemical Ltd., Tochigi, Japan). SAA was purified from pooled sera using high concentrations of C-reactive protein by sequential gel filtration as described previously [24]. Rabbit anti-COX-2–specific antibody was purchased from IBL Co. (Fujioka, Japan). Anti-β_2m–specific antibody was purchased from Dako Japan (Tokyo, Japan). All other reagents were obtained from Sigma.

Synovial cell culture

The experimental protocol was approved by the local ethics committee, and a signed consent form was obtained from each patient. Synovial tissue samples were obtained from patients with rheumatoid arthritis and osteoarthritis (OA) during synovectomy. The synovial membranes were minced aseptically and were then dissociated enzymatically with collagenase (4.0 mg/ml; Sigma) in RPMI 1640 for four hours at 37°C. The obtained cells were plated on culture dishes and were allowed to adhere. To eliminate nonadherent cells from synovial cell preparations, the plated cells were cultured for 18 hours with RPMI 1640 supplemented with 10% fetal calf serum at 37°C in humidified 5% CO_2 in air. The cells were then washed thoroughly with phosphate-buffered saline (PBS) solution. Adhering synovial cells were removed by adding trypsin-ethylenediaminetetraacetic acid and were washed with PBS containing 2% fetal calf serum. The collected synovial cells were used at the third or fourth passages for subsequent experiments. Synovial cell preparations were less than 1% reactive with monoclonal antibodies CD3, CD20, CD68 (Coulter Immunology, FL, USA), and anti-human von Willebrand factor (Immunotech, Marseille, France), which respectively define an antigen on mature T lymphocytes, B lymphocytes, monocytes/macrophages, and vascular endothelial cells.

COX-2 expression analysis by Western blot

The expression of COX-2 was analyzed by the Western blot method as Crofford et al previously described [20]. For this purpose, the cells were washed with cold PBS and lyzed by the addition of a lysis buffer containing 1% nonidet P-40, 50 mM Tris (pH 7.5), 100 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 20 minutes at 4°C. Insoluble material was removed by centrifugation at 15,000 × g for 15 minutes at 4°C. The supernatant was saved, and the protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). An identical amount of protein (50 μg) for each lysate was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The fractionated proteins were transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL, USA), and the membranes were blocked in a solution containing 5% bovine serum albumin, 10 mM Tris-HCl (pH 8.2), and 140 mM NaCl for 16 hours. The blots were incubated with an anti-COX-2–specific antibody (IBL Corp.) for two hours at room temperature. The membranes were further incubated with peroxidase-conjugated antirabbit IgG antibodies (Amersham) for 60 minutes and were developed using an enhanced chemiluminescence system (Amersham).

RNA preparation and reverse transcriptase–polymerase chain reaction (RT-PCR) assay

Total cellular RNA was extracted from synovial fibroblasts cultured with or without β_2m using guanidium thiocyanate and phenol (RNAzol B; Cinna/Bioek Labs Intl. Inc., Friendswood, TX, USA). First-strand cDNA was synthesized by RT at 42°C for 45 minutes in a 50 μl reaction mixture containing 1 μg of total RNA and MuLV RT (Gibco BRL, Gaithersburg, ND, USA). After heating at 99°C for five minutes for denaturing, followed by cooling at 5°C, the cDNA was used for amplification. For the PCR reactions, 2 μl of denatured cDNA was amplified in a 20 μl final volume with 1 U Taq DNA polymerase (Gibco BRL), 1 mM of both primers, and Taq polymerase buffer contained 1.5 mM $MgCl_2$ with 1.5 mM of each dNTP. PCR was performed in a thermal cycler (Perkin Elmer Cetus, Foster City, CA, USA) using a program of 25 cycles at 94°C for one minute, 60°C for one minute, 72°C for one minute, followed by a 10-minute extension at 72°C. The amplified products were subjected to electrophoresis on 1.5% agarose gel.

The specific primers used were as follows: COX-2 [25], 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′ (forward primer), 5′-AGATCATCTCTGCCTGAGTA TCTT-3′ (reverse primer). The predicted size of the fragment was 301 bp. For β-actin, the primer was 5′-GAC GAGGCCCAGA GCA AGAGAG-3′ (forward primer), 5′-ACGTACA TGGCTGGGGTGTTG-3′ (reverse primer). The predicted size of the fragment was 284 bp.

