β₂-microglobulin induces MMP-1 but not TIMP-1 expression in human synovial fibroblasts

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Background. β₂-Microglobulin (β₂m) amyloidosis is a destructive articular disease that causes significant morbidity in patients undergoing hemodialysis. The amyloid deposits contain β₂m, some of which is altered with advanced glycation end products (AGE-β₂m). The deposits are located principally in joint structures, with adjacent degradation of cartilage and bone. We hypothesized that one of the mechanisms by which β₂m induces joint destruction is to induce the release of matrix metalloproteinase-1 (MMP-1), but not tissue inhibitor of metalloproteinase-1 (TIMP-1), from synovial fibroblasts.

Methods. To test this hypothesis and determine the role of AGE-β₂m, we incubated human osteoarthritic synovial fibroblasts in the presence and absence of β₂m and AGE-β₂m and measured the release of interstitial collagenase (MMP-1) and/or TIMP-1 by enzyme-linked immunosorbent assay and Northern blot analysis.

Results. β₂m and AGE-β₂m at 10 and 25 μg/mL induced the release of MMP-1 from human osteoarthritic synovial fibroblasts at 24 hours. In contrast, there was no increased release of TIMP-1, leading to an increase in the MMP-1/TIMP-1 ratio indicative of uncontrolled collagenolysis. A similar dose response was observed at 48 hours, except that AGE-β₂m had no effect over control cultures. MMP-1 mRNA expression by Northern blot analysis paralleled these findings. The source of the fibroblasts did not alter the results. Finally, we demonstrated that doxycycline, a treatment for arthritis, can inhibit the release of MMP-1 from synovial fibroblasts incubated with β₂m.

Conclusion. β₂m, at physiologically relevant concentrations, induces the release of MMP-1 without concomitant release of TIMP-1 from human synovial fibroblasts, leading to uncontrolled collagenolysis. The alteration of β₂m with AGE did not alter this effect at 24 hours, but blocked the effect at 48 hours. These findings may account for the tissue destruction seen in β₂m amyloidosis.

β₂-Microglobulin (β₂m) amyloidosis is a destructive articular disease that causes significant morbidity and mortality in patients receiving dialytic therapy [1]. Recent autopsy series have demonstrated that 90% of patients on hemodialysis (HD) for over five years had pathologic evidence of the disease [2, 3]. The disease initially manifests with symptoms and findings localized to joints: carpal tunnel syndrome, bone cysts near joints, synovial hypertrophy, and spondyloarthropathy [1, 4]. Lesions in the shafts of long bones are not observed, implying that structures in the joint such as synovium are important for amyloid formation. Pathologic analysis reveals the earliest sites of deposition to be the surface of articular cartilage, followed by the subintima of the synovial membrane and collagenous connective tissue of the joint capsule. With increasing time, an erosive arthropathy develops: loss of articular cartilage, fibrous and fibrocartilaginous replacement of the articular surface, and subchondral bone sclerosis [5, 6]. On microscopic analysis, β₂m is the major constituent of the amyloid fibrils, although recent data have shown that some of the β₂m is altered with advanced glycation end products (AGEs) [7, 8].

Advanced glycation end-products are a heterogeneous group of proteins that have been altered with glucose or carbohydrate adducts. The alteration of proteins with AGE is increased in diabetes, renal failure, and aging and is hypothesized to account for a variety of disease states [reviewed in 9]. In vitro studies have demonstrated that AGE-β₂m stimulates the chemotaxis of macrophage/microcytes preferentially over non-AGE-β₂m, with subsequent release of cytokines [7, 8]. In contrast, AGE-β₂m inhibited collagen synthesis in skin fibroblasts, mediated by the receptor for AGE (RAGE) [10]. Autopsy studies of dialysis patients have demonstrated the presence of AGE-β₂m only late in the disease course, indicating that AGE alteration of β₂m is likely to occur in situ [11]. Thus, the exact role of AGE-β₂m in β₂m amyloidosis remains unknown, but it may be important in late inflammatory reactions.

