Degradation of the extracellular matrix (ECM) is a prominent feature in osteoarthritis (OA), which is mainly because of the imbalance between anabolic and catabolic processes in chondrocytes resulting in cartilage and bone destruction. Various proteases act in concert to degrade matrix components, e.g. type II collagen, MMPs, ADAMTS, and cathepsins. Protease-generated collagen fragments may foster the destructive process. However, the signaling pathways associated with the action of collagen fragments on chondrocytes have not been clearly defined. The present data demonstrate that the N-terminal telopeptide of collagen type II enhances expression of cathepsins B, K, and L in articular chondrocytes at mRNA, protein, and activity levels, mediated at least in part through extracellular calcium. We also demonstrate that the induction is associated with the activation of protein kinase C and p38 MAP kinase.

The participation of proteolytic enzymes in the pathogenesis of osteoarthritis was suggested already 30 years ago (1). The key event in arthritic lesions is the degradation of cartilage matrix proteins, predominantly consisting of collagen type II and aggrecan. Therefore, the regulation of matrix-degrading protease activities in resting and in activated chondrocytes has been extensively studied during the past two decades. Matrix metalloproteinases (MMPs) and ADAMTS 4 and 5 have been reported to be mainly responsible for matrix destruction in degenerative joint diseases (2–6). However, cysteine proteases may also play an essential role in this catabolic process (5–8). Among them cathepsin K (CK) is the only protease cleaving native triple-helical collagen (9). It is mainly expressed in osteoclasts (10–12) but occurs also in activated chondrocytes (5, 13–15) and in synovial fibroblasts of patients suffering from rheumatoid arthritis (16). Konttinen et al. (15) detected the pH in the immediate surrounding of degraded cartilage areas to be between pH 6.2 and 5.5. This milieu is acidic enough for autocatalytic activation of procathepsin K released from activated cells. In the presence of chondroitin-4-sulfate active CK may then efficiently attack triple-helical collagen (9). Because osteoarthritis is a chronic disease, the question remains for the reason of the persistence of degradative processes outside of inflammatory episodes, the so-called “chondrocytic chondrolysis” (17). This concept was further evolved implicating matrix autolysis products into the process of constant hyperactivation of chondrocytic matrix degrading enzymes (18–21).

Degradation products of collagen type II are secreted into the urine and into the synovial fluid. Collagen-derived peptides, mainly the C-terminal telopeptide, are used as diagnostic tools to follow progressive cartilage breakdown (22–26). In this context it is important to note that not only inflammatory reactions but also mechanical injuries may lead to an enhanced release of such peptides (27). According to literature data, about 1–3% of the total collagen dry mass in normal cartilage occurs as partially degraded collagen peptides (21, 28, 29) whereas this percentage is much larger in OA cartilage, namely up to 6% (28) or even 20% (29). Those peptides are not only diagnostic markers of cartilage degradation but may also initiate a cascade of matrix-degrading proteolytic activities (20, 21). Fichter et al. have shown levels of MMPs-2, -3, -9, and -13 mRNA, and protein to be elevated after stimulation by collagen degradation products and, in particular, by a sequence from the N-terminal telopeptide of collagen type II (Ntelo). Thus, collagen peptides may contribute to the regulation of matrix turnover (20). It has been shown that pro-inflammatory cytokines like interleukin-1 (IL-1) and tumor necrosis factor α (TNFα) contribute substan-
tially to the liberation of matrix-degrading enzymes from OA chondrocytes (30, 31). Whereas the biological effects initiated by these pro-inflammatory cytokines are mediated by MAP kinases (p38 and JNK) and nuclear factor κB (NF-κB) (32), little is known of the signaling cascade triggered by collagen type II degradation products. In this report, we show that besides MMPs the biosynthesis of some cysteine proteases may also be selectively stimulated by the N-terminal telopeptide of collagen type II. Moreover, our findings also indicate that at least one member of the protein kinase C family and the p38 MAP kinase are involved in Ntelo-dependent signaling events in articular chondrocytes.

**EXPERIMENTAL PROCEDURES**

**Materials**—The substrates Z-Arg-4-methoxy-β-naphthylamide (Z-RR-4-MβNA), Z-Phe-Arg-4-methoxy-β-naphthylamide (Z-FF-4-MβNA), Z-Gly-Pro-Arg-4-methoxy-β-naphthylamide (Z-GPR-4-MβNA), and the inhibitors t-trans-epoxysuccinyl-Ile-Pro-OMe propylamide (CA-074Me) and Z-Phe-Phe-diacylmet-hylketone (Z-FF-CHN₂) were purchased from BACHEM (Bubendorf, Switzerland). 5-Nitro-salicyl aldehyde (NSA) was purchased from Sigma (Buchs, Switzerland). The inhibitor benzylxoxy carbonyl-Leu-Gly nitrile (Z-LG-[CN]) was synthesized as previously described (33). A synthetic 29-mer peptide (Ntelo) was used, corresponding to the N-terminal telopeptide of human type II collagen (20). It was activated from JPT Peptide Technologies (Berlin, Germany). The activator of protein kinase C (PKC) phorbol-12,13-dibutyrate (PDBu) was purchased from Biomol (Hamburg, Germany). The pan-PKC inhibitor InSolution™ Bisindolylmaleimide I (BIM L, GF109203X, Gö 6850) was purchased from Calbiochem (Darmstadt, Germany). The p38 MAP Kinase inhibitor SB203580 was purchased from Jena Bioscience GmbH (INH-007; Jena, Germany).

**Cells**—Articular cartilage was harvested from human knee joints and the tissue samples were used with the patient’s informed consent and institutional approval (University of Jena Medical Faculty Ethics Committee approval 1772-04/06). Human cartilage (normal looking tissue) was obtained from patients undergoing total knee replacement surgery and was collected from tibial and femoral surfaces of the knee joints. Chondrocytes were extracted from tissue by sequential pronase/collagenase digestion (34).

