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Effects of Ceramide on Apoptosis, Proteoglycan Degradation, and Matrix Metalloproteinase Expression in Rabbit Articular Cartilage

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Cartilage loss in osteoarthritis is characterized by matrix degradation and chondrocyte death. The lipid messenger ceramide is implicated in signal transduction of the catabolic cytokines tumor necrosis factor (TNF) and interleukin-1 (IL-1), as well as in apoptosis. The aim of this study was to examine the *in vitro* effects of ceramide on proteoglycan degradation, matrix-metalloproteinase (MMP) expression and activity, and chondrocyte apoptosis in rabbit articular cartilage. Cell-permeant ceramide C_2 stimulated proteoglycan degradation in cartilage explants starting from 3×10^{-5} M, with 100% increase at the dose of 10^{-4} M. This effect was probably due to MMPs since it was blocked by the MMP inhibitor batimastat. Furthermore, in isolated chondrocytes, C_2 stimulated the expression of MMP-1, 3, and 13 at the mRNA level, MMP activity, and MMP-3 production. Ceramide also caused chondrocyte apoptosis at doses ranging from 10^{-5} to 10^{-4} M. This study supports the hypothesis that ceramide might play a mediatory role in both matrix degradation and apoptosis in processes of cartilage loss such as those observed in osteoarthritis. © 2000 Academic Press

Progressive loss of articular cartilage, finally leading to subchondral bone exposure, is the main feature of osteoarthritis (OA). MMPs likely play a major role in cartilage matrix degradation (1). Their production is stimulated by various cytokines and growth factors, among which IL-1, whose involvement in OA is suggested by growing evidence. *Ex vivo* production of IL-1 is increased in samples of diseased synovium (2) and cartilage (3) and higher levels of the cytokine are detected by immunohistochemistry in joint tissues of OA patients (4). Furthermore, development of experimental OA is inhibited by IL-1 receptor antagonist (IL-1 ra), either injected (5) or locally produced by synoviocytes transduced with IL-1 ra gene (6). In a variety of cell types, signal transduction of IL-1, as well as other catabolic cytokines such as TNF, involves activation of sphingomyelinases, which degrade the membrane phospholipid sphingomyelin into phosphocholine and the intracellular messenger ceramide (7). Reunanen *et al.* showed that ceramide stimulates mRNA expression of collagenase-1/MMP-1 and stromelysin-1/MMP-3 in human fibroblasts (8), through activation of three different mitogen-activated protein kinases (MAPK), ERK1/2, SAPK/JNK and p38. The transduction cascade initiated by ceramide ends with the induction of Jun and Fos and the AP-1-dependent transcription of MMP genes. This effect is not restricted to a single cell type because also in keratinocytes ceramide stimulates MMP production, namely of gelatinase B/MMP-9 (9).

Besides its involvement in cytokine signaling and MMP expression, ceramide mediates apoptosis induced by Fas, a member of the TNF-receptor superfamily (10). Ceramide mimics Fas pathway through Ras and MAPK phosphorylation (11), caspase activation, GD3 ganglioside formation and cell death (12). Fas is also present on the surface of chondrocytes and its activation *in vitro* by Fas-ligand (Fas-L) or Fas-binding antibodies leads the cells to apoptosis (13). That this mechanism could also function *in vivo,* is suggested by the fact that Fas-L is present in the synovial fluid of osteoarthritic patients and expressed by infiltrating lymphocytes (14). Another inducer of apoptosis in cartilage is nitric oxide (NO), which is produced by NO synthase (NOS) II in response to IL-1 and TNF (15). Even though NOS inhibitors do not block Fas-induced chondrocyte death (13), the two pathways are otherways connected since ceramide generation is observed in HL-60 leukemia cells undergoing NO-induced apop-

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tosis (16). Cells with characteristic features of apoptosis are present in sections of human articular cartilage and their number increases in OA (17). Distribution and frequence of apoptosis among chondrocytes correlate with OA grade and proteoglycan depletion, suggesting a link between cell death and matrix degradation (18). The possibility that sphingomyelin pathway plays a mediatory role in MMP-dependent matrix degradation and chondrocyte apoptosis prompted us to examine the effects of ceramide on articular cartilage.

The aim of this *in vitro* study was therefore to determine whether ceramide (a) stimulates cartilage degradation through the induction of MMPs and (b) causes chondrocyte apoptosis.

