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Lipoxygenase Inhibitors Abolish Proliferation of Human Pancreatic Cancer Cells

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Epidemiologic and animal studies have linked pancreatic cancer growth with fat intake, especially unsaturated fats. Arachidonic acid release from membrane phospholipids is essential for tumor cell proliferation. Lipoxygenases (LOX) constitute one pathway for arachidonate metabolism, but their role in pancreatic cancer growth is unknown. The expression of 5-LOX and 12-LOX as well as their effects on cell proliferation was investigated in four human pancreatic cancer cell lines (PANC-1, MiaPaca2, Capan2, and ASPC-1). Expression of 5-LOX and 12-LOX mRNA was measured by nested RT-PCR. Effects of LOX inhibitors and specific LOX antisense oligonucleotides on pancreatic cancer cell proliferation were measured by ^{3}H-thymidine incorporation. Our results showed that (1) 5-LOX and 12-LOX were expressed in all pancreatic cancer cell lines tested, while they were not detectable in normal human pancreatic ductal cells; (2) both LOX inhibitors and LOX antisense markedly inhibited cell proliferation in a concentration-dependent and time-dependent manner; (3) the 5-LOX and 12-LOX metabolites 5-HETE and 12-HETE as well as arachidonic and linoleic acids directly stimulated pancreatic cancer cell proliferation; (4) LOX inhibitor-induced growth inhibition was reversed by 5-HETE and 12-HETE. The current studies indicate that both 5-LOX and 12-LOX expression is upregulated in human pancreatic cancer cells and LOX plays a critical role in pancreatic cancer cell proliferation. LOX inhibitors may be valuable for the treatment of pancreatic cancer.

Pancreatic carcinoma is characterized by a poor prognosis and lack of response to conventional therapy (1–3). The incidence of this disease has significantly increased in recent years (3). The 5-year survival rate for this disease is less than 2%, and median survival after diagnosis is 4-6 months (3). Epidemiologic and animal studies have linked pancreatic cancer growth with fat intake, particularly unsaturated fats (4–5). For example, dose-dependent promotion of pancreatic cancer cell growth by linoleic acid was shown by Fisher et al and ingestion of high levels of fats, especially unsaturated fats enhanced carcinogen-induced pancreatic carcinogenesis in both hamsters and rats (4, 5, 6).

Arachidonic acid (5,8,11,14-eicosatetraenoic acid), an ω-6 polyunsaturated essential fatty acid, is released from cellular membrane phospholipids in response to external stimuli such as EGF receptor activation through phospholipase A2 (7–9). Inhibition of this enzyme blocks the growth response to exoenous EGF (10). Free arachidonic acid is a substrate for three distinct enzymatic pathways; the cylcooxygenase (COX), lipoxygenase (LOX) and epoxygenase pathways. Among these, the LOX pathway catalyzes the incorporation of an oxygen molecule into polyunsaturated fatty acids containing 1,4-cic, *cis*-pentadiene structure to yield a 1-hydroperoxy-2, 4-*trans, cis*-pentadiene product (11). Mammalian lipoxygenases possess regiospecificity during interaction with substrate and on that basis have been designated as arachidonate 5-, 12-, 15-LOX which produce 5S-, 12S- or 15S-hydroperoxyeicosa-tetraenoic acid (5-, 12-, 15-HPETE) and hydroeicosatetranoic acid (5-, 12-, 15-HETE) (11). 5-HPETE is noteworthy, in that it is the only HPETE that can be further metabolized to leukotrienes. Activation of 5-LOX is dependent upon a second factor termed 5-lipoxygenase-activating protein (FLAP) (11). Three types of mammalian 12-LOX isoenzymes have been reported (11). The first, human platelet-type 12-LOX is expressed in platelets, human erythroleukemia cells, and umbilical vein endothelial cells (11, 12). The second is the porcine leukocyte-type 12-LOX, which metabolizes both arachidonic acid and linoleic acid, thus generating 12(S)-HETE (hydroxyeicosatetraenoic acids) as well as small amounts of 15-(S)-HETE (11, 12, 13). The third 12-LOX (sometimes termed epithelial 12-LOX), which shares more homology with 15-lipoxygenase and leukocyte-type 12-LOX than with platelet-type 12-LOX, was isolated from bovine tracheal epithelial cells

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and rat brain (11). This type of 12-LOX, like reticulocyte 15-lipoxygenase and leukocyte-type 12-LOX, catalyzes the formation of both 12(S)-HETE and 15(S)-HETE (11).

