A Relationship between 5-Lipoxygenase-activating Protein and bcl-xL Expression in Murine Pro-B Lymphocytic FL5.12 Cells*

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Inhibitors of 5-lipoxygenase-activating protein (FLAP) have been found to induce apoptosis. The current study examined the expression of FLAP and bcl family proteins and the induction of apoptosis in interleukin-3-dependent control and bcl-xL-overexpressing FL5.12 cell lines after treatment with MK886, a specific FLAP inhibitor. FL5.12 cells contained a substantial amount of FLAP protein and mRNA but surprisingly had no measurable 5-lipoxygenase protein or 5-, 12-, or 15-lipoxygenase activity. The basal level of FLAP protein in cells overexpressing bcl-xL was 70% less than in controls. FLAP disappeared 4 h after withdrawal of interleukin-3 in bcl-xL cells but not in control cells, which underwent apoptosis. A dose- and time-response study revealed that 5 nmol of MK886/10⁶ cells was sufficient to induce apoptosis both in control and bcl-xL cells, respectively, but to different degrees. bcl-xL and bcl-2 proteins, but not bax or FLAP, were decreased by 4 h after 5 nmol of MK886/10⁶ cells in both cell lines, although the higher levels of bcl-xL in overexpressors took longer to disappear. This early loss of bcl-xL and bcl-2 was not attributable to generalized proteolysis, as shown by Coomasie Blue staining and by the maintenance of bax. Caspase-3 was activated 2 h after MK886 treatment in control cells but not in bcl-xL cells. Inhibition of caspase-3 decreased MK886-induced apoptosis by 50% in control cells. Inhibition of this caspase after MK886 treatment was unable to prevent the loss of bcl-xL in control cells but did provide partial protection for the loss of the transfected form, but not the endogenous form, in overexpressing cells. These data indicate that MK886 induces extensive apoptosis that is partially caspase-3 dependent and may be related to a rapid loss of bcl-xL. Although caspase-3 inhibitors had no effect on the loss of bcl-xL, other caspases or protease systems may still be involved. The absence of 5-lipoxygenase in cells containing FLAP, the lower level of FLAP in bcl-xL cells, the apoptosis-inducing activity of MK886, and the rapid loss of bcl-xL and bcl-2 proteins after treatment with MK886 strongly indicate that FLAP has activities unrelated to lipoxygenase and suggest a possible functional or regulatory link between these proteins, which share similar subcellular localizations.

Apoptosis is a highly controlled form of cell death that plays an important role in embryonic development, the maintenance of tissue and organ homeostasis, and various pathologic conditions, including cancer and xenobiotic toxicity. Several molecular entities, e.g. oncogenes, signal transducers, cell cycle proteins, and free radicals, have been associated with the induction of apoptosis (1, 2). Free radical species are of particular interest, because they may mediate various biochemical processes leading to altered cell growth, differentiation, and death (3, 4), and an association between free radicals and several effectors of apoptosis has been reported. Furthermore, proteins encoded by some of the bcl-2 family proto-oncogenes (e.g. bcl-2 and bcl-xL) suppress apoptosis induced by various oxidative processes (5, 6), whereas different antioxidants are capable of preventing apoptosis spurred by a variety of agents (7, 8). However, the molecular mechanisms underlying the morphological and biochemical hallmarks of apoptosis induced by oxidants are not known.

Reactive oxygen species and oxidized lipid products are considered crucial elements in both tumor cell proliferation (9) and apoptosis (10). Different stimuli, including the activation of CD28 (11) and CD95 (12) cell surface receptors, and tumor necrosis factor-α-mediated cytotoxicity (13) are capable of inducing apoptosis while generating reactive oxygen species and/or lipid hydroperoxides. In each case, the involvement of arachidonic acid metabolism mediated by lipoxygenase (LOX)1 was identified, indicating a possible role for LOX in regulating apoptosis and cell survival. Recently, some evidence has shown that both lipid hydroperoxides (14, 15) and LOX inhibitors, specific as well as nonspecific (16–18), are capable of inducing apoptosis in some cell systems. LOX has been further related to apoptosis because of the ability of a LOX antisense oligonucleotide to cause apoptosis in W256 cells (17). In contrast to these proapoptotic effects, some LOX inhibitors blocked apoptosis in systems in which induction of LOX activity by CD95 receptor ligand (12), hydrogen peroxide (19), or γ radiation (20) occurred. The conflicting information about the effects of LOX inhibitors in apoptosis indicates that the exact mechanism(s) by which LOX affects this form of cell death is not yet understood.