RESULTS

Induction of COX-2 in β_2m–treated synovial cells

To study whether β_2m up-regulates COX-2 biosynthesis in human synovial cells, lysates of synovial cells stimulated with or without β_2m (2 μg/ml) were analyzed by Western blot using anti-COX-2 antibody. In untreated synovial cells, COX-2 was not detected. However, treatment of synovial cells with β_2m for 12 hours resulted in

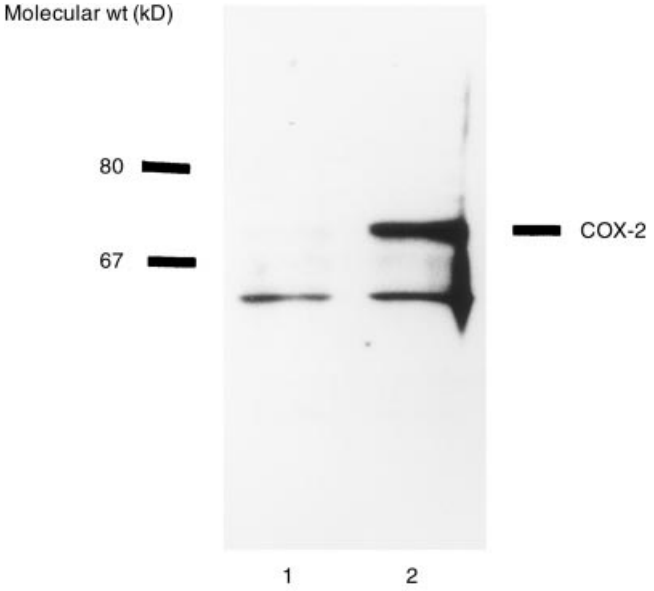

Fig. 1. Western blot demonstrating the COX-2 protein expression in rheumatoid synovial cells after stimulation with β2-microglobulin (β_2m; 2 μg/ml). Synovial cells were cultured with (lane 2) or without (lane 1) β_2m (2 μg/ml) in serum-free RPMI media for 12 hours. The cells were lysed, and lysates were analyzed by anti-COX-2 Western blot as described in the **Methods** section. Prestained molecular markers are shown in the far left lane.

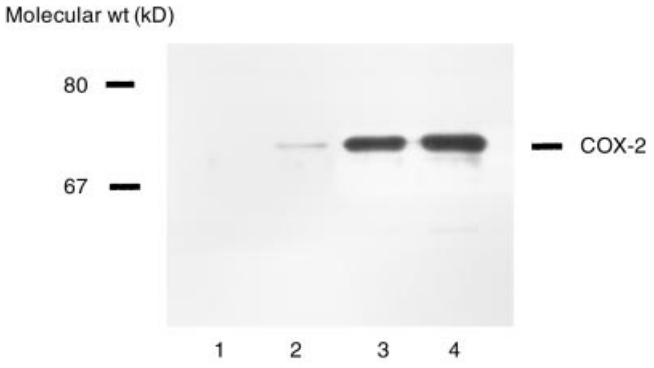

Fig. 2. β_2m induces COX-2 protein expression on synovial cells. Synovial cells were cultured with various concentrations of β_2m in serum-free RPMI media for 12 hours. The cells were lysed, and lysates were analyzed by anti-COX-2 Western blot (lane 1, without β_2m; lane 2, cultured with 0.2 μg/ml of β_2m; lane 3, cultured with 2 μg/ml of β_2m; lane 4, cultured with 10 μg/ml of β_2m).

a marked expression of COX-2 polypeptide (Fig. 1). To examine the dose response of β_2m on COX-2 expression, synovial cells were treated with different concentrations of β_2m (0.2 to 10 μg/ml) for 12 hours, and cellular lysates were analyzed by anti-COX-2 Western blot. As shown in Figure 2, β_2m induced COX-2 expression on synovial cells in a dose-dependent manner and reached a plateau at 2 μg/ml of β_2m. We also examined the effects of β_2m on COX-2 expression using the synovial cells isolated

