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Activation of SAPK and Increase in Bak Levels during Ceramide and Indomethacin-Induced Apoptosis in HT29 Cells

Ju Ho Kim, Sae Ock Oh, Sung Sook Jun, Jin Sup Jung, Jae Suk Woo, Yong Keun Kim, and Sang Ho Lee

Department of Physiology, College of Medicine, Pusan National University, Pusan 602−739, Korea

It has been reported that activation of sphingomyelin pathway and nonsteroidal anti-inflammatory drugs (NSAIDS) inhibit the promotion of colon carcinoma. Ceramide, a metabolite of sphingomyelin, and indomethacin were shown to induce apoptosis in colon carcinoma cells. However, the mechanisms of ceramide- and indomethacin-induced apoptosis in the colon carcinoma cells are not clearly elucidated. Recent studys showed that indomethacin-induced apoptosis in colon cancer cells through the cyclo-oxygenase-independent pathways, and that may be mediated by generation of ceramide. In this study, we compared effects of ceramide and indomethacin on important modulators of apoptotic processes in HT29 cells, a human colon cancer cell line. Ceramide and indomethacin induced apoptosis dose- and time-dependently. Ceramide and indomethacin increased stress-activated protein kinase (SAPK) activity, and decreased mitogen-activated protein kinase (MAPK) activity. The expression of Bak was increased by the treatment of ceramide and indomethacin. The expression of other Bcl-2 related proteins (Mcl-1, Bcl-X_L, Bax) which were known to be expressed in colon epithelial cells was not changed during the ceramide- and indomethacin-induced apoptosis. Our results suggest that ceramide and indomethacin share common mechanisms for induction of apoptosis in HT29 cells.

Key Words: Apoptosis, Ceramide, Indomethacin, HT29 cells

INTRODUCTION

The sphingomyelin pathway is one of important signal transduction pathways in regulation of cellular functions. A number of agents and insults such as tumor necrosis factor (TNF), Fas ligands, chemotherapeutic agents and UV irradiation cause the activation of sphingomyelinase, which acts on membrane sphingomyelin and subsequently releases ceramide. The ceramide can be phosphorylated into ceramide-1-phosphate or deacylated to sphingosine. Recent studies suggested that ceramide may be implicated in regulating diverse responses such as cell cycle arrest, apoptosis and cell senescence (Hannun, 1996).

Administration of a colon carcinoma carcinogen, 1,2-dimethyl hydrazine, in rats induced an accumulation of membrane sphingomyelin (Dudeja et al, 1986) and decreased neutral sphingomyelinase activity in colon mucosa. Also dietary sphingomyelin could inhibit the promotion of colon carcinoma induced by 1,2-dimethyl hydrazine in mice (Dillehay et al, 1995). It was reported that the activities of acidic, neutral and alkaline sphingomyleinase which differs from the known acidic and neutral sphingomyelinase were reduced in human colon carcinoma (Hertervig et al, 1997). These results indicate that alterations in sphingomyelinase activity may be involved in carcinogenesis of the colon.

Apoptosis is a strictly regulated process which removes unnecessary, aged or damaged cells (Thompson, 1995). An apoptotic process is characterized by DNA fragmentation, clumping of chromatin, nuclear disruption and formation of cytosol containing apoptotic bodies (Wyllie et al, 1980). The sphingomyelin pathway, initiated by hydrolysis of the phospholipid sphingomyelin in the cell membrane to generate the second messenger ceramide, is thought to mediate apoptosis in response to tumor-necrosis factor (TNF)-α, to Fas ligand and to X-rays in various cells

Corresponding to: Sang Ho Lee, Department of Physiology, College of Medicine Pusan National University, Ami-Dong, Suh-Gu, Pusan 602-739, Korea. (Tel) 051-240-7731, (Fax) 051-246-6001, E-mail: sangho89@hyowon.pusan.ac.kr

including colon carcinoma cells (Kolesnick & Kronke, 1998; Veldman et al, 1998).

It has been reported that activation of SAPK/JNK plays a critical role in ceramide-induced apoptosis (Verheij et al, 1996). Several reports showed specific roles for the mitogen-activated protein kinase cascade in the maintenance of proliferation and/or differentiation. Accordingly, the balance between the SAPK and MAPK systems has been proposed as a fundamental determinant of cell survival (Xia et al, 1995).