5-Lipoxygenase-activating protein (FLAP) is considered to be an integral part of the 5-LOX pathway of arachidonic acid metabolism (21). FLAP transfers arachidonic acid to 5-LOX protein, thereby enabling this enzyme to efficiently produce oxidized lipid products. Inhibition of this process can be achieved at nanomolar doses of MK886. However, at higher doses, MK886 appears to further disrupt arachidonic acid bind-

1 The abbreviations used are: LOX, lipoxygenase; FLAP, 5-lipoxygenase activating protein; NDGA, nordihydroguaiaretic acid; IL, interleukin; RT-PCR, reverse transcription-polymerase chain reaction.
ing to FLAP, thereby altering metabolism of this fatty acid, reducing cell proliferation, and causing apoptosis (22).

Interestingly, FLAP is localized to the nuclear membrane (23), a site where bcl antiapoptotic proteins are also found (24). Alterations in LOX and FLAP expression or activity in connection with apoptosis and the role of the bcl family of oncogenes in this process have not been examined. In the current study, the effects of MK886 (Scheme 1), a specific FLAP inhibitor, on cell survival, apoptosis, bcl-xL, bcl-2, and FLAP expression, and caspase activity in both control and bcl-xL-overexpressing murine pro-B lymphocytic FL5.12 cell lines have been investigated. The results show a much lower expression of FLAP in bcl-xL gated. The results show a much lower expression of FLAP in bcl-xL and streptomycin (100 units/ml and 100 μM; Calbiochem, La Jolla, CA) for 1 h before the treatment was measured according to the method described by Ioannou and Chen (30). Fluorescence microscope to differentiate early apoptotic cells from late apoptotic, normal, and necrotic cells. The number of apoptotic cells (both early and late) was counted in 10 different fields (magnification ×40) in each case.

Western Blot Assays—Cells (10⁶ cells/treatment) were pelleted, lysed in 250 μl of ice-cold buffer containing 10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 1 mM EDTA, 0.1% (v/v) Nonidet P-40, and anti-protease mixture (1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 30 μM/ml aprotinin) by repeated pipetting and incubated for 15 min at 4 °C. Cell extracts were centrifuged at 16,000 × g for 10 min at 4 °C, and total protein content in the supernatant was measured (30). Supernatants were run on either 8% (5-LOX) or 15% (FLAP, bax, bcl-2, and bcl-xL) reducing SDS-polyacrylamide gels (buffer composition: 0.2 M Tris (pH 6.8), 4% (w/v) SDS, 20% (w/v) glycerol, 4% (w/v) β-mercaptoethanol, and 0.02% (w/v) bromophenol blue). Protein was transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and blocked for at least 1 h in 5% (w/v) nonfat dry milk (Bio-Rad). The membrane was then incubated with anti-bcl-xL (rabbit), anti-bcl-2 (rat), or anti-bax (rabbit) monoclonal or polyclonal antibodies (1:1500 dilution each; Santa Cruz Biotechnology, Santa Cruz, CA), anti-FLAG M2 mouse monoclonal antibody (1:1000 dilution; Sigma), FLAP, or 5-LOX rabbit polyclonal antiserum (1:1500 dilution each; generous gifts from Dr. Jilly F. Evans, Merck Institute for Therapeutic Research, Quebec, Canada) for 1 h. The membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibody, anti-rabbit, anti-rat, or anti-mouse IgG (1:3000 dilution each; Amersham Pharmacia Biotech), for 1 h, and the protein of interest was visualized using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech). The film was scanned, and individual band densities were integrated using NIH software with an electronic densitometer.

cDNA Preparation, RNA Isolation, and Northern Blot Assay—bcl-xL cDNA was purified from the corresponding plasmid (a generous gift from Dr. Gabriel Nuñez) using a Qiagen (Santa Clara, CA) kit. Total cellular RNA (10⁶ cells/treatment) was isolated using Ultraspec RNA isolation reagent (Biotex Laboratories, Houston, TX). Isolated RNA was separated in a 1% agarose-formaldehyde gel and transferred to a Zeta Probe membrane (Bio-Rad) by diffusion. A 2.1 kb cDNA fragment was labeled with [32P]dCTP (ICN, Irvine, CA) by the random primer method (31) using a kit (Boehringer Mannheim). The membrane was hybridized in 0.25 × Na₂HPO₄ (pH 7.2) and 7% (w/v) SDS overnight at 65 °C. The membrane was then washed twice with 20 mM Na₂HPO₄ (pH 7.2) and 5% (w/v) SDS for 5 min and twice with 20 mM Na₂HPO₄ (pH 7.2) and 1% (w/v) SDS for 5 min. Autoradiography was performed either electronically with a Packard Instrument Co. Instant Imager or using Eastman Kodak Co. XAR-5 film (Sigma) exposed overnight at –80 °C. Radiometric quantitation was carried out using Packard Imaging software (version 2.02). Band densities were compared after correction against the constant level of 5 S RNA expression.

