RESEARCH COMMUNICATION

The 5-lipoxygenase-activating protein (FLAP) inhibitor, MK886, induces apoptosis independently of FLAP

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The ability of various inhibitors of lipoxygenase (LOX) enzymes and 5-lipoxygenase-activating protein (FLAP) to induce apoptosis has implicated these pathways in the mechanism(s) of this form of cell death. Although FLAP plays an important role in 5-LOX activity, this protein is found at high levels in some cells lacking LOX, suggesting it might mediate other effects. Furthermore, the concentration of MK886, a FLAP inhibitor, required to induce apoptosis is $\approx$ 100-fold more than that required to inhibit LOX, and this compound remains effective in cells lacking LOX. The present study examines the role of FLAP in MK886-induced apoptosis. MK886 induced apoptosis in WSU cells, a human chronic lymphocytic leukaemia cell line that lacks FLAP protein and mRNA, suggesting that this agent is acting independently of FLAP. This conclusion was further supported by the fact that a more specific FLAP inhibitor, MK591, induced only minimal apoptosis in FL5.12 cells, a murine prolymphoid cell line containing FLAP. The role of FLAP was examined more directly by decreasing its expression by more than 50% in FL5.12 cells treated with 10 μM of an antisense oligonucleotide for 48 h. This change in FLAP was not accompanied by any increase in apoptosis. Furthermore, FLAP-depleted cells exhibited the same level of apoptosis 8 h after treatment with 10 μM MK886, as did control cells. The increased fluorescence seen in MK886-treated cells loaded with carboxydichlorofluorescein indicates that oxidative reactions are stimulated by this compound, possibly via the release of fatty acids from fatty acid-binding proteins and their subsequent oxidation.

Key words: antioxidants, apoptosis, bax, bcl-x_L.

INTRODUCTION

Apoptosis, a form of cell death involving a series of well-organized events requiring active cell participation, is the basis for normal tissue remodelling, as well as the end result of certain toxic insults [1]. It differs morphologically from necrotic cell death [2], and several genes have been identified as potentially controlling apoptosis in different species [3]. The possibility that reactive oxygen species play a significant role in apoptosis has been reviewed, and a relationship between free radicals and several effectors of apoptosis has been reported [4–7].

The mechanism by which oxidants induce apoptosis is unknown, but it is likely that some signalling factor is generated. Since polyunsaturated fatty acids are highly susceptible to oxidation, lipid messengers [8] or lipid peroxides [9–11] that can induce apoptosis are reasonable candidates. Fatty acid metabolites are known to be involved in various signalling pathways, and some data suggest that lipoxygenase (LOX) enzymes play an important role in apoptosis [12]. However, there is a significant amount of conflicting information, largely because of the reliance on LOX inhibitors with unclear specificity that induce apoptosis in some systems [13–16], while inhibiting it in others [8,17,18].

The ability of a purportedly specific 5-LOX-activating protein (FLAP) inhibitor, MK886, to induce apoptosis has been used in several studies implicating LOX [19–21]. FLAP was named for the function initially identified for this protein, and the effects of MK886 have been assumed to be directly related to LOX. However, since MK886 induces apoptosis in cells lacking LOX [22] and the concentration needed (10 μM) [19–22] is $\approx$ 100 times greater than that required to affect LOX [23], the mechanism would appear to involve other pathways.

The goal of the present work was to determine whether the pro-apoptotic effects of MK886 are mediated via FLAP. The results showing that MK886 is active in cell lines lacking FLAP, and that decreasing FLAP levels via antisense oligonucleotide treatment has no effect on the ability of this compound to induce apoptosis, support the hypothesis that MK886, the most studied FLAP inhibitor, induces apoptosis independently of FLAP.