The articular predilection of β₂m amyloidosis suggests a specific interaction of β₂m with articular structures. β₂m freely fluxes into the synovial fluid, such that synovial fluid levels of β₂m in dialysis patients are very ele-
vated, even during the first few years of dialysis [12, 13]. The synovial lining is composed, based on morphology and function, of two cell types: macrophage-like (type A) and fibroblast-like (type B) [14]. Macrophage-like synoviocytes have been shown to produce cytokines, especially interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). Fibroblast-like synoviocytes produce matrix proteins and metalloproteinases, both of which have been colocalized to βm amyloid deposits [15–17]. Metalloproteinases are zinc-dependent enzymes that degrade matrix proteins. Interstitial collagenase, or metalloproteinase-1 (MMP-1), degrades the interstitial collagen at neutral pH, with resultant destruction of articular cartilage and subchondral bone. The actions of MMP-1 are inhibited, in part, by the naturally occurring inhibitor, tissue inhibitor of metalloproteinase-1 (TIMP-1). There is increased expression of both MMP-1 and, to a lesser extent, TIMP-1 in the synovium of patients with osteoarthritis (OA) and rheumatoid arthritis [18, 19]. The importance of MMP in arthritis is supported by studies showing that inhibition of MMPs results in an improvement in joint pathology and inflammation [20, 21]. Because of the importance of MMPs in subsequent joint destruction, some investigators feel that the synovial fibroblast, which produces most of the MMPs, may be the initial cell activated in rheumatoid arthritis [14, 22]. Interestingly, there is some similarity between the adjacent joint destruction observed in rheumatoid arthritis and βm amyloidosis, suggesting a similar pathophysiologic mechanism of joint destruction, despite different inciting events.

We therefore hypothesized that the synovial fibroblast may be an important cell in the early pathogenesis of βm amyloidosis. The synovial fibroblast is exposed to high levels of βm in the synovial fluid and produces all of the proteins (such as glycosaminoglycans) present in amyloid deposits. Further supporting a role of the synovial fibroblast in the early pathogenesis of βm amyloid is that Nakashima et al were able to generate intact βm amyloid in tissue cultures of human synovium (predominantly fibroblasts) incubated for three months in a collagen gel matrix [23]. To test our hypothesis and examine the role of AGE alteration of βm, we determined the effect of physiologic concentrations of βm and AGE-βm on synovial fibroblast expression of MMP-1 and its inhibitor TIMP-1.

**METHODS**

**Isolation and purification of βm and AGE-βm**

Ultrafiltrate from a chronic (25 years) HD patient with documented βm amyloidosis was collected during hemofiltration with an F-80 polysulfone hollow fiber dialyzer (Fresenius, Ogden, UT, USA). The ultrafiltrate was then concentrated and desalted through a regenerated cellulose cartridge (Millipore, Bedford, MA, USA), eliminating solutes with a molecular weight of less than 1 kD. The filtrate was separated with isoelectric focusing using five to seven ampholytes (Bio-Rad, Richmond, CA, USA). Some fractions containing βm were eluted through an anion exchange column (Econo Q Cartridge; Bio-Rad) with a NaCl step gradient. The pure βm, separated into nonglycated and endogenous early glycated βm (AGE-βm) fractions by affinity chromatography on m-boronate columns normally used for the detection of glycated hemoglobin, through an adaptation of the methods of Silver et al [24], using a 200 mmol/L glycine buffer, pH 8.5. The resultant glycated βm (gβm; less than 0.1% of total) was removed, and the remaining nonglycated βm (ngβm) was separated into three fractions. The first was incubated in the presence of glycating buffer [1 × phosphate-buffered saline (PBS), 200 mmol/L D-glucose, 1.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L ethylenediaminetetraacetic acid (EDTA)] at 37°C for 60 days and represents gβm. The second was similarly incubated but without the glucose to serve as a control for the long incubation, and is termed ngβm. The last fraction was neither incubated nor glycated, and is termed fresh βm (fβm). All three fractions were then run on a Toxigel column (Pierce, Rockford, IL, USA) to remove any potential contamination from lipopolysaccharide (LPS). For comparison, samples from each isolation step were run on a 10 to 20% SDS-polyacrylamide gradient gel and then electrophoretically transferred to a nitrocellulose membrane for Western blot analysis with polyclonal rabbit antihuman βm IgG (Boehringer Mannheim, Indianapolis, IN, USA) or a monoclonal mouse anti-AGE antibody (Alteon, Inc., Ramsey, NJ, USA).

**Isolation of human synovial fibroblasts**

Human synovium was obtained from patients undergoing total knee or hip replacement for osteoarthritis. Synovial tissue was minced and incubated in collagenase (Wako, Richmond, VA, USA), followed by trypsin. The supernatant was collected. Collagenase activity was quenched, and cells were pelleted and placed in a T-25 flask in Dulbecco’s modified Eagle’s medium (DMEM) with 20% fetal bovine serum (FBS) [25]. To characterize the cells further, histochemistry for uridine diphosphoglucose dehydrogenase activity (UDPGH) and immunofluorescence for markers specific to fibroblast [vascular cell adhesion molecule-1 (VCAM-1)] and macrophage (CD68) were done at the first and third passes.

