Arachidonate lipoxygenases as essential regulators of cell survival and apoptosis

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Communicated by K. Frank Austen, Harvard Medical School, Boston, MA, January 2, 1996 (received for review October 25, 1995)

ABSTRACT Arachidonic acid (AA) metabolites derived from both cyclooxygenase (COX) and lipoxygenase (LOX) pathways transduce a variety of signals related to cell growth. Here, we report that the AA LOX pathway also functions as a critical regulator of cell survival and apoptosis. Rat Walker 256 (W256) carcinosarcoma cells express 12-LOX and synthesize 12(S)- and 15(S)-hydroxyeicosatetraenoic acids as their major LOX metabolites. W256 cells transfected with 12-LOX-specific antisense oligonucleotide or antisense oligonucleotides directed to conserved regions of LOXs underwent time- and dose-dependent apoptosis. Likewise, treatment of W256 cells with various LOX but not COX inhibitors induced apoptotic cell death, which could be partially inhibited by exogenous 12(S)- or 15(S)-hydroxyeicosatetraenoic acids. The W256 cell apoptosis induced by antisense oligos and LOX inhibitors was followed by a rapid downregulation of bcl-2 protein, a dramatic decrease in the bcl-2/bax ratio, and could be suppressed by bcl-2 overexpression. In contrast, p53, which is wild type in W256 cells, did not undergo alterations during apoptosis induction. The results suggest that the LOX pathway plays an important physiological role in regulating apoptosis.

Apoptosis, or programmed cell death, plays an indispensable role in embryonic development, maturation of the immune system, and maintenance of tissue and organ homeostasis. A wide spectrum of molecular entities including oncogenes, tumor suppressor genes, signal transducers, cell cycle proteins, free radicals, cations, and proteases (1, 2) have been implicated in apoptosis. Arachidonic acid (AA) metabolites derived from both lipoxygenase (LOX) and cyclooxygenase (COX) pathways have been shown to transduce growth-related signals and regulate cell proliferation (3, 4). However, no information is available as to whether these potent biological metabolites are also involved in the regulation of cell survival. Studies presented here show that the AA LOX system also plays a significant, physiological role in regulating cell survival and apoptosis.

MATERIALS AND METHODS

Cell Culture and Materials. W256 cells, which arose spontaneously in the mammary gland of a pregnant albino rat and recently were shown to be of hematopoietic (i.e., monocytoid) origin (5), were maintained in minimum essential medium supplemented with antibiotics and 5% fetal calf serum (FCS) as described (6). All chemicals and inhibitors were purchased from Biomol (Plymouth Meeting, PA). Various eicosanoids were bought from either Cayman Chemicals (Ann Arbor, MI) or Oxford and their specified purities were always >99%. Lipofectin was obtained from Life Technologies (Grand Island, NY).

DNA Fragmentation Assay and Quantitation of Fragmented DNA. Fragmented DNA was extracted using SDS/RNase/Proteinase K method (7) and 20 μ g of DNA was run on a 1.2% agarose gel. To quantitate DNA fragmentation, W256 cells

labeled with ³H-thymidine (1 μ Ci/ml, 18 h; 1 Ci = 37 GBq) were treated as described in *Results and Discussion*. Subsequently, both fragmented DNA in the supernatants and intact genomic DNA were isolated and ethanol precipitated. The % of the DNA fragmentation was obtained using the formula [% = cpm of fragmented DNA/(cpm of fragmented DNA + cpm of intact genomic DNA)].

Determination of Cell Survival. The number of living W256 cells following treatment with antisense oligonucleotides or inhibitors was determined by counting on a Coulter counter or by the trypan blue dye exclusion assay.

Oligonucleotide Synthesis, Purification, and Characterization. Various oligonucleotides of 15–20 bases were synthesized either in our own lab on a Gene Assembler Plus (Pharmacia LKB) or by Integrated DNA Technology (Coralville, IA). All oligos were purified by size exclusion chromatography and the purity was characterized by PAGE and/or HPLC analysis.

Transfection of Oligonucleotides and Plasmid Expression Vectors into W256 Cells. W256 cells were transfected with various concentrations of oligonucleotides in the presence of 10 μ g/ml Lipofectin (BRL) in serum-free MEM for 4 h, after which the oligonucleotides were washed off and cells were cultured in MEM supplemented with 5% FCS for up to 24 h (day 1). The treatment was repeated every 24 h until day 5. To construct bcl-2 expression vector, the full-length human bcl-2 cDNA [1.9 kb; provided by S. J. Korsmeyer (Washington University, St. Louis)] was cloned into a eukaryotic expression vector that carries the cytomegalovirus promoter and a minicassette conferring neomycin resistance (pCMVbcl-2). The empty vector without the insert (pCMVneo) was used as the control. pCMVneo or pCMVbcl-2 (1 μ g) purified by PEG precipitation was transfected into W256 cells using Lipofectin (10 μ g/ml). Selection was initiated 48 h after the transfection with 800 μ g/ml G418 (Life Technologies). Individual resistant clones were isolated using the limiting dilution method and propagated in serum-containing MEM supplemented with 300 μ g/ml G418.