**Treatment of Cells**—For stimulation experiments, chondrocytes were cultured in DMEM (containing 1 mM CaCl₂, Sigma) plus 5% FCS for 24 h in 96-well microtiter plates at a density of 10⁵ cells per well. Subsequently, chondrocytes were cultured in DMEM plus 1% FCS in the presence of varying concentrations (0; 0.2; 0.4; 0.6; 0.8; 1; 4 mg/ml) of the synthetic 29-mer N-telopeptide of type II collagen (Ntelo) for 24 or 72 h. The culture media were collected and analyzed for released cathepsins by immunoblotting, mRNA levels of cathepsins were measured by Real-Time PCR, and the enzyme activities were measured by an in vivo cathepsin activity assay (35). To assess the effect of extracellular Ca²⁺ on the Ntelo effect, chondrocytes were cultured with essentially calcium-free DMEM (US Biological, Swampscott, MA) or the same medium containing 1 mM CaCl₂.

For the experiments with the pan PKC inhibitor, BIM I, human OA chondrocytes were preincubated in the absence or presence of BIM I (240 nm) for 1 h. Thereafter, the cells were stimulated with PDBu (100 ng/ml) or Ntelo (0.5 mg/ml) for different periods of time. The cathepsin activities were measured as described below.

For inhibition of the p38 MAP kinase pathway, chondrocytes were precultured in DMEM plus 5% FCS for 24 h. Subsequently, chondrocytes were cultured in DMEM plus 1% FCS in the presence of Ntelo (0.5 mg/ml, 24 h) and the p38 MAP kinase inhibitor SB203580 (1 µM, 24 h).

**cDNA Synthesis and Real-Time (RT)-PCR**—Total RNA from human chondrocytes was extracted with the Trizol® Reagent (Invitrogen) according to the supplier’s instruction. RNA was redissolved in RNase-free water. The quality of the isolated RNA was controlled using non denaturing 1.25% agarose gel electrophoresis and the determination of the A₂₆₀/A₂₈₀ ratio (SmartSpec 3000 Spectrophotometer, Bio-Rad). Only samples exhibiting no RNA degradation and showing an A₂₆₀/A₂₈₀ ratio equal or above 1.8 were used for further application. 1 µg of total RNA was used for transcription into complementary DNA (cDNA) using OmniScript Reverse Transcription kit (Quiagen), oligo-dT primer (Amersham Biosciences), and RNAseOUT (Invitrogen).

1 µl of the cDNA was used as template to specifically amplify CB, CK, and CL messages. RT-PCR was carried out using a MyiQ Cycler with the iQ SYBR Green Supermix, as described by the manufacturer (Bio-Rad). Normalization of the signal was based on the expression of the housekeeping gene β-actin. A standard curve was prepared by serial dilutions of the respective PCR products. All samples that had to be compared for expression differences were run in the same assay as duplicates together with the standards. After completion of PCR amplification, data were analyzed by MyiQ Software, version 1.0. After amplification was complete, the PCR products were analyzed by agarose gel electrophoresis. The primers used and the resulting PCR product sizes are given in the following as previously published (36).

For the experiments with the p38 MAP kinase inhibitor, BIM I, the primers were designed for MAPK13 and MAPK14, we designed specific oligonucleotide primers using the public domain Primer3 program. β-Actin (resulting in a 635-bp fragment): forward: 5'-acc aag gcc gag cgg gaa atc-3', reverse: 5'-gag ccc cag atc cac acg gag ta-3'. CB (resulting in a 539-bp fragment): forward: 5'-gca gcc tca gcc acc cag at-3', reverse: 5'-cca cca tta cag ccc tcc cca cac-3'. CK (resulting in a 514-bp fragment): forward: 5'-gtg tgg ttc ctt ggc ttc tag-3', reverse: 5'-tcc ttt gtt ccc cca gtc tcc-3'. CL (resulting in a 612-bp fragment): forward: 5'-ccg ggg agg gca gtt gac g-3', reverse: 5'-ctc tga ggc cca gag gag cat tc-3'. MAPK13 (resulting in a 243-bp fragment): forward: 5'-tcc acc cat ccc ttc gga gaa g-3', reverse: 5'-ata ctt gtc ctt ggg cag tc-3'. MAPK14 (resulting in a 241-bp fragment): forward: 5'-gcc cca gta gta aga acc ag-3', reverse: 5'-tag ggg ctt aag aga gtt ga-3'.

**Proteinase Analysis**—The human proteinase DNA microarray in the ArrayTube® format (36), representing 45 genes of proteinases and proteinase inhibitors, plus two housekeeping genes and three controls, was used to analyze the effects of extracellular calcium and/or Ntelo on proteinase gene expression in human articular chondrocytes.
Total RNA from differently stimulated human chondrocytes (calcium-free media: with or without Ntelo; calcium-containing media: with and without Ntelo) was extracted with the Trizol® reagent in accordance with the manufacturer’s manual. The following steps generating double-stranded cDNA according to the Eberwine protocol, labeling during the linear amplification reaction, and hybridization were previously described in detail (36). The peroxidase precipitation reaction (100 μl peroxidase substrate, Clondiag, Jena, Germany) was monitored by the ATR01 array tube reader (Clondiag) at 25 °C. Data analysis was carried out using IconoClust software, version 2.2 (Clondiag) determining the signal intensity and the local background value of each spot. Only the average values of spot hybridization signals with amounts above 0.05 (1 minus mean divided by local background values) were considered as positive. Both spotted oligos of an examined gene had to be “positive.” If these conditions were met, the signal resulting from the hybridization with the oligo sequence situated closer to the 3’-end of the RNA sequence was used for calculation.

**MAP Kinase Signaling Analysis**—The RT2 Profiler™ PCR Array Human MAP Kinase Signaling Pathway (SuperArray, Biomol GmbH, Hamburg, Germany), representing 84 genes related to the MAP kinase signaling pathways, plus five housekeeping genes and three controls, was used to analyze the effect of Ntelo on signaling related gene expression in human articular chondrocytes.

