Biochemical and Biophysical Research Communications **269,** 70–75 (2000)
doi:10.1006/bbrc.2000.2239, available online at http://www.idealibrary.com on IDEAL®

Regulation of Rheumatoid Synovial Cell Growth by Ceramide

Kiyoshi Migita,*[,1] Seiyo Honda,† Satoshi Yamasaki,* Yasuko Hirai,* Takaaki Fukuda,† Takahiko Aoyagi,‡ Masako Kita,* Hiroaki Ida,* Toshiaki Tsukada,* Atsushi Kawakami,* Yojiro Kawabe,* and Katsumi Eguchi*

**First Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki, Japan; †First Department of Internal Medicine, Kurume University School of Medicine, Kurume, Japan; and ‡Department of Orthopedic Surgery, National Ureshino Hospital, Saga, Japan*

Received January 24, 2000

Overgrowth of rheumatoid synoviocytes, which results in joint destruction, is due to impaired balance between cell proliferation and cell death (apoptosis). Ceramide is an important lipid messenger involved in mediating a variety of cell functions including apoptosis. We investigated the effects of ceramide on growth-promoting anti-apoptotic signals in rheumatoid synovial cells. Human synovial cells isolated from patients with rheumatoid arthritis (RA) were stimulated with platelet-derived growth factor (PDGF) in the presence or absence of C2-ceramide. The kinase activity of Akt, MEK, and ERK1/2 was analyzed in PDGF-stimulated synovial cells by Western blot analysis. Pretreatment with C2-ceramide completely inhibited PDGF-induced cell cycle progression of rheumatoid synovial cells. PDGF stimulation induced phosphorylation and activation of Akt, MEK, and ERK1/2 in rheumatoid synovial cells. C2-ceramide inhibited the activation of Akt, MEK and ERK1/2 in PDGF-stimulated synovial cells. Our data demonstrated that inhibition of anti-apoptotic kinases, such as Akt and ERK1/2, may play an important role in ceramide-mediated apoptosis of rheumatoid synovial cells. © 2000 Academic Press

Marked proliferation of synovial cells is a primary feature of rheumatoid arthritis (1). Apoptosis, or programmed cell death, is a physiological process, characterized by chromatin condensation and shrinkage (2). Induction of apoptosis eliminates extraneous or dangerous cells and previous studies have demonstrated that mice with genetic defects affecting the apoptotic process develop autoimmune diseases similar to systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (3, 4). This might suggest that rheumatoid synovial cells have a defect in apoptotic processes.

Recent studies suggest that activated sphingomyelinase and subsequent generation of ceramide play an important role in the regulation of apoptosis in many systems (5). We and other groups have demonstrated that cell permeable C2-ceramide induces apoptosis of cultured synovial fibroblasts *in vitro* and inhibits synovial overgrowth *in vivo* (6, 7). Accumulating evidence suggests that apoptosis develops when an imbalance between anti-apoptotic and pro-apoptotic signaling occurs within the cells (8, 9). Effector kinases having a pro-apoptotic role include stress-activated protein kinase (SAPK)/c-Jun N-terminal kinases (JNK) and P38 mitogen-activated kinase (10). On the other hand, it has been shown that activation of Akt kinase is important for growth factor-mediated suppression of apoptosis (11, 12). In addition, the potential role of extracellular signal-regulated kinases (ERKs) in preventing apoptosis has emerged in recent years (13).

As an extension to our recent study (6), we examined here the effects of C2-ceramide on growth factor-induced synovial mitogenic pathways and investigated the possibility that this apoptotic lipid second messenger may affect cell growth by inhibiting mitogenic signal pathways. Our results showed that C2-ceramide blocked the activation of various kinases such as Akt, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) and ERK1/2 in platelet-derived growth factor (PDGF)-stimulated rheumatoid synovial fibroblasts. Our results indicate that ceramide induces apoptosis of rheumatoid synovial cells by regulating multiple signaling pathways that involve inhibiting anti-apoptotic signals in addition to stimulating pro-apoptotic signals.