Terminal Deoxynucleotidyltransferase-Mediated UTP End Labeling (TUNEL) of Apoptotic Cells and Confocal Microscopy. Apoptotic W256 cells following treatment with either antisense oligos or inhibitors were detected using the TUNEL method (8) with the ApopTag kit (Oncor). The labeled cells were examined using a confocal microscope (9, 10).

Immunoblotting. Immunoblotting was performed essentially as described using the enhanced chemiluminescence system (6, 9).

RESULTS AND DISCUSSION

Previous studies showed that W256 cells express platelet-type 12-LOX and biosynthesize 12(S)- and 15(S)-hydroxyeicosatetraenoic acids (HETEs) as their major LOX products (11–13).

Abbreviations: AA, arachidonic acid; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; NDGA, nordihydroguaiaretic acid; BHPP, *N*-benzyl-*N*-hydroxy-5-phenylpentanamide; COX, cyclooxygenase; LOX, lipoxygenase; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling.

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FIG. 1. Antisense oligonucleotide to 12-LOX inhibits W256 cell growth by inducing apoptosis. (A) Time course. Forty thousand W256 cells (indicated by the horizontal bar) cultured in 24-well plates were transfected with 2.5 μ M 12-LOX-specific antisense oligonucleotide (AP1; 5'-CTCAGGAGGGTGTAAACA-3') or corresponding sense oligonucleotide (SP1) in the presence of 10 μ g/ml Lipofectin in serum-free MEM for 4 h, after which the oligonucleotides were washed off and cells were cultured in MEM supplemented with 5% FCS for up to 24 h (day 1). The treatment was repeated every 24 h up to 72 h (day 3). Dead cells were removed by gentle washing at the end of each day and the number of live cells determined with a Coulter counter. Each condition was run in triplicate and the bars represent mean $\pm$ SE obtained from three independent experiments. (B and C) Phase contrast photomicrographs showing W256 cells treated with 2.5 μ M of either SP1 (B) or AP1 (C) for 2 days. The treatment protocol was as in A. Arrowheads indicate apoptotic cells or apoptotic bodies. (D and E) Detection of apoptosis in W256 cells treated with SP1 (D) or AP1 (E) using the TUNEL labeling method with the ApopTag kit (Oncor). The treatment conditions were as in B and C. Note the majority of the apoptotic cells were washed off during sample processing. Shown are imposed pictures of fluorescein-labeled apoptotic (arrowheads in E) and phase contrast images taken on a Zeiss LSM 30010 confocal microscope. (F) DNA fragmentation. W256 cells were treated with different doses of AP1 or 2.5 μ M of SP1, the scrambled sequence (5'-AAGATTGCGCGACGATGA-3') or an antisense oligonucleotide derived from the 3' untranslated region (3'-UTN; GTGACTATGCGGT-CAGCA-3'; nucleotide 2189–2206 of 12-LOX) (left panel; 48 h treatment). For the time course, cells were harvested at the indicated time points (Right). (B and C, $\times 200$; D and E, $\times 400$.)

To explore the role of the W256 LOX(s) in cell growth, W256 cells were transfected with an antisense oligonucleotide (AP1) directed specifically to platelet-type 12-LOX. It was found that AP1 strongly inhibited W256 cell growth by inducing apoptosis (Fig. 1). The growth-inhibitory effect was observed at 0.1 μ M and became apparent in a dose-dependent fashion, as determined by [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]/phenazine methosulfate (MTS/PMS) proliferation assay (ref. 4; data not shown). Dose studies revealed a maximum effect of the oligonucleotide at ≈ 2.5 μ M (data not shown; also see Fig. 1F). By 24 h and 48 h, $\approx 40\%$ and $\approx 80\%$ of the cells were killed, respectively, by AP1 (Fig. 1A). The growth inhibition induced by AP1 was the consequence of apoptosis induction, as revealed by phase contrast microscopy (Fig. 1B and C), *in situ* apoptosis labeling using the TUNEL method and confocal microscopy (Fig. 1D and E), and DNA fragmentation assay (Fig. 1F). Electron microscopy, PI (propidium iodide) staining/flow cytometric analysis, and PI-acridine orange double