Uridine diphosphoglucose dehydrogenase activity is
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an enzyme that is necessary for the production of proteoglycans and is increased several-fold in synovial fibroblasts compared with other fibroblasts [26]. Slides were incubated for 30 minutes in 3 mg/mL UDPG, 0.3 mg/mL β-nicotinamide adenine dinucleotide (NAD), 0.05 mol/L glycylglycine buffer, 3 mg/mL nitro blue tetrazolium (NBT), in 30% wt/vol polyvinyl alcohol, pH 7.8, and were saturated with nitrogen. Negative controls were run simultaneously by omission of NBT [26]. Immunofluorescence was done by fixing cells with 4% paraformaldehyde, washed in phosphate-buffered saline, Triton X-100 extracted and blocked with 2% defatted bovine serum albumin. Goat polyclonal antihuman VCAM-1 (R&D Systems, Minneapolis, MN, USA) and mouse monoclonal antihuman CD68 (Dako Corp., Carpinteria, CA, USA) were used as the primary antibodies (1:100 for 2 h at 37°C) as markers for fibroblast and macrophage activity, respectively [14]. After washing, secondary antibodies (Texas Red-conjugated donkey antigoat and FITC-conjugated sheep antimouse; both from Jackson ImmunoResearch Labs, West Grove, PA, USA) were added at 1:200 and incubated for an additional 45 minutes at 37°C. Cells were washed. Nuclei were stained with To-Pro-3 stain (Molecular Probes, Eugene, OR, USA). The cells were visualized with a Bio-Rad MRC-1024 laser scanning confocal microscopy with Kr/Ar laser, and images were processed on a metamorph image analysis system.

For some experiments, cells were isolated from human foreskin (the kind gift of Dr. Dan Spandau, Indiana University Department of Dermatology, Indianapolis, IN, USA) or from patients with inflammatory arthritis (systemic lupus erythematosus or rheumatoid arthritis) and compared with the osteoarthritis (OA) cells. Finally, cells were incubated in the presence and absence of doxycycline (Sigma) to determine whether this anti-arthritis medication inhibits βm-induced MMP-1 release.

**Measurement of MMP-1 and TIMP-1**

Human synovial fibroblast-like cells isolated from each patient were plated at 5 × 10⁴ cells per well in a 24-well plate and grown in DMEM, with 20% FBS for three days until confluent at 37°C and 5% CO₂. The media were then changed to DMEM with 3% FBS for 18 hours. The cells were then incubated in the same media in the presence and absence of 1, 10 and 25 μg/mL fβm, gβm, and ngβm for 24 or 48 hours. These concentrations were chosen to simulate actual synovial fluid concentrations of βm in normal individuals, patients with severe rheumatoid arthritis, and dialysis patients, respectively [12, 13]. MMP-1 and TIMP-1 were measured by two-site enzyme-linked immunosorbent assay (ELISA; Biotrak, Inc., Arlington Heights, IL, USA) in the culture supernatant. The MMP-1 assay recognizes total human MMP-1, including pro-MMP-1, free MMP-1, and that complexed to specific inhibitors such as TIMP-1. However, it does not recognize MMP-1 bound to nonspecific inhibitors such as α-2-macroglobulin, and there is no cross-reactivity with MMP-3, MMP-5, or MMP-9. The TIMP-1 assay recognizes total TIMP-1, including free TIMP-1 and that bound to MMP-1. It does not cross-react with TIMP-2.