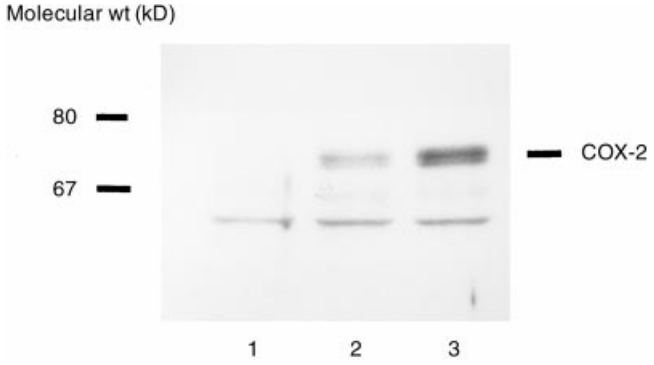

Fig. 3. β_2m induces COX-2 protein expression on synovial cells isolated from osteoarthritis (OA) patients. Synovial cells isolated from OA patients were cultured with various concentrations of β_2m in serum-free RPMI media for 12 hours. The cells were lysed, and lysates were analyzed by anti-COX-2 Western blot (lane 1, without β_2m; lane 2, cultured with 0.2 μg/ml of β_2m; lane 3, cultured with 2 μg/ml of β_2m).

from OA patients. In synovial cells isolated from OA patients, β_2m induced COX-2 expression (Fig. 3). In contrast, SAA protein, another amyloid protein, did not induce the COX-2 expression on synovial cells (Fig. 4). These results indicate that COX-2 induction is not a generalized effect of amyloid proteins.

To determine whether β_2m directly induces COX-2 expression in synovial cells, we performed immunoprecipitation analysis using a specific antibody against β_2m. Culture of synovial cells with β_2m–containing media immunoabsorbed by anti-β_2 antibody abolished β_2m–mediated COX-2 expression on synovial cells. In contrast, immunoabsorption using control rabbit IgG did not influence β_2m–mediated COX-2 expression (data not shown).

RT–PCR analysis of mRNA for COX-2

To confirm the effects of β_2m on COX-2 induction, we examined COX-2 mRNA in control and β_2m–treated synovial cells by RT–PCR analysis. Reverse transcription was performed on total RNA from synovial cells incubated with 10 U/ml of interleukin-1β for six hours. β-actin (lanes 1 to 5) and COX-2 (lanes 6 to 10) cDNA were amplified by PCR for 19 to 31 cycles. As shown in Figure 5A, there was a linear correlation between the number of cycles (19 to 31), and the yield of PCR productions of both COX-2 and β-actin, indicating that within these cycles, our PCR condition can be used for semiquantitation of the respective mRNA.

The results of COX-2–specific RT–PCR analysis corresponded well to those of the Western blot analysis. COX-2 mRNA was not detected in synovial cells under basal conditions. In contrast, stimulation of these cells with β_2m for six hours induced COX-2 mRNA expression in a dose-dependent manner. There was no difference in the levels of mRNA for β-actin, a control house-

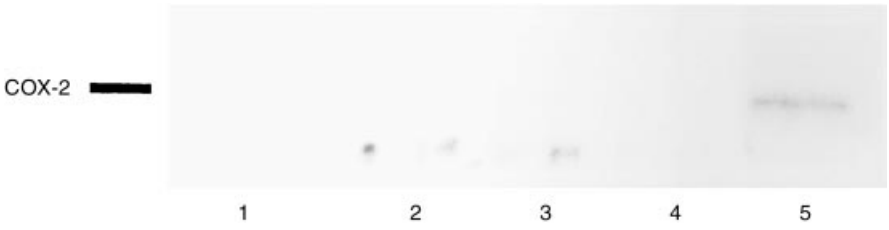

Fig. 4. Serum amyloid A protein (SAA) did not induce COX-2 protein expression on synovial cells. Synovial cells were cultured with various concentrations of SAA in serum-free RPMI media for 12 hours. The cells were lysed, and lysates were analyzed by anti-COX-2 Western blot (lane 1, without SAA; lane 2, cultured with 0.5 μg/ml of SAA; lane 3, cultured with 5 μg/ml of SAA; lane 4, cultured with 50 μg/ml of SAA; lane 5, cultured with 2 μg/ml of β_2m).