MATERIALS AND METHODS

Cell culture and treatments

An IL-3-dependent murine prolymphoid progenitor cell line (FL5.12) and a human chronic lymphocytic leukaemia cell line (WSU) [24] were used. Cells were maintained in RPMI 1640 media (Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Summit Biotechnology, Ft. Collins, CO, U.S.A.), penicillin (100 units/ml), streptomycin (100 μg/μl) and 10% (v/v) WEHI-3B-conditioned medium (FL5.12 cells only) in an air/CO_2 (19:1) atmosphere at 37 °C. WEHI-3B cells were grown under similar conditions to produce IL-3-conditioned medium, as described previously [25]. Cultures were passaged on alternate days with fresh medium, and cell counts were determined with a T-890 Coulter counter (Miami, FL, U.S.A.). Cells were treated with either DMSO (0.1% as vehicle control), MK886 (Biomol Re-

Abbreviations used: carboxy-DCF, carboxydichlorofluorescein; carboxy-H_2DCFDA, carboxydichlorofluorescin diacetate; FLAP, 5-lipoxygenase-activating protein; (5-)LOX, (5-)lipoxygenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; NAC, *N*-acetylcysteine; RT, reverse transcriptase.

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search, Plymouth Meeting, PA, U.S.A.) or MK591 (a gift from Merck Frosst Canada, Inc., Pointe Claire-Dorval, Quebec, Canada).

Western blot assay

SDS/PAGE and Western blots for FLAP protein were done by established procedures. Briefly, cells (5×10^6) were lysed with 150 μl of RIPA buffer [10 mM sodium phosphate/150 mM NaCl (pH 7.4)/0.5 % (w/v) sodium deoxycholate/0.1 % (w/v) SDS/ 1 mM sodium orthovanadate] containing 100 μg/ml PMSF and 30 μl/ml aprotinin by repeatedly pipetting the cell suspension and incubating for 15 min at 4 °C. The lysed cells were centrifuged at 400 ***g*** for 10 min, and supernatants were subjected to SDS/ PAGE (15 % polyacrylamide gels) [buffer composition: 20 % (v/v) glycerol/4 % (w/v) 2-mercaptoethanol/4 % (w/v) SDS/0.2 M Tris/HCl (pH 6.8)/0.02 % Bromophenol Blue]. Protein was transferred on to PVDF membranes and blocked for 1 h. The membrane was then incubated with FLAP rabbit polyclonal antiserum (1:1500 dilution) (Merck Frosst Center for Therapeutic Research, Quebec, Canada). After membrane washing, horseradish-peroxidase-conjugated anti-rabbit secondary antibodies were used (1:3000 dilution; Sigma Chemical Co., St. Louis, MO, U.S.A.). Bound antibodies were detected using enhanced chemiluminescence with a kit from Amersham (Arlington Heights, IL, U.S.A.). Band intensities were determined using NIH Image software on scanned images. Protein content was determined by the method of Lowry et al. [26], using BSA as a standard.

Measurements of apoptosis

Acridine Orange/ethidium bromide

Cells were pelleted by centrifugation at 300 ***g*** for 10 min at 4 °C. The cells were resuspended in 40 μl of fresh media. Apoptosis was assessed using fluorescence microscopy by mixing 2 μl of Acridine Orange (100 μg/ml), 2 μl of ethidium bromide (100 μg/ml) and 20 μl of the cell suspension. A minimum of 200 cells were counted in at least five random fields. 'Live' apoptotic cells were differentiated from 'dead' apoptotic, necrotic and normal cells by examining the changes in cellular morphology on the basis of distinctive nuclear and cytoplasmic fluorescence [27].

ELISA

A characteristic event in apoptosis is DNA fragmentation and release of nucleosomes into the cytoplasm, processes which can be detected and are quite specific for apoptosis relative to necrosis [28]. Cells were first washed to remove histone-associated DNA released by necrotic cells. Cell membranes were then gently lysed, releasing nucleosomes from apoptotic cells. A one-step ELISA kit using streptavidin-coated microplates, biotinylated anti-histone and anti-DNA antibodies was used to complete the assay (Roche Molecular, Indianapolis, IN, U.S.A.). Results were determined as the $\Delta A_{405-490}$ relative to control cells, which indicates the enrichment of nucleosomes in the cytoplasm.