Apoptotic processes are modulated by Bcl-2 related proteins. Bcl-2 is known to belong to a growing family of apoptosis regulatory gene products, which may either be death antagonists (Bcl-2, Bcl-X_L, Bcl-w, Bfl-1, Brag-1, Mcl-1 and A1) or death agonists (Bax, Bak, Bcl-X_S, Bad, Bid, Bik and Hrk). The balance of antagonistic and agonistic Bcl-2 related proteins is important in regulation of apoptosis (Reed, 1994). Overexpression of Bcl-2 inhibited ceramide-induced apoptosis (Herrmann et al, 1997). However, changes in expression of Bcl-2 related proteins during ceramide-induced apoptosis have not been examined.

Nonsteroidal antiinflammatory drugs (NSAIDs) are among the most commonly used medications. They have been utilized as an antipyretics, as a preventive drugs for myocardic infarction and cerebrovascular diseases, or for the treatment of chronic inflammatory diseases such as rheumatoid arthritis (Insel, 1990). Recent studies have demonstrated that NSAIDs profoundly inhibit colorectal tumorigenesis (Giardiello et al, 1993; Reddy et al, 1993; Giovannucci et al, 1994).

Shiff et al (1996) suggested NSAIDs-induced apoptosis of colon cancer cells is the mechanism that NSAIDs reduces the incidence of colon cancer. Hanif et al (1996) demonstrated that the NSAIDs-induced apoptosis is independent of the prostaglandin pathway, of which inhibition is the well known pharmacological action mechanism of NSAIDs. NSAIDs induced apoptosis in HCT-15 cells that do not express cyclooxygenase, and addition of prostaglandins failed to reverse NSAIDs-induced apoptosis in HT-29 cells that express cyclooxygenase (Hanif et al, 1996). Mechanisms of intracellular signaling in NSAIDs-induced apoptosis are not clear. Recently, Chan et al (1998) reported that ceramide, a product of sphingomyelin pathway, is involved in NSAIDS-mediated apoptosis in colon carcinoma cells, suggesting that NSAIDS and ceramide have common mechanisms for apoptosis induction in colon carcinoma cells.

HT29 cells were derived from human colon carcinoma and have been widely employed in researches of colon cancer and epithelial physiology (von Kleist et al, 1975; Matthews et al, 1998; Mallo et al, 1998). This study was undertaken to examine whether ceramide and indomethacin affect SAPK and MAPK activity and expression of Bcl-2 related proteins in HT29 cells, a colon carcinoma cell line.

METHODS

HT29 cell culture

The human colon adenocarcinoma cell line HT29 (ATCC, HTB 38) was obtained from the American Type Culture Collection (ATCC). Culture medium consisted of Dulbeco's modified Eagle's Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco BRL), penicillin (50 U/ml, Sigma) and streptomycin (50 g/ml, Sigma). HT29 cells were grown as monolayers in 60- or 100-mm plates. The cells were incubated at 37°C in 5% CO_2/95% air. Cell proliferation was determined by counting the cells in a hemocytometer. To document morphologic alterations induced by ceramide, cells were photographed in the culture dishes using an inverted microscope (Olympus, IX50).

Tunnel assay

Tunnel assay was performed by use of a commercial kit provided by Boehringer Mannheim (Sandhofer, Germany). HT29 cells were fixed by 4% paraformaldehyde at room temperture. After washing with Dulbeco's phosphate buffered saline (PBS, Sigma) the cells were permeabilized by permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 5 min at room temperature. DNA strand breaks were labelled with fluorescein-labelled nucleotides by use of terminal deoxynucleotidyl transferase (TdT) in a humidified chamber for 60 min at 37°C. And then the cells were washed by PBS, observed by fluorescence microscope, and photographed (Moss et al, 1996).

DNA degradation

HT29 cells were counted with a hemocytometer, plated at a density 3×10^6 cells/60 mm dish and treated with control or drug-supplemented medium for 48 h. Genomic DNAs were obtained from cells by lysis in 10 mM EDTA, 50 mM Tris (pH 8.0), 0.5% (weight/volume) sodium dodecyl sulfate (SDS). These extracts were digested with 100 μg/ml boiled RNase (Sigma) for 1 h at 37°C followed by treatment with proteinase K (Boehringer-Mannheim) at a concen-

tration of 1 mg/ml for 18 hrs at 50°C. Isolated genomic DNA was extracted with phenol : chloroform (1 : 1, vol/vol) then chloroform, followed by ethanol precipitation. The resulting DNA was dissolved in TE buffer and its amount was quantified by measuring its absorbance at 260 nm. 10 μg of DNA was mixed with sample buffer (final concentration: 0.025% bromophenol blue/0.025 xylene cyanole/3.0% glycerol) and resolved in 2% agarose gels impregnated with ethidium bromide (0.1 μg/ml). DNA was visualized by UV transillumination and photographed using Polaroid 667 Film (Hanif et al, 1996).