Total RNA from differently treated human chondrocytes (unstimulated and stimulated with Ntelo) was extracted with the Trizol® reagent in accordance with the manufacturer’s manual. The DNase treatment was performed by Amplification Grade I DNase I (AMPD1, Sigma-Aldrich) in accordance with the manufacturer’s instructions. Each total RNA preparation (5 μg) was digested with 1 μl of DNase I (1 unit/μl) and 1 μl of 10X reaction buffer in a volume of 10 μl. After incubation and adding the Stop Solution, the DNase I was denatured by incubation at 70 °C for 10 min. The RNA samples were kept on ice for another 5 min, then converted with the RT2 PCR Array First Strand Kit (SuperArray) in accordance with the manual. The template was combined with the RT2 SYBR Green/Fluorescein PCR master mix (SuperArray). Equal amounts of this mixture (25 μl) were added to each well of the RT2 Profiler™ PCR plate containing the predispensed gene-specific primer sets, and the reaction was started. Data analysis was based on the ΔΔCt method with normalization to β-actin.

**Western Blot Analysis**—For detection of procathepsins, cell culture media were separated by SDS-PAGE under reducing conditions in MES buffer using 4–12% NuPAGE Bis-Tris gradient gels (Invitrogen). After protein transfer onto nitrocellulose membranes (Invitrogen) by electroblotting, membranes were blocked with 1% soy proteins (Alpro Soja, Düsseldorf, Germany) in phosphate-buffered saline for 1 h and probed overnight with one of the following polyclonal antibodies (Santa Cruz Biotechnology): goat anti-human cathepsin B (S-12), goat anti-human cathepsin K (C-16), and goat anti-human cathepsin L (C-18) antibody, respectively, on separate blots each. Secondary antibody was rabbit anti-goat IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Detection was performed by enzyme-linked chemiluminescence (Pierce SuperSignal West Femto Maximum Sensitivity Substrate, Perbio Science, Bonn, Germany).

For detection of PKC, cell lysates were isolated with the radioimmune precipitation lysis buffer (Santa Cruz Biotechnology) as described in the manual. Protein content was determined using the Bradford reagent (Sigma). Equal amounts of protein were separated by SDS-PAGE as mentioned above and transferred onto nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin (Merck) in TBST (TBS plus Tween-20, Santa Cruz) for 1 h.

Both phosphorylated and non-phosphorylated PKC levels were detected by monoclonal rabbit anti-human phospho-PKC (pan) antibody (Cell Signaling Technology; Beverly, MA) and monoclonal mouse anti-human PKC (pan: α, β, γ) antibody (Biomol, Hamburg, Germany). Secondary antibodies were goat anti-rabbit IgG and goat anti-mouse IgG conjugated to peroxidase (Perbio Science). Detection was performed by enzyme-linked chemiluminescence (see above) or 4-chloronaphthol staining (Sigma).

**Assay for Cathepsin B, K, and L Activities**—Cathepsin activity determination was performed as previously published (35). Briefly, adherent cells (about 10⁵ cells per well) were seeded in black-walled, clear-bottom 96-well microtiter plates. The culture medium was replaced by 100 μl of serum-free and phenol red-free medium, and cells were incubated for 2 h at 37 °C with 10 μm nitrosalicylaldehyde and the following substrate or inhibitor mixtures: 1 mM Z-RR-4MβNA with or without 1 μM CA-074Me for cathepsin B; 1 mM Z-GPR-4MβNA with or without 1 μM Z-LG-ψ (C≡N) for cathepsin K; 0.1 mM Z-FR-4MβNA with or without 1 μM Z-FF-CHN₂ for cathepsin L. After 2 h, cells were covered with 100 μl of phosphate-buffered saline (37 °C). The generated fluorescent product was read using a microplate reader (Fluor-S™ Multimager, BioRad) at λ₄₅₈ nm for excitation and λ₅₂₀–₅₃₀ nm for emission. Controls were run without substrates. Activity was calculated using the Quantity One® software version 4.2.1 by subtracting the fluorescence in the presence of inhibitor from the fluorescence in the absence of inhibitor resulting in “artificial fluorescence units” (AFU).

**Statistical Analysis**—Analyses were performed with statistical software SPSS 10.0 for Windows (SPSS Inc., Chicago, IL). Data were analyzed with Mann-Whitney U test. Values of p < 0.05 (*) were considered significant.

Some results are indicated as “fold increase,” which means the activities/mRNA concentrations of the stimulated cultures divided by the activities/mRNA concentrations of the non-stimulated controls.

**RESULTS**

**Ntelo Induces Cathepsins B, K, and L in a Dose-dependent Manner**—The potential of type II collagen fragments, especially of the N-terminal telopeptide, to activate the cathepsin metabolism in articular chondrocytes was tested based on two different observations. On the one hand Morko et al. and others showed that at least CK is enhanced in arthritic cartilage (14, 15). Otherwise Billinghamurst and others delineate in great detail that articular cartilage contains accumulated quantities of type II collagen fragments (28, 29).
Collagen Fragments Induce Cathepsins via p38 in Articular Chondrocytes

Whereas previous studies analyzing matrix degradation determined mainly mRNA and protein levels, more tightly focused on matrix metalloproteinases and ADAMTS (2–6, 29), we examined the mRNA, protein and activity levels of cysteine proteases. Changes in CB, CK, and CL activities were investigated in viable cells using different fluorogenic peptide substrates (4M-ATP, Z-Arg-Arg-MCA, and Z-Phe-Arg-MCA) plus 1% FCS in the presence of increasing amounts of Ntelo. The data shown represent the mean of three experiments (means ± S.D.). Significant changes in the expression (mRNA or enzyme activity) are indicated by asterisks (∗, p < 0.05). RT-PCR was performed using total RNA isolated from human articular chondrocytes treated without or with Ntelo to characterize changes in expression levels (fold increase) of CB (middle panel), CK (left panel), and CL (right panel). The housekeeping gene β-actin was used for normalization. Determination of cathepsin activities was performed using the cathepsin assay (35). Activities are given in relative AFU.

As shown in Fig. 1, after treatment of chondrocytes with Ntelo (synthetic peptide representing the N-terminal telopeptide of collagen type II) (20, 37), we observed enhanced CB, CK, and CL mRNA expression and activities as compared with control, untreated cultures.

