Ceramide, a mediator of interleukin 1, tumour necrosis factor α, as well as Fas receptor signalling, induces apoptosis of rheumatoid arthritis synovial cells

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Abstract

***Objectives*—To examine the effects of ceramide, which is a lipid second messenger of cell surface receptors, including tumour necrosis factor α (TNFα), interleukin 1 (IL1), and Fas receptors, on rheumatoid arthritis (RA) synovial cells.**

***Methods*—Synovial cells from RA patients and normal skin fibroblasts were cultured with cell permeable ceramide (C2-ceramide). Apoptosis was assessed by microscopic observation of morphological changes, nuclear staining, and DNA electrophoresis. DNA synthesis was examined by thymidine incorporation.**

***Results*—C2-ceramide induced reversible morphological changes of synovial cells such as cell rounding within four hours. Subsequently, irreversible nuclear changes characteristic to apoptosis were observed at 48 hours. DNA synthesis was not promoted. The addition of ceramide exerted similar effects on cultured dermal fibroblasts.**

***Conclusion*—Ceramide induced apoptosis in RA synovial cells. Ceramide could be a second messenger specific for apoptosis of RA synovial cells.**

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Rheumatoid arthritis (RA) is a chronic inflammatory disease mainly characterised by marked synovial hyperplasia. Synovial cells in the inflamed joints produce proinflammatory cytokines such as tumour necrosis factor α (TNFα) and interleukin 1 (IL1). They appear to contribute to synovial cell proliferation[1–4] and tissue damage through induction of various enzymes.[5] In addition to remarkable synovial proliferation, apoptotic cells were observed commonly in the RA synovium.[6 7] It was shown that RA synovial cells express Fas antigens, crosslinking of which by antibodies induces apoptosis.[6 8]

Accumulated data suggest that the effects of both TNFα[9–12] and IL1[13 14] are mediated by a recently identified second messenger, ceramide. It is a hydrolytic product of sphingomyelin by sphingomyelinases.[15 16] It promotes growth of some cell lines such as 3T3 fibroblastoid cells,[17 18] IL1 mediated PGE_2 production,[13] and IL6 gene expression in human fibroblasts.[19] These effects are analogous to those of TNFα and IL1, supporting that ceramide is one of the key molecules mediating TNFα and IL1 signals.

Ceramide, on the other hand, also mediates apoptosis and cell cycle arrest.[15 16] Acidic sphingomyelinase deficient human and mouse cells are resistant to radiation induced apoptosis.[20] Furthermore, ceramide delivers an apoptotic signal upon crosslinking of Fas (Apo1/CD95)[21–24] and TNF receptor.[9–12] These findings suggest that ceramide plays differential parts in cell turnover, depending on the cell type and the activation status.

IL1 and TNF receptors and Fas molecules are all expressed on the RA synovial cells.[1–7] Although their stimulations all activate the sphingomyelinases and generate ceramide as a second messenger, they exert distinct effects on the RA synovial cells.

The role of the sphingomyelin-ceramide pathway in the RA synovium has not been elucidated. It is unclear how the synovial cells react to upregulated intracellular ceramide. In this study, we investigated the direct effects of ceramide on cultured synovial cells from RA patients. Normal dermal fibroblasts were also studied for comparison because they displayed distinct responses on cytokine stimulation,[25] and ceramide induced proliferation of murine fibroblastoid cells.[17 18]

Methods

CELL CULTURE

Synovial tissue samples were obtained from eight patients with active RA at synovectomy or total knee joint replacement surgery. RA was diagnosed according to the American College of Rheumatology criteria.[26] The synovial tissues were minced and treated with 0.5 mg/ml collagenase (Sigma, St Louis, MO) and 0.15 mg/ml DNase-I (Sigma) for one hour at 37°C. The isolated synovial cells were washed and cultured with RPMI 1640 supplemented with 10% fetal calf serum (FCS). Dermal fibroblasts were obtained from normal skin regions of three non-RA patients. Both synovial cells and dermal fibroblasts used in this study were from the third to fifth passages. At this stage, most of cultured cells were fibroblastoid B type synovial cells.