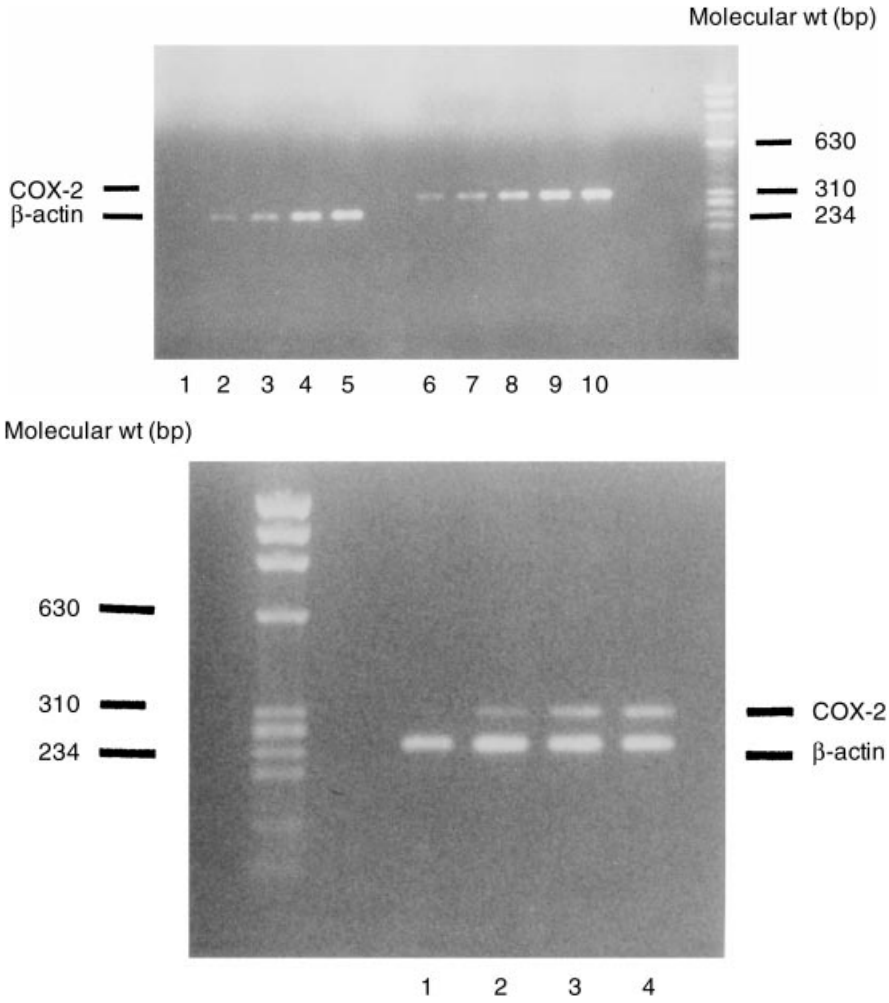

Fig. 5. RT–PCR analysis of mRNA for COX-2 in synovial cells treated with β_2m. (*A*) Reverse transcription was performed on total RNA from synovial cells incubated with 10 U/ml of interleukin-1β for six hours as described in the **Methods** section. β-actin (lanes 1–5) and COX-2 (lanes 6–10) cDNA were amplified by PCR for 19–31 cycles. Lanes 1–5 (β-actin) correspond to 19, 22, 25, 28, and 31 cycles, and lanes 6–10 (COX-2) correspond to 19, 22, 25, 28, and 31 cycles, respectively. (*B*) Synovial cells were treated with various concentrations of β_2m for six hours. Total RNA was reverse transcribed following PCR amplification with primers for COX-2 and β-actin. Ethidium bromide staining of PCR products after 25 cycles of amplification and separated in a 1.5% agarose gel (lane 1, without β_2m; lane 2, cultured with 0.2 μg/ml of β_2m; lane 3, cultured with 2 μg/ml of β_2m; lane 4, cultured with 10 μg/ml of β_2m).

keeping gene, between control and β_2m–treated synovial cells (Fig. 5B).