To better explore the mechanism of CB, CK, and CL induction on mRNA and activity levels, cultured human articular chondrocytes were treated with Ntelo at varying concentrations (0; 0.2; 0.4; 0.6; 0.8; 1 mg/ml). The concentration range of Ntelo from different patients (see “Discussion”).

**FIGURE 1. Dose dependence of the Ntelo effect on CB, CK, and CL mRNA and activities.** Primary human chondrocytes were cultured in DMEM (containing 1 mM CaCl2) plus 1% FCS in the presence of increasing amounts of Ntelo. The data shown represent the mean of three experiments (means ± S.D.). Significant changes in the expression (mRNA or enzyme activity) are indicated by asterisks (∗, p < 0.05). RT-PCR was performed using total RNA isolated from human articular chondrocytes treated without or with Ntelo to characterize changes in expression levels (fold increase) of CB (left panel), CK (middle panel), and CL (right panel). The housekeeping gene β-actin was used for normalization. Determination of cathepsin activities was performed using the cathepsin assay (35). Activities are given in relative AFU.

Enhancement of the PKC Activity by Ntelo—In previous work on the regulation of annexin V in chondrocytes, PKC was identified as one of the signal cascade-related components involved in annexin V-related metabolism (39). To explore the signal

**FIGURE 2. Effect of Ntelo on secretion of procathepsins.** Primary human chondrocytes were cultured in DMEM (containing 1 mM CaCl2) plus 1% FCS in the presence of increasing concentrations of Ntelo for 72 h. Procathepsins B, K, and L (proCB, proCK, and proCL, respectively) secreted into the culture media were determined mainly mRNA and protein levels, more tightly controlled by Ntelo. The molecular weight standards, in kDa, are indicated at the right margin. The lanes –FCS/+FCS represent cell-free culture media controls.

The data shown represent the mean of three experiments (means ± S.D.). Significant changes in the expression (mRNA or enzyme activity) are indicated by asterisks (∗, p < 0.05). RT-PCR was performed using total RNA isolated from human articular chondrocytes treated without or with Ntelo to characterize changes in expression levels (fold increase) of CB (left panel), CK (middle panel), and CL (right panel). The housekeeping gene β-actin was used for normalization. Determination of cathepsin activities was performed using the cathepsin assay (35). Activities are given in relative AFU.

The data shown represent the mean of three experiments (means ± S.D.). Significant changes in the expression (mRNA or enzyme activity) are indicated by asterisks (∗, p < 0.05). RT-PCR was performed using total RNA isolated from human articular chondrocytes treated without or with Ntelo to characterize changes in expression levels (fold increase) of CB (left panel), CK (middle panel), and CL (right panel). The housekeeping gene β-actin was used for normalization. Determination of cathepsin activities was performed using the cathepsin assay (35). Activities are given in relative AFU.

The data shown represent the mean of three experiments (means ± S.D.). Significant changes in the expression (mRNA or enzyme activity) are indicated by asterisks (∗, p < 0.05). RT-PCR was performed using total RNA isolated from human articular chondrocytes treated without or with Ntelo to characterize changes in expression levels (fold increase) of CB (left panel), CK (middle panel), and CL (right panel). The housekeeping gene β-actin was used for normalization. Determination of cathepsin activities was performed using the cathepsin assay (35). Activities are given in relative AFU.

The data shown represent the mean of three experiments (means ± S.D.). Significant changes in the expression (mRNA or enzyme activity) are indicated by asterisks (∗, p < 0.05). RT-PCR was performed using total RNA isolated from human articular chondrocytes treated without or with Ntelo to characterize changes in expression levels (fold increase) of CB (left panel), CK (middle panel), and CL (right panel). The housekeeping gene β-actin was used for normalization. Determination of cathepsin activities was performed using the cathepsin assay (35). Activities are given in relative AFU.

The data shown represent the mean of three experiments (means ± S.D.). Significant changes in the expression (mRNA or enzyme activity) are indicated by asterisks (∗, p < 0.05). RT-PCR was performed using total RNA isolated from human articular chondrocytes treated without or with Ntelo to characterize changes in expression levels (fold increase) of CB (left panel), CK (middle panel), and CL (right panel). The housekeeping gene β-actin was used for normalization. Determination of cathepsin activities was performed using the cathepsin assay (35). Activities are given in relative AFU.

The data shown represent the mean of three experiments (means ± S.D.). Significant changes in the expression (mRNA or enzyme activity) are indicated by asterisks (∗, p < 0.05). RT-PCR was performed using total RNA isolated from human articular chondrocytes treated without or with Ntelo to characterize changes in expression levels (fold increase) of CB (left panel), CK (middle panel), and CL (right panel). The housekeeping gene β-actin was used for normalization. Determination of cathepsin activities was performed using the cathepsin assay (35). Activities are given in relative AFU.

The data shown represent the mean of three experiments (means ± S.D.). Significant changes in the expression (mRNA or enzyme activity) are indicated by asterisks (∗, p < 0.05). RT-PCR was performed using total RNA isolated from human articular chondrocytes treated without or with Ntelo to characterize changes in expression levels (fold increase) of CB (left panel), CK (middle panel), and CL (right panel). The housekeeping gene β-actin was used for normalization. Determination of cathepsin activities was performed using the cathepsin assay (35). Activities are given in relative AFU.
transduction pathway leading to the enhanced synthesis, activation, and secretion of the cathepsins under the influence of Ntelo and calcium we studied the phosphorylation state of PKC. Incubation of chondrocytes with Ntelo for 60 min led to an increased phosphorylation of PKC comparable to that seen after incubation with the phorbol ester phorbol-12,13-dibutyrate (PDBu), a general activator of PKC (see Fig. 4A) (40). The Ntelo effect was concentration-dependent (Fig. 4B). The phosphorylation state of PKC was highest already after 10 min of Ntelo addition, and this activation remained constant for about 30 min before declining (Fig. 4C).