Preparing cell lysate for western blotting of Bcl-2 family and determination of SAPK and MAPK

Cells were lysed in 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 15 min at 4°C. The lysates were centrifuged at 10,000× g for 15 min and the supernatants were used as a source for western blotting of Bcl-2 family and determination of SAPK and MAPK.

Determination of SAPK and MAPK activities

SAPK and MAPK activities were determined with commersial kits (New England Biolab). The 250 μl of cell lysate (~250 μg total protein) were added with 2 μg of c-Jun fusion protein beads or 2 μg of monoclonal antibodies to p44/42 MAPK and incubated with gentle rocking overnight at 4°C. In case of MAPK, after incubation at 4°C 20 ml protein G (50% bead, Sigma) was added and incubated with gentle rocking for 3 h at 4°C. The beads were centrifuged for 30 s at 4°C, and then the pellet washed twice with 500 μl of lysis buffer and twice with kinase buffer (20 mM Tris, pH 7.5, 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) on ice. Kinase assay was performed by incubating the suspended pellet with kinase buffer containing 100 mM ATP and c-Jun as a substrate of SAPK or Elk-1 protein as a substrate of p44/42 MAPK. The reactions were terminated with 25 μl 3 × sample buffer (62.5 mM Tris, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.1% w/v bromophenol blue) and the samples were analyzed by 12% SDS Polyacrylamide gel electrophoresis (Coroneos et al, 1996). Phosphorylated c-Jun and Elk-1 were detected with phospho-c-Jun and antibodies, respectively.

Western blotting

Proteins were transferred onto Hybond ECL (Amersham) membrane. The blots were blocked in Tris-buffered solution-T (TBST, 1 × TBS, 0.1% Tween-20) containing 5% nonfat dry milk for 2 h at room temperature and were incubated with primary antibodies (1 : 1000) in blocking buffer with gentle agitation overnight at 4°C. The primary antibodies were removed, and then the membranes were washed 3 times with TBST and then incubated with horse radish peroxidase-conjugated anti-rabbit secondary antibody (1 : 2000) for 1 h and detected by chemiluminescent reaction (Jung et al, 1996).

Materials

C2-ceramide was purchased from the Calbiochem-Novabiochem Corporation (La Jolla, CA). Rabbit polyclonal antibodies to Bak, Bcl-X_L, Bax and Mcl-1 were purchased from Santa Crutz (Delaware, CA). Tunnel assay kit was purchased from the Boehringer Mannheim (Sandhofer, Germany). MAPK and SAPK kits were purchased from the New England Biolabs (Bevely, MA). ECL western blotting kits were purchased from the Amersham International (Amersham, UK). All other chemicals were the highest quality available.

RESULTS

Apoptosis by ceramide and indomethacin in HT29 cells

To identify whether ceramide and indomethacin induce apoptosis, we performed Tunnel assay and examined degradation of genomic DNA by agarose gel electrophoresis. For the Tunnel assay HT29 cells were cultured on the coverslips for 72 hrs. Thereafter, cells were treated with 50 μM C2-ceramide for 24 hrs or 600 μM indomethacin for 48 hrs. Because terminal deoxytransferase labels DNA strand breaks with FITC-conjugated dUTP, the fluorescence can be observed in the apoptotic nuclei. Cells containing apoptotic condensed nuclei could be observed in the ceramide- and indomethacin-treated cells, not in control cells (Fig. 1). The degradation of genomic DNA was analyzed by agarose gel electrophoresis of genomic DNA of HT29 cells. DNA fragmentation by ceramide was time- and dose-dependent. DNA fragmentation appeared at 24 hrs after 50 μM C2-ceramide treatment and at 48 hrs after 600 μM