**Northern blot analysis**

Human foreskin fibroblasts were incubated in the presence or absence of phorbol 12-myristate 13-acetate (PMA; 10⁻⁸ mol/L), and fresh gβm and ngβm at 10 μg/mL for 12, 24, and 48 hours. RNA was isolated with TRIzol Reagent (Gibco BRL, Grand Island, NY, USA) per an adaptation of the methods of Chomczynski and Sacchi [27]. Twenty micrograms of total RNA were run on each lane of an agarose/formaldehyde gel, and the integrity was confirmed by visualizing 18S and 28S ribosomal bands with ultraviolet light. The RNA was transferred to nitrocellulose membranes (Nitrobind, MSI, MA, USA) and hybridized under high stringency conditions to radiolabeled cDNA probe for human MMP-1 (kind gift of Dr. Connie Brinckerhoff, Dartmouth University) in 50% formamide, 5 × Denhardt’s solution, 5 × SSPE, and salmon sperm DNA (200 μg/mL) at 42°C overnight. The cDNA probe was labeled with ³²P by random priming using Promega Prime-A-Gene Labeling System. After hybridization, the membranes were washed twice with 2 × standard saline citrate (SSC) and 0.1% SDS at room temperature for 30 minutes each, and twice with 0.1 × SSC and 0.1% SDS at 50°C for 15 minutes each. The membranes were exposed to an x-ray film (Hyperfilm; Amersham, Arlington Heights, IL, USA) for different times. The autoradiographs were scanned, and densitometric analysis was performed using metatoward software (Universal Imaging Corporation). The membranes were stripped and reprobed with GAPDH to compare loading.

**Statistical analysis**

A mixed-effects analysis of variance for a randomized complete block design model was used to test the effects of βm and the βm concentration and their interaction. Since the fibroblast samples were obtained from several different patient subjects for each experiment, the subject was included in the model as a random effect. To control for differences among the subjects, MMP-1 and TIMP-1 results were expressed as the percentage of the mean result of the control run for each subject (an example of patient to patient variation is in Fig. 7). For each experiment presented, the results were combined from data obtained from two to four different patients, with three to six per group for each of the two to four different patients. Pair-wise comparisons among the effect levels were adjusted using Tukey’s procedure for multiple com-
parisons. For the MMP-1/TIMP-1 ratio, results were compared with control or PMA using one-way analysis of variance (ANOVA) with Fisher’s test for pair-wise comparisons. The analyses were done using SPSS software (Chicago, IL, USA) and Statview (Abacus Concepts, Inc., Berkeley, CA, USA). All results are expressed as mean ± SEM. An overall significance level of 0.05 was used to test all hypotheses.

RESULTS
Isolation and purification of β₂m

The results of the SDS-PAGE and immunoblotting of the β₂m, gβ₂m, and ngβ₂m purified from human serum ultrafiltrate as described previously in this article are shown in Figure 1. By SDS-PAGE, the gβ₂m had a slightly higher molecular weight compared with fβ₂m and ngβ₂m because of the formation of glucose adducts. All three types of β₂m reacted with the anti-human β₂m, but only the glycated reacted with the anti-AGE Ab (Fig. 1). In addition to these results, the gβ₂m, but not the ngβ₂m or fβ₂m, fluoresced at 400 nm with excitation at 325 nm, typical for glycated proteins (data not shown) [9]. In addition to its apparent purity by SDS-PAGE, subsequent Western blots with appropriate controls failed to demonstrate any contamination of the β₂m with IL-1β, IL-6, or TNF-α. For comparison purposes, β₂m purified from human urine (Cortex Biochemicals, San Leandro, CA, USA) was also used and glycated as discussed previously in this article. The two sources of β₂m had a similar appearance on SDS-PAGE (Fig. 1). Furthermore, there was no difference in the results obtained from the β₂m purified from plasma ultrafiltrate or that obtained from urine for MMP-1 release, TIMP-1 release, or Northern analysis. Thus, the results were pooled, but the β₂m isolated from ultrafiltrate accounted for greater than 95% of the results for each experiment presented in this manuscript.

Isolation and characterization of primary human synovial fibroblasts

Human synovial fibroblasts were isolated from OA synovium. By immunofluorescent microscopy, the localization of the antibodies were characteristic: CD68 localized to the cytoplasm characteristic of macrophage-like cells and VCAM-1 to dendritic processes of fibroblast like cells. At pass one, approximately 80% of cells were characteristic of macrophage-like cells and 20% characteristic of fibroblast-like cells. As passages continued, the proportion of fibroblasts increased to 80 to 90% by pass 3 (Fig. 2). Supporting these immunohistochemical findings were the results of the UDPGH enzyme assay: Very few cells stained positive at first pass, whereas almost all cells were positive by the third pass (data not shown). As a final test of cell purity, fourth-pass synovial cells were examined by flow cytometry using the monocyte/macrophage marker CD14. No CD14 expression was found, confirming pure fibroblast cultures from fourth pass on.

As a result of these findings, all subsequent experiments were done with fourth- to seventh-pass cells to ensure pure fibroblast cell populations.