RT-PCR—Total cellular RNA was isolated using a RNeasy kit (Ambion, Austin, TX). 3 μg of total RNA were transcribed with 100 units of Moloney murine leukemia virus reverse transcriptase, 300 ng of oligo(dT), 1 mM dNTPs, and 10 units of plasental RNase inhibitor (RETROscript kit, Ambion) in reaction buffer supplied by the manufacturer, in a total volume of 20 μl at 37 °C for 1 h. PCR was performed using 1 μl of the resulting cDNA, 2.5 units of AmplTag DNA polymerase (Perkin-Elmer), reverse and forward primers (250 μM each), and 250 μM dNTP in a final volume of 50 μl. The conditions in the amplification of FLAP were as follows: 5′-‘ATGGATCAAGAGG-CGTGGGGC-3′ and 3′-‘5-GTCGTCGCTCAGCTGTG-3′. The reaction mixture was heated to 94 °C for 3 min, and amplification was performed for 30 sequential cycles at 94 °C for 50 s, 58 °C for 40 s, and 72 °C for 40 s. After the last cycle, the reactions were incubated at 72 °C for an additional 5 min. 10 μl of PCR products were electrophoretically

![Scheme 1](image-url)
Nordihydroguaiaretic acid (NDGA), a nonspecific LOX inhibitor, induced apoptosis in control FL5.12 cells (Fig. 1). This occurred at low (0.5 μM, probably non-antioxidant) concentrations. Apoptotic cells were represented by ∼20% of the total cell population in the control cells 3 and 6 h after 1 μM NDGA treatment, whereas only 10–14% of bcl-x<sub>L</sub>-transfected cells underwent apoptosis, an amount comparable to untreated controls (Fig. 1). Protection was also evident in bcl-x<sub>L</sub> cells using 1 μM NDGA.

NDGA is an effective nonspecific LOX inhibitor but has numerous other effects. Thus, NDGA could induce apoptosis by other mechanisms, although available data suggest its action on LOX is the critical one (18). Because not all cells have LOX activity, FL5.12 cells were examined for the presence of LOX.

Identification of LOX Metabolites of Arachidonic Acid—Cells (2 × 10<sup>6</sup>) were pelleted, resuspended in 500 μl of 100 mM Tris (pH 7.8) containing 1 mM EDTA, and sonicated on ice using at least three 30-s pulses. Phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM. The disrupted cells were centrifuged at 16,000 × g for 10 min at 4 °C, and the supernatant was incubated with 100 μM 3H-labeled arachidonic acid (10 μCi/ml; DuPont) at 37 °C for 20 min. The reaction was halted by adding 2 ml of methanol, and cellular protein was removed by centrifugation. The methanol layer was evaporated to dryness under vacuum, reconstituted in 200 μl of methanol, and injected into a C<sub>18</sub> reverse-phase column (Whatman). 3H-Labeled arachidonic acid metabolites, particularly the level of different hydroxyeicosatetraenoic acids, were analyzed to assess cellular LOX activity (32). Peaks were identified by comparison with authentic standards (Cayman Chemical Co., Ann Arbor, MI).

RESULTS

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Immunoblot analysis revealed that FL5.12 cells do not have any measurable 5-LOX protein (Fig. 2A). The faint bands evident do not match the positive control obtained from mouse white blood cells. Confirmation of this finding was achieved using a LOX activity assay, which indicated there is no measurable 5-, 15-, or 12-LOX activity present in either the control or transfected cell lines. Based on the apparent absence of 5-LOX, the effect of caffeic acid, a specific 5-LOX inhibitor (34), was also examined. This compound was unable to induce ap-
optosis in FL5.12 cells, indicating that the effect of NDGA is unrelated to 5-LOX.