LOX products are considered biologically to be at least as important as prostaglandins produced through the cyclooxygenase pathway, because of their involvement in many aspects of inflammation (13). Compared to cyclooxygenase, however, reports on the regulation of specific LOX in cancer growth and development are limited although several lines of evidence have shown the importance of LOX in regulating human lung cancer and rat melanoma cancer cells (13–18). There is also a report of inhibition of proliferation of a single pancreatic cancer cell line by a FLAP inhibitor (19). With this background, we examined the expression of LOX expression in human pancreatic cancer cells and their role in the regulation of human pancreatic cancer cell growth.

MATERIALS AND METHODS

Materials. RPMI1640, DMEM, McCoy's 5A media, penicillin-streptomycin solution, trypsin-EDTA solution, 5-HETE, 12-HETE, 15-HETE, and arachidonic acid were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA). NDGA (nordihydroguaiaretic acid) a non-specific lipoxygenase inhibitor (20), baicalein (5,6,7-Trihydroxyflavone) a specific 12-LOX inhibitor (21), MK886 (3-[1-(p-Chlorobenzyl)-5-(isopropyl)-3-t-butylthioindol-2-yl]-2,2-dimethylpropanoic acid) a 5-LOX activating protein inhibitor (22), and Rev5901 (α-pentyl-4-(2-quinolinylmethoxy benzenemethanol) 5-LOX inhibitor (23), were purchased from CalBiochem (La Jolla, CA). Methyl-[^{3}H]-thymidine was a product of Amersham Life Science Company (Arlington Heights, IL).

Human pancreatic cancer cell lines and cell culture. Two well-differentiated (Capan2 and ASPC1) and two poorly differentiated (PANC-1 and MiaPaCa2) human pancreatic cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD). PANC-1 and MiaPaCa2 were cultured in DMEM medium and Capan2 and ASPC1 were grown in McCoy's 5A media. Media were supplemented with 10% FBS and cells grown as monolayers in a humidified atmosphere at 37°C.

Nested RT-PCR analysis of human 5-LOX and platelet-type 12-LOX. RNA from PANC-1, MiaPaCa2, ASPC-1, Capan2 cells was isolated using RNAzol B (TEL-TEST Inc, Friendswood, TX), extracted with chloroform and precipitated with isopropanol. The RNA pellets were washed with 75% ethanol and the integrity confirmed on a 1% agarose gel. Cellular RNA was reverse transcribed into cDNA using each specific downstream primer and reverse transcriptase according to the manufacturer's protocol (Perkin Elmer GeneAmp kit, Foster City, CA). The total volume of the reaction was 20 μl containing the following: 2 μg of total RNA, 25 pmol of reverse primer with 25 mM $MgCl_2$, 10X PCR buffer II, RNase/DNase-free water, 0.2 mM of dNTPs, RNase inhibitor (20 units) and the reverse transcriptase (5 units). The thermocycler protocol for the RT phase is one hold at 42°C for 60 min, one hold at 99°C for 5 min and one hold at 5°C for 5 min.

After the RT reaction, nested PCR was used to examine 5-LOX and 12-LOX mRNA expression using human platelet RNA and leukocyte RNA as positive controls. PCR primers were designed based on the sequences of human 5-LOX and platelet-type 12-LOX cDNA. For 12-LOX, the first run PCR profile was 94°C, 15 s to denature; 61°C, 30 s for annealing and extension for 35 cycles with upstream (5′-CTTCCCGTGCTACCGCTG-3′) and downstream (5′-TGGGGT-TGGCACCATTGAG-3′) primers. 5 μl of first run PCR product was used for the nested PCR with the profie of 94°C, 15 s to denature; 61°C, 30 s for annealing and extension for 25 cycles with nested primers (upstream 5′-CCAGGAGACAATGCTTTGGACA-3′; downstream 5′-GAACAACTCATCATCCTGCCAG-3′). For 5-LOX, the first run PCR profile was 94°C, 15 s; 61°C, 30 s and 72°C, 1 min for 35 cycles with upstream 5′-CCAGGAACAGCTCGTTTTCCT-3′ and downstream 5′-CCCGGGGCATGGAGAGCAA-3′ primers. 5 μl of first run PCR product was used for the nested PCR with the profile of 94°C, 15 s; 61°C 30 s and 72°C 1 min, for 35 cycles with nested primers (upstream 5′-GTCCACGATCTGCTCAATGGT-3′; downstream 5′-ATCAGGACGTTCACGGCCGA-3′). The final PCR product was separated on 2% agarose gel and visualized with ethidium