To exclude the possibility that activation of PKC and up-regulation of cathepsin expression are independent processes, we performed two further experiments. First, treatment of chondrocytes with PDBu, a general PKC activator, for 1 h resulted in a stimulation of CB, CK, and CL. Prolonged treatment for 2 h with PDBu further increased the activities of CB and CL but not of CK. However, after 12 h of incubation with PDBu, the activities of the three cathepsins were lower than before the treatment with PDBu started (Fig. 5, left panel, black bars). This effect is probably caused by the degradation of PKC after prolonged exposure of cells with phorbol esters (41). Pretreatment of chondrocyte cultures with the inhibitor bisindolylmaleimide I (BIM I) reduced the effect of the PDBu treatment on the cathepsin activities significantly (Fig. 5, left panel, open bars), suggesting that activation of PKC and the increase of cathepsin activities are not independent processes.

In a second experiment chondrocytes were incubated with Ntelo for 1, 2, and 24 h in presence (open bars) or in absence (black bars) of the PKC inhibitor BIM I. The up-regulation of CK activity was already visible after 1 h, whereas the effect on the CB and CL activities took more time and was highest after 24 h of incubation. Again, the effect was significantly inhibited by BIM I (Fig. 5, right panel). This indicates the involvement of PKC activity in the signaling of the Ntelo effect on CB, CK, and CL expression and activation in cultured chondrocytes.

Enhancement of p38 MAP Kinase-related Signaling Components by Ntelo—Stimulation of CK transcription, e.g. in osteoclasts, has been shown to involve the activation of p38 MAP kinase (42). To explore which signaling intermediates of the MAP kinase cascade are involved in the activation of CB, CK, and CL by Ntelo the RT² Profiler™ PCR Array Human MAP Kinase Signaling Pathway was applied. As shown in Supplemental Table S2, chondrocytes treated with Ntelo for 24 h exhibited a high induction of MAPK13 (p38α), MAPK14 (p38β), MAPKAP kinase 2, HRAS, and Egr-1 gene expression (mRNA levels). These results suggest that the MAP kinase p38, but not JNK or ERK, is involved in the Ntelo-mediated induction of CB, CK, and CL. Detailed information on the effects of Ntelo on all MAP kinase-related signaling components are provided in Supplemental Table S2. As shown in Fig. 6, the results are validated for MAPK13 and MAPK14 by Real-Time PCR.

p38 Inhibitor Blocks Ntelo-induced Cathepsin B, K, and L Up-regulation in Articular Chondrocytes—We used the selective inhibitor SB203580, which inhibits p38α and β MAP kinase activities, to study whether the Ntelo effect was induced by the activation of this signaling pathway. Fig. 7 clearly shows that inhibition of p38 MAP kinase α and β interrupts this signaling pathway. There is no induction of CB, CK, and CL expression in presence of SB203580.

DISCUSSION

In the present study, the downstream effects of chondrocyte-matrix interactions were investigated by the use of the collagen
type II-derived peptide fragment Ntelo. Fragmentation of matrix components like collagen type II is the result of the action of various proteases. Chondrocytes may release enzymes that mediate the normal turnover of extracellular matrix but may also lead to its uncontrolled pathologic degradation. Recently, it has been shown that fibronectin fragments induce proteoglycan and collagen type II degradation and that this pathway may substantially contribute to the maintenance of arthritic processes and finally to chondrocytic chondrolysis (43–45). Fibronectin fragments induce the release of catabolic cytokines in cartilage explants (46). Most likely collagen type II-derived peptides as well as fibronectin fragments do not directly influence the expression, secretion, and activation of the procathepsins and pro-MMPs even though some fibronectin fragments bind to integrin α5β3 (47). The present investigation offers insights into the signaling events in articular chondrocytes mediated by collagen fragments and thus eventually to the contribution of cytostatic and cathepsin degradation. Recently, some of us reported MMP-2, -3, -9, and -13 to be up-regulated by the treatment of cultured chondrocytes with collagen type II-derived peptides (20). Similar findings were also reported by others (48, 49), and the results of the protease array shown here in Fig. 3B confirm them again. Whereas these effects on matrix metalloproteinases have been studied in more detail, similar studies on the action of collagen-derived peptides on the expression, translation, and activation of cathepsins are lacking. The present report describes for the first time the up-regulation of collagen-degrading CB, CK, and CL by the N-telopeptide of collagen type II (Fig. 1). Whereas CB probably does not attack native insoluble collagen (50), the two other cathepsins, L and K, have been shown to degrade collagen (9).

In cartilage, collagens comprise about 20% of wet weight. Type II collagen (1059 amino acids, SwissProt accession number P02458) is the major component (~95% of total collagen). Healthy articular cartilage contains about 1–3% of collagen fragments because of normal ECM turnover (21, 28, 29). In osteoarthritic cartilage, this amount is dramatically increased: up to 20% of whole collagen is fragmented (29). In terms of the N-telopeptide (29 amino acids), these 20% may result in up to 1–1.3 mg N-telopeptide per gram of articular cartilage. Because 1 g of cartilage has a water content of about 0.6 ml, the maximally reached concentration of N-telopeptide may be close to...
1–2 mg/ml tissue fluid. For our studies, Ntelo was normally added at concentrations ranging from 0.1 to 1 mg/ml. Consequently, the added concentrations were comparable with the in vivo situation.

All experiments were performed with primary chondrocytes cultured not longer than 24 h before addition of the Ntelo, and the other reagents to keep the native state of the cells as good as possible. We had to consider that the activation state of the primary cells were different because of differences in the extent of joint damage of individual patients. Such primary cells cannot be standardized as it is possible in established cell lines. Therefore, the results of stimulation are indicated in the figures mainly as "fold increase" related to the non-stimulated cells of the same preparation.