MMP-1 and TIMP-1 protein expression

Human synovial fibroblasts from four different OA patients incubated for 24 hours in media with β₂m at 1 μg/mL had no effect on the release of MMP-1 compared with control (control = 100%; fβ₂m, 114.2 ± 9.2; gβ₂m, 126.8 ± 8.7; ngβ₂m, 115.3 ± 9.2; P = NS). In contrast, both 10 and 25 μg/mL of β₂m induced a significant increase in the release of MMP-1 at 24 hours compared with control (P < 0.01; Fig. 3). The release of MMP-1 at 25 μg/mL was slightly greater than that observed at 10 μg/mL, although this did not reach statistical significance. There was no difference between fβ₂m, gβ₂m, and ngβ₂m at any concentration (at 10 μg/mL: fβ₂m, 183.2 ± 13.8; gβ₂m, 170.2 ± 19.7; ngβ₂m, 163.8 ± 18.6% control; at 25 μg/mL: fβ₂m, 211.3 ± 18.4; gβ₂m, 196.6 ± 20.3; ngβ₂m, 190.7 ± 32.8% control; Fig. 3). PMA, a known potent stimulator of MMP-1, was used as a positive control for each experiment and increased the release of MMP-1 to a similar magnitude as 25 μg/mL of β₂m (PMA = 243.6 ± 16.1; Fig. 3).

There was no difference in the release of TIMP-1 from synovial fibroblasts in the presence or absence of β₂m at any concentration nor any difference among β₂m, gβ₂m, and ngβ₂m at equivalent concentrations. However, there was a significant release of TIMP-1 in synovial fibroblasts incubated in the presence of 10⁻⁴ mol/L PMA compared with control (Fig. 4). As a result, compared with control, the ratio of MMP-1/TIMP-1 was significantly increased at both 10 and 25 μg/mL of β₂m (Fig. 5), but not in the presence of PMA.

At 48 hours, the type of β₂m, the concentration of β₂m, and their interaction had a significant effect on MMP-1 release. Post hoc analyses demonstrated that the incubation of synovial fibroblasts from OA patients in the presence or absence of 10 μg/mL fβ₂m and ngβ₂m similarly increased the release of MMP-1, but there was no effect observed in the presence of gβ₂m (10 μg/mL: fβ₂m, 123.6 ± 5.6; gβ₂m, 99.7 ± 5.3; ngβ₂m, 126.5 ± 4.5% control; P < 0.02, fβ₂m and ngβ₂m compared with control and gβ₂m). There was a similar, but greater response observed at 25 μg/mL (fβ₂m, 153.8 ± 5.4; gβ₂m, 98.1 ± 8.3; ngβ₂m, 149.9 ± 9.5% control, P < 0.001, fβ₂m and ngβ₂m compared with control and gβ₂m; Fig. 6). Thus, there was a similar dose response to fβ₂m and ngβ₂m at 24 and 48 hours. However, gβ₂m only elicited a release of MMP-1 at 24 hours. Similar to the results observed at 24 hours, there was no change in the release of TIMP-1.
Fig. 1. Isolation and purification of β₂-microglobulin (β₂m) from human serum ultrafiltrate. Ultrafiltrate was collected from a long-standing dialysis patient, and β₂m was purified as detailed in the text. The pure β₂m was then left unaltered (fβ₂m), incubated for 60 days at 37°C in the presence of 200 mmol/L d-glucose (gβ₂m) or incubated for 60 days at 37°C in the absence of glucose as a control for the incubation process (ngβ₂m). The three proteins were then loaded at 20 μg/lane and run on a 15% SDS-PAGE and transferred to nitrocellulose and incubated with anti-β₂m and anti-AGE antibodies. As positive controls, fresh, glycated, and nonglycated bovine serum albumin (fBSA, gBSA, ngBSA), which were similarly prepared, were run for comparison purposes. In addition, β₂m isolated from urine (Cortex, Inc.) was also run for comparison of purity. The resultant Coomassie stained gel and Western blots are shown (A–C). All three forms of the β₂m isolated from ultrafiltrate were detectable by the anti-β₂m antibody, and the glycated β₂m showed the expected slight increase in molecular weight. In contrast, only the gβ₂m and gBSA were detected by the anti-AGE antibody.
Fig. 2. Isolation of human synovial fibroblasts. Human synovium obtained from operative specimens were minced and digested, and the resultant cells were cultured. The cells were then immunostained with anti-human CD68 as a marker for monocyte/macrophage-like cells or anti-human VCAM-1 as a marker for fibroblast-like cells. Controls indicate immunostaining without the primary antibodies. The results demonstrate the predominance of monocyte-like cells in first passage and fibroblast-like cells by third pass. (Reproduction of this figure in color was made possible by Amgen, Inc., Thousand Oaks, CA, USA).

at 48 hours in the presence of fβ2m, gβ2m, or ngβ2m at any concentration, leading to an increase in the MMP-1/TIMP-1 ratio for both 10 and 25 μg/mL of fβ2m and ngβ2m, but not gβ2m (data not shown).