MATERIALS AND METHODS

Reagents

All the media, and supplements for tissue culture were from GIBCO BRL (Cergy-Pontoise, France), unless noted. Recombinant mouse IL-1β was from Genzyme (Cergy-St Christophe, France). *N*-Acetyl-D-sphingosine (ceramide C_2), sphingomyelinase (from *Staphilococcus aureus*), bovine serum albumin (BSA), ethidium bromide, propidium iodide (PI), ribonuclease A, dimethyl sulfoxide (DMSO), Dithiothreitol (DTT), ethylenediamino tetraacetic acid (EDTA) and papain were from Sigma (Saint Quentin Fallavier, France). *N*-Acetyl-D-sphinganine (dihydroceramide C_2) was from Biomol (Plymouth Meeting, PA). Ceramides were freshly dissolved at 10^{-2} M in DMSO and then diluted in culture medium to the final concentration. Hyaluronidase, trypsin and collagenase were from Boehringer Mannheim (Meylan, France). Annexin-V coupled to fluorescein isothiocyanate (FITC) and the cell binding buffer were from Pharmingen (San Diego, CA). RNA NOW reagent was purchased from Biogentex (Seabrook, TX), VENT polymerase from Biolabs (Massachusetts), oligo $d(T)_{12\text{-}18}$ from Pharmacia (Orsay, France) and Superscript II from Gibco. The fluorogenic peptide Dnp-Pro-β-cyclohexyl-Ala-Gly-Cys(Me)-His-Ala-Lys(N-Me-ABz)-NH_2 was purchased from Bachem (Voisins-les-Bretonneux, France) and the reaction buffer (LC2671) from Novex (San Diego, CA). Batimastat was synthesized in the Division of Medicinal Chemistry at the Institut de Recherches Servier (IdRS) (Suresnes, France).

Animals

Male New Zealand rabbits (500–600 g, Charles River, Cléon, France) were anesthesized under isoflurane and nitrogen protoxide and killed by cervical dislocation. Ethical guidelines for experimental investigation in animals were followed, and the experimental protocol was used after acceptance by the IdRS animal experimentation ethics committee.

Cartilage Degradation

Fragments (1–2 mg) of articular cartilage were isolated from the knees of one rabbit and labeled with 3.7 MBq (100 μCi) of $^{35}SO_4^-$ (Amersham, Les Ulis, France) in 25 ml of Dulbecco's minimal essentiam medium (DMEM)/Ham's F12 media supplemented with 10% fetal calf serum (FCS) and 1% of a stock solution of 10^4 IU/ml penicillin and 10 mg/ml streptomycin (PS). After 3 days, the unincorporated radioactivity was removed by 6 media changes over 24 h using DMEM/Ham's F12 supplemented with 0.1% BSA and 1% PS. Each fragment was then transferred to 48-well plates into 0.5 ml of media containing the treatment or its vehicle. Each group was made of 8 fragments. After 3 more days, the fragments were collected and digested in 0.5 ml of 0.6 mg/ml papain, 1 mM EDTA, 0.25 mg/ml DTT 20 mM sodium phosphate, pH 6.8 at 56°C for 16 h. Radioactivity in the culture media and in the tissue digest was measured by liquid scintillation using a β-counter (Beckman, Gagny, France). Proteoglycan degradation in each fragment was expressed as the percentage of released radioactivity.

Chondrocyte Studies

Articular chondrocytes were isolated from knee cartilage by sequential enzymatic digestion with hyaluronidase, trypsin and collagenase as described (19). Cells were then cultured in DMEM/Ham's F12 media supplemented with 10% FCS and 1% PS.

For studies of MMP expression and apoptosis, chondrocytes were plated in Petri dishes of 10 cm diameter at the density of 5×10^5 cells/dish/10 ml media. After reaching confluence, the cells were rinsed twice with Hanks' balanced salt solution (HBSS), then fed with 10 ml of serum-free media supplemented with 0.1% BSA and 1% PS. Cells were treated with IL-1, ceramide or its vehicle (DMSO), and incubated for various times before assay.

RT-PCR Studies

Total RNA was extracted using RNA NOW reagent. Five μg of total RNA was loaded onto a 1% agarose gel containing ethidium bromide, in order to check for RNA integrity and loading. 2.5 μg to total RNA was reverse transcribed (RT) for 1 h at 37°C, using oligo $d(T)_{12\text{-}18}$ and Superscript II, in a total reaction volume of 20 μl. Two μl of the RT reaction was used for each PCR reaction. Gene-specific oligonucleotide primers were designed from the reported cDNA sequences of rabbit MMP-1 (20), MMP-3 (21), MMP-13 (communicated by D. Herbage, Lyon, France) and 18S rRNA (22), used as a housekeeping gene. The amplification profile involved 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min in a Gene Amp PCR system 2400 (Perkin-Elmer, Courtaboeuf, France) using VENT polymerase. The PCR products were then separated by electrophoresis on a 1% agarose gel and photographed under UV light.

MMP Production

Cells were plated in 12-well plates at the density of 1.6×10^5 cells/well/2 ml of 10% FCS, 1% PS, DMEM/Ham's F12 media. At confluence cells were rinsed twice with HBSS, then refed with 0.6 ml/well of serum-free, 0.1% BSA media containing the treatment or its vehicle; each treatment group consisted of 4 wells. After 3 or 6–7 days, the conditioned media were collected and assayed for either gelatinase/collagenase activity or pro-MMP-3 concentration.