Despite the absence of 5-LOX, both control and bcl-xL-overexpressing FL5.12 cells expressed FLAP (Fig. 2B). Interestingly, the basal level of FLAP detected in bcl-xL overexpressors was only 30% of that present in the control cells. The growth of FL5.12 cells depends on IL-3, and significant apoptosis (30%) occurs in control but not in bcl-xL cells (13%) 6 h after withdrawal of this growth factor (35). The expression of FLAP was maintained at basal levels in control cells up to 8 h after withdrawal of IL-3 (Fig. 2B). However, FLAP completely disappeared at 4 h after IL-3 withdrawal in the bcl-xL-transfected cells (Fig. 2B). In contrast, bcl-xL expression did not change in either control or bcl-xL cells after IL-3 withdrawal (Fig. 2C).

Immunoblot analyses of different subcellular fractions obtained from both control and bcl-xL cell lines indicated that FLAP was mainly present in the nuclear fraction (Fig. 2D), as has been documented previously in different cell lines (23). The presence of FLAP mRNA in both control and bcl-xL cell lines was identified by RT-PCR (Fig. 2E). Although quantitative RT-PCR was not performed, it appeared that FLAP mRNA expression in control cells was greater than that in bcl-xL overexpressors.

Mobilization of esterified arachidonic acid from membrane lipid pools has been postulated to contribute to tumorigenesis and to the progression of certain cancers by promoting cell proliferation (36, 37). Because FLAP assists in arachidonic acid mobilization through its binding activity, we studied its role in apoptosis using a specific FLAP inhibitor, MK886 (21, 38). Apoptosis was not evident in human lung adenocarcinoma A549 cells that do not contain FLAP (data not shown). However, consistent with the presence of FLAP, a dose of MK886 of as low as 5 nmol/10^6 cells (≈10 μM) was able to induce significant apoptosis in control FL5.12 cells at 8 h, as assessed by DNA fragmentation (Fig. 3, lanes 1–6). bcl-xL-overexpressing cells showed a degree of resistance against apoptosis at 8 h caused by the same concentration of MK886, although DNA fragmentation was clearly evident using 10 nmol/10^6 cells (Fig. 3, lanes 7–12).

After a time-response study using 5 nmol of MK886/10^6 cells, DNA fragmentation was first evident in control cells at 8 h. bcl-xL overexpression largely prevented DNA ladder formation by this dose of MK886 up to 24 h (data not shown). However, a quantitative analysis of apoptosis using acridine orange/ethidium bromide revealed a time-dependent increase using MK886 treatment at 5 nmol/10^6 cells in both cell lines, with controls significantly increased by 4 h and bcl-xL cells by 8 h (Fig. 4). Overall, bcl-xL overexpression reduced the apoptotic cell count by ~55% over 8–24 h compared with control cells treated with MK886.

The induction of apoptosis by MK886 in bcl-xL-overexpressing cells was surprising based on the broad resistance to this form of cell death normally seen in the presence of this protein. Immunoblot data indicated that endogenous bcl-xL protein levels in control cells were reduced 35% (Fig. 5A, lane 2) relative to untreated cells (lane 1) by 2 h after MK886 treatment (5 nmol/10^6 cells), decreased further by 4 h (lane 3), and disappeared at 8 h (lane 4). Similar changes in both endogenous and transfected bcl-xL protein levels in bcl-xL overexpressors were observed after MK886 treatment. The transfected human form of bcl-xL (FLAG-bcl-xL, which is slightly smaller than the murine form and thus runs faster) was decreased 30, 40, and 75% relative to controls by 2, 4, and 8 h, respectively, after MK886 treatment (Fig. 5A, lanes 5–8). Detectable levels of the transfected form remained even after 8 h, possibly explaining the lesser apoptosis after MK886 treatment in bcl-xL-transfected cells compared with controls. Endogenous murine bcl-xL protein was, however, completely lost by 8 h (Fig. 5A, lane 8). Similarly, endogenous bcl-2 expression in both the cells was gradually decreased with time after MK886 treatment (Fig. 5B). It was interesting to note that the expression of bax (Fig. 5C) and FLAP (Fig. 5D) proteins was unaltered 8 h after MK886 treatment compared with control in both cell lines.

The rapid loss of bcl-xL protein after MK886 appeared to be unrelated to transcription, because bcl-xL mRNA in both of the cell lines was not changed with time compared with controls (data not shown). This information, and the rapidity of the change in proteins, suggested that a proteolytic mechanism might be involved. The rapid and massive induction of apoptosis by MK886 in both control and bcl-xL-transfected cells would be expected to be accompanied by a generalized proteolysis, at least at later time points. However, the early loss of bcl-xL was not attributable to a generalized loss in protein, because up to 4 h (when a significant decrease in bcl-xL protein was already evident; Fig. 5A) only minimal changes were evident in total.
protein bands in Coomassie Blue-stained gels (data not shown), and bax and FLAP did not change (Fig. 5, C and D). In addition, there was no loss of bcl-xL after induction of apoptosis in FL5.12 cells by withdrawal of IL-3, indicating that these changes in bcl proteins were not a general consequence of apoptosis.