fluorescence microscopy also corroborated apoptotic cell death (data not shown). The sense control oligonucleotide (SP1), a scrambled oligonucleotide with the same nucleotide composition as AP1, and an antisense oligonucleotide derived from the 3' untranslated region (3'-UTN) all failed to cause DNA fragmentation (Fig. 1F) or morphologic apoptosis. Time studies indicated that AP1-induced DNA fragmentation was observed 24 h posttreatment and became more prominent with time (Fig. 1F, Right). To confirm the AP1 effect, three pairs of sense and antisense oligonucleotides derived from the conserved regions of three mammalian LOXs (i.e., 5-, 12-, and 15-LOX; refs. 14 and 15) were transfected into W256 cells. As presented in Table 1, all three antisense oligonucleotides demonstrated stronger effects than AP1, inducing fulminant W256 cell apoptosis and leading to significantly lower cell survival. The nature of the apoptosis was substantiated by microscopy and fragmentation assay (data not shown).

So far, 5-, 12-, and 15-LOXs have been cloned from mammalian cells and at least two isoforms of 12-LOX (i.e., the

Table 1. Effects of LOX antisense oligonucleotides on W256 cell survival

Oligo-nucleotide	Sequence*	Identity with		W256 cell survival (%)†
		5-LOX	15-LOX	
Lipofectin	—	—	—	95 ± 5
Sense	5'-GACGCGTGGTTCTGC-3'	10/15	14/15	98 ± 11
Antisense	5'-GCAGAACACGCGTC-3' (nt 220–234)	(67%)	(93%)	13 ± 7
Sense	5'-CACCCATCTTCAAG-3'	14/15	13/15	91 ± 12
Antisense	5'-CTTGAAGATGGGGTG-3' (nt 1147–1161)	(93%)	(87%)	8 ± 3
Sense	5'-TGTCCTCTGACGACCTG-3'	12/18	15/18	99 ± 19
Antisense	5'-CAGGTCGTGAGGAGACA-3' (nt 1324–1341)	(67%)	(83%)	5 ± 3

W256 cells were treated with various oligonucleotides (2.0 μ M) in the presence of 10 μ g/ml of Lipofectin in serum-free MEM for 4 h, after which oligonucleotides were washed off and cells were put back to MEM with 5% FCS. The treatment was repeated at 24 h and the experiment was terminated by 48 h.

*The sequence was from 12-LOX (14, 15).

†The number of live cells was determined by trypan blue assay and the % of cell survival was determined by comparing to the control (i.e., not any addition). Each condition was run in triplicate and the values represent mean $\pm$ SE derived from three repeat experiments.

leukocyte-type and the platelet-type) have been identified (11, 14, 15). W256 cells have been shown to express platelet-type 12-LOX (11–13). It is not known whether they also express 15- and 5-LOX. The results obtained with the antisense oligonucleotides as described above suggest that 12-LOX and probably 15- and/or 5-LOXs play a vital role in sustaining the viability of W256 cells. This hypothesis was tested using a panel of general or selective LOX inhibitors. *N*-benzyl-*N*-hydroxy-5-phenylpentanamide (BHPP), a compound preferentially inhibiting 12-LOX activity (16), demonstrated a dose-dependent inhibitory effect on W256 cell growth (Fig. 2A). The DNA fragmentation was observed with 10 μ M BHPP treatment (12 h) and the inhibitor revealed a dose- and time-dependent effect (Fig. 2B). NDGA, a general LOX inhibitor, demonstrated the most potent apoptosis-inducing effect, inducing apoptosis at 0.1 μ M 24 h after drug application. Two h after 2.5 μ M NDGA treatment, numerous apoptotic cells with typical membrane blebbing could easily be seen (Fig. 2C). At 4 h, the majority of cells in culture were dead and released membrane-bound apoptotic bodies (Fig. 2D). DNA fragmentation assays revealed a similar time- and dose-dependent relationship (Fig. 2E). NDGA-induced apoptosis declined at ≥ 10 μ M. At 25 μ M, a mixed apoptosis and necrosis was observed, while at ≥ 50 μ M, NDGA induced a rapid necrotic response that affected the whole cell population. Another general LOX inhibitor, 5,8,11-eicosatrienoic acid (ETI) (17) also induced potent cell death, with obvious effects observed at 5 μ M (Fig. 2F). Of the three selective 5-LOX inhibitors tested, caffeic acid (18) and 5,6-dehydro-arachidonic acid (19) did not induce apoptosis at the doses tested (5–100 μ M and 1–50 μ M, respectively; Fig. 2 E and F; data not shown). Another select and potent 5-LOX inhibitor, 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadienyl)-1,4-benzoquinone (AA-861) (20) induced W256 cell apoptosis only at ≥ 20 μ M. It is not known whether this effect of AA-861 resulted from a specific inhibition of 5-LOX or from inhibition of other LOXs. In addition to BHPP, two other selective 12-LOX inhibitors, baicalein (5,6,7-trihydroxyflavone; ref. 21) and cinnamyl-3,4-dihydroxy- α -cyanocinnamide (CDC) (22), also induced dose-dependent DNA fragmentation and apoptosis of W256 cells (Fig. 2 E and F). In general, selective 12-LOX inhibitors (BHPP, baicalein, and CDC) required higher doses and longer treatment than NDGA and ETI (general LOX inhibitors) in