In this report using quantitative Real-Time-PCR, DNA microarray and cathepsin activity analyses, we were able to demonstrate that Ntelo induces CB, CK, and CL in the presence of extracellular calcium (Fig. 3). Furthermore we were able to underline, that stimulation with Ntelo assists the imbalance between catabolic (proteinases) and anabolic (proteinase inhibitors) factors in human OA chondrocytes (Fig. 3B). Using the proteinase DNA microarray we identified the Ca2+/H11001-dependent Ntelo effect also for cathepsin S (CS) mRNA. CS shows collagenolytic (50) and strong elastinolytic (51, 52) activity, however, was never reported to occur in chondrocytes. The expression of CS detected in OA joints was discussed as originating from synoviocytes (53). Both cathepsin genes, S and K, are localized close to each other on the same chromosome (1q21) and originate probably from gene duplication. Therefore, similar activation mechanisms are not unexpected. Further investigation about the implication of CS in matrix degeneration and its role in OA is necessary.

The omission of Ca2+ from the culture medium was obviously of influence on the expression of some enzymes, however did not detach the chondrocytes from the culture vessel. The addition of 0.5 mg of Ntelo/ml in the presence of Ca2+ induced the expression to a similar extent as previously shown (Fig. 1).

The calcium-dependence of the CB, CK, CL, and CS induction under Ntelo stimulation was not identical in all cases. CK was already stimulated by Ca2+ alone, MMP-1 and -3 were 2-fold increased by Ntelo without Ca2+ (Fig. 3B and Supplemental Table S1), indicating that different signaling pathways may be involved.

Moreover, the Ntelo-mediated increase of the intracellular Ca2+ concentration also leads to the release of vesicular contents (51–53) and may thus be responsible for the occurrence of procathepsins B, K, and L in the culture medium of chondrocytes as shown in Fig. 2.

The involvement of calcium allows independent interpretations with respect to the interaction of annexin V with different matrix components and/or matrix receptors. First, it should be mentioned that annexin V is not only a calcium entry gate into various cells contributing to matrix mineralization (38) but also selectively binds to the N-telopeptide region of collagen type II (37). Upon binding, the calcium influx into chondrocytes is significantly stimulated (37). Second, there are some reports dealing with the interaction of annexin V and integrins (39, 54). The interaction between

Collagen Fragments Induce Cathepsins via p38 in Articular Chondrocytes

FIGURE 6. Validation of calcium-dependent Ntelo-induced up-regulation of p38 MAPK14 and MAPK14 mRNA expression. Articular human chondrocytes were cultured in DMEM (containing 1 mM CaCl2) plus 5% FCS for 24 h. Thereafter, the chondrocytes were cultured for further 24 h either in calcium-free DMEM (−Ca2+) or in DMEM containing 1 mM CaCl2 (+Ca2+) in the absence (−Ntelo) and in the presence of Ntelo (+Ntelo, 0.5 mg/ml), respectively. RT-PCR was performed using total RNA isolated from human articular OA chondrocytes. The housekeeping β-actin gene was used for normalization.

FIGURE 7. Inhibition of Ntelo-induced activation of cathepsin expression by the selective p38 MAP kinase α and β inhibitor SB203580. Primary human chondrocytes were cultured in Ca2+-containing (1 mM) DMEM plus 1% FCS in the absence (−) and in the presence (+) of Ntelo (0.5 mg/ml) and of the p38 MAP kinase inhibitor SB203580 (1 μM) for 24 h. Inhibition of the Ntelo-induced activation of CB, CK, CL activities (left panel) and mRNA expression (right panel) by the p38 MAP kinase inhibitor SB203580. Cathepsin activities were determined using the cathepsin assay (35). The results were given in relative AFU. The mRNA expression was analyzed using Real-Time PCR.

JANUARY 11, 2008•VOLUME 283•NUMBER 2 JOURNAL OF BIOLOGICAL CHEMISTRY 1049
those two molecules also influences the PKC pathway and leads to PKC phosphorylation and to apoptosis in growth plate chondrocytes (39). Alternatively to the annexin link, α10β1 integrin function may also be affected by the treatment with collagen peptides. This integrin has been shown to bind to triple-helical collagen type II (55), and it may also interfere with the same signaling pathway. Putative strategies to further clarify the role of the peptide feedback loop in chondrocytes to control ECM turnover will have to consider the various options. In this study using Western blot analyses of protein extracts of Ntelo-treated chondrocytes, we demonstrated that this peptide increased the level of active protein kinase C, illustrated by its phosphorylation (Fig. 4).

However, we observed some differences between the effects of PDBu, which acts as a non-selective activator and that of the Ntelo peptide, indicating different time periods for activation. The PDBu effect is quick and short lasting; long term treatment of chondrocytes with the phorbolester reverses the effect (Fig. 5). In contrast, the effect of Ntelo is a slow process, and the effect can still be observed after 24 h of treatment (Fig. 5).

PDBu is a diacylglycerol analogue and activates PKC directly. The induction of CB, CK, and CL by Ntelo was also preferentially blocked by the pan PKC inhibitor BIM I. Together these results demonstrate that Ntelo induce CB, CK, and CL expression by controlled activation of protein kinase C.

The pivotal role of MAP kinases in the chondrocyte differentiation was recently summarized by Stanton et al. (56). Activation of p38 MAP kinase by various stimuli induces chondrocyte senescence and apoptosis (57–59), and it plays a role in the maintenance of osteoarthritis (60). Therefore, we tested the impact of Ntelo on signaling events downstream of protein kinase C stimulating catabolic processes by activation of the MAP kinase signaling pathway.

Our results revealed a high up-regulation of H-Ras, MAPK13, and MAPK14 in Ntelo-stimulated articular chondrocytes. The p38 MAP kinase is known to induce the expression of various proteases such as CK as well as MMP-3, -9, and -13 (32, 42, 61–63). In contrast, the addition of the p38 MAP kinase α and β inhibitor SB203580 strongly inhibited the cytokine-induced MMP-13 expression in chondrosarcoma cells (64). Our findings suggest on the one hand the implication of p38 MAP kinase in the induction of CB, CK, and CL by Ntelo (Fig. 6 and Supplemental Table S2), and they confirm on the other hand the inhibition of this induction by the selective p38 MAP kinase inhibitor SB203580 (Fig. 7). Exposure of chondrocytes to Ntelo also induced the up-regulation of p38 MAP kinase targets like MAPKAP kinase 2. Recently, Hegen et al. (65) underlined the role of MAPKAP kinase 2 in the stress pathway and postulated that inhibition of MAPKAP kinase 2 results in a prevention of degenerative processes. Our findings are consistent with other studies underlining the pivotal role of p38 MAP kinase in osteoarthritis (60, 63). In summary, our results suggest that interactions of collagen type II-derived peptides with collagen receptors maintain an induction of CB, CK, and CL in articular chondrocytes mediated by protein kinase C and p38 MAP kinase in a calcium-dependent manner.