Comparison of fibroblasts from different sources

To determine whether the underlying disease of patients from whom synovial fibroblasts were isolated affected the response to 10 μg/mL β2m, synovial fibroblasts isolated from patients with various surgical diagnoses were compared with each other and with primary skin fibroblasts. The basal release of MMP-1 was different depending on the underlying cell source or disease state (Fig. 7). The greatest basal release was seen in a patient with rheumatoid arthritis, although one patient with a diagnosis of OA also had elevated basal release of MMP-1. Thus, the basal release may be more of a factor of severity of arthritic changes, rather than of the actual diagnosis. Despite the variation in basal release, these results demonstrate that β2m has a similar effect on the release of MMP-1 from all types of human synovial and skin fibroblasts (P < 0.02 for each patient, except the


Effect of β₂m on mRNA expression for MMP-1

To determine whether the β₂m-induced increase in MMP-1 protein release was due to an increase in mRNA, Northern blot analysis was performed on human primary skin fibroblasts. The skin fibroblasts were used because of the ability to grow faster and thus obtain more RNA. The results of a representative experiment (Fig. 8) indicate that fβ₂m, ggβ₂m, and ngβ₂m increased the expression of MMP-1 at 12, 24, and 48 hours compared with control. The magnitude of increased expression, when calculated as the MMP-1/GAPDH ratio, was less for glycated as compared with fresh and nonglycated at all time points. PMA also induced a large increase in the mRNA for MMP-1 that was much greater than that observed for β₂m. Thus, PMA induced a more potent increase in

dialysis patient, by ANOVA). In addition, in experiments with skin fibroblasts and synovial fibroblasts from the systemic lupus erythromatosus (SLE) patient and OA patient 3, there was also a difference between β₂m and ggβ₂m despite an incubation period of only 24 hours. One dialysis patient with known β₂m amyloid disease underwent a hip-replacement surgery for a pathologic fracture. The results of cells from this HD patient are also shown. These cells were difficult and slow to grow, and attempts at culturing cells from two other dialysis patients were unsuccessful. Despite these difficulties, there was a trend toward a response to β₂m in the synovial fibroblasts isolated from a HD patient.

Fig. 3. Release of MMP-1 at 24 hours. Human osteoarthritic synovial fibroblasts were incubated for 24 hours in the presence of fresh, glycated, and nonglycated β₂m at 1, 10, and 25 μg/mL, or the known MMP-1 stimulator PMA (10⁻⁸ mol/L), and the concentration of MMP-1 release into the culture supernatant was measured. β₂m induced an increase in MMP-1 release at 10 and 25 μg/mL that was unaffected by glycation. The MMP-1 released by 25 μg/mL of β₂m was similar to that released from PMA by t-test. Symbols are: *, compared with control; +, compared with 1 μg/mL of same group (fresh, glycated, or nonglycated β₂m), both P < 0.05; N = 9 to 15 per group pooled from three to four different patients. The dotted line represents values for control (no β₂m).

Fig. 4. Release of TIMP-1 at 24 hours. Human osteoarthritic synovial fibroblasts were incubated for 24 hours in the presence of fresh, glycated, and nonglycated β₂m at 1, 10, and 25 μg/mL, or the known TIMP-1 stimulator PMA (10⁻⁸ mol/L), and the concentration of TIMP-1 release into the culture supernatant was measured. There was no difference in the release of TIMP-1 in the presence or absence of β₂m at any concentration, nor any difference among the types of β₂m at equivalent concentrations. However, PMA induced a large increase in TIMP-1 release (P < 0.05 compared with control by t-test). The results are from three different patients with a combined N = 9 to 15 per group. The dotted line represents values for control (no β₂m).