inducing W256 cell apoptosis, thus strengthening the notion that, in addition to 12-LOX, other LOXs (most probably 15-LOX) may also be involved in regulating W256 cell survival. In contrast to the results obtained with LOX inhibitors, two COX inhibitors, indomethacin (preferentially inhibiting COX-1 over COX-2) and ibuprofen (with comparable inhibition for both COX-1 and COX-2), did not induce cell death at the doses tested (5–100 μ M) (Fig. 2E; data not shown). W256 cell apoptosis induced by NDGA and other LOX inhibitors were confirmed and studied in detail by electron microscopy, which revealed typical morphological features (membrane blebbing, chromatin condensation, etc.) associated with apoptosis. Apoptosis induced by NDGA and other LOX inhibitors involved *de novo* protein synthesis because it could be efficiently blocked by pretreatment of W256 cells with cycloheximide (D.G.T. and K.V.H., unpublished results).

NDGA is a promiscuous, general LOX inhibitor that also possesses many LOX-unrelated effects such as blocking voltage-activated Ca^{2+} currents (23), inhibiting P450 monooxygenase activity (24), and working as an antioxidant (25). However, several lines of evidence suggest that the NDGA-induced W256 cell apoptosis is mediated primarily via its effects on LOX. First, at low concentrations (i.e., <10 μ M) NDGA is known to demonstrate a preferential inhibitory effect on LOX activity (26). In this study, only NDGA at 1–10 μ M induced W256 cell apoptosis. Second, 1 h after NDGA treatment and before the morphologic manifestation of apoptosis, decreased biosynthesis of 12- and 15-HETE in W256 cells was observed (data not shown). Third, the NDGA-induced DNA fragmentation and W256 cell death could be inhibited by exogenous 12(S)- and 15-HETEs but not by 5(S)-HETE (Fig. 3A). The apoptosis-blocking effects of 12(S)- and 15(S)-HETEs could not be mimicked by their metabolic precursors [i.e., 12(S)- and 15(S)-HPETE, respectively] nor by isomeric enantiomers such as 12(R)-HETE or 5-LOX products including lipoxin A4 and B4 and leukotriene C4 (Fig. 3B). In fact, 12(S)-HPETE slightly enhanced the NDGA-induced apoptosis (Fig. 3B). Interestingly, leukotriene B4 appeared to prevent NDGA-induced cell death (Fig. 3B), for which the reason remains to be explored. Finally, NDGA is widely known as an antioxidant (e.g., ref. 19). However, in the present case, apoptosis of W256 cells triggered by NDGA appeared to involve free radical generation since the process could be effectively blocked by carazostatin, an inhibitor of lipid peroxidation catalyzed by free radicals (D.G.T. and K.V.H., unpublished results).

We next explored the potential biochemical mechanisms involved in the LOX-regulated apoptosis. Specifically, the role of three important molecules, i.e., bcl-2, bax, and p53, was examined in the apoptosis of W256 cells induced by LOX inhibitors and antisense oligonucleotides. Twenty-four hours after AP1 treatment, the 12-LOX protein was slightly downregulated (≈ 3 -fold; Fig. 4A). By 48 h, 12-LOX protein decreased to nearly undetectable levels when normalized to corresponding actin protein levels (Fig. 4A). This observation provided evidence that the antisense oligonucleotide was specifically blocking the 12-LOX gene expression. In contrast to the relatively slow downregulation in the 12-LOX protein, the bcl-2 protein level markedly decreased (≈ 20 -fold) and the level of bax, a protein known to complex with and antagonize the effect of bcl-2 (27), only marginally decreased (<2 -fold) by 24 h. By 48 h, the bcl-2 protein dropped to an undetectable level. However, the bax protein level decreased by only ≈ 3 -fold (Fig. 4A). Similar blotting experiments with various LOX inhibitors (data not shown) revealed that NDGA, BHPP, baicalein, and CDC treatment all led to a more dramatic decrease in bcl-2 (5–20-fold) than in bax (1–3-fold). The effect of NDGA on bcl-2 and bax protein levels was also confirmed by flow cytometric analysis. The decrease of bcl-2 protein occurred temporally before the microscopic manifestation of W256 cell apoptosis. Fifteen minutes after NDGA treatment, bcl-2 protein level decreased by ≈ 3 -fold; by 60 min it decreased further by >5 -fold (Fig. 4B). The end result, following oligonucleotide or