Annexin V test (phosphatidylserine externalization)

Phosphatidylserine exposure on the plasma membrane was measured directly by the binding of annexin V–FITC to phosphatidylserine in the presence of Ca^{2+} using the annexin V–FITC kit (Immunotech, Miami, FL, U.S.A.). Together with annexin V–FITC, cells were stained with propidium iodide (100 μg/ml), which allows the differentiation between necrotic and non-necrotic cell populations. Cells (0.5×10^6/ml) were treated with MK886 for 8 h. The level of apoptosis showing positive annexin V–FITC binding and cells permeable to propidium iodide were analysed in 10000 processed cells using a Coulter flow cytometer equipped with an argon laser.

Flow cytometry

Carboxydichlorofluorescin diacetate (carboxy-H_2DCFDA) is a lipid soluble probe that, following deacetylation, can be oxidized to the fluorescent product carboxydichlorofluorescein (carboxy-DCF). Although not specific for any oxidized species, it does provide a qualitative index of the overall oxidation status of a cell. Cells (0.5×10^6/ml) were loaded with 10 μM carboxy-H_2DCFDA for 1 h at 37 °C. At that time, cells were treated with MK886 for 1 h. Cells were then pelleted, washed and resuspended (10^6 cells/ml) in fresh medium. Carboxy-DCF fluorescence was measured in 10000 cells in a Coulter flow cytometer with excitation and emission settings of 495 and 525 nm respectively.

Cell viability

This was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay, which measures the reduction of the tetrazolium to purple formazan crystals by metabolically active cells. WSU cells (10000/well) were plated in 90 μl of RPMI 1640 medium in a 96-well, flat-bottom microtitre plate. Cells were then treated with either 10 μl of medium containing either 0.1 % (v/v) DMSO (vehicle control) or MK886. After 24 h, MTT labelling reagent (10 μl) was added to each well, and the plate was incubated for 4 h at 37 °C. Solubilizing solution (100 μl) was then added to each well, and the plate was incubated overnight at 37 °C. The A_{595} in each well was measured using a microplate reader.

Antisense treatment

A 20-mer phosphorothioate (to decrease hydrolysis by nucleases) oligonucleotide was obtained from Oligos Etc (Willsonville, OR, U.S.A.) directed against the start codon of mouse FLAP mRNA (TTC TTG ATC CAT GTT TGC TT). Samples (0.5×10^6 cell/ml) were plated in each well of a six-well plate, centrifuged at 600 ***g*** and the media was discarded. Cells were then resuspended in 1 ml of opti-MEM containing 6.6 μl of lipofectin and treated with either non-sense or antisense FLAP oligonucleotide (10 μM each) for 4 h at 37 °C. After 4 h, 1 ml of fresh IL-3 medium was added to each well and left for 20 h at 37 °C. Some cells were pelleted after 24 h, washed and lysed; other cells received a second, identical oligonucleotide treatment and were studied at 48 h.

Reverse transcriptase (RT)-PCR

Total cellular RNA was isolated using a RNAqueous kit (Ambion, Austin, TX, U.S.A.). Samples (5 μg) of the total RNA were transcribed with 100 units of Moloney-murine-leukaemia virus RT, 300 ng of oligo dT, 1mM dNTPs and 10 units of placental RNase inhibitor (RETROscript kit; Ambion) in reaction buffer supplied by the manufacturer, in a total volume of 20 μl at 41 °C for 1 h. PCR was performed using 1 μl of the resulting cDNA, 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, CT, U.S.A.), reverse and forward primers (250 μM each), and 250 μM dNTPs in a final volume of 50 μl. The cDNA sequences used in the amplification of human FLAP were: 5′ (5′-ATGGATCAAGAAACTGTAGGC-3′) and 3′ (5′-ATGAGAAGTAGAGGGGGAGATG-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 sec, 55 °C for

30 sec and 72 °C for 35 sec. After the last cycle, the reactions were incubated at 72 °C for an additional 7 min. PCR products (10 μl) were electrophoretically separated on 1.5 % (w/v) agarose gels and stained with ethidium bromide (0.5 μg/ml).