Caspases have been implicated both in initiation and execution stages of apoptosis (39). In the control cells, caspase-3 activity was increased by 10-fold 2 h after MK886 treatment. The activity level remained significantly higher than the basal level to 8 h (Fig. 6). Caspase-3 activity was unchanged by MK886 in bcl-xL-overexpressing cells (Fig. 6). Caspase-3 activity was completely blocked by the peptide inhibitors zVAD-FMK and Ac-DEVD-CHO (Table I). In addition, zVAD-FMK and Ac-DEVD-CHO inhibited MK886-induced apoptosis in control cells by 50% (Table II), indicating the partial involvement of caspase-3 in causing apoptosis. Caspase-1 activity in both control and MK886-treated cells was measured in the lysate as described under "Materials and Methods." Results are expressed as means ± S.E. (n = 3).

### DISCUSSION

Diverse apoptotic stimuli including growth factor withdrawal, ionizing and UV radiation, and ceramide treatment have been associated with elevated intracellular generation of reactive oxygen species and peroxides (40). The role of bcl-2 in protecting different cell types from apoptosis caused by various stimuli including oxidative stress is well established (41, 42). Similarly, bcl-xL rescues B-lymphocytes from apoptosis induced by different oxidative insults (40). Although these two bcl family proteins can prevent oxidant-induced cell death, they are not part of any classical antioxidant system (14), and the precise biochemical mechanism by which bcl-2/bcl-xL functions to block apoptosis remains obscure. One possibility that has not yet been studied is that bcl-2/bcl-xL, after stress, may control the expression of protein(s) involved in fatty acid mobilization and metabolism, respectively. The

### Table I

| Treatments | Caspase-3 activity % of total cell count |
|------------|----------------------------------------|
| Control    | 5.1 ± 0.6                              |
| MK886      | 25.5 ± 2.7                             |
| MK886 + zVAD-FMK (200 μM) | 10.7 ± 0.6 |
| MK886 + Ac-DEVD-CHO (50 μM) | 11.9 ± 1.6 |

*Significantly different from other treatments (p < 0.05).

### Table II

| Treatments | % of apoptosis |
|------------|----------------|
| Control    | 14.1 ± 1.4 |
| MK886      | 100 ± 7.6 |
| MK886 + zVAD-FMK (50 μM) | 15.0 ± 1.0 |
| MK886 + zVAD-FMK (200 μM) | 4.9 ± 0.8 |
| MK886 + Ac-DEVD-CHO (25 μM) | 3.8 ± 0.2 |
| MK886 + Ac-DEVD-CHO (50 μM) | 3.2 ± 0.7 |

*Significantly different from other treatments (p < 0.05).
name FLAP was derived from the activity first identified for this protein. Because nanomolar concentrations of the FLAP inhibitor MK886 are sufficient to inhibit 5-LOX activity, whereas micromolar concentrations are required to induce apoptosis, it would appear that the effect of FLAP on LOX is distinct from its ability to induce apoptosis. Several factors identified in the current study support the concept that activities of this protein other than its ability to activate 5-LOX may be physiologically significant. First, a specific 5-LOX inhibitor (caffeic acid) was unable to induce apoptosis, similar to a previous report in another cell line (19). Second, there was no detectable 5-LOX protein or any LOX activity detected in either control or bcl-xL-overexpressing FL5.12 cells. This dichotomy between the presence of FLAP protein or mRNA and 5-LOX has been reported previously in different mammalian cell lines of hematopoietic origin (43–45). It seems possible, therefore, that a profound disruption of fatty acid transport after treatment with a FLAP inhibitor may yield abnormal lipid metabolites that induce apoptosis.