MMP activity. MMP activity was measured using the fluorogenic substrate peptide Dnp-Pro-β-cyclohexyl-Ala-Gly-Cys(Me)-His-Ala-Lys(N-Me-ABz)-NH_2, cleaved by gelatinase-B/MMP-9 (k_{cat}/K_m = 1.3×10^4 $M^{-1}s^{-1}$) and collagenase-1/MMP-1 (k_{cat}/K_m = 8.7×10^4 $M^{-1}s^{-1}$) (23). Tests in our laboratory showed that this substrate is also cleaved by gelatinase-A/MMP-2, collagenase-2/MMP-8 and collagenase-3/MMP-13 but not by stromelysin-1/MMP-3 (data not shown). In order to assess the proteolytic activity due to MMPs only, each sample was assayed both in the absence and presence of 10^{-5} M batimastat. Briefly, 60 μl/well of Novex reaction buffer was pipetted in microtiter plates together with 10 μl of 2×10^{-3} M substrate in buffer, 20 μl of conditioned media and 10 μl of 10^{-4} M batimastat. After 24 h of incubation at 37°C, the plates were read at excitation and emission wavelengths of 360 and 460 nm respectively, using a Cytofluor 2350 from Millipore (St Quentin-Yvelines, France). For each sample, MMP-dependent activity was obtained by using as a blank the fluorescence measured in the presence of batimastat.

MMP-3 assay. pro-MMP-3 was measured in the culture medium by an EIA kit (Amersham, Orsay, France) specific for the rabbit protein.

Apoptosis

Apoptotic cells were detected by flow cytometry using annexin-V-FITC (24) and PI (25) labeling. In both cases, cells in the supernatant were pooled with those harvested by trypsin/EDTA.

Annexin-V labeling. After collection, the cells were washed twice with culture media, pelleted by centrifugation and resuspended in 20% FCS medium at the final concentration of 10^6 cells/ml. After 1 h at 37°C, the cells were washed twice with cold PBS and resuspended in 200 μl binding buffer containing 10 μl annexin-V-FITC and 5 μg/ml PI. After 15 min at room temperature, 800 μl binding buffer was added before flow cytometric analysis. For each sample, 10^4 cells were analysed on an EPICS XL/MCL flow cytometer (Coulter, Margency, France). FITC and PI fluorescences were collected through 520 and 630 nm bandpass filters, respectively.

PI labeling. Cells were sedimented by centrifugation then fixed in cold (−20°C) 70% ethanol for at least 1 h. The cells were then washed twice with PBS, pelleted, and their DNA was labeled by addition of 1 ml of 50 μg/ml PI in the presence of 100 μg/ml ribonuclease A. After 30 min at room temperature the DNA content was analysed by flow cytofluorimetry using a 630 nm bandpass filter. For each sample 10^4 cells were analysed, and the results were expressed as the percentage of cells in sub-G1 state.

Statistics

Control and treated groups were statistically compared by analysis of variance followed by Dunnett's test. Significance was noted as follows: $***P < 0.001$; $**P < 0.01$; $*P < 0.05$. Data shown are averages ± standard error of the mean (SEM) unless otherwise noted.

RESULTS

Effect of Ceramide on Cartilage Degradation

The effect of ceramide was first tested on proteoglycan degradation in explants of rabbit articular cartilage. Synthetic cell-permeant ceramide C_2 dose-dependently increased proteoglycan release starting at the concentration of 30 μM (Fig. 1A). Increased production of endogenous ceramides, following addition of bacterial sphingomyelinase, exerted the same degradative action (Fig. 1B). The effect of ceramide C_2 was specific, as the analog dihydroceramide C_2, which lacks the double bond between C_4 and C_5 in the sphingoid base, was inactive (Fig. 1C). Since MMPs are thought to play a major role in cartilage degradation, we examined the effect of the MMP-inhibitor batimastat on ceramide action. Batimastat is a broad-spectrum inhibitor with potent activity on MMPs-1, 2, 3, 7, and 9, in the nanomolar range (26). The increase of proteoglycan degradation caused by 100 μM ceramide C_2 was significantly decreased by 0.1 μM, and completely blocked by 1 μM batimastat (Fig. 2A), which also inhibited cartilage degradation caused by 10 ng/ml IL-1β (Fig. 2B). These results suggest that increased production of MMPs mediates the degradative action of ceramide on cartilage.