RESULTS

In order to assess the role of FLAP in apoptosis, several different haematopoietic cell lines were examined to identify those with different levels of FLAP. As shown in Figure 1(A), FL5.12 cells (lane 1) express a substantial amount of FLAP, whereas WSU cells (lane 5) appear to be completely lacking in expression of this protein. Other cell lines shown in this blot expressed variable amounts of FLAP. Constitutive levels of bcl-x_L expression were identical in these cell lines (Figure 1B), demonstrating that not all proteins differ. Further studies of FLAP expression using RT-PCR to assess mRNA content revealed that WSU cells lacked this mRNA (Figure 1C), while Raji (a human B-cell lymphoma line) (Figure 1C) and FL5.12 cells [22] contained this message.

Despite the absence of FLAP, WSU cells were sensitive to MK886. At 24 h after 10 or 20 μM MK886 was added, viability (as determined by the MTT assay) declined from > 98 % to 74 % and 11 % respectively. At 8 h after treatment with 10 and 20 μM MK886 respectively, apoptosis using ELISA was increased by 14.6- and 30-fold relative to control cells. Enhanced apoptosis was confirmed using flow cytometry to detect the exposure of phosphatidylserine with Annexin V (results not shown). The apoptotic response of WSU cells to MK886 was comparable with FL5.12 cells that contain FLAP [22], which was confirmed in the present study (results not shown).

MK591 is a FLAP inhibitor considered to have greater specificity, and somewhat greater potency, for FLAP than MK886. When tested to determine its ability to induce apoptosis, MK591 was less effective than MK886. At 8 h after treating FL5.12 cells with either 10 μM MK886 or 10 μM MK591, 27.0 and 12.3 % of the cells respectively were apoptotic. This compares with control levels of apoptosis of 2.8 % (as determined by Acridine Orange/ethidium bromide staining).

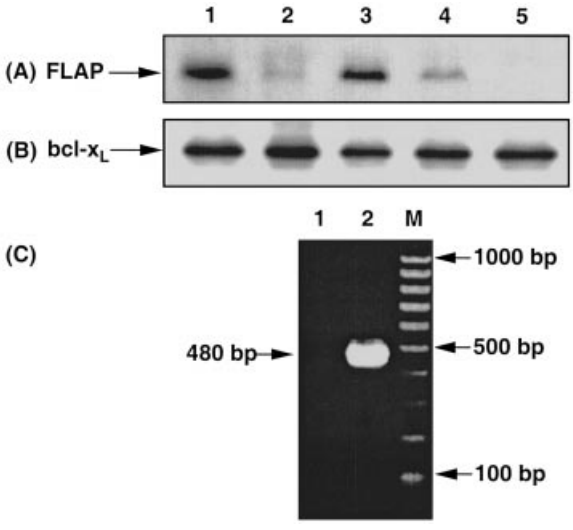

Figure 1 FLAP (A) and bcl-x_L (B) expression in various haematopoietic cell lines

Cells were lysed and the lysate was subjected to SDS/PAGE. Each lane was loaded with either 50 (**A**) or 25 (**B**) μg of total protein. Lane 1: FL5.12; lane 2: Jurkat; lane 3: Raji; lane 4: JVM-2; lane 5: WSU. **(C)** RT-PCR for human FLAP mRNA in WSU (lane 1) and Raji (lane 2) cells. M, molecular-mass markers. Raji cells, a human B cell lymphoma line that expresses FLAP protein (**B**), also express FLAP mRNA and were used as a positive control.

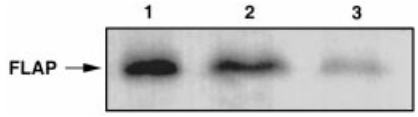

Figure 2 Effect of FLAP antisense on FLAP expression in FL5.12 cells

Samples (0.5×10^6 cell/ml per well) were treated with either non-sense or antisense FLAP oligonucleotide (10 μM each) at 37 °C. After 4 h, 1 ml of fresh IL-3 medium was added to each well and left for 20 h at 37 °C. At the end of 24 h, cells received a second identical oligonucleotide treatment and were lysed at 48 h. Lysate was used for SDS/PAGE as described in the Materials and Methods section. Each lane was loaded with 50 μg of total protein. Lane 1, untreated control; lane 2, non-sense oligonucleotide-treated; lane 3, FLAP antisense oligonucleotide-treated.