[1] To whom correspondence should be addressed at First Department of Internal Medicine, Nagasaki University School of Medicine, Sakamoto 1-7-1, Nagasaki 852-8501, Japan. Fax: +81-95-849-7270. E-mail: eguchi@net.nagasaki-u.ac.jp.

0006-291X/00 $35.00

MATERIALS AND METHODS

Antibodies and reagents. Rabbit anti-phospo-Akt1, anti-phospho-MEK1 and anti-Phospho-ERK1/2 were purchased from New England Biolabs (Boston, MA). Rapamycin and recombinant human PDGF-BB were obtained from GIBCO, BRL (Gaithersburg, MD). PD09805, a selective inhibitor of ERK-activating kinase, MEK (14), was purchased from New England Biolabs. Other reagents were purchased from Sigma Chemicals (St. Louis, MO).

Synovial cell culture. The experimental protocol was approved by the local ethics committee and a signed consent form was obtained from each patient. Synovial tissue samples were obtained from patients with RA during synovectomy. The synovial membranes were minced aseptically, then dissociated enzymatically with collagenase (4.0 mg/ml, Sigma) in RPMI 1640 for 4 h at 37°C. The obtained cells were plated on culture dishes and allowed to adhere. To eliminate non-adherent cells from synovial cell preparations, the plated cells were cultured for 18 h with RPMI 1640 supplemented with 10% FCS at 37°C in humidified 5% CO_2 in air. Cells were then washed thoroughly with phosphate-buffered saline (PBS) solution. Adherent synovial cells were removed by adding trypsin-EDTA followed by washing with PBS containing 2% FCS. Collected synovial cells were used at the third or fourth passages for subsequent experiments. Synovial cell preparations were less than 1% reactive with monoclonal antibodies CD3, CD20, CD68 (Coulter Immunology, FL), and anti-human von Willebrand factor (Immunotech, Marseille, France), indicating that these preparations were almost free of mature T lymphocytes, B lymphocytes, monocytes/macrophages, and vascular endothelial cells.

Analysis of cell cycle. Synovial cells (5×10^5) were plated in culture dishes (Falcon 3003, Becton Dickinson) in RPMI 1640 supplemented with 10% FBS. For serum starvation, cells were washed with PBS and maintained in RPMI 1640 with 0.3% BSA for 24 h. C2-ceramide was added to this medium during the final 30 min. After starvation and washing the medium, synovial cells were stimulated with PDGF (50 ng/ml) for 24 h. Synovial cells were fixed at −20°C with 70% ethanol, then washed and incubated at 37°C for 30 min with ribonuclease (100 μg/ml, Sigma). After centrifugation, cells were resuspended in 2.0 ml of propidium iodide (100 μg/ml, Sigma) in PBS for at least 1.0 h then analyzed by flow cytometry. An argon-ion laser flow cytometer (Profile model, Coulter) was used, with an excitation of 488 nm. Red fluorescence was collected with a photomultiplier masked with 610 nm long-band pass filter. Cells (2×10^4) were collected at a sample flow rate of 10 μl/min.

Western blot analysis. Synovial cells were grown to subconfluence on culture dishes and starved by serum-free medium for 24 h. After starvation, synovial cells were stimulated with PDGF (50 ng/ml) for 5 min in the presence or absence of C2-ceramide. Cells were washed with cold PBS and lysed by the addition of a lysis buffer (1% Nonidet P 40, 0.1% SDS, 50 mM Tris, pH 7.5, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 20 mM β-glycerophosphate, 1.0 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 20 min at 4°C. Insoluble material was removed by centrifugation at $15{,}000 \times g$ for 15 min at 4°C. The supernatant was saved and the protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). An identical amount of protein (50 μg) for each lysate was subjected to 10% SDS-PAGE. Western blotting was performed with the following primary antibodies at indicated dilutions: anti-phosphotyrosine (4G10, 1:1,000), anti-phospho-Akt1 (1:2,000), anti-phospho-MEK1(1:2,000), and anti-phospho-ERK1/2 (1:4,000), followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and were developed with ECL Western blotting kit (Amersham, Arlington Heights, IL).