LIPIDS

N-acetyl ceramide (C2-ceramide) (Wako, Osaka, Japan) and dioctanoylglycerol (Sigma) were dissolved in ethanol. C2-dihydroceramide (Calbiochem, Cambridge, MA) was dissolved in dimethyl sulphoxide (DMSO). The final concentrations of ethanol and DMSO in the culture medium were less than 0.1% and 0.5%, respectively.

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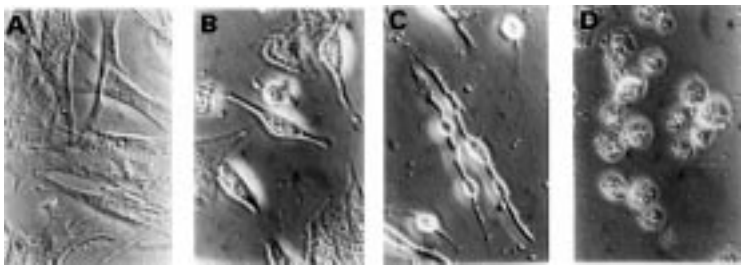

Figure 1 Induction of cell death in RA synovial cells by C2-ceramide. RA synovial cells were incubated with 10 μM of C2-ceramide in serum free RPMI 1640 medium (supplemented with 40 ng/ml PDGF) for 0 hours (A), 4 hours (B), 24 hours (C), and 48 hours (D). Cells were observed by phase contrast microscopy (original magnification × 400).

CRYSTAL VIOLET ASSAY

Synovial cells or dermal fibroblasts were plated at 1×10^4 cells per well of 96 well microplates and allowed to adhere for 16 hours. The cells were then placed in serum free RPMI 1640 for 24 hours. Subsequently, the culture medium was changed to RPMI 1640 with 5% FCS or with 40 ng/ml recombinant human platelet derived growth factor (PDGF)-BB (Genzyme, Cambridge, MA) to maintain cell growth. At this time, various concentrations of the lipids were added to the medium. After 48 hours of culture, the cells were washed with PBS and incubated with 50 μl of 0.1% crystal violet for 15 minutes. The cells were then washed five times with distilled water and dried. Incorporated crystal violet was eluted with 100 μl of 0.5% SDS and the optical density of each well was determined by a microplate reader at 590 nm.