A possible relationship between FLAP and the bcl family of antiapoptotic proteins is suggested by several findings in the current study. Most importantly, the baseline expression of FLAP was decreased in FL5.12 cells overexpressing bcl-xL compared with control. This change is the first report of another protein being altered in bcl-xL-overexpressing cells. RT-PCR data confirmed the presence of FLAP and also suggested a decrease in FLAP mRNA levels in bcl-xL cells compared with control. Interestingly, FLAP expression entirely disappeared from bcl-xL-overexpressing cells after IL-3 withdrawal, a condition that induces apoptosis in control but not the bcl-xL-overexpressing cell line. This change in FLAP expression was presumably not caused by apoptosis, because bcl-xL-overexpressing cells are resistant to apoptosis after IL-3 withdrawal, and FLAP was unchanged in control cells treated with MK886 to induce apoptosis. It seems possible that bcl-xL in some way affects FLAP expression both under basal conditions and in response to apoptotic stresses. This would imply that FLAP is a critical component of the apoptosis pathway, and by eliminating this protein bcl-xL can inhibit apoptosis.

An additional indication that FLAP has an important role in apoptosis comes from the finding that the FLAP inhibitor MK886 rapidly induced massive apoptosis in both control and bcl-xL-overexpressing FL5.12 cells. Although overexpressing bcl-xL somewhat diminished apoptosis, it was much less effective against this process with MK886 than any other agent tested, including NDGA.

The mechanism by which MK886 induces apoptosis remains unclear. Immunoblot analyses showed that endogenous bcl-xL protein levels were completely eliminated in both control and bcl-xL-overexpressing cells, whereas the transfected form was profoundly decreased shortly after MK886 treatment. Because bax levels were unaffected, it is suggested that the disappearance of bcl-xL may predispose these cells to apoptosis. Sphingosine also induces apoptosis with a concomitant decrease in both bcl-xL mRNA and protein levels but without any change in bax expression in DU-145 cells (46).

Changes in caspase activities may be important in the pro-apoptotic effects of MK886. MK886 significantly increased caspase-3 activity, and the caspase-3 inhibitors zVAD-FMK and Ac-DEVD-CHO partially reduced MK886-induced apoptosis in control cells. However, the failure by these peptide inhibitors to prevent endogenous bcl-xL breakdown in control cells, and to only modestly protect the transfected form suggests the involvement of other caspase(s) or non-caspase proteases in the degradation of bcl-xL. Although MK886 treatment reduced bcl-xL protein levels in the overexpressing cells, it is possible that the remaining protein level was sufficient to block caspase-3 activation through one or more of its reserved functions, such as pore formation and protein-protein interactions (47, 48). This idea is further supported by the data in bcl-xL cells in which, although caspase-3 was not activated, partial protection of transfected bcl-xL levels by zVAD-FMK and Ac-DEVD-CHO after MK886 treatment was evident.

MK886 treatment of chronic myelogenous leukemia cells increases the intracellular Ca$^{2+}$ concentration (22). Suppression of apoptosis by bcl-2 has been correlated with the regulation of nuclear and cytosolic Ca$^{2+}$ levels (49), although other data indicate that Ca$^{2+}$ is not a critical mediator of this form of cell death (50). It is possible that Ca$^{2+}$ influx into the nucleus may be controlled by bcl-xL. In this case, disappearance of bcl-xL could increase Ca$^{2+}$ concentration in the nucleus followed by endonuclease activation-mediated internucleosomal DNA cleavage (51). Ca$^{2+}$-dependent proteases have been implicated in apoptosis (52), supporting this concept.

A recent study suggests that bcl-xL may control GSH efflux in FL5.12 cells (35). The loss of GSH that occurs before apoptosis may prevent efficient removal of lipid hydroperoxides, thereby enabling signals for apoptosis to predominate. Although GSH levels after treatment with MK886 have not yet been determined, together with the loss of bcl-xL with MK886 treatment, this may explain the rapid onset of apoptosis.

The mechanism by which NDGA induces apoptosis remains unclear, but given the apparent absence of LOX in these cells, it seems unlikely to be related to inhibition of this enzyme. Although an antioxidant at higher concentrations, the levels found to induce apoptosis are unlikely to have any significant antioxidant activity. Like NDGA, other antioxidants, e.g. N-acetylcysteine and pyrrolidinedithiocarbamate, can induce apoptosis in vascular smooth muscle cells (53) (although in most systems antioxidants inhibit apoptosis). However, NDGA-induced apoptosis is associated with lipid peroxidation and depletion of GSH (18), suggesting some pro-oxidant activity at these low doses.

Because arachidonic acid is capable of inhibiting NDGA-induced cell death (54), it has been postulated that arachidonic acid, without metabolic transformation, may exert an anti-apoptotic effect by initiating distinct signal transduction pathway(s). Because arachidonic acid can modulate the activation of mitogen-activated protein kinase through a protein kinase C-dependent mechanism (55), this may be related to the stimulation of cell proliferation. Arachidonic acid has also been documented to induce cell proliferation in a dose-dependent manner (56). Assuming that FLAP inhibition in FL5.12 cells prevents the mobilization of arachidonic acid, this may hinder cell survival and proliferation, followed by apoptosis. Alternatively, acute FLAP inhibition may displace bound fatty acids.