FIG. 2. Induction of W256 cell apoptosis by LOX but not COX inhibitors. (A) Time- and dose-studies of W256 cell apoptosis induced by BHPP. Sixty thousand W256 cells (indicated by the horizontal bar) cultured in 24-well plates were treated with solvent control (ethanol; labeled as "0") or increasing doses of BHPP in regular serum-containing MEM. The experiments were terminated at 24 h (day 1), 48 h (day 2), and 72 h (day 3) after the initiation of treatment. Dead cells were removed and cell survival enumerated with a Coulter counter. Each condition was run in triplicate and the results represent mean $\pm$ SE obtained from three independent experiments. (B) Dose and time studies of DNA fragmentation induced by BHPP. (C and D) Morphological alterations of W256 cells after nordihydroguaiaretic acid (NDGA) (2.5 μ M) treatment for 2 h (C) or 4 h (D). Numerous apoptotic cells with membrane blebbing were seen in the early phase (arrowheads in C) and apoptotic bodies were prevalent in the culture at a later stage (arrowheads in D). Quantitation revealed that at the end of 4-h treatment, NDGA caused a $60 \pm 4.7\%$, $87 \pm 6.8\%$, and $98 \pm 3.4\%$ apoptosis at 1.0, 2.5, and 5.0 μ M, respectively. (E) DNA fragmentation induced by various LOX inhibitors. W256 cells were treated with NDGA or other inhibitors (at indicated doses; 24 h) in serum-containing MEM and fragmented DNA prepared as described above. IN, indomethacin; Dehydro-AA, 5,6-dehydro-arachidonic acid. (F) Quantitation of W256 cell apoptosis induced by LOX inhibitors. The surviving cells were quantitated as described in A. The treatment was for 24 h and the results represent mean $\pm$ SE derived from three independent experiments. CA, caffeic acid; D-AA, 5,6-dehydro-arachidonic acid. (C and D, $\times 200$.)

inhibitor treatment, is a time-dependent decrease in the bcl-2/bax ratio, which is a critical determinant of apoptosis (27), thus shifting the homeostasis toward a preference for apoptosis. It is unlikely that the different protein levels of bcl-2 and bax observed above were due to their differential turnover rates since bcl-2 is a long-lived protein with a half-life of 10–14 h (28, 29).

The above results prompted us to perform overexpression experiments. W256 cells were stably transfected with human bcl-2 cDNA under the control of cytomegalovirus early enhancer/promoter (pCMVbcl-2) or with the empty vector (pCMVneo) as a control. Three clones of transfectants that had been confirmed to express increased levels of bcl-2 and 2 control clones (Fig. 4C) were treated with NDGA or AP1 to induce apoptosis. As shown in Fig. 4E, all three bcl-2 overexpressers were significantly more resistant to apoptosis induction by either inducer. W256 cells appeared to express

wild-type p53. Untreated W256 cells express barely detectable levels of p53 protein (Fig. 4D). Treatment of W256 cells with the genotoxic drug Adriamycin induced a significant increase by 24 h (>5 -fold) in the p53 protein (Fig. 4D). The protein level of P21^{WAF-1}, a downstream target of the p53, did not undergo any significant alterations 24 h after Adriamycin treatment. However, by 48 h it was dramatically induced (>10 -fold; Fig. 4D). It is known that, in response to DNA damage, p53 protein induces P21^{WAF-1} only when p53 is wild type (30). Therefore, the above experimental findings suggest that p53 is wild type in W256 cells. This conclusion is further supported by the single strand conformation polymorphism analysis of exons 5–8 using primers derived from the rat p53 (31) sequence (data not shown). In sharp contrast to bcl-2, the p53 protein in W256 cells did not undergo apparent alterations following treatment with LOX