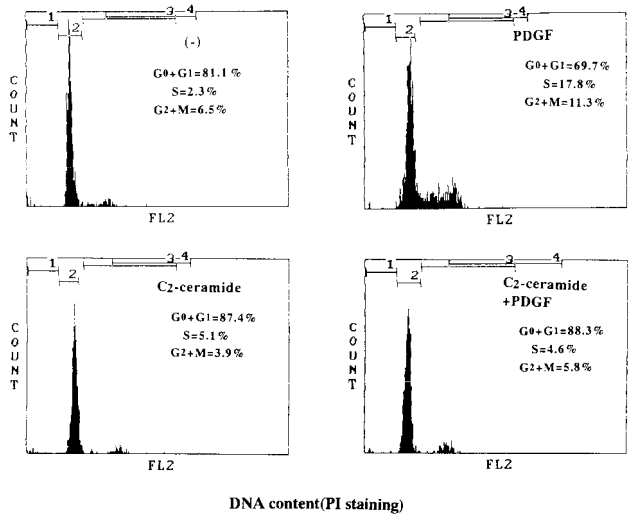

FIG. 1. C2-ceramide arrests platelet-derived growth factor-(PDGF)-stimulated cell cycle progression of rheumatoid synovial cells. Quiescent synovial cells were pretreated with C2-ceramide (50 μM) for 30 min. The cells were stimulated with PDGF (50 ng/ml) for 24 h. Cell cycle analysis was perfomed as described under Materials and Methods.

RESULTS

C2-Ceramide Inhibits PDGF-Stimulated Synovial Cell Growth

We have previously shown that cell permeable ceramide (C2-ceramide) induces apoptosis of synovial cells *in vitro* and *in vivo* (6). To expand our original findings, we investigated the effects of C2-ceramide on PDGF-induced growth-promoting signal in rheumatoid synovial cells. We examined the cell cycle analysis of synovial cells treated with C2-ceramide and PDGF. Synovial cells were synchronized in early G1 phase by starvation for 24 h. Quiescent synovial cells were pretreated with or without C2-ceramide for 30 min and after washing the medium, cells were stimulated with 50 ng/ml of PDGF for 24 h. PDGF stimulation induced cell cycle progression of serum-starved synovial cells (Fig. 1). In contrast, pretreatment with C2-ceramide for 30 min before stimulation by PDGF produced a virtual inhibition of S-phase entry and arrested the cell cycle progression in G1 phase. C2-ceramide alone had no effect on synovial cells not treated with PDGF (Fig. 1).

C2-Ceramide Inhibits Akt Kinase Activation in PDGF-Stimulated Synovial Cells

To determine the cellular mechanisms of the inhibitory effects of C2-ceramide on PDGF-mediated signal transduction, we examined the early biochemical events in PDGF-stimulated synovial cells. Stimulation of synovial cells with PDGF resulted in tyrosine phos-

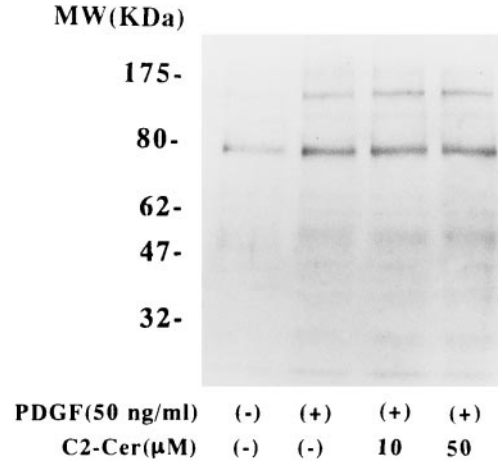

FIG. 2. Effects of C2-ceramide on tyrosine phosphorylation of PDGF-stimulated synovial cells. Quiescent synovial cells were stimulated with PDGF (50 ng/ml) in the presence or absence of C2-ceramide for 5 min. Equal amounts (50 μg) of cell lysates were electrophoresed on 8% polyacrylamide gel and analyzed by Western blot using anti-phosphotyrosine antibody. A representative example of three independent experiments.

phorylation of cellular proteins. However, C2-ceramide did not influence such effect of PDGF (Fig. 2).