Effect of Ceramide on MMP Expression and Activity

RT-PCR was then used to study ceramide effect on the expression of MMP at mRNA level in isolated chondrocytes. Twenty four and 48-h treatments with ceramide C_2 at 10 to 30 μM, dose-dependently increased the messages of MMP-1 and MMP-3 (Fig. 3). Higher ceramide concentrations did not further increase the effect (data not shown), which remained lower than that of 10 ng/ml IL-1 β. Forty eight h treatment with ceramide C_2 at 20 and 30 μM also increased collagenase-3/MMP-13 expression, to a level in this case comparable to that obtained with IL-1 (Fig. 4). That stimulation of MMP expression at mRNA level resulted in increased proteolytic activity was verified by a fluorogenic substrate that is cleaved by gelatinases and collagenases. At concentrations of 30 and 40

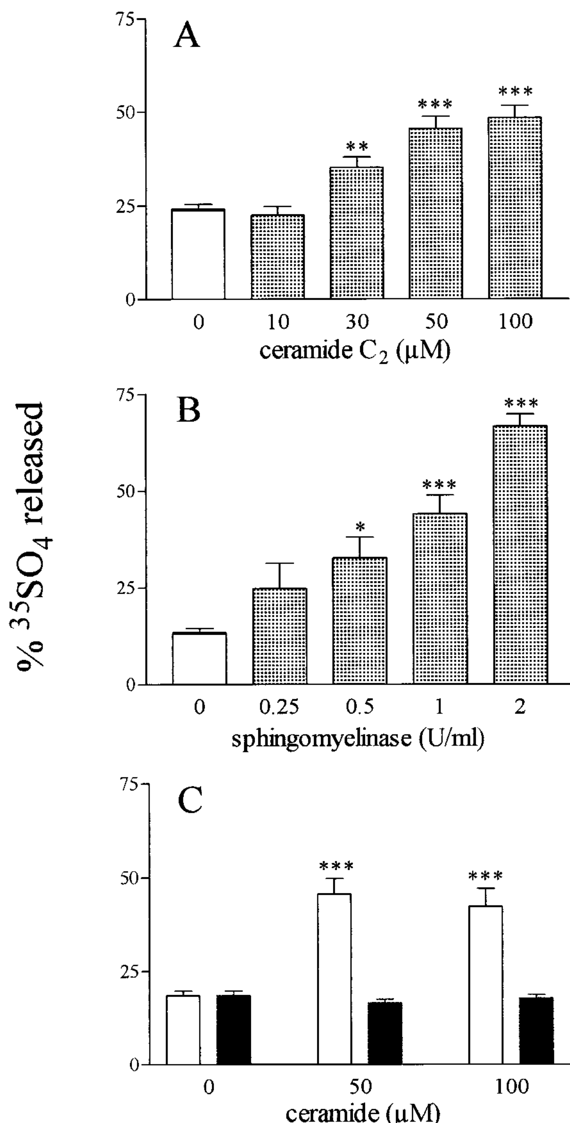

FIG. 1. Effects of ceramides and sphingomyelinase on proteoglycan degradation of cartilage explants after 3-day treatment. (A), ceramide C_2; (B), sphingomyelinase; (C), ceramide C_2 (open boxes) and dihydroceramide C_2 (black boxes). Asterisks indicate a significant difference with the vehicle: $***P < 0.001$; $**P < 0.01$; $*P < 0.05$. Data are averages ± SEM; $n = 8$.

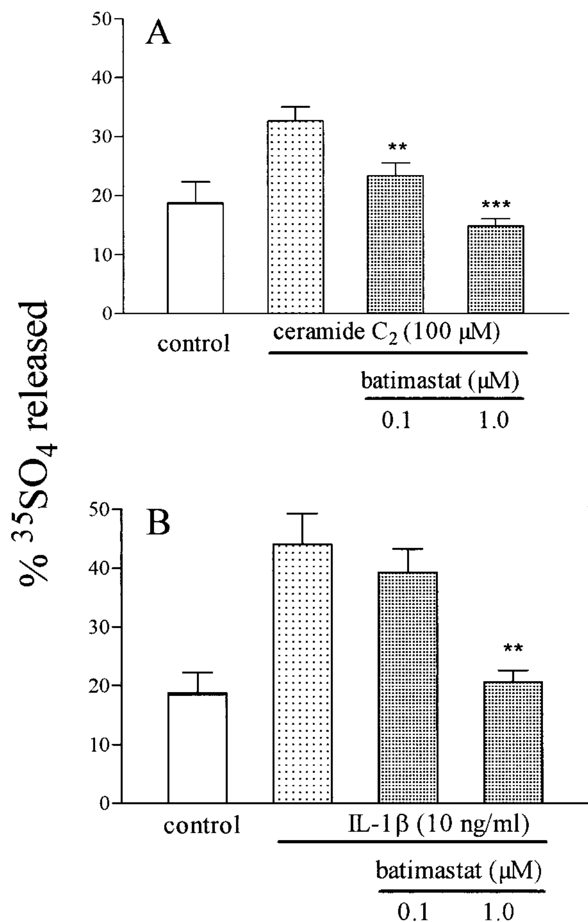

FIG. 2. Effect of batimastat on proteoglycan degradation of cartilage explants after 3-day treatment with ceramide C_2 (A) or IL-1β (B). Asterisks indicate a significant difference between batimastat plus ceramide (or IL-1) vs ceramide (or IL-1) alone: ***$P < 0.001$; **$P < 0.01$. Data are averages ± SEM; $n = 8$.