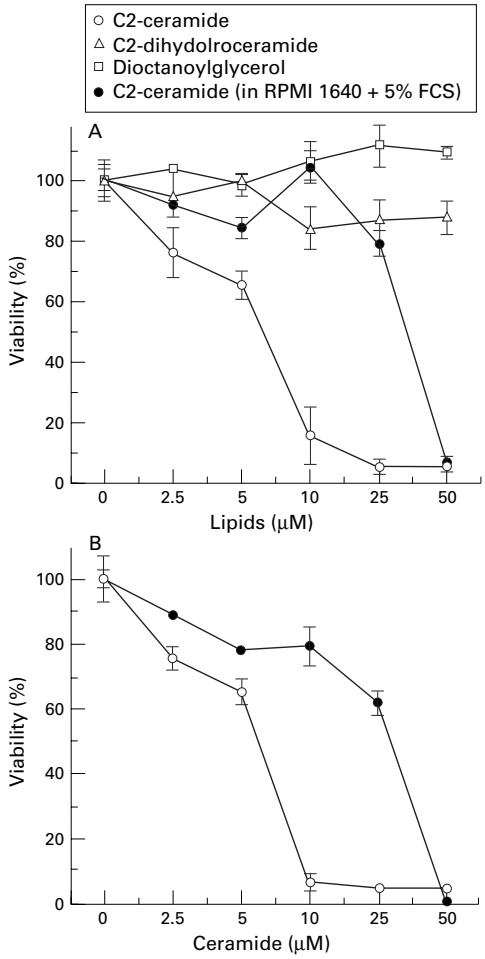

Figure 2 Cytotoxicity of C2-ceramide on RA synovial cells and dermal fibroblasts. RA synovial cells (A) and normal dermal fibroblasts (B) were treated with C2-ceramide, C2-dihydroceramide, and dioctanoylglycerol in serum free RPMI 1640 medium (supplemented with 40 ng/ml PDGF), C2-ceramide in RPMI 1640 containing 5% FCS for 48 hours. Cell viability was determined by crystal violet assay. Values are the mean (SD) of triplicate cultures. The data were representative of synovial cells from eight RA patients and dermal fibroblasts from normal skin of three controls.

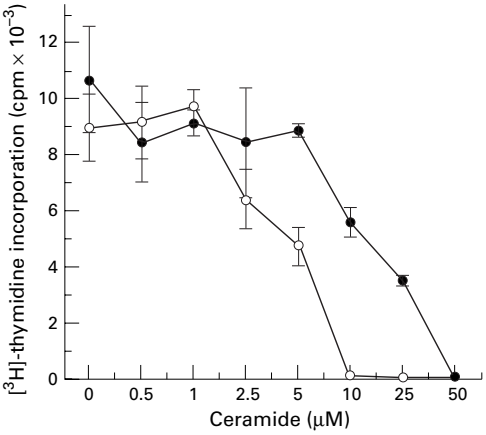

Figure 3 Inhibitory effect of ceramide on synovial cell DNA synthesis. RA synovial cells were treated with C2-ceramide in serum free RPMI 1640 medium (supplemented with 40 ng/ml PDGF, open symbols) or in RPMI 1640 containing 5% FCS (closed symbols) for 48 hours. DNA synthesis was determined by ^{3}H-thymidine incorporation assay. Values are the mean (SD) of triplicate cultures. The data were representative of synovial cell samples from eight RA patients.

PROLIFERATION ASSAY

Synovial cells and dermal fibroblasts were treated with or without lipids for 48 hours as described above. The cells were pulsed with 0.5 μCi/well ^{3}H-thymidine (ICN Pharmaceuticals, Irvine, CA) for 24 hours. The cells were detached with 50 μl of 0.25% trypsin - 0.2% EDTA, and harvested onto glass fibre filters. The incorporation of ^{3}H-thymidine was measured by liquid scintillation counting.

ASSESSMENT OF APOPTOSIS

Cells (5×10^5) treated with the lipids were detached from a dish with trypsin-EDTA solution, and fixed with 1% glutaraldehyde in PBS. After washing with PBS, cells were stained with 0.2 mM Hoechst 33258 (Molecular Probes, Eugene, OR) and examined by fluorescence microscopy (Provis AX80, Olympus, Japan). For DNA electrophoresis, cells were lysed with 20 μl of lysis buffer (10 mM EDTA, 50 mM TRIS-HCL (pH 8), 0.5% (w/v) sodium lauroyl sarcosinate (Wako)). The lysates were incubated with 0.5 mg/ml RNase A for 30 minutes at 50°C, and with 0.5 mg/ml proteinase K for one hour at 50°C. Ten μl of the samples were electrophoresed on 2% agarose gel, and stained with ethidium bromide.

Results

SYNOVIAL CELL DEATH INDUCED BY CERAMIDE

The effects of ceramide were analysed using C2-ceramide, which is a synthetic cell permeable ceramide. RA synovial cells, cultured in