PI-3 kinase pathway has been shown to be involved in PDGF-mediated signaling (15). Furthermore, recent studies indicate that the growth-promoting effect of PI-3 kinase is primarily mediated through the activation of a downstream target, the serine/threonine kinase Akt (16, 17). Therefore, we investigated whether C2-ceramide might interfere with activation of Akt kinase. To measure Akt kinase activation, we analyzed phosphorylation of Akt by western blot with anti-phospho-Akt kinase antibody. Quiescent synovial fibroblasts were stimulated with PDGF in the presence or absence of exogenous C2-ceramide for 5 min. PDGF stimulation induced phosphorylation of Akt indicating the activation of Akt. On the other hand, simultaneous addition of C2-ceramide inhibited PDGF-induced Akt kinase activation (Fig. 3). A structural analogue of C2-ceramide, C2-dihydroceramide, slightly increased the PDGF-induced Akt kinase activation compared to

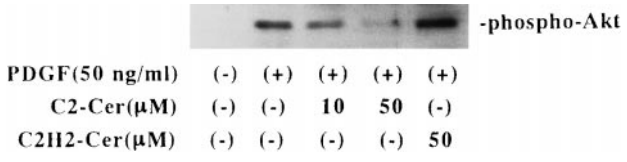

FIG. 3. Effects of C2-ceramide on Akt kinase activation of PDGF-stimulated synovial cells. Quiescent synovial cells were stimulated with PDGF (50 ng/ml) in the presence or absence of C2-ceramide for 5 min. Equal amounts of cell lysates were electrophoresed on 8% polyacrylamide gel and analyzed by Western blot using anti-phospho-Akt antibody. A representative example of three independent experiments.

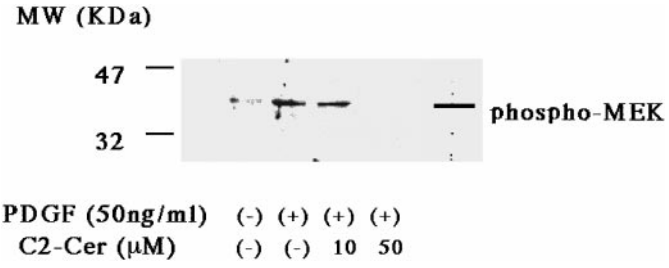

FIG. 4. Effects of C2-ceramide on MEK kinase activation of PDGF-stimulated synovial cells. Quiescent synovial cells were stimulated with PDGF (50 ng/ml) in the presence or absence of C2-ceramide for 5 min. Equal amounts of cell lysates were electrophoresed on 8% polyacrylamide gel and analyzed by Western blot using anti-phospho-MEK antibody. A representative example of three independent experiments.

that of PDGF treatment alone. Although C2-ceramide activates cytosolic phosphatase 2A, C2-dihydroceramide was shown to inhibit its enzymatic activity (18). These differential effects of C2-dihydroceramide may contribute to the increased Akt kinase phosphorylation. These results indicate that C2-ceramide inhibits PDGF-stimulated Akt kinase activation, and therefore interferes with this important anti-apoptotic signaling pathway.