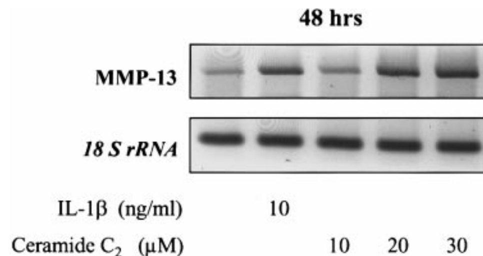

FIG. 4. Effects of ceramide C_2 and IL-1β on MMP-13 mRNA expression by chondrocytes treated for 48-h. Transcripts were amplified by RT-PCR, and processed as in Fig. 3. Representative results are shown of 1 out of 2 experiments.

μM, ceramide C_2 increased MMP activity in the culture media of isolated chondrocytes (Fig. 5A). Maximum activity was reached by day 3 in the presence of 40 μM and by day 7 in the presence of 30 μM ceramide C_2. Ceramide C_2 (50 μM) also increased media concentration of pro-MMP-3, measured by an immunoassay, by 12 and 20 times after 3 and 6 days of treatment, respectively (Fig. 5B).

Effect of Ceramide on Chondrocytes Apoptosis

It was then examined if ceramide caused apoptosis in isolated chondrocytes. Translocation of phosphatidyl serine from the inner to the outer leaflet of the plasma membrane is an early event in apoptosis (27), which can be detected by flow cytometry using the Ca^{2+}-dependent, phospholipid-binding protein annexin-V. Furthermore, the simultaneous use of PI allows discrimination between apoptotic cells with an intact membrane from cells undergoing necrosis. Figure 6A shows typical biparametric histograms of annexin-V-FITC binding of chondrocytes incubated with PI. In the control culture, only a few cells (5%) showed annexin-V labeling, without PI uptake. In contrast, 23% of the cells treated for 5 h with 50 μM ceramide C_2 were annexin-V positive, thus entering apoptosis. Figure 6B shows the average results of 3 separate experiments, in which chondrocytes treated for 5 h with ceramide C_2 at 25 and 50 μM underwent a dose-dependent increase of

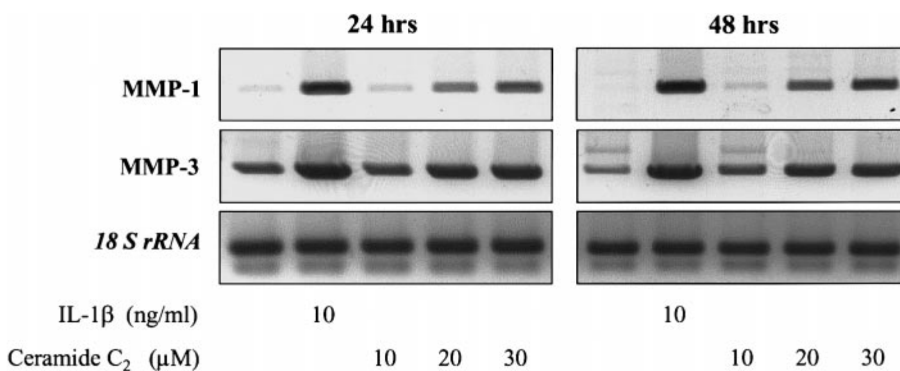

FIG. 3. Effects of ceramide C_2 and IL-1β on MMP-1 and MMP-3 mRNA expression by chondrocytes treated for 24 or 48 h. Transcripts were amplified by RT-PCR, electrophoresed and visualized by ethidium bromide staining under UV light; 18S rRNA was taken as a house-keeping gene. Representative results are shown of 1 out of 3 experiments.