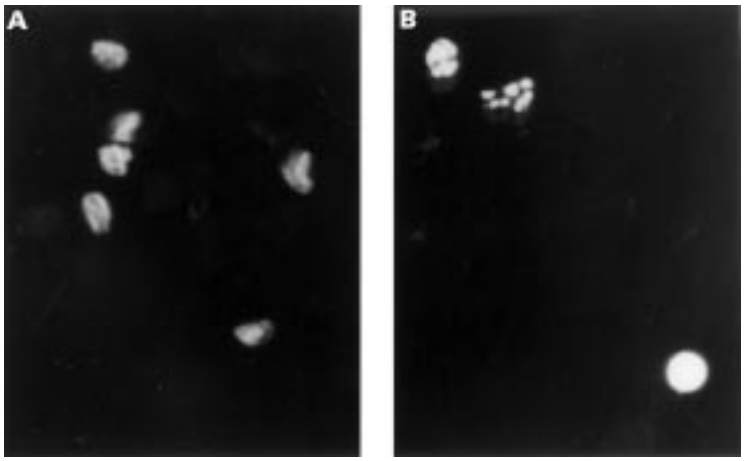

Figure 4 Nuclear condensation and fragmentation of RA synovial cells induced by C2-ceramide. RA synovial cells were treated with 0.1% ethanol, as a control (A) or 10 μM C2-ceramide (B) for 48 hours. Nuclei were stained with Hoechst 33258 and observed by fluorescence microscopy (original magnification × 400).

RPMI 1640 supplemented with 40 ng/ml of PDGF to maintain the cell growth, were treated with 10 μM of C2-ceramide. Cell rounding was observed as early as four hours after the addition, and followed by contraction of the cells (fig 1A to C). At 48 hours, most cells were detached from the dish (fig 1D).

As is shown in fig 2A, viability of the synovial cells was lost at more than 10 μM ceramide added in serum free RPMI 1640 medium supplemented with PDGF alone. Less than 2.5 μM ceramide had no effect. On the other hand, in the medium with 5% FCS, 50 μM ceramide was required for complete cytotoxicity. It is probable that the ceramide, as a water insoluble lipid, bound to proteins in FCS and was kept from shifting into the cytoplasm of the synovial cells. The experiments were repeated with the samples from different donors and yielded the essentially same results. Normal dermal fibroblasts had similar sensitivity when cultured with or without the serum in the medium (fig 2B).

Structural analogues of C2-ceramide, C2-dihydroceramide, which is different from C2-ceramide only in the absence of the double bond between carbons 4 and 5 of the sphingoid back bone, and dioctanoylglycerol, which is a cell permeable diacylglycerol, had no cytotoxicity (fig 2). Thus, ceramide did not lyse the cells as an amphipathic detergent. The results suggest that ceramide acted specifically on target molecule(s) in the synovial cells.

INHIBITION OF DNA SYNTHESIS OF THE SYNOVIAL CELLS BY CERAMIDE

Although ceramide inhibits growth of various type of cells, it promotes growth of 3T3 fibroblasts at sub-cytotoxic doses.[17 18] Therefore, we assessed the effect of sub-cytotoxic doses of ceramide on synovial cell proliferation. As shown in fig 3, C2-ceramide with or without FCS in the culture medium inhibited DNA synthesis of the synovial cells in a dose dependent manner. DNA synthesis was not augmented in any concentrations. All synovial cells from eight RA patients showed similar sensitivities to ceramide (IC_{50}: 7.2 (2.8) μM (mean (SD)) without serum and 38.8 (17.3) μM with 5% FCS). C2-ceramide exerted an equivalent inhibitory effect on the DNA synthesis of dermal fibroblasts (IC_{50}: 8.1 (0.9) μM without serum and 41.5 (3.0) μM with 5% serum).

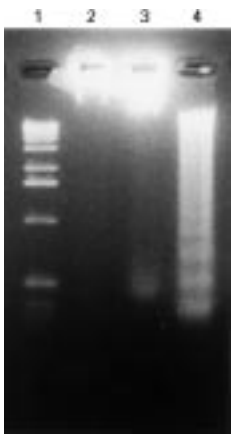

Figure 5 DNA fragmentation of ceramide treated RA synovial cells. RA synovial cells were treated with 10 μM C2-ceramide for the indicated periods. Extracted DNA was electrophoresed on a 2% agarose gel, and stained with ethidium bromide. Lane 1:1kb ladder DNA molecular weight marker, lane 2:0.1% ethanol, lane 3:C2-ceramide for 24 hours, lane 4:C2-ceramide for 48 hours.