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**Fig. 7.** Effect of caspase inhibitors on bcl-xL expression in control and bcl-xL-transfected FL5.12 cells after MK886 treatment. Cells (10$^7$) were treated with ethanol (final concentration, 0.2%), MK886 (5 nmol/10$^6$ cells), and either zVAD-FMK (200 μM) or Ac-DEVD-CHO (50 μM) in the presence of MK886. Cells were lysed after 4 h, and the lysate was used to perform SDS-polyacrylamide gel electrophoresis as described under "Materials and Methods." Lanes 1–4 and 5–8 represent control and bcl-xL-transfected cells, respectively. Each lane was loaded with 25 μg of total protein.
increasing free levels in the cell and leading to inappropriate oxidation and the generation of apoptosis-inducing species.

In summary, bcl-xL transfection significantly decreased the basal level of FLAP in FL5.12 cells. The dramatic changes in bcl-xL expression along with apoptosis after treatment with the FLAP inhibitor MK886 are the most profound ever reported for basal level of FLAP in FL5.12 cells. The dramatic changes in membranes suggest a functional or regulatory relationship.

REFERENCES

1. Vaux, D. L., Haecker, G., and Strasser, A. (1994) Cell 76, 777–779
2. Corcoran, G. B., Fix, L., Jones, D. P., Moslen, M. T., Nicotera, P., Oberhammer, F. A., and Buttyan, R. (1994) Toxicol. Appl. Pharmacol. 267, 169–181
3. Sen, C. K., and Packer, L. (1996) Free Radical Biol. Med. 22, 269–285
4. Zhou, L-T., Sarafin, R., Kane, D. J., Charles, A. C., Mah, S. P., Edwards, R. H., and Reden, D. E. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 4533–4537
5. Minn, A. J., Rudin, C. M., Boise, L. H., and Thompson, C. B. (1995) Blood 86, 1903–1910
6. Minn, A. J., Rudin, C. M., Boise, L. H., and Thompson, C. B. (1995) Toxicol. Appl. Pharmacol. 127, 22764–22772
7. Tao, J., and Honn, K. V. (1997) J. Biol. Chem. 272, 773–776
8. Tao, J., and Honn, K. V. (1997) J. Biol. Chem. 272, 385–417
9. Minn, A. J., Rudin, C. M., Boise, L. H., and Thompson, C. B. (1995) Cancer Res. 55, 2155–2160
10. Korsmeyer, S. J. (1995) J. Exp. Med. 182, 821–828
11. Korsmeyer, S. J. (1995) J. Biol. Chem. 270, 22764–22772
12. Lee, J. C., Hapel, A. J., and Igle, J. N. (1982) Cell 227, 773–776
13. Korsmeyer, S. J. (1995) J. Exp. Med. 182, 2035–2046
14. Sandstrom, P. A., Pardi, D., Tebbey, P. W., Dudek, R. W., Terrian, D. M., Folks, T. D., and Behrens, T. W. (1995) J. Immunol. 155, 66–75
15. Korystov, Y. N., Dobrovinskaya, O. R., Shaposhnikova, V. V., and Eidus, L. K. (1995) FEBS Lett. 353–357
16. Takemoto, M., Shirahama, T., Sweeney, E. A., Ozawa, M., Takemoto, M., Zhivotovsky, B., Burgess, D. H., and Orrenius, S. (1996) FEBS Lett. 383–417
17. Ondrey, F. G., Jiun, S. K., and Adams, G. L. (1996) Laryngoscope 106, 129–134
18. Tang, D. G., and Honn, K. V. (1997) Cancer Res. 57, 353–42
19. Korsmeyer, S. J. (1995) Cancer Surv. 310, 665–670
20. Korystov, Y. N., Dobrovinskaya, O. R., Shaposhnikova, V. V., and Eidus, L. K. (1996) FEBS Lett. 388, 238–241
21. Vickers, P. J. (1995) Biochim. Biophys. Acta 1223, 247–254