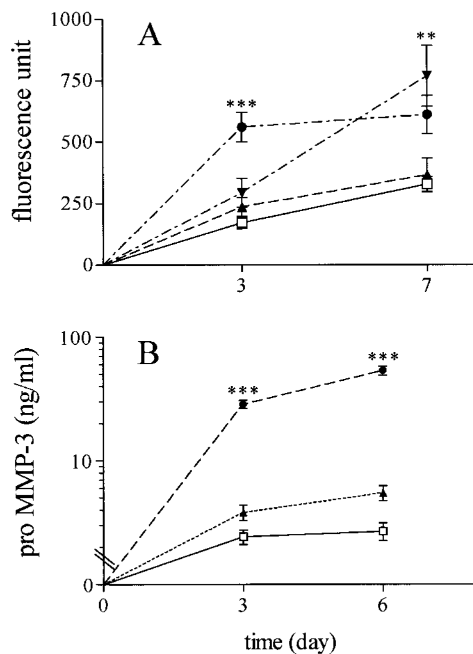

FIG. 5. Effect of ceramide C_2 on MMP production. (A) MMP activity of conditioned media of chondrocytes cultured for 3 and 7 days in the presence of vehicle alone (□) or ceramide at concentrations of 20 (▲), 30 (▼) and 40 (●) μM. (B) pro-MMP-3 concentration in conditioned media of chondrocytes cultured for 3 and 6 days in the presence of vehicle alone (□) or ceramide at concentrations of 25 (▲) and 50 (●) μM. Asterisks indicate a significant difference between ceramide and vehicle: $^{***}P < 0.001$; $^{**}P < 0.01$. Data are averages ± SEM; $n = 4$.

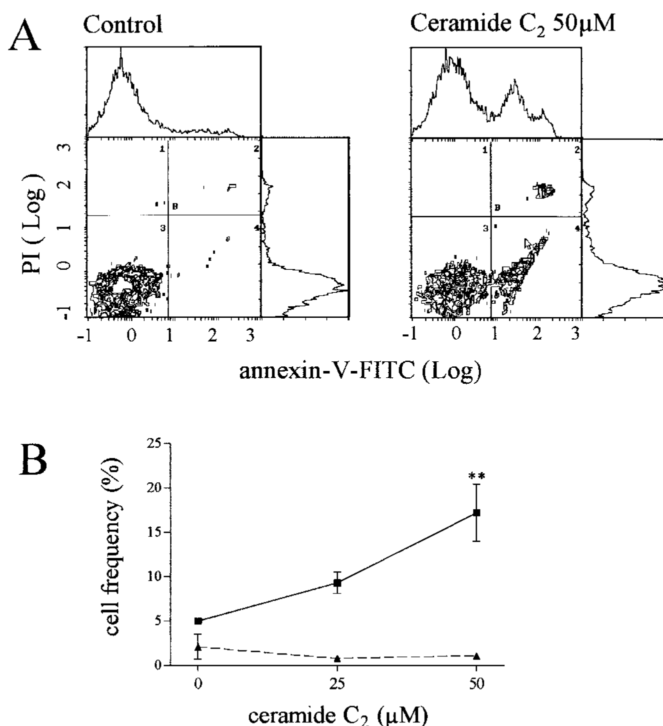

FIG. 6. Effect of ceramide on chondrocyte apoptosis. (A) Representative biparametric histograms of chondrocytes cultured for 5 h in the presence of vehicle or 50 μM ceramide C_2. Cells were analysed by flow cytometry using annexin-V-FITC (x axis) and PI (y axis) double labeling. (B) Percentage of chondrocytes annexin-V-positive (PI-negative) (■), and PI-positive (annexin-V-negative) (▲). Data are averages ± SEM of data of 3 experiments. Asterisks indicate a significant difference between ceramide and vehicle: $^{**}P < 0.01$. Data are averages ± SEM; $n = 3$.

annexin-V labeling. No increase was observed of cells labeled with PI alone (necrotic cells). A later event in apoptosis is chromatin degradation by endonucleases, which progressively cleave DNA in fragments small enough to diffuse out of fixed, permeabilized cells. The amount of intact or partially degraded DNA remaining inside the cells can then be measured by flow cytometry on PI labeled cells. Treatment with ceramide C_2 at concentrations between 20 and 80 μM induced a time- and dose-dependent increase of the fraction of chondrocytes with an hypodiploid (sub-G1) content of DNA (Fig. 7).

DISCUSSION

This study shows that exogenous synthetic as well endogenous ceramides cause matrix degradation in cultures of articular cartilage. This effect is specific to ceramide, because dihydroceramide C_2, a synthetic analog lacking the C_4-C_5 double bond in the sphingoid base is inactive. Ceramide-induced degradation seems to depend on MMPs, since it is antagonized by a broad-spectrum inhibitor of this type of enzymes. More importantly, ceramide stimulates chondrocyte expression of MMP-1, 3, and 13, three of the main enzymes involved in matrix degradation of cartilage. It also increases MMP activity due to gelatinases/collagenases and pro-MMP-3 concentration in chondrocyte media. Maximum effect of ceramide C_2 on MMP expression