FIG. 3. (A) Exogenous 12(S)- and 15(S)-HETE, but not 5(S)-HETE, partially block the DNA fragmentation and W256 cell apoptosis induced by NDGA. W256 cells were treated with NDGA (2.5 μ M) together with either ethanol (control) or 1.0 μ M HETEs for 4 h. Apoptosis was assessed by DNA fragmentation assays and cell viability. The quantitation of DNA fragmentation was detailed in *Materials and Methods*. For cell survival, the viability was determined by the trypan blue dye exclusion assay and the number of live cells divided by the cell number initially plated gives the % of cell survival. Each experimental condition was run in triplicate or quadruplicate and the results represent the mean $\pm$ SE from three independent experiments. The difference between the control and 1.0 μ M of 12(S)- or 15(S)-HETE is statistically significant ($P < 0.01$) for both cell survival and DNA fragmentation. (B) The apoptosis-blocking effects of 12(S)-HETE and 15(S)-HETE are not mimicked by other eicosanoids. W256 cells treated with NDGA (5 μ M $\times$ 2 h) alone and NDGA with 1 μ M of various eicosanoids for 2 h. Cell survival was determined by the trypan blue dye exclusion assay and the results expressed as the % of control (i.e., ethanol-treated). Shown is the mean $\pm$ SE from two independent experiments. $P < 0.001$ for 12(S)-HETE/NDGA and 15(S)-HETE/NDGA and NDGA alone. $P < 0.05$ for 12(S)-hydroperoxyeicosatetraenoic acid (HPETE)/NDGA and leukotriene B4 (LTB4)/NDGA and NDGA alone.

inhibitors or antisense LOX oligonucleotides, as analyzed by immunoblotting (data not shown).

The present study provides evidence for a novel class of regulators of cell survival and apoptosis, i.e., the LOX pathways (in particular, the 12-LOX pathway) of AA metabolism. Using rat W256 carcinosarcoma of hematopoietic origin as a model, we observed that downregulating the 12-LOX gene by specific antisense oligonucleotides or abrogating the LOX enzymatic activity with inhibitors both trigger apoptosis. Although lack of specific 15-LOX inhibitor(s) did not allow us to directly assess the involvement of 15-LOX in W256 cell survival, several lines of evidence suggest that 15-LOX may

also have such a regulatory role. First, antisense oligonucleotides directed to the conserved regions of 12- and 15-LOX induced stronger apoptosis of W256 cells than 12-LOX-specific (i.e., AP1) oligonucleotide (Table 1). Second, general LOX inhibitors demonstrated a stronger apoptosis-inducing effect than preferential 12-LOX inhibitors (Fig. 2). Third, W256 cells are known to express platelet-type 12-LOX (11, 13) that metabolizes AA almost exclusively to 12(S)-HETE (14, 15). Previous experiments (12, 13) also documented that W256 cells biosynthesize both 12(S)-HETE and 15(S)-HETE, thus suggesting that W256 cells might express a 15-LOX activity. Fourth, supplementation of W256 cells with exogenous 12- or 15-HETE could partially block both oligonucleotide- and inhibitor-induced apoptosis. By contrast, the current studies suggest that 5-LOX may not be involved in regulating W256 cell survival, as evidenced by the inability of 5-LOX inhibitors to trigger apoptosis and of 5(S)-HETE to overcome NDGA-induced W256 cell death, although this LOX system has been implicated in regulating apoptosis of other tumor cells (32).

The present study suggests that in W256 cells, the LOX system (i.e., 12-LOX and probably 15-LOX) is tightly or inherently coupled to cell survival and the cellular apoptotic machinery. This phenomenon is not unique to W256 cells; several cell lines such as human erythroleukemia (HEL) and rat MTLn-3 mammary adenocarcinoma cells also undergo apoptosis in response to treatment with oligonucleotides or LOX inhibitors (D.G.T. and K.V.H., unpublished results). The precise mechanism whereby the LOX pathway is coupled to apoptosis is unknown. However, the data presented points to a role of LOX pathway as a critical regulator of intracellular oxidation process in modulating cell survival. Intracellular oxidants (free radicals, oxidized intermediates of lipid and protein molecules, etc.) are closely involved in the apoptotic process. AA metabolism and mitochondrial electron transport are the two major sources for the generation of reactive oxidants. These reactive oxidation species not only directly damage important cellular components, but also regulate the expression of many other genes, including those implicated in cell death. Intriguingly, it was observed in this study that, after treatment of W256 cells with LOX antisense oligonucleotides or LOX inhibitors, there was a rapid decrease in the bcl-2 protein level. Based on the observations that W256 cell apoptosis induced by NDGA and other LOX inhibitors requires *de novo* protein synthesis and can be inhibited by peroxidation inhibitors (D.G.T. and K.V.H., unpublished results), it is tempting to hypothesize that the LOX system may directly or indirectly (e.g., via reactive free radicals) regulate certain cell death genes (e.g., Interleukin 1 Converting Enzyme-related proteases), which in turn regulate the bcl-2 protein. Future studies will be directed to dissecting the role and molecular mechanisms of individual LOXs in regulating cell survival and apoptosis, identifying the genes regulated by the LOX system, and elucidating the cause-and-effect relationship between the LOX, intracellular redox homeostasis, and important apoptosis-regulatory molecules such as bcl-2, bax, and proteases.