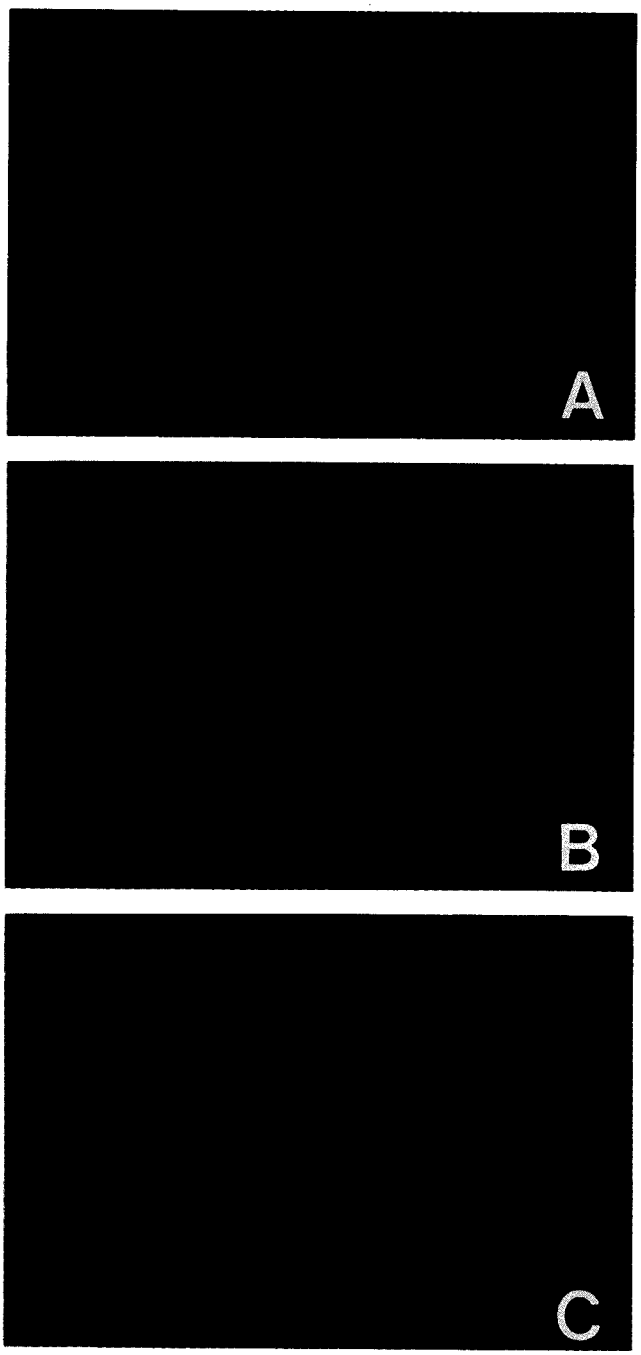

Fig. 1. Evaluation of ceramide- or indomethacin-induced apoptosis in HT29 cells by Tunnel assay. HT29 cells were treated with control medium (A) or 50 μM C2-ceramide for 30 hrs (B) and 600 μM indomethacin for 48 hrs (C). Apoptotic nuclei are seen in cells treated with C2-ceramide (B) and indomethacin (C) but not in those treated with control medium (A) (40× Magnification).

indomethacin treatment. The minimum dose of C2-ceramide to induce DNA fragmentation at 30 hrs after treatment was 30 μM, and that of indomethacin to induce at 48 hrs after treatment was 300 μM (Fig. 2). Measurement of apoptosis in this study were performed in the presence of 10% FBS, the normal growth conditions for cell culture, and with the cells in logarithmic phase because removal of serum deprivation was reported to cause apoptosis and to increase intracellular formation of ceramide (Jayadev et al, 1996). In the present study, removal of serum in the medium accelerated the ceramide- and indomethacin-induced apoptosis (data not shown).

Effects on SAPK and MAPK activity

Signaling pathways of apoptosis are various according to apoptosis-inducing stimuli. Activations of SAPK and inhibition of MAPK pathway are one of important pathways in apoptosis (Xia et al, 1995). Therefore, to identify the involvement of these pathways in ceramide- and indomethacin-induced apoptosis of HT29 cells, SAPK and MAPK activity were measured. 50 μM C2-ceramide and 600 μM indomethacin activated SAPK at 15 min after treatment of the reagents and its activation lasted for 60 min (Fig. 3). The time course of SAPK activation was similar with other studies using ceramide or agents that induce intracellular formation of ceramide (Verheij et al, 1996). MAPK activity was decreased at 15 min after treatment of 50 μM C2-ceramide and 600 μM indomethacin with respect to the control and the effect lasted for more than 180 min (Fig. 3).