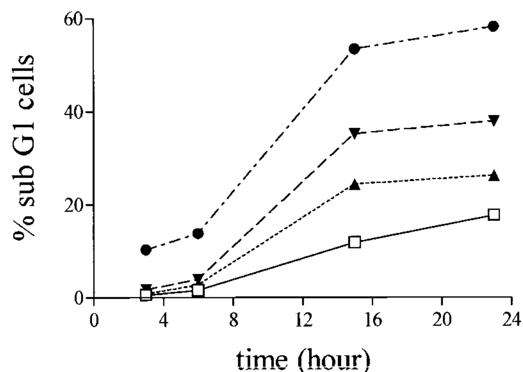

FIG. 7. Effect of ceramide C_2 on chondrocyte apoptosis. Cells were treated with vehicle alone (□) or ceramide C_2 at 20 μM (▲), 40 μM (▼) and 80 μM (●) for the indicated times, then fixed and analysed by flow cytometry after PI labeling. Data are from 1 of 2 experiments with similar results.

and activity in chondrocytes cultured in monolayer, was seen at lower concentration than that necessary for strongest proteoglycan degradation in tissue explants. This discrepancy is probably due to the lipophilic nature of ceramide, which limits its diffusion to the chondrocytes embedded in the highly-charged extracellular matrix of cartilage. As already mentioned, catabolic cytokines such as IL-1 and TNF activate sphingomyelinases (7). In our experiments though, the effect of synthetic exogenous ceramide C_2 on MMP-1 and 3 expression, assessed by RT-PCR, was lower than that caused by IL-1, suggesting that the sphingomyelinase pathway may just account for part of the transduction mechanisms of this cytokine. Our results showing stimulation of MMP expression by ceramide do not exclude that enzymes other than MMPs participate to the increased degradation observed on cartilage explants. It is in fact possible that, similarly to what observed with IL-1, ceramide-induced proteoglycan degradation is also mediated by aggrecanases (28), zinc-metalloproteinases belonging to the ADAMTS (a disintegrin and a metalloproteinase) family (29). Experiments are currently under way to clarify this point. The catabolic activity of ceramide C_2 was otherwise confirmed with another synthetic, cell-permeant analog, ceramide C_6, which also stimulated proteoglycan degradation and expression of MMPs-1 and -3 (data not shown).

Ceramide can also cause apoptosis of cultured chondrocytes, as shown by two independent parameters, annexin-V binding and DNA content, which reflect early and late phases of programmed cell death. Even if the concentrations of ceramide that stimulate MMP-1, 3 and 13 expression are in the same range as those that cause apoptosis, this does not allow to distinguish if stimulation of MMP expression and apoptosis are concomitant events in the same cell or alternative responses that depend on the differentiation and activation state of the chondrocyte. It was previously shown that fibroblasts and keratinocytes increased their MMP production without signs of apoptosis (8, 9), suggesting that stimulation of MMP expression by ceramide is not necessarily linked to programmed cell death.

This paper extends to chondrocytes the notion that ceramide activates MMP expression and links it to degradation of extracellular matrix and cell death in cartilage. Ceramide could act as intracellular signal in chondrocytes targeted by different stimuli such as Fas-L, TNF and IL-1. As already mentioned, Fas-L was detected in the synovial fluid of osteoarthritic patients, where it derives from infiltrating lymphocytes (14), while articular chondrocytes cannot produce it (13). Although evidence for a role of TNF in osteoarthritis is less clear than for IL-1, an increase of TNF and its receptors was reported in articular cartilage with mild degenerative changes (30).

Taken together, the results of this study suggest a role of the sphingomyelinase pathway in both chondrocyte apoptosis and matrix degradation during cartilage degeneration. Availability of specific inhibitors of sphingomyelinase would allow further investigation to test this hypothesis and determine in which measure ceramide is involved in cytokine-induced cartilage degradation. On this background the sphingomyelin pathway could also represent an additional target for pharmacological intervention on cartilage loss in arthritic diseases.