C2-Ceramide Inhibits Signaling Pathway of MEK/ERK Kinase

MEK/ERK kinase cascade, which participates in the transduction of growth-promoting signals to the nucleus, is involved in the anti-apoptotic signaling pathway (19). ERK1/2 are activated by phosphorylation of tyrosine and threonine residues by MEK (20). We measured the activity of these kinases in PDGF-stimulated synovial cells. PDGF stimulation significantly induced phosphorylation and activation of MEK in synovial cells. Simultaneous addition of C2-ceramide inhibited MEK kinase activation in response to PDGF (Fig. 4). We next examined ERK kinase activity using the same cell lysates. Similarly, PDGF-induced ERK kinase activation was blocked by the addition of C2-ceramide (Fig. 5).

To determine whether C2-ceramide block anti-apoptotic signal pathway induced by other stimulations than PDGF, we stimulated quiescent synovial fibroblasts using 10% FCS containing media. Similarly, serum-stimulated kinase activations of Akt (Fig. 6A) and ERK1/2 (Fig. 6B) were inhibited by C2-ceramide.

Finally, to determine whether inhibition of PI-3 kinase or MEK might modulate Akt kinase activation, we investigated the effects of wortmannin and PD-98059 toward PDGF-stimulated synovial cells. Treatment with PI-3 kinase inhibitor, wortmannin, blocked the activation of Akt kinase in PDGF-

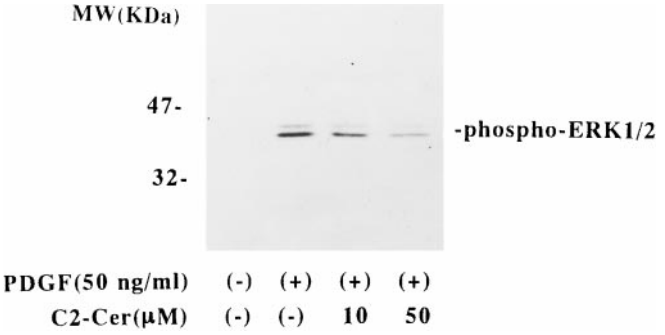

FIG. 5. Effects of C2-ceramide on ERK1/2 kinase activation of PDGF-stimulated synovial cells. Quiescent synovial cells were stimulated wit PDGF (50 ng/ml) in the presence or absence of C2-ceramide for 5 min. Equal amounts of cell lysates were electrophoresed on 8% polyacrylamide gel and analyzed by Western blot using anti-phospho-ERK1/2 antibody. A representative example of three independent experiments.

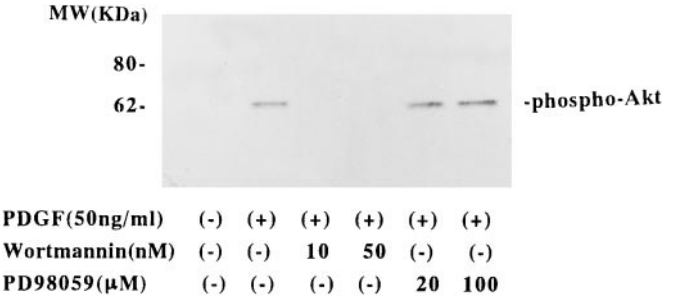

FIG. 7. Effects of wortomannin and PD09805 on Akt kinase activation of PDGF-stimulated synovial cells. Quiescent synovial cells were pretreated with wortmannin and PD09805 for 60 min, then stimuated with PDGF (50 ng/ml). Equal amounts of cell lysates were electrophoresed on 8% polyacrylamide gel and analyzed by Western blot using anti-phospho-Akt antibody. A representative example of three independent experiments.

stimulated synovial cells. In contrast, PD-98059, a synthetic MEK inhibitor, did not affect PDGF-induced Akt kinase activation (Fig. 7). These results indicate that PI-3 kinase is required for PDGF-induced Akt kinase activation, whereas MEK is not necessary for Akt activation.

It is possible that ERKs can be activated by PDGF stimulation independent of Akt or MEK. We studied the effects of wortmannin and PD-98059 on ERK kinase activation induced by PDGF. Inhibition of Akt kinase by wortmannin blocked the ERKs activation of PDGF-stimulated synovial cells (Fig. 8A). Also, pretreatment of the cells with PD-98059 inhibited ERKs activation (Fig. 8B). Taken together, our results suggest that PDGF-mediated activation of ERKs requires upstream kinases such as Akt and MEK.