Fig. 5. MMP-1/TIMP-1 ratio at 24 hours. Human osteoarthritic synovial fibroblasts were incubated for 24 hours in the presence of fresh, glycated, and nonglycated β₂m at 1, 10, and 25 μg/mL, or the known MMP-1/TIMP-1 stimulator PMA (10⁻⁸ mol/L) and the concentration of MMP-1 and TIMP-1 release into the culture supernatant was measured. The ratio was calculated using the actual measurements of MMP-1 and TIMP-1. As a result of the discordant release of MMP-1 and TIMP-1, the ratio of MMP-1/TIMP-1 was increased at 10 and 25 μg/mL of β₂m, but was unaltered in the presence of PMA. These results indicate that β₂m induces collagenolysis from human synovial fibroblasts. Symbols are: *, compared with control; +, compared with 1 μg/mL of same group (fresh, glycated, or nonglycated β₂m), both P < 0.05; N = 9 to 15 per group pooled from 3 to 4 different patients.
mRNA MMP-1 expression, but a similar increase in MMP-1 protein expression. The latter is likely due to concomitant increase in TIMP-1 protein, which may degrade some of the released MMP-1. Northern blot analysis was repeated a total of two to five times at each time point, with similar results.

Effect of doxycycline on \( \beta_m \)-induced MMP-1 release

Tetracyclines inhibit MMP-1 activity through unclear post-translational effects and have proved useful for the treatment of rheumatoid and OA [20, 21]. To determine whether doxycycline can inhibit the release of MMP-1 induced by 10 \( \mu \)g/mL \( \beta_m \), we incubated OA synovial fibroblasts in the presence of increasing concentrations of doxycycline for 24 hours. As previously demonstrated, \( \beta_m \) increased the expression of MMP-1 over basal (control) values (Fig. 9). Again, there was no difference between the effects observed in the presence of \( \beta_m \), \( \beta_{g\beta_m} \), and \( ng\beta_m \). Doxycycline inhibited this release in a dose-dependent manner, regardless of the type of \( \beta_m \) (\( P < 0.001 \)).

DISCUSSION

The results of this study demonstrate that \( \beta_m \), in physiologically relevant concentrations, induces the release of MMP-1 protein and gene expression. However, there is not a concomitant increase in its naturally occurring inhibitor, TIMP-1. Metalloproteinases are known to enhance the degradation of bone and cartilage [28, 29], and are thus believed to be one of the main etiologic factors of bone destruction in rheumatoid arthritis [30, 31]. Synovial fluid analysis from patients with rheumatoid arthritis reveals that the ratio of MMP-1/TIMP-1 is elevated and that the ratio correlated with collagenolytic activity in vitro assays [31]. In cultured synovial explants taken from rabbits with proliferative arthritis, the induction of the lesion correlated with no reduction in the synthesis of TIMP-1 but an increase in the synthesis of MMP-1 [32]. In \( \beta_m \) amyloid deposits, there is erosion of cartilage, joint capsule, and bone with increased immunostaining for proteinase inhibitors [5, 6], implying increased collagenolytic/ proteolytic activity. Ohashi et al. examined metalloproteinase immunostaining in dialysis patients’ \( \beta_m \) amyloid deposits and found that MMP-1 was the most abundantly expressed metalloproteinase. By comparison, there was little expression of TIMP-1 [16]. These in vivo findings support our in vitro findings of increased MMP-1/TIMP-1 protein ratio from human synovial fibroblasts in response to \( \beta_m \). Thus, in dialysis patients, \( \beta_m \) may stimulate the release of MMP-1 from synovial fibroblasts, saturating the existing inhibitor TIMP-1. This uncontrolled collagenolysis may be at least partly responsible for the bone cysts and erosions observed in \( \beta_m \) amyloidosis.

Our results in human synovial fibroblasts expand on earlier work by Brinkerhoff et al. in rabbit synovial fibroblasts treated with phorbol esters [33]. These studies found an increase in collagenase transcription and synthesis in response to phorbol esters and the production
of two proteins: one 80% homologous to human β2m and
the other 60% homologous to human serum amyloid A (SAA) protein. These latter two proteins functioned as
autocrine factors, further stimulating the release of MMP-1 from rabbit fibroblasts [33]. Human β2m and human
SAA also stimulated the release of MMP-1 from the
rabbit fibroblasts [33]. Similar to our findings of increased
MMP-1, Migita et al found increased MMP-3 (stromely-
sin) without increased TIMP-1 in human synovial fibro-
blasts incubated with β2m. However, they were unable
to find increased MMP-2 (gelatinase) release, which may,
in part, be due to the low concentrations of β2m used (1
to 5 μg/mL) [34].