We thank Ms. S. Stojakovic for her devoted and consistent technical help. We also thank Dr. S. J. Korsmeyer for providing the human bcl-2 cDNA sequence, Dr. S. A. Belinsky for communicating the rat p53 primer sequences used in single strand conformation polymorphism analysis, and Drs. I. Butovich, R. Sheikhejad, and M. Trika for helpful discussions and invaluable advice. We gratefully acknowledge Dr. K. Szekeres and Ms. S. Stojakovic for performing RIA, Mr. S. Lundy for flow cytometry experiments, Dr. K. Moin for help in confocal microscopy, Dr. M. Haddad for help in electron microscopy, and Drs. I. Butovich and C. A. Diglio for critically reading the manuscript. This work was supported by National Institutes of Health Grant CA-29997 (K.V.H.) and a Developmental Grant from Harper Hospital.

FIG. 4. Mechanistic studies on W256 cell apoptosis regulated by LOX. (A) Immunoblotting studies of the effect of 12-LOX antisense oligonucleotide (AP1) on the protein levels of 12-LOX, bcl-2, and bax. Protein (200 μ g) of whole cell lysates prepared from W256 cells with no treatment (lanes 1 and 5), or from W256 cells treated with Lipofectin alone (10 μ g/ml; lanes 2 and 6), or Lipofectin plus 2.5 μ M of sense (SP1; lanes 3 and 7) or antisense (AP1; lanes 4 and 8) oligonucleotides for 24 h (lanes 1–4) or 48 h (lanes 5–8), were fractionated on a 12% denaturing SDS/PAGE under reducing conditions. The membrane was probed with polyclonal anti-12-LOX and then stripped and reprobed with polyclonal anti-bcl-2 (Santa Cruz Biotechnology sc-492), polyclonal anti-bax (Santa Cruz Biotechnology sc-526), or monoclonal anti-actin (ICN) for loading control. Shown is one of three experiments with similar results. In these experiments, 12-LOX, bcl-2, bax, and actin were detected as $\approx$ 76-, $\approx$ 26-, $\approx$ 22-, and $\approx$ 41-kDa proteins, respectively. Note some lower bands in the bcl-2 blots that might represent degradation products. (B) The downregulation of bcl-2 protein occurs rapidly in NDGA-treated W256 cells. Whole cell lysates (20 μ g) of W256 cells treated with 2.5 μ M of NDGA for the time intervals indicated were separated on a denaturing 12% minigel under reducing conditions. The membrane was probed and reprobed as described above. (C) Overexpression of human bcl-2 protein in W256 cells. Two stably transfected pCMVneo clones and 3 pCMVbcl2 clones were used in immunoblotting as described in B. (D) W256 cells express wild-type p53. Whole cell lysates (20 μ g) prepared from W256 cells treated with 0.2 μ g/ml of Adriamycin for 0, 24, and 48 h (lanes 1–3, respectively) were separated on a 12% denaturing minigel. The membrane was probed and reprobed with monoclonal anti-p53 (Ab-1; Oncogene Science), polyclonal anti-p21 (Santa Cruz Biotechnology sc-397), or with monoclonal anti-actin. (E) Overexpression of bcl-2 partially blocks apoptosis of W256 cells induced by NDGA and AP1. Untransfected (control) or transfected (see C) W256 cells were treated with either NDGA (2 μ M for 3 h) or AP1 (2.5 μ M $\times$ 48 h). The surviving cells were enumerated by a Coulter counter. Presented is the % of cell survival of NDGA-treated groups vs. the vehicle control, or of AP1 treated groups vs. the Lipofectin control. The sense oligonucleotide control (i.e., SP1) gave very similar results as the Lipofectin control. Each condition was run in triplicate and the results represent the mean $\pm$ SE obtained from three separate experiments. All three clones of bcl-2 transfectants are significantly more resistant ($P < 0.01$) to both AP-1 and NDGA-induced apoptosis.