Acknowledgments—We thank Reik Löser and Michael Gütschow, Pharmaceutical Institute Poppelsdorf, University of Bonn for providing us the cathepsin K inhibitor benzylxoycarbonyl-Leu-Gly nitrile (Z-LG-CN). The development of the human proteinase DNA microarray in the ArrayTube® format was performed in cooperation with Clonadiag chip technologies GmbH Jena, whose assistance is thankfully acknowledged. The authors are grateful to Dirk Pohlers, Experimental Rheumatology Unit, Department of Orthopedics, Eisenberg, University of Jena for providing us the p38 MAP kinase inhibitor SB203580. We thank Jana Schönburg and Waltraud Seul for excellent technical assistance.

REFERENCES

1. Saposky, A., and Howell, D. (1976) Compr. Ther. 2, 33–40
2. Glasson, S. S., Askew, R., Sheppard, B., Carito, B., Blanchet, T., Ma, H. L., Flannery, C. R., Peluso, D., Kanki, K., Yang, Z., Majumdar, M. K., and Morris, A. E. (2005) Nature 434, 646–648
3. Mort, J. S., and Billington, C. I. (2001) Arthritis Res. 3, 337–341
4. Helio Le Graverand, M. P., Eggerger, J., Sciore, P., Reno, C., Vignon, E., Ottersen, I., and Hart, D. A. (2000) Matrix Biol. 19, 431–441
5. Uusitalo, H., Hiltunen, A., Soderstrom, M., Aro, H. T., and Vuorio, E. (2000) Calcif. Tissue Int. 67, 382–390
6. Keyszer, G., Redlich, A., Haupl, T., Zacher, J., Sparrmann, M., Enguthem, U., Gay, S., and Burmester, G. R. (1998) Arthritis Rheum. 41, 1378–1387
7. Lang, A., Horler, D., and Baici, A. (2000) J. Rheumatol. 27, 1970–1979
8. Baici, A., Lang, A., Horler, D., Kissling, R., and Merlin, C. (1995) Ann. Rheum. Dis. 54, 289–297
9. Li, Z., Hou, W. S., and Bromme, D. (2000) Biochemistry 39, 529–536
10. Velasco, G., Ferrando, A. A., Puente, X. S., Sanchez, L. M., and Lopez-Otin, C. (1994) J. Biol. Chem. 269, 27136–27142
11. Bromme, D., Okamoto, K., Wang, B. B., and Biroc, S. (1996) J. Biol. Chem. 271, 2126–2132
12. Drake, F. H., Dodds, R. A., James, I. E., Connor, J. R., Debouck, C., Richardson, S., Rykaczewski, E., Coleman, L., Riemann, D., Barthlow, R., Hings, G., and Gowen, M. (1996) J. Biol. Chem. 271, 12511–12516
13. Rantakokko, J., Aro, H. T., Savontaus, M., and Vuorio, E. (1996) FEBS Lett. 393, 307–313
14. Morko, J. P., Soderstrom, M., Saamanen, A. M., Salminen, H. J., and Vuo, R. I. (2004) Ann. Rheum. Dis. 63, 649 – 655
15. Konttinen, Y. T., Mandelin, J., Li, T. F., Salo, J., Lassus, J., Liljestrom, M., Hukkanen, M., Takagi, M., Virtanen, I., and Santavirta, S. (2002) Arthritis Rheum. 46, 953–960
16. Hummel, K. M., Petrow, P. K., Franz, J. K., Muller-Ladner, U., Aicher, W. K., and Gay, R. E. (1998) J. Rheumatol. 25, 1887–1894
17. Riede, U. N., and Staubesand, J. (1977) Beitr. Pathol. 160, 3–37
18. Homandberg, G. A., Meyers, R., and Xie, D. L. (1992) J. Biol. Chem. 267, 3597–3604
19. Knudson, W. C. B., Nishida, Y., Eger, W., Kuettner, K. E., and Knudson, C. B. (2000) Arthritis Rheum. 43, 1165–1174
20. Fichter, M., Korner, U., Schomburg, J., Jennings, L., Cole, A. A., and Molchenau, J. (2006) J. Orthop. Res. 24, 63–70
21. Jennings, L., Wu, L., King, K. B., Hammerle, H., Cs-Szabo, G., and Molchenau, J. (2001) Connect. Tissue Res. 42, 71–86
22. Hein, G., Franke, S., Muller, A., Fraenig, E., Eidner, T., and Stein, G. (1997) Clin. Rheumatol. 16, 167–172
23. Downs, J. T., Lane, C. L., Nestor, N. B., McLellan, T. J., Kelly, M. A., and Karam, G. A. (2001) J. Immunol. Methods 247, 25–34
24. Christgau, S., Garnero, P., Fledelius, C., Moniz, C., Ensig, M., and Gineyts, E. (2001) J. Immunol. Methods 252, 27136–27142
25. Jung, M., Christgau, S., Lukoschek, M., Henriksen, D., and Richter, W. (2004) Pathobiology 71, 70–76
26. Bank, R. A., Krikken, M., Beekman, B., Stoop, R., Maroudas, A., Lafeber, F. P., and. (1997) Matrix Biol. 16, 233–243
27. Thibault, M., Poole, A. R., and Buschmann, M. D. (2002) J. Orthop. Res. 20, 1265–1273
28. Hollander, A. P., Heathfield, T. F., Webber, C., Iwata, Y., Bourne, R., Rorabeck, C., and Poole, A. R. (1994) *J. Clin. Investig.* 93, 1722–1732