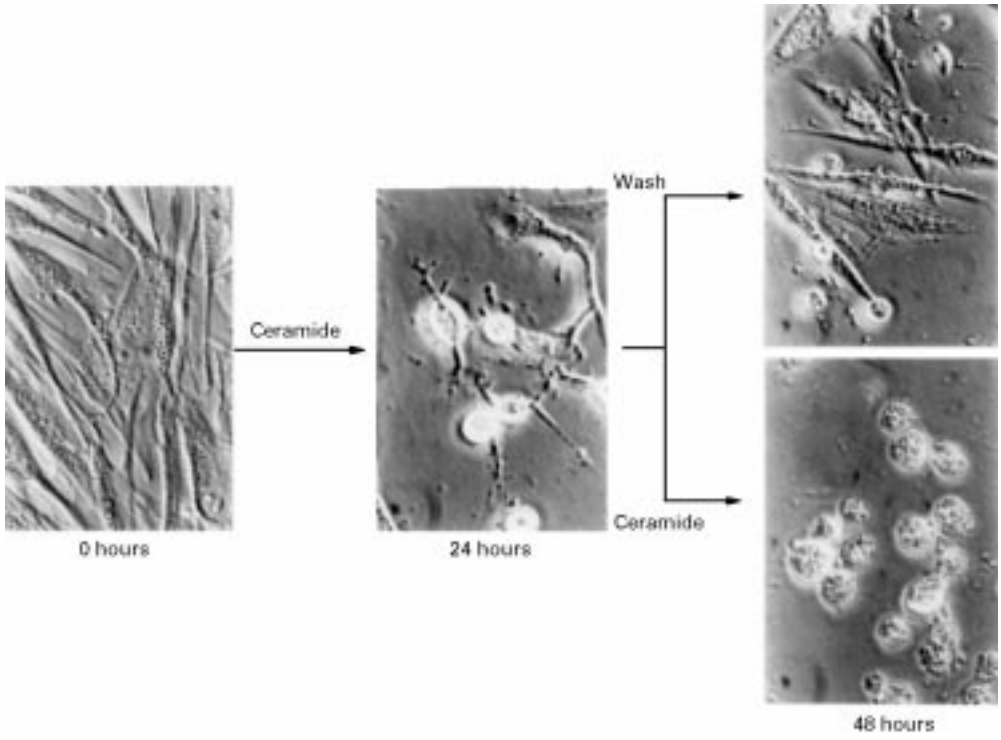

Figure 6 Reversibility of early morphological changes induced by ceramide. RA synovial cells were incubated with 10 μM of C2-ceramide in serum free RPMI 1640 medium (supplemented with 40 ng/ml PDGF) for 24 hours. The medium was changed to ceramide free medium, and the culture was continued for additional 24 hours. The cells were observed by phase contrast microscopy (original magnification × 400).

CERAMIDE INDUCED APOPTOSIS OF SYNOVIAL CELLS

Incubation of synovial cells with C2-ceramide specifically induced massive cell death at 48 hours. Hoechst 33258 staining of the cells showed morphological changes of nuclei such as condensation and fragmentation (fig 4). Increase of hypodiploid nuclei was also observed by flow cytometry (data not shown). Agarose gel electrophoresis of the nuclear DNA showed nucleosomal ladder formation (fig 5). These results demonstrated that the synovial cells treated with ceramide underwent apoptosis. Nuclear staining and electrophoresis assay of the normal dermal fibroblasts treated with C2-ceramide showed their death in a similar fashion (data not shown).

Although C2-ceramide clearly induced morphological changes of the cell by four hours (fig 1), DNA fragmentation was not observed at 24 hours (fig 5, lane 3). The morphological changes were reversed by washing out the ceramide from the medium within 24 hours, but not later than 48 hours (fig 6). Thus, the reversible changes in the cell morphology preceded irreversible nuclear changes.