DISCUSSION

Rheumatoid arthritis is a chronic inflammatory disease characterized by synovial hyperplasia. Although apoptosis of the synovial lining cells have been demonstrated in RA (21, 22), abnormal events within the apoptotic process may potentially be involved in rheumatoid synovial overgrowth (23). We have previously demonstrated that cell permeable ceramide is a potent inducer of apoptosis of proliferative synovial cells *in vitro* and *in vivo,* suggesting that this lipid messenger might

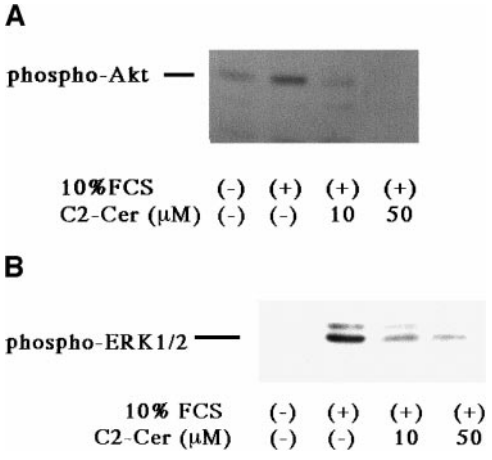

FIG. 6. Effects of C2-ceramide on Akt and ERK1/2 kinase activation of serum-stimulated synovial cells. Quiescent synovial cells were stimulated with 10% FCS containing RPMI media in the presence or absence of C2-ceramide for 5 min. Equal amounts of cell lysates were electrophoresed on 8% polyacrylamide gel and analyzed by Western blot using anti-phospho-Akt (A) and anti-phospho-ERK1/2 (B) antibody. A representative example of three independent experiments.

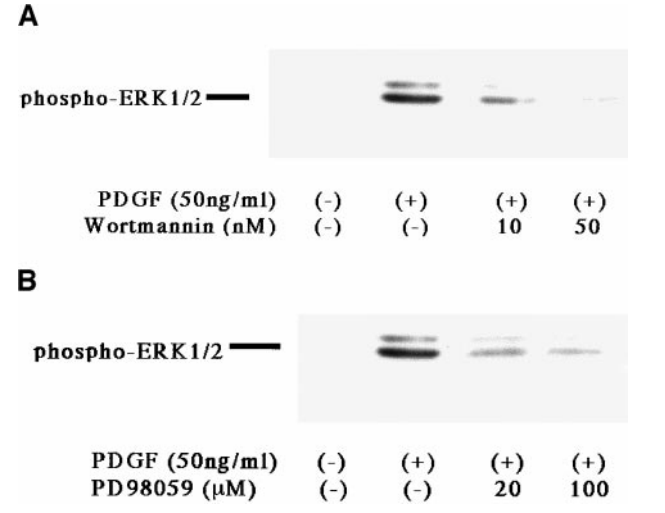

FIG. 8. Effects of wortmannin and PD09805 on ERK1/2 kinase activation of PDGF-stimulated synovial cells. Quiescent synovial cells were pretreated with wortomannin (A) and PD09805 (B) for 60 min, then stimuated with PDGF (50 ng/ml). Equal amounts of cell lysates were electrophoresed on 8% polyacrylamide gel and analyzed by Western blot using anti-phospho-ERTK1/2 antibody. A representative example of three independent experiments.

inhibit synovial overgrowth (6). Ceramide is known to activate the stress-activated protein kinase/c-jun N-terminal kinase which is involved in the apoptotic signal pathway (10). Since survival and growth of rheumatoid synovium depend on a balance between apoptosis and proliferation, we postulated that this apoptotic lipid second messenger might affect the cell proliferative signal in addition to the apoptotic pathway. Here we showed that C2-ceramide completely blocked PDGF-induced Akt kinase activation in rheumatoid synovial cells. In addition, C2-ceramide inhibited downstream kinases, such as MEK and ERK1/2, in PDGF-stimulated synovial cells. C2-ceramide-mediated inhibition of these kinases coincided with the blockage of cell cycle progression of PDGF-stimulated synovial cells.