Effects of dexamethasone on COX-2 expression

Recent studies have suggested that glucocorticoids inhibit COX-2 mRNA expression by modulating the transcription factors that are located upstream of COX-2 gene [26, 27]. We therefore investigated the effects of dexamethasone on β_2m–induced COX-2 expression on synovial cells. Synovial cells were stimulated with β_2m in the absence or presence of dexamethasone (10^{-9} to 10^{-7} M) for 12 hours, and cellular lysates were analyzed by anti-COX-2 Western blot. As shown in Figure 6, dexamethasone completely inhibited COX-2 polypeptide expression at physiologically relevant concentrations (more than 10^{-8} M).

To confirm further the inhibitory effects of dexamethasone on COX-2 expression, we evaluated the effects of dexamethasone on β_2m–induced COX-2 mRNA expression using the RT–PCR method. COX-2 mRNA was

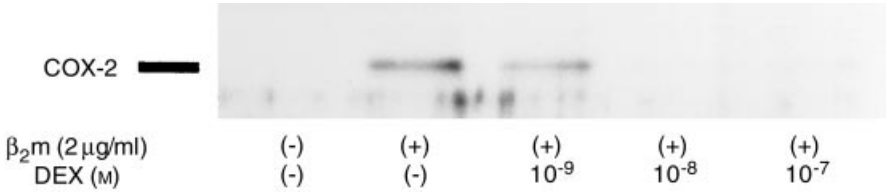

Fig. 6. Effect of dexamethasone on COX-2 protein expression in β_2m–stimulated synovial cells. Synovial cells were treated with β_2m (2 μg/ml) in the presence or absence of dexamethasone for 12 hours. The cells were lysed, and lysates were analyzed by anti-COX-2 Western blot.

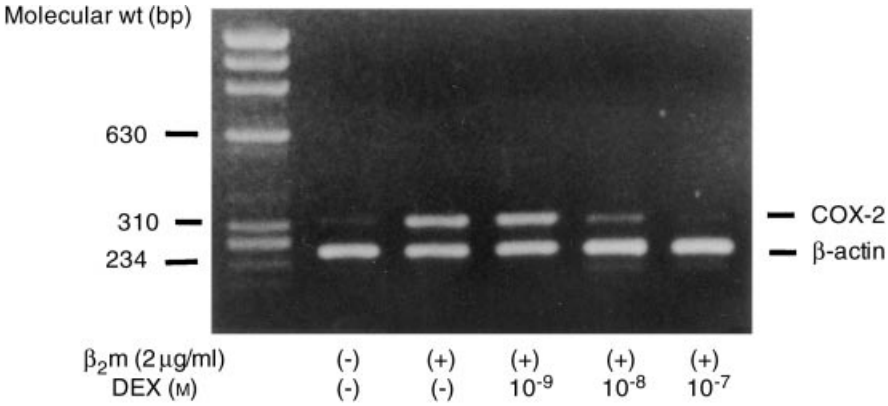

Fig. 7. Effect of dexamethasone on COX-2 mRNA expression in β_2m–stimulated synovial cells. Synovial cells were treated with β_2m (2 μg/ml) in the presence or absence of dexamethasone for six hours. Total RNA was reverse transcribed following PCR amplification with primers for COX-2 and β-actin. Ethidium bromide staining of PCR products after 25 cycles of amplification and separated in a 1.5% agarose gel.

detected after treatment of synovial cells by β_2m (2 μg/ml) for six hours. This β_2m–mediated COX-2 mRNA induction was inhibited by 10^{-7} to 10^{-8} M of dexamethasone. This concentration of dexamethasone is similar to the concentrations that inhibited COX-2 polypeptide synthesis. No change was noted in 284 bp amplified fragment of β-actin in synovial cells treated with or without dexamethasone (Fig. 7).