Identification of the related members of Bcl-2 family in the ceramide- and indomethacin-induced apoptosis

To identify whether the expression of the related members of Bcl-2 family is changed in ceramide- and indomethacin-induced apoptosis, we performed western blotting with Bcl-X_L, Bax, Mcl-1 and Bak rabbit polyclonal antibodies. The expression levels of Bcl-X_L, Bax and Mcl-1 were not changed by the treatment of 50 μM C2-ceramide and 600 μM indomethacin during the period to be examined. However, the level of Bak was significantly increased after the treatment of C2-ceramide and indomethacin (Fig. 4).

DISCUSSION

The long-term administration of NSAIDs reduces the incidence of colon cancer, which is related to NSAIDs-induced apoptosis of colon cancer cells. However, NSAIDs-induced apoptosis is independent of inhibition of cyclooxygenase pathway in HT-29 and HCT-15 cells. Chan et al (1998) suggested that NSAIDS-induced apoptosis is mediated by the generation of ceramide through the activation of sphin-

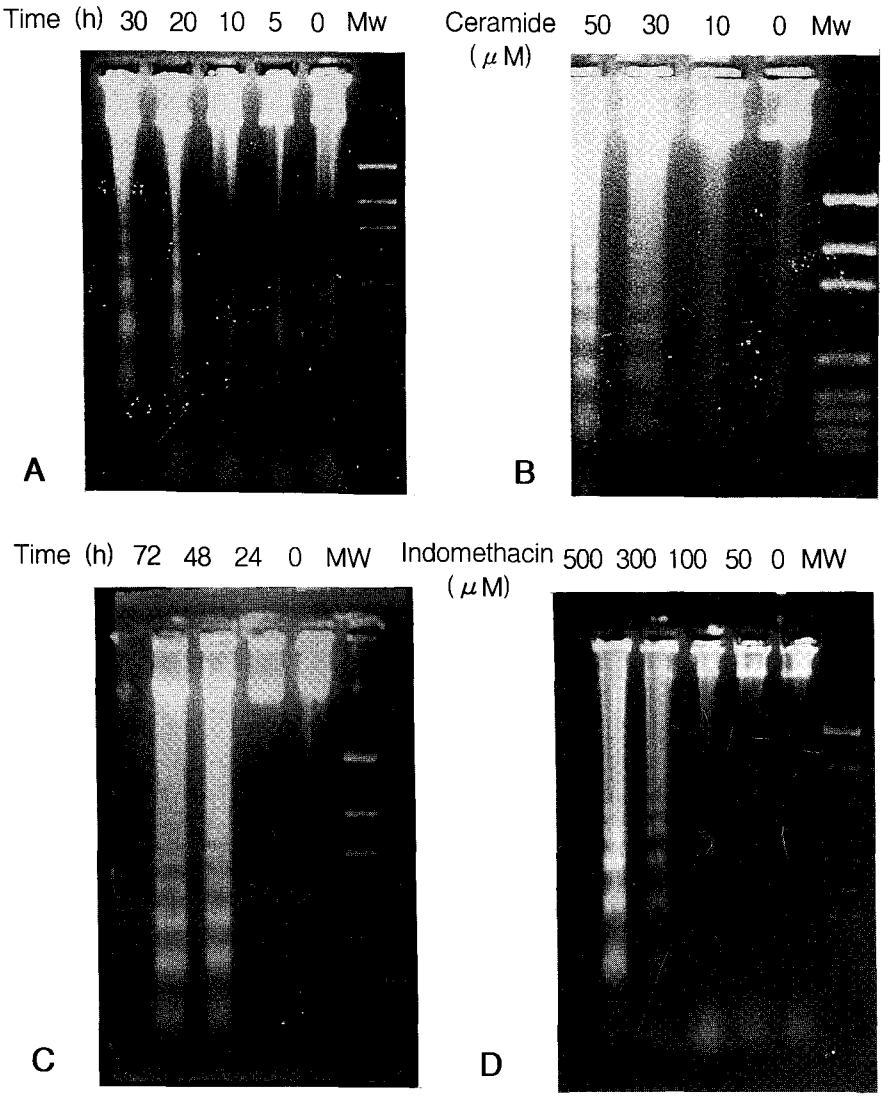

Fig. 2. Effects of ceramide and indomethacin on DNA fragmentation in HT29 cells. Genomic DNA was isolated from HT29 cells and fractionated on 2% agarose gels, as described in Materials and Methods. A and C. HT29 cells were treated with 50 μM C2-ceramide (A) or 600 μM indomethacin (C) for the indicated times. B and D. HT29 cells were treated for 24 hrs with ethanol vehicle or with the indicated concentrations of C2-ceramide (B) or indomethacin (D). Mw: Molecular weight marker.

gomyeline pathway.