Synovial fibroblasts secrete MMP-1 in response to a
number of stimuli such as IL-1 and phorbol esters (PMA)
[35–37]. The tissue specificity of MMP-1 gene activation
in response to these cytokines and PMA involves the
interaction of activator protein-1 (AP-1) with other cis-
acting sequences in the promoter and with transcription
factors, c-fos and c-jun, that bind these sequences [38, 39].
MMP-1 gene expression is regulated at multiple points
that require both transcriptional and post-transcriptional
mechanisms [39]. In addition, some cytokines also increase the stability (half-life) of the MMP-1 transcripts [40]. The mechanism by which βm induces MMP-1 is likely equally complex, with the potential differences between βm and gβm further complicating the issue.

The mechanism of uptake of βm in fibroblasts is unknown, but uptake in the proximal tubule and hematopoietic cells is mediated through endocytosis [41]. In contrast, proteins altered with AGE are taken up by the receptor for AGE (RAGE) and appear to activate nuclear factor-κB [42]. Owen et al found that skin fibroblasts had a decrease in collagen production in response occurs in nonarticular tissues late in the disease course. In conclusion, we have demonstrated that AGE-

Fig. 9. Human OA synovial fibroblasts were incubated for 24 hours in the presence of 10 μg/mL of fresh, glycated, or nonglycated βm at 10 μg/mL, with and without increasing concentrations of the inhibitor of MMP-1, doxycycline (doxy). The concentration of MMP-1 release into the culture supernatant was measured. βm without doxycycline induced a significant release of MMP-1 over basal (control = no βm) values with no difference among the type of βm. Doxycycline inhibited the βm induced release of MMP-1 in a dose-dependent manner (P < 0.01). This inhibition was observed irrespective of whether the βm was fresh, glycated, or nonglycated. Symbols are: *, compared with without doxycycline for same type of βm. N = 7 to 8 per group for 2 patients. The dotted line represents values for control (no βm or doxycycline).

deue MMP-1 in response to βm. However, we also found that skin fibroblasts release MMP-1 in response to βm.

Why then are articular manifestations more common in βm amyloidosis? One potential explanation is that βm is trapped in the synovial space, exposing synovial cells to high concentrations of βm. The daily production rate of βm is approximately 230 mg/day, and unfortunately, attempts at removal of βm with high-flux dialysis cannot keep up with the production rate [43]. Furthermore, no significant extrarenal sites of catabolism are known. Given this positive mass balance, approximately 2100 g of βm is retained over 25 years [43], and thus, serum levels of βm should rise substantially with years on dialysis. However, serum βm levels do not rise with time on dialysis and do not correlate with disease activity [1, 4, 43, 44]. Therefore, βm must accumulate over time in extravascular compartments, such as the synovial space. The synovial space is separated from the extravascular space by a simple membrane that allows free flux of fluid into the synovial space. Synovial fluid levels of βm in dialysis patients are 25 to 50 μg/mL and 5 to 10 μg/mL in rheumatoid arthritis patients [12, 13], concentrations stimulating MMP-1 in the present study. Some of the βm may become trapped in the synovium because of the binding of βm to collagen [17] and hyaluronan (personal observations). Thus, early manifestations may be due to this “trapping” of βm, with release of MMP-1 (as in the present study), and release of IL-1 [45, 46], both leading to bone destruction. In addition, we hypothesize that similar to rheumatoid arthritis, the stimulation of synovial fibroblasts by βm may also induce expression of adhesion molecules that may attract macrophages to the joint. The presence of macrophages, observed histologically late in the disease course, may augment this cascade, leading to even more cytokine release and bone destruction. Clearly, further work is required to understand how βm amyloid deposition occurs, the precise sequence of events, and why it occurs in nonarticular tissues late in the disease course.

Common therapies for articular diseases, such as methotrexate and corticosteroids, are known to inhibit the release of MMP-1 [30]. Of note is the dramatic improvement of clinical symptoms in βm amyloidosis observed immediately post-renal transplant, perhaps in response to steroids [47]. One other therapy that has gained recent recognition is tetracyclines. These agents block the release of MMP-1 by unknown mechanisms. However, therapy with doxycycline in dogs [20] and minocycline in humans [21] has proved effective in not only reducing joint erosions, but in improving symptoms as well. We have demonstrated that doxycycline can inhibit the release of MMP-1 induced by βm. Whether this exciting finding has clinical implications in patients suffering from βm amyloidosis remains to be seen.

In conclusion, we have demonstrated that βm induces collagenolytic activity in human synovial fibroblasts. We

Our studies demonstrate that synovial fibroblasts pro-
propose that this effect of β₂m may be key in the tissue destruction observed adjacent to β₂m amyloid deposits.

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