DISCUSSION

Hemodialysis-associated amyloidosis is a serious complication in patients on long-term dialysis therapy and may lead to carpal tunnel syndrome, destructive osteoarthropathy, or cystic bone lesions [5–8]. Amyloid deposits are mainly located in joint structures and periarticular bones, and β_2m was identified as the major constituent protein of this unique type of amyloidosis [4, 5]. However, the pathological role of β_2m in the development of articular and bone lesions is poorly understood.

The production of PGs is an important regulatory mechanism of bone turnover and is involved in bone resorption of inflammatory joint disease [28]. COX is an important regulatory enzyme in PG biosynthesis [10, 11]. COX-1 is considered to be expressed constitutively [14], whereas COX-2 is highly inducible by cytokines and growth factors, and is responsible for the enhanced PG synthesis at inflammatory sites [15–17]. It has been demonstrated that COX-2 is expressed in human synovial tissues, and treatment with inflammatory cytokines such as interleukin-1β enhanced the expression of COX-2 in synovial cells [20].

Recent studies have shown that β_2m has several biological functions in a various types of cells. Several investigators provided sufficient evidence indicating that β_2m is involved in the development of osteoarticular diseases [21–24]. We have also recently reported that β_2m directly induces matrix metalloproteinase production from human synovial fibroblasts [22]. In this study, we examined the impact of β_2m on COX-2 expression in human synovial fibroblasts. These results showed an absence of COX-2 protein in human synovial cells under basal conditions. However, COX-2 synthesis was markedly increased when these cells were treated with β_2m. RT–PCR analysis demonstrated that COX-2 mRNA was also enhanced in β_2m–treated synovial cells. It is possible that COX-2 expression on synovial cells was induced by contaminating substances present in the β_2m solution, such as endotoxin. However, no endotoxin was detected under our experimental culture conditions. Furthermore, our results also showed that immunoabsorption of β_2m–containing media by anti-β_2m–specific antibody abrogated the COX-2–inducing activity. These results indicate that the expression of COX-2 on β_2m–treated synovial cells is the direct effect of β_2m rather than being mediated by other factors.

Moe et al reported that β_2m did not stimulate the release of prostaglandin E_2 (PGE_2) from mouse calvariae and that β_2m–induced bone mineral dissociation is independent to PGE_2 [29]. This discrepancy between their

data and ours may be related to the cell types used for the β_2m treatment. Also, it was demonstrated that COX-2 induction is not sufficient for PGE_2 synthesis in a particular experimental condition. For example, PGE_2 cannot be produced in the serum-free or low-serum condition, despite the COX-2 expression [30].

Our data also indicated that dexamethasone dramatically inhibited the transcription of COX-2 in β_2m–stimulated synovial cells. Glucocorticoids cross the cell membrane to interact with the intracellular glucocorticoid receptor. Ligand-binding glucocorticoid receptor translocates to the nucleus and modulates the target gene expression either positively and negatively [31]. Although the exact mechanism of β_2m–mediated signaling for the expression of COX-2 in synovial cells is unknown at present, whether β_2m–mediated COX-2 expression is regulated at a transcription level requires further evaluation.

Recently, several lines of evidence suggest the possible involvement of AGE (advanced glycation end products) modified β_2m in HAA [32]. AGE-modified β_2m exhibited more biological activity in monocyte/macrophage chemotaxis and cytokine production compared with β_2m [33]. Further study examining the effects of AGE-modified β_2m on synovial COX-2 expression is also necessary.

In summary, our data show that β_2m increased the expression of COX-2 in human synovial cells. Furthermore, we confirmed that dexamethasone inhibits β_2m–induced expression of COX-2 mRNA and protein. Our findings suggest that modulation of synovial COX-2 expression by β_2m may be an important mechanism of osteoarthropathy in HAA.

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APPENDIX

Abbreviations used in this article are: β_2m, β2-microglobulin; COX, cyclooxygenase; CRP, C-reactive protein; ECL, enhanced chemiluminescence; HAA, hemodialysis-associated amyloidosis; OA, osteoarthritis; PBS, phosphate buffered saline; PGs, prostaglandins; RA, rheumatoid arthritis; SAA, serum amyloid A protein.

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