In this study, indomethacin and ceramide induced DNA fragmentation, a typical hallmark of apoptosis, in HT29 cells. Induction of apoptosis by these reagents were further supported by Tunnel assay. Ceramide- and indomethacin-induced apoptosis has been also reported by other investigators (Hanif et al, 1996; Shiff et al, 1996; Chan et al, 1998; Kolesnick & Kronke, 1998; Veldman et al, 1998).

Apoptosis is mediated or modulated by several intracellular mediators (Bosman et al, 1996; Leist & Nicotera, 1998). p53 is an important mediator of apoptosis (Steele et al, 1998). However, HT29 cells have a mutation of p53 gene and overexpress nonfunctional mutants (Rodrigues et al, 1990). Therefore, role of p53 in ceramide- or indomethacin-induced apoptosis can be excluded.

SAPK is the dominant c-Jun amino terminal protein kinase activated in response to a variety of cellular stresses, including treatment with TNF-α and interferon-γ, ultraviolet radiation and serum deprivation (Sanchez et al, 1994). The importance of SAPK in the process of apoptosis was reported in several studies using dominant-negative c-jun mutant (Xia et al, 1995), SAPK antisense oligonucleotide (Seimiya et al, 1997) or neutralizing antibody to

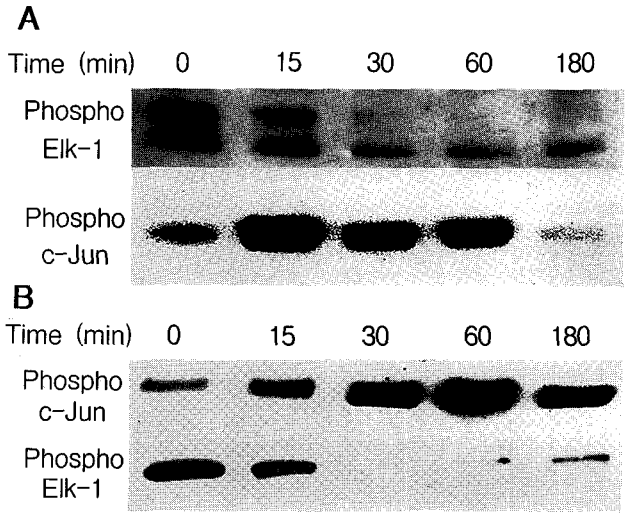

Fig. 3. Activation of SAPK and inhibition of MAPK by ceramide and indomethacin in HT29 cells. HT29 cells grown for 24 hrs after plating on a 60 mm dish were treated with 50 μM C2-ceramide (A) and 600 μM indomethacin (B) before lysis and subsequent immunoprecipitation/immunoblotting. Time after exposure to C2-ceramide or indomethacin is indicated. The immunoblot is a representative of three separate experiments. SAPK activity was determined in aliquots containing equal amounts of total protein (200 μg) and c-Jun fusion protein bead as substrate as described in Materials and Methods. The phosphorylated c-jun fusion protein band is indicated. MAPK activity was determined in aliquots containing equal amounts of total protein (200 μg) and Elk-1 fusion protein as substrate as described in Materials and Methods. The phosphorylated Elk-1 fusion protein band is indicated.