29.Billinghurst, R. C., Dahlberg, L., Ionescu, M., Reiner, A., Bourne, R., Rorabeck, C., Mitchell, P., Hambor, J., Diekmann, O., Tschesche, H., Chen, J., Van Wart, H., and Poole, A. R. (1997) *J. Clin. Investig.* 99, 1534–1545
30. Goldring, S. R., and Goldring, M. B. (2004) *Clin. Orthop. Relat. Res.* 427, (suppl.) S27–S36
31. Fernandes, J. C., Martel-Pelletier, J., and Pelletier, J. P. (2002) *Biorheology* 39, 237–246
32. Liacini, A., Sylvester, J., Li, W. Q., and Zafarullah, M. (2002) *Matrix Biol.* 21, 251–262
33. Loser, R., Schilling, K., Dimmig, E., and Gutschow, M. (2005) *J. Med. Chem.* 48, 7688–7707
34. Kuettner, K. E. M. V., Pauli, B. U., Wrobel, N. C., Thonar, E. J., and Daniel, J. C. (1982) *J. Cell Biol.* 93, 751–757
35. Ruettger, A., Mollenhauer, J., Loeser, R., Guetschow, M., and Wiederanders, B. (2006) *BioTechniques* 41, 469–472
36. Schuler, S., Wenz, L., Wiederanders, B., Slickers, P., and Ehricht, R. (2006) *BMC Genomics* 7, 144
37. Lucic, D., Mollenhauer, J., Kilpatrick, K. E., and Cole, A. A. (2003) *Connect. Tissue Res.* 44, 225–239
38. Gerke, V., and Moss, S. E. (2002) *Physiol. Rev.* 82, 331–371
39. Wang, W., and Kirsch, T. (2006) *J. Biol. Chem.* 281, 30848–30856
40. Soderholm, H., Olsson, A., Lavenius, E., Ronnstrand, L., and Nanberg, E. (2001) *Cell. Signal.* 13, 95–104
41. Peng, Z., Grimberg, E., and Sagi-Eisenberg, R. (2002) *J. Cell Sci.* 115, 3083–3092
42. Matsumoto, M., Kogawa, M., Wada, S., Takayanagi, H., Tsujimoto, M., Katayama, S., Hisatake, K., and Nogi, Y. (2004) *J. Biol. Chem.* 279, 45969–45979
43. Homandberg, G. A., Hui, F., Wen, C., Purple, C., Bewsey, K., Koepp, H., Huch, K., and Harris, A. (1997) *Biochem. J.* 321, 751–757
44. Yasuda, T., and Poole, A. R. (2002) *Arthritis Rheum.* 46, 138–148
45. Barilla, M. L., and Carsons, S. E. (2000) *Semin. Arthritis Rheum.* 29, 252–265
46. Dreier, R., Wallace, S., Fuchs, S., Bruckner, P., and Grassel, S. (2001) *J. Cell Sci.* 114, 3813–3822
47. Homandberg, G. A., Costa, V., and Wen, C. (2002) *Osteoarthritis Cartilage* 10, 938–949
48. Yasuda, T., Tchetina, E., Ohshima, K., Roughley, P. J., Wu, W., Mousa, A., Ionescu, M., Pidoux, I., and Poole, A. R. (2006) *Matrix Biol.* 25, 419–429
49. Tchetina, E. V., Kobayashi, M., Yasuda, T., Meijers, T., Pidoux, I., and Poole, A. R. (2007) *Matrix Biol.* 26, 247–258
50. Maciewicz, R. A., and Etherington, D. J. (1988) *Biochim. J.* 256, 433–440
51. Stojilkovic, S. S. (2005) *Trends Endocrinol. Metab.* 16, 81–83
52. Jans, R., Sartor, M., Jaid, M., and Pannem, Y. (2004) *Arch. Dermatol. Res.* 296, 30–41
53. Rodriguez, A., Webster, P., Ortego, J., and Andrews, N. W. (1997) *J. Cell Biol.* 137, 93–104
54. Kurtis, M. S., Tu, B. P., Gaya, O. A., Mollenhauer, J., Knudson, W., Loeser, R. F., Knudson, C. B., and Sah, R. L. (2001) *J. Orthop. Res.* 19, 1122–1130
55. Camper, L., Hellman, U., and Lundgren-Akerlund, E. (1998) *J. Biol. Chem.* 273, 20383–20389
56. Stanton, L. A., Underhill, T. M., and Beier, F. (2003) *Dev. Biol.* 263, 165–175
57. Wang, H., Wang, Z., Chen, J., and Wu, J. (2007) *Cell Biol. Int. 31*, 1027–1035
58. Wei, L., Sun, X. J., Wang, Z., and Chen, Q. (2006) *Arthritis Res. Ther.* 8, R37
59. Zwerina, J., Hayer, S., Redlich, K., Bobacz, K., Kollias, G., Smolen, J. S., and Schett, G. (2006) *Arthritis Rheum.* 54, 463–472
60. Afif, H., Brenderdour, M., Mbuma-Endam, L., Martel-Pelletier, J., Pelletier, J. P., Duval, N., and Fahmi, H. (2007) *Arthritis Res. Ther.* 9, R31
61. Troen, B. R. (2006) *Ann. N. Y. Acad. Sci.* 1068, 165–172
62. Nguyen, J., Gogusev, J., Knapsnouge, P., and Bauvios, B. (2006) *Immunol. Lett.* 106, 34–41
63. Kim, H. A., Cho, M. L., Choi, H. Y., Yoon, C. S., Ihun, J. Y., Oh, H. J., and Kim, H. Y. (2006) *Arthritis Rheum.* 54, 2152–2163
64. Pei, Y., Harvey, A., Yu, X. P., Chandrasekhar, S., and Thirunavukkarasu, K. (2006) *Osteoarthritis Cartilage* 14, 749–758
65. Hegen, M., Gaestel, M., Nickerson-Nutter, C. L., Lin, L. L., and Telliez, J. B. (2006) *J. Immunol.* 177, 1913–1917

Collagen Fragments Induce Cathepsins via p38 in Articular Chondrocytes

JANUARY 11, 2008•VOLUME 283•NUMBER 2
JOURNAL OF BIOLOGICAL CHEMISTRY

1051