Table 1 Apoptosis in FL5.12 cells after FLAP antisense oligonucleotide

Cells (0.5×10^6/ml per well) were treated with either non-sense or antisense FLAP oligonucleotides (10 μM each) at 37 °C. After 4 h, 1 ml of fresh IL-3 medium was added to each well and left for 20 h at 37 °C. At the end of 24 h, cells received a second identical oligonucleotide treatment. After 48 h, cells were treated with 10 μM MK886 and apoptosis was quantified 8 h later, using distinctive nuclear and cytoplasmic fluorescence staining with Acridine Orange/ethidium bromide. Data are expressed as means ± S.E. ($n = 3$).

	% Apoptosis (at 8 h)	
Treatment	− MK886	+ MK886
Control	4.4 ± 2.8	27.4 ± 2.2
Non-sense	8.6 ± 1.7	32.4 ± 3.4
Antisense	10.7 ± 1.9	33.6 ± 3.3

Table 2 NAC inhibits MK886-induced apoptosis in FL5.12 cells

Cells (10^6/ml per well) were pretreated with 10 mM NAC for 2 h, followed by 10 μM MK886 for 8 h at 37 °C. Experiments were performed in triplicate. Results were determined as the mean $\Delta A_{405-490}$ ± S.E. ($n = 3$) relative to control cells, which indicates the enrichment of nucleosomes in the cytoplasm. NAC (10 mM) treatment alone did not cause apoptosis.

Treatment	$\Delta A_{405-490}$	Enrichment factor (ΔA treated : ΔA control)
Control	0.076 ± 0.005	1.0
MK886	0.368 ± 0.041	4.8
MK886 + NAC	0.124 ± 0.017	1.6

To assess more directly the importance of FLAP in MK886-induced apoptosis, FL5.12 cells were treated with 10 μM of a FLAP antisense phosphorothioate oligonucleotide. At 24 h of treatment, FLAP levels were modestly lowered (results not shown), while after 48 h FLAP expression was decreased by ≈ 70 % (Figure 2). The non-sense control oligonucleotide also seemed to have a negative effect (of 17 %) on FLAP expression (Figure 2), and both non-sense and antisense treatments slightly increased basal levels of apoptosis (Table 1). However, relative to basal levels, the extent of apoptosis induced in FL5.12 cells by 10 μM MK886 alone was almost identical with with that of the cells treated with antisense oligonucleotide followed by 10 μM MK886 (Table 1).

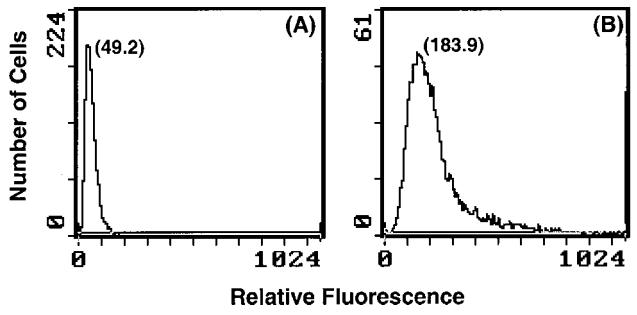

Figure 3 Oxidative reactions following treatment of FL5.12 cells with MK886

Cells (10^6/ml) were loaded with 10 μM carboxy-H_2DCFDA for 1 h at 37 °C. Cells were then treated with 10 μM MK886. After 1 h, cells were pelleted, washed and resuspended in fresh medium. Carboxy-DCF fluorescence was measured using a Coulter flow cytometer with excitation and emission maximum settings at 495 and 525 nm respectively.

Oxidative reactions have been implicated in many forms of apoptosis. MK886-induced apoptosis could be prevented by the antioxidant, *N*-acetylcysteine (NAC; Table 2). The possibility that MK886 enhances intracellular oxidative reactions was further studied in cells loaded with carboxy-H_2DCFDA, which provides a non-specific indication of oxidative stress. Oxidation to carboxy-DCF was examined 1 h after 10 μM MK886 treatment. The results showed a 4-fold increase in oxidation (Figure 3), which did not further increase after either 2 or 4 h (results not shown).