- Vaux, D. L., Haecker, G. & Strasser, A. (1994) *Cell* **76**, 777–779.
- Korsmeyer, S. J., Yin, X.-M., Oltvai, Z. N., Veis-Novack, D. J. & Linette, G. P. (1995) *Biochim. Biophys. Acta* **1271**, 63–66.
- Needleman, P., Turk, J., Jakschik, B. A., Morrison, A. R. & Lefkowitz, J. B. (1986) *Annu. Rev. Biochem.* **55**, 69–102.
- Tang, D. G., Renaud, C., Stojakovic, S., Diglio, C. A., Porter, A. & Honn, K. V. (1995) *Biochem. Biophys. Res. Commun.* **211**, 462–468.
- Simpkins, H., Lehman, J. M., Mazurkiewicz, J. E. & Davis, B. H. (1991) *Cancer Res.* **51**, 1334–1338.
- Tang, D. G., Grossi, I. M., Chen, Y. Q., Diglio, C. A. & Honn, K. V. (1993) *Int. J. Cancer* **54**, 102–111.
- Herrmann, M., Lorenz, H.-M., Voll, R., Grunke, M., Woith, W. & Kalden, J. R. (1994) *Nucleic Acids Res.* **22**, 5506–5507.
- Gavrieli, Y., Sherman, Y. & Ben-Sasson, S. A. (1992) *J. Cell Biol.* **119**, 493–501.
- Tang, D. G., Diglio, C. A. & Honn, K. V. (1994) *Cancer Res.* **54**, 1119–1129.
- Tang, D. G., Diglio, C. A., Bazaz, R. & Honn, K. V. (1995) *J. Cell Sci.* **108**, 2629–2644.
- Tang, D. G. & Honn, K. V. (1994) *Ann. N.Y. Acad. Sci.* **744**, 199–215.
- Liu, B., Timar, J., Howlett, J., Diglio, C. A. & Honn, K. V. (1991) *Cell Regul.* **2**, 1045–1055.
- Chen, Y., Duniec, Z. M., Liu, B., Hagmann, W., Gao, X., Shimoji, K., Marnett, L. J., Johnson, C. R. & Honn, K. V. (1994) *Cancer Res.* **54**, 1574–1579.
- Toh, H., Yokoyama, C., Tanabe, T., Yoshimoto, T. & Yamamoto, S. (1992) *Prostaglandins* **44**, 291–315.
- Funk, C. D., Furci, L. & FitzGerald, G. A. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5638–5642.
- Liu, B., Marnett, L. J., Chaudhary, A., Ji, C., Blair, I. A., Johnson, C. R., Diglio, C. A. & Honn, K. V. (1994) *Lab. Invest.* **70**, 314–323.
- Hammarstrom, S. (1977) *Biochim. Biophys. Acta* **487**, 517–519.
- Koshihara, Y., Neichi, T., Murota, S., Lao, A.-N., Fujimoto, Y. & Tatsuno, T. (1984) *Biochim. Biophys. Acta* **792**, 92–97.
- Sok, D. E., Han, C. Q., Pai, J. K. & Sih, C. J. (1982) *Biochem. Biophys. Res. Commun.* **107**, 101–108.
- Yoshimoto, Y., Yokoyama, C., Ochi, K., Yamamoto, S., Maki, Y., Ashida, Y., Terao, S. & Shiraishi, M. (1982) *Biochim. Biophys. Acta* **713**, 470–473.
- Sekiya, K. & Okuda, H. (1982) *Biochem. Biophys. Res. Commun.* **105**, 1090–1095.
- Cho, H., Ueda, M., Tamaoka, M., Hamaguchi, M., Aisaka, K., Kiso, Y., Inoue, T., Ogino, R., Tatsuoka, T., Ishihara, T., Noguchi, T., Morita, I. & Murota, S. (1991) *J. Med. Chem.* **34**, 1503–1505.
- Korn, S. & Horn, R. (1990) *Mol. Pharmacol.* **38**, 524–530.
- Agarwal, R., Wang, Z. Y., Bik, D. P. & Mukhtar, H. (1991) *Drug Metab. Disposition* **19**, 620–624.
- Jackson, W. F. (1988) *Am. J. Physiol.* **255**, H711–H716.
- Goodman, Y., Steiner, M. R., Steiner, S. M. & Mattson, M. P. (1994) *Brain Res.* **654**, 171–176.
- Oltvai, Z., Millman, C. L. & Korsmeyer, S. J. (1993) *Cell* **74**, 609–619.
- Yang, T., Kozopas, K. M. & Craig, R. W. (1995) *J. Cell Biol.* **128**, 1173–1184.
- Merino, R. L., Ding, L., Veis, D., Korsmeyer, S. J. & Nunez, G. (1994) *EMBO J.* **13**, 683–691.
- Cox, L. S. & Lane, D. P. (1995) *BioEssays* **17**, 501–508.
- Nickell-Brady, C., Hahn, F. F., Finch, G. L. & Belinsky, S. A. (1994) *Carcinogenesis* **15**, 257–262.
- Anderson, K. M., Levin, J., Jahaj, A., Seed, T. & Harris, J. E. (1993) *Prostaglandin Leukot. Essen. Fatty Acids* **8**, 675–684.