22. Chao, D. T., Linette, G. P., Boise, L. H., White, L. S., Thompson, C. B., and Korsmeyer, S. J. (1995) J. Exp. Med. 182, 821–828
23. Simonian, P. L., Grillet, D. A., Merino, R., and Nuñez, G. (1996) J. Biol. Chem. 271, 22764–22772
24. Lee, J. C., Hapel, A. J., and Igle, J. N. (1982) J. Immunol. 128, 2393–2398
25. Pouliot, M., McDonald, P. P., Krump, E., Mancini, J. A., McColl, S. R., Weech, P. K., and Borget, P. (1996) Eur. J. Biochem. 238, 250–258
26. Ioannou, Y. A., and Chen, F. W. (1996) Nucleic Acids Res. 24, 992–993
27. Duke, R. C., and Cohen, J. J. (1992) in Current Protocols in Immunology (Coligan, J. E., and Kruisbeak, A. M., eds) pp. 3.17.1–3.17.16, John Wiley & Sons, New York
28. Dahl, O. H., Roseborough, N. J., Far, A. L., and Randall, R. J. (1951) J. Biol. Chem. 153, 265–275
29. Feinburg, A. P., and Vogelstein, B. (1985) Anal. Biochem. 136, 266–267
30. Fair, A., and Pritchard, R. A. (1994) Biochem. Biophys. Res. Commun. 201, 1014–1020
31. Mizushima, N., Koike, R., Kohsaka, H., Kushi, Y., Hanada, S., Yagita, H., and Miyazaki, N. (1996) FEBS Lett. 385, 267–271
32. Ford-Hutchinson, A. W. (1994) Adv. Prostaglandin Thromboxane Leukotriene Res. 22, 13–21
33. Salvesen, G. S., and Dixit, V. M. (1997) Cell 91, 443–446
34. Ford-Hutchinson, A. W., Gresser, M., and Young, R. N. (1996) Annu. Rev. Biochem. 63, 83–347
35. Bojes, H. K., Datta, K., Xu, J., Chin, A., Simonian, P., Nuñez, G., and Kehrer, J. P. (1997) Biochem. J. 325, 315–319
36. Flamm, B. A., and Cook, J. (1994) J. Cell Biol. 124, 1–6
37. Korsmeyer, S. J. (1992) Cancer Surv. 10, 105–118
38. Reed, J. C. (1994) J. Cell Biol. 124, 1–6
39. Korsmeyer, S. J. (1995) J. Cell. Physiol. 193, 28169
40. Keynan, O., and Cook, J. (1994) J. Immunol. 155, 172–177
41. Korsmeyer, S. J. (1995) J. Cell. Physiol. 193, 22764–22772
42. Korsmeyer, S. J. (1995) J. Exp. Med. 182, 2035–2046
43. Nicotera, P., Bellomo, G., and Orrenius, S. (1992) Annu. Rev. Pharmacol. Toxicol. 32, 449–470
44. Zhivotovsky, B., Burgess, D. H., and Orrenius, S. (1996) Experientia 52, 968–978
45. Tsai, J.-C., Jain, M., Hsieh, C.-M., Lee, W.-S., Yoshizumi, M., Patterson, C., Perrelli, M. A., Cooke, C., Wang, H., Haber, E., Schlegel, R., and Lee, M.-E. (1996) J. Biol. Chem. 271, 3667–3670
46. Tang, D. G., Guan, K.-L., Li, L., Honn, K. V., Chen, Y. Q., Rice, R. L., Taylor, J. D., and Porter, A. T. (1997) Int. J. Cancer 72, 1078–1087
47. Tso, J.-C., Jain, M., Hsieh, C.-M., Lee, W.-S., Yoshizumi, M., Patterson, C., Perrelli, M. A., Cooke, C., Wang, H., Haber, E., Schlegel, R., and Lee, M.-E. (1996) J. Biol. Chem. 271, 3667–3670
48. Tang, D. G., Guan, K.-L., Li, L., Honn, K. V., Chen, Y. Q., Rice, R. L., Taylor, J. D., and Porter, A. T. (1997) Int. J. Cancer 72, 1078–1087
49. Hii, C. S. T., Ferrante, A., Edwards, W. S., Huang, Z. H., Hartfield, P. J., Rathjen, D. A., Poulos, A., and Murray, A. W. (1996) J. Biol. Chem. 270, 4201–4204
50. Hii, C. S. T., Ferrante, A., Edwards, W. S., Huang, Z. H., Hartfield, P. J., Rathjen, D. A., Poulos, A., and Murray, A. W. (1996) J. Biol. Chem. 270, 4201–4204
51. Nishio, E., and Watanabe, Y. (1997) Br. J. Pharmacol. 121, 665–670