REFERENCES

1. Nagase, H., and Woessner, J. F., Jr. (1993) *in* Joint Cartilage Degradation (Woessner, J. F., Jr., and Howell, D. S., Eds.), pp. 159–185, Marcel Dekker, New York, NY.
2. Pelletier, J. P., McCollum, R., Cloutier, J. M., and Martel-Pelletier, J. (1995) *J. Rheumatol.* **22**(Suppl 43), 109–114.
3. Attur, M. G., Patel, I. R., Patel, R. N., Abramson, S. B., and Amin, A. R. (1998) *Proc. Assoc. Am. Phys.* **110,** 65–72.
4. Sasha, N., Moldovan, F., Tardif, G., Pelletier, J.-P., Cloutier, J.-M., and Martel-Pelletier, J. (1999) *Arthritis Rheum.* **42,** 1577–1587.
5. Caron, J. P., Fernandes, J. C., Martel-Pelletier, J., Tardif, G., Mineau, F., Geng, C., and Pelletier, J.-P. (1996) *Arthritis Rheum.* **39,** 1535–1544.
6. Pelletier, J.-P., Caron, J. P., Evans, C., Robbins, P. D., Georgescu, H. I., Jovanovic, D., Fernandes, J. C., and Martel-Pelletier, J. (1997) *Arthritis Rheum.* **40,** 1012–1019.
7. Serhan, C. N., Haeggström, J. Z., and Leslie, C. C. (1996) *FASEB J.* **10,** 1147–1158.
8. Reunanen, N., Westermarck, J., Häkkinen, L., Holmström, T. H., Elo, I., Eriksson, J. E., and Kähäri, V.-M. (1998) *J. Biol. Chem.* **273,** 5137–5145.
9. Buisson-Legendre, N., Bernard, P., Bobichon, H., Emonard, H., Schneider, C., Maquart, F. X., Haye, B., and Hornebeck, W. (1999) *Biochem. Biophys. Res. Commun.* **260,** 634–640.
10. Smith, C. A., Farrah, T., and Goodwin, R. G. (1994) *Cell* **76,** 959–962.
11. Gulbins, E., Bissonnette, R., Mahboubi, A., Martin, S., Nishioka, W., Brunner, T., Baier, G., Baier-Bitterlich, G., Byrd, C., Lang, F., *et al.* (1995) *Immunity* **2,** 341–351.
12. De Maria, R., Lenti, L., Malisan, F., D'Agostino, F., Tomassini, B., Zeuner, A., Rippo, M. R., and Testi, R. (1997) *Science* **277,** 1652–1655.
13. Hashimoto, S., Setareh, M., Ochs, R. L., and Lotz, M. (1997) *Arthritis Rheum.* **40,** 1749–1755.
14. Hashimoto, H., Tanaka, M., Suda, T., Tomita, T., Hayashida, K., Takeuchi, E., Kaneko, M., Takano, H., Nagata, S., and Ochi, T. (1998) *Arthritis Rheum.* **41,** 657–662.
15. Blanco, F. J., Ochs, R. L., Schwarz, H., and Lotz, M. (1995) *Am. J. Pathol.* **146,** 75–85.
16. Takeda, Y., Tashima, M., Takahashi, A., Uchiyama, T., and Okazaki, T. (1999) *J. Biol. Chem.* **274,** 10654–10660.
17. Blanco, F. J., Guitian, R., Vàzquez-Martul, E., deToro, F. J., and Galdo, F. (1998) *Arthritis Rheum.* **41,** 284–289.
18. Hashimoto, S., Ochs, R. L., Komiya, S., and Lotz, M. (1998) *Arthritis Rheum.* **41,** 1632–1638.
19. Corvol, M. T., Carrascosa, A., Tsagris, L., Blanchard, O., and Rappaport, R. (1987) *Endocrinology* **120,** 1422–1429.

20. Fini, M. E., Plucinska, I. M., Mayer, A. S., Gross, R. H., and Brinckeroff, C. E. (1987) *Biochemistry* **26,** 6156–6165.
21. Fini, M. E., Karmilowicz, M. J., Ruby, P. L., Beeman, A. M., Borges, K. A., and Brinckeroff, C. E. (1987) *Arthritis Rheum.* **30,** 1254–1264.
22. Sasaki, K., Hattori, T., Fujisawa, T., Takahashi, K., Inoue, H., and Takigawa, M. (1998) *J. Biochem.* **123,** 431–439.
23. Bickett, D. M., Green, M. D., Berman, J., Dezube, M., Howe, A. S., Brown, P. J., Roth, J. T., and McGeehan, G. M. (1993) *Anal. Biochem.* **212,** 58–64.
24. Koopman, G., Reutelingsperger, C. P., Kuijten, G. A., Keehnen, R. M., Pals, S. T., and van Oers, M. H. (1994) *Blood* **84,** 1415–1420.
25. Darzynkiewicz, Z., Bruno, S., Del Bino, G., Gorczyca, W., Hotz, M. A., Lassota, P., and Traganos, F. (1992) *Cytometry* **13,** 795–808.
26. Rasmussen, H. S., and McCann, P. P. (1997) *Pharmacol. Ther.* **75,** 69–75.
27. Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M. (1992) *J. Immunol.* **148,** 2207–2216.
28. Hughes, C. E., Little, C. B., Büttner, F. H., Bartnik, E., and Caterson, B. (1998) *J. Biol. Chem.* **273,** 30576–30582.
29. Tortorella, M. D., Burn, T. C., Pratta, M. A., Abbaszade, I., Hollis, J. M., Liu, R., Rosenfeld, S. A., Copeland, R. A., Decicco, C. P., Wynn, R., *et al.* (1999) *Science* **284,** 1664–1666.
30. Kammermann, J. R., Kincaid, S. A., Rumph, P. F., Baird, D. K., and Visco, D. M. (1996) *Osteoarthritis Cartilage* **4,** 23–34.