DISCUSSION

FLAP is an 18 kDa protein considered to play a pivotal role in the 5-LOX pathway of arachidonic acid metabolism [23]. FLAP transfers arachidonic acid to 5-LOX protein, thereby enabling this enzyme to efficiently produce oxidized lipid products (mainly eicosanoids). Although it seems clear that eicosanoids can be mediators of oxidative stress-related events during apoptosis in cell lines of haematopoietic origin, much of these supporting data have made use of various specific and non-specific LOX inhibitors [13–16]. On the basis of our data, it is now apparent that MK886, as well as the general LOX inhibitor nordihydroguaiaretic acid, can induce apoptosis independently of LOX activity [22]. In accordance with these findings, a specific 5-LOX inhibitor (caffeic acid) was unable to induce apoptosis [12,22], further supporting the fact that inhibition of LOX is not required to induce apoptosis.

A dichotomy between the presence of FLAP protein or mRNA and 5-LOX has been reported previously in different mammalian cell lines of haematopoietic [29–31] and intestinal origin [32], and it appears that FLAP might not be specific for LOX, since it can also increase cyclo-oxygenase activity [32]. While not necessarily conflicting with FLAP having important function(s) relating to 5-LOX, taken together with our data it indicates in terms of apoptosis either that FLAP can act independently of this enzyme or that MK886 might act independently of FLAP. The latter possibility is supported by the facts that (1) the IC_{50} of MK886 for LOX activity is only $\sim$ 3 nM [23], whereas doses of 10 μM are required to induce apoptosis, and (2) the extent of apoptosis induced by MK886 appears to be independent of the cellular levels of FLAP (present work).

Arachidonic acid competes with a synthetic analogue for binding to FLAP with an IC_{50} of 10–20 μM [23], interestingly close to the dosage of MK886 that leads to apoptosis. As an arachidonic acid-binding protein [33], FLAP exhibits many characteristics similar to other fatty acid-binding proteins, perhaps providing a key to the apoptotic effects of FLAP inhibitors. Fatty acid-binding proteins have been postulated to participate in signal-transduction pathways and in fatty acid regulation of gene expression [34,35]. Some fatty acid-binding proteins also appear to be involved in cell growth and differentiation [36], and it is possible that disrupting these pathways might initiate apoptosis. Given the ability of free fatty acids and lipid hydroperoxides to cause apoptosis in some cells [9–11,37], the release and oxidation of polyunsaturated fatty acids could generate potent apoptotic mediators. The increase in oxidation (or reactive oxygen species generation) observed by flow cytometry, and the ability of NAC to block apoptosis induced by MK886 ([21] and Table 2), are consistent with such a mechanism.

MK886 is an indole and, although considered a 'specific' FLAP inhibitor, contains many sites for metabolic modifications. Thus MK886 or its products could interact with other sites within a cell, thereby inducing apoptosis. MK591 is a structural analogue of MK886 that differs in the nature of the indoyl-5-substituent (it is quinoline based). Both compounds are considered to have the same mechanism of action, although MK591 might be somewhat more potent, with an IC_{50} value of 1.6 nM in a FLAP-binding assay [38]. If the inhibition of FLAP was the critical apoptosis-inducing feature of MK886, a more specific inhibitor would be expected to yield as much, or more, apoptosis. However, since MK591 was actually substantially less potent in this regard, any direct role for FLAP inhibition in cell death must be minimal.

Our previous data showing that MK886 and nordihydroguaiaretic acid induce apoptosis in the absence of measurable amounts of LOX protein or activity have supported the hypothesis that the effects of LOX inhibitors on apoptosis have a mechanism unrelated to their ability to inhibit LOX. The present data extend this finding to FLAP by demonstrating that FLAP inhibitors induce apoptosis at doses which are both far above those required to affect LOX and independent of the levels of this protein. The mechanism by which FLAP inhibitors induce apoptosis remains to be determined, but might be related to more general effects on fatty acid transport and oxidation.

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