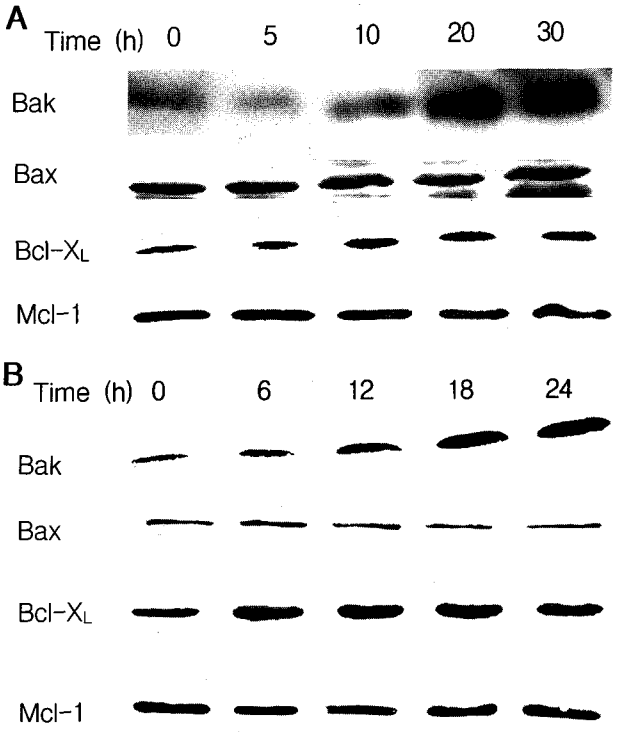

Fig. 4. Western blots of Bcl-2 related proteins in HT29 cells. HT29 cells grown for 24hrs after plating on a 60 mm dish were treated with 50 μM C2-ceramide (A) or 600 μM indomethacin (B). Bak expression was increased in a time-dependent manner during ceramide- or indomethacin-induced apoptosis without affecting the expression of other members of Bcl-2 related proteins that have known to be expressed in HT29 cells. Time after exposure to C2-ceramide and indomethacin is indicated.

SAPK (Estus et al, 1994). A recent study (Verheij et al, 1996) showed that activation of SAPK plays an critical role in ceramide-induced apoptosis. This study also showed that activation of SAPK preceded apoptotic cell death in HT29 cells, supporting an important role of SAPK on the process.

Activation of Ras-MAPK pathway is known to promote cell survival (Mansour et al, 1994). Xia et al (1995) suggested that inactivation of MAPK together with activation of SAPK or P38 or both is critical for apoptosis. In the present study ceramide did not affect the basal activity of MAPK under the serum-free condition, but inhibited serum-induced activation of MAPK. The effect of ceramide on MAPK activity is still controversial. Ceramide stimulated MAPK in human promyelocytic leukemia HL-60 cells (Raines et al, 1993), but inhibited in human monoblastic leukemia U937 cells (Jarvis et al, 1997). The variation of the responses may be resulted from the differences of cell lineage. Therefore, there is a possibility that inhibition of cytoprotective MAPK activity by ceramide and indomethacin may enhance ceramide-induced apoptotic processes as well as inhibition of proliferation of HT29 cells.

Bcl-2 related proteins, which are predominantly located in the outer membrane of mitochondria, regulate translocation of cytochrome C or apoptosis inducing factor (AIF) from mitochondria to cytosol (Yang et al, 1997; Kroemer et al, 1997; Susin et al, 1996). Some of Bcl-2 related proteins (Bak, Bax etc) have proapoptotic activities and the others (Bcl-2, Bcl-X_L, Mcl-1 etc) have antiapoptotic activities. Bcl-2 related proteins are known to heterodimerize each other, and the balance of pro-apoptotic and anti-apoptotic Bcl-2 related proteins is important in the regulation of apoptosis (Kroemer, 1997). Smyth et al (1996) reported that the overexpression of Bcl-2 inhibited ceramide-induced apoptosis in human leukemic Molt-4 cells, suggesting that Bcl-2 related proteins are involved during the ceramide-induced

apoptosis. Changes in expression of Bcl-2 related proteins were variable according to apoptotic stimuli. In HL-60 and U-937 cells ionizing radiation decreased Bcl-X_L, whereas ceramide and TNF-α failed to affect it (Chen et al, 1995). In the present study, ceramide and indomethacin increased the expression of pro-apoptotic Bak without affecting the expression of other members of Bcl-2 related proteins. Hong et al (1998) also reported that the expression of Bcl-2 and Bax was not affected by indomethacin and excluded the possibility of involvement of Bcl-2 proteins in indomethacin-induced apoptosis. However, they did not examine changes in expression of Bak. Changes in expression of Bak protein among Bcl-2 related proteins were most positively correlated with apoptotic cell death in colon cancer cells (Moss et al, 1996; Partik et al, 1998). Moss et al (1996) reported changes in Bak expression played an important role in the butyrate-induced apoptosis in HT29 cells.

The data in this study indicated that ceramide- and indomethacin-induced apoptosis in HT29 cells is induced by changes in expression of Bcl-2 related proteins as well as activation of SAPK and decrease in MAPK activity, suggesting that indomethacin and ceramide share common mechnisms for apoptosis.

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