Akt kinase, a downstream target of PI3 kinase, is a key serine/threonine kinase in growth factor-mediated inhibition of apoptosis (11, 12). On the other hand, the potential role of MEK/ERK pathway in preventing apoptosis has emerged in recent years (13, 19). In the present study, we showed that PDGF-stimulated activation of Akt kinase was inhibited by PI3 kinase inhibitor, wortmannin, but not by MEK specific inhibitor PD98059. Furthermore, PDGF-stimulated activation of ERK kinase was blocked by wortmannin. These results indicated that activation of PI-3 kinase is necessary for activation of Akt kinase and MEK/ERK signal pathway located downstream of Akt kinase. Activated ERK, which may translocate from the cytoplasm to the nucleus, induces phosphorylation and increases the transactivating activity of nuclear transcription factors (24). Thus, ERKs could potentially directly regulate gene expression. In this regard, diverse signals are known to activate ERKs *in vivo*, including activated receptor tyrosine kinases, Ras, PKC, and Raf (25). We conclude that C2-ceramide interferes with PDGF-mediated mitogenic signal at the upstream process of Akt kinase activation and that this process was associated with the disruption of downstream MEK/ERK activation pathway.

The mechanisms by which C2-ceramide inhibits PDGF-stimulated Akt phosphorylation are not clear at present. PDGF stimulation induces tyrosine phosphorylation of the intracellular domain of PDGF receptor and subsequently, other signal transduction molecules associated with these phosphorylated tyrosine residues (26). C2-ceramide did not inhibit tyrosine phosphorylation of cellular proteins in PDGF-stimulated synovial cells. Therefore, ceramide does not seem to interfere with this early event of PDGF signaling. Whether ceramide acts directly on Akt or indirectly through other upstream pathways is clearly of interest. In agreement with our study, Summers *et al.* (27) reported that C2-ceramide decreases insulin or serum-stimulated Akt kinase activation. They demonstrated that C2-ceramide did not affect insulin-stimulated PI-3 kinase activation, suggesting that the effects of C2-ceramide on Akt kinase are not mediated through modulation of PI-3 kinase. Olivera *et al.* (28) have recently reported that PDGF stimulates mitogenic sphingolipid-derived second messengers, including sphingosine and sphingosine-1-phosphate, by degrading ceramide. Furthermore, sphingosine-1-phosphate not only stimulates ERK-pathways, it also counteracts ceramide-induced apoptotic signals (29). Hence, the balance between intracellular levels of ceramide and sphingosine-1-phosphate may modulate the mitogenic signal pathway.

Rheumatoid synovial overgrowth depends on the balance between apoptosis and proliferation. In this report, we provide a novel action for ceramide; it affects the growth-promoting signal pathway in addition to its effect on the apoptotic pathway. Furthermore, our study may offer a novel therapeutic approach for inhibiting synovial overgrowth by modulating the kinase activation cascade involved in promotion of synovial cell growth.

In summary, we have demonstrated that C2-ceramide blocked the activation of Akt kinase in PDGF-stimulated rheumatoid synovial cells. Furthermore, C2-ceramide also interfered with the MEK/ERK signal pathway, which participate in the transduction of growth-promoting signals to the nucleus. Our data suggest that inhibition of these kinases may contribute to the apoptotic effects of ceramide by eliminating proliferative signals in the rheumatoid synovium.