Discussion

Membrane lipids are precursors of second messengers that include ceramide, diacylglycerol, and phosphatidylinositol (3,4,5) triphosphate (PIP_3). Ceramide is generated through hydrolysis of sphingomyelin by sphingomyelinases, which are activated by a variety of stimuli such as vitamin D3,[27] TNFα,[9–12] IL1,[13 14] nerve growth factor,[28] Fas ligation,[21–24] serum starvation,[29] and CD28 ligation.[30 31] The addition of cell permeable ceramide in culture medium increases intracellular ceramide concentration of the cultured cells and mimics the sphingomyelinases activation.[32] It has been shown that ceramide has a wide spectrum of effects on cell growth, death, and function. It induces cell differentiation, cell cycle arrest, and apoptosis in leukaemic cell lines.[15] It also promotes IL6 and PGE_2 production from fibroblasts,[13 19] and increases DNA synthesis of 3T3 cells.[17 18] These findings suggest that ceramide exerts distinctive effects depending on the cell type and the cellular context.[33]

The effects of ceramide on synovial cells have remained to be elucidated. Both IL1 and TNFα stimulate fibroblasts and synovial cells to induce proliferation.[1–4 34 35] As both activate sphingomyelinase, ceramide may function as a positive mediator in growth of these cells. Indeed, it promotes growth of 3T3 fibroblastoid cells.[17 18] In this study we have demonstrated that ceramide induced apoptosis of RA synovial cells, but not their proliferation of RA synovial cells. The cytotoxic effect of ceramide was dose dependent. Although the crystal violet assay used in this study does not necessarily count apoptotic cells, the chromatin morphology (fig 4), chromosomal DNA electrophoresis (fig 5), and flow cytometry analysis (data not shown) demonstrated that the cell death resulted from apoptosis. The results suggest that ceramide could be a specific mediator for apoptosis, and mimics the effect of Fas receptor ligation, but not of TNFα or IL1. However, it should be carefully interpreted, as complex signal transduction pathways other than sphingomyelin-ceramide pathway are involved in response to TNFα and IL1. Further studies are necessary to define the role of ceramide in the synovial cells. Quantification of ceramide in the cultured synovial cells after anti-Fas, TNFα, and IL1 stimulation will be informative. Also, TNF receptor associated factor (TRAF) 2,[36 37] TRAF6[38] and their associated molecules probably play some part in TNFα and IL1 signalling in the RA synovial cells.

Cell permeable ceramide first induced reversible morphological changes of the cytoplasm, which preceded irreversible nuclear changes and ultimate cell death of RA synovial cells. Recently, we found that exogenous ceramide leads to CPP32 (caspase-3) activation, one of the ICE family proteases, in Jurkat cells.[39] It is known that ICE-like proteases including CPP32 play a major part in cytoskeletal reorganisation during apoptosis.[40–44] Thus, caspase activation might be responsible for cytoskeletal alteration and subsequent morphological changes observed in this study.

Marked proliferation of synovial cells is a primary feature of RA. This led to an early hypothesis that the RA synovial cells have defects in apoptotic processes.[45] However, recent studies showed that apoptotic changes were observed in the RA synovial cells more frequently than those from osteoarthritis and normal joints.[6 7] We found that synovial cells and dermal fibroblasts had similar sensitivity to ceramide induced apoptosis. Thus, the effect of ceramide is not specific for RA synovial cells, suggesting that apoptosis signal transduction downstream of ceramide is intact in RA synovial cells.

We have shown that ceramide, which may be involved in TNFα, IL1, and Fas receptor signalling pathway induced apoptosis of RA synovial cells. Ceramide would be a second messenger specific for apoptosis in RA synovial cells. Thus, the sphingomyelin-ceramide pathway could be a novel therapeutic target to modulate synovial hyperplasia.

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