REFERENCES

1. Harris, E. D., Jr. (1990) *N. Engl. J. Med.* **322,** 1277–1289.
2. Cohen, J. J. (1993) *Immunol. Today* **14,** 126–130.
3. Watanabe-Fukunaga, R., Brannan, C. I., Copeland, N. G., Jenkins, N. A., and Nagata, S. (1992) *Nature* **356,** 314–317.
4. Takahashi, T., Tanaka, M., Brannun, C. J., Jenkins, N. A., Capeland, N. G., Suda, T., and Nagata, S. (1994) *Cell* **76,** 969–976.
5. Hannun, Y. A., and Obeid, L. M. (1995) *Trends. Biol. Sci.* **20,** 73–77.
6. Ichinose, Y., Eguchi, K., Migita, K., Kawabe, Y., Tsukada, T., Koji, T., Aoyagi, T., Nakamura, H., and Nagataki, S. (1998) *J. Lab. Clin. Med.* **131,** 410–416.
7. Mizushima, N., Kohsaka, H., and Miyasaka, N. (1998) *Ann. Rheum. Dis.* **57,** 495–499.
8. Steller, H. (1995) *Science* **267,** 1445–1449.
9. Thompson, C. B. (1995) *Science* **267,** 1456–1462.
10. Verheiji, M., Bose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Bierrer, M. J., Szabo, E., Zon, L., Kyriakis, J. M., Haimovitz-Friedman, Fuks, Z., and Kolesnick, R. (1996) *Nature* **380,** 75–79.
11. Kauffmann-Zeh, A., Rodriguez-Vlciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J., and Evan, G. (1997) *Nature* **385,** 544–548.
12. Dudek, H., Datta, S., Franke, T., Birnbaum, M., Yao, R., Cooper, G., Segal, R., Kaplan, D., and Greenberg, M. (1997) *Science* **275,** 661–665.
13. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. (1995) *Science* **270,** 1326–1331.

14. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7686–7689.
15. Fantl, W. J., Escobedo, J. A., Martin, G. A., Turck, C. W., del Rosario, M., McCormick, F., and Williams, L. T. (1992) *Cell* **69,** 412–423.
16. Burgering, B. M., and Coffer, P. J. (1995) *Nature* **376,** 599–602.
17. Franke, T. F., Yang, S. I., Chan, T. O., Tatt, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) *Cell* **81,** 727–736.
18. Dobrowsky, R. T., Kamibayashi, C., Mumby, M. C., and Hannun Y. A. (1993) *J. Biol. Chem.* **268,** 15523–15530.
19. Jarpe, M. B., Widmann, C., Knall, C., Schlesinger, T. K., Gibson, S., Yujiri, T., Fanger, G. R., Gelfand, E. W., and Johnson, G. L. (1998) *Oncogene* **17,** 1476–1482.
20. Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, J., and Johnson, G. L. (1993) *Science* **260,** 315–317.
21. Nakajima, T., Aono, H., Hasunuma, T., Yamamoto, K., Shirai, T., Hirohata, K., and Nishioka, K. (1995) *Arthritis Rheum.* **38,** 485–491.
22. Firestein, G. S., Yeo, M., and Zvaifler, N. J. (1995) *J. Clin. Invest.* **96,** 1631–1638.
23. Nishioka, K., Hasunuma, T., Kato, T., Sumida, T., and Kobata, T. (1998) *Arthritis Rheum.* **41,** 1–9.
24. Davis, R. J. (1995) *Mol. Reprod. Dev.* **42,** 459–467.
25. Ahn, N. G., Seger, R., and Krebs, E. G. (1992) *Curr. Opin. Cell. Biol.* **4,** 992–999.
26. Claesson-Welsh, L. (1994) *J. Biol. Chem.* **269,** 32023–32026.
27. Summers, S. A., Garza, L. A., Zhou, H., and Birnbaum, M. J. (1998) *Mol. Cell. Biol.* **18,** 5457–5464.
28. Olivera, A., and Spiegel, S. (1993) *Nature* **365,** 557–560.
29. Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P. G., Coso, O. A., Gutkind, J. S., and Spiegel, S. (1996) *Nature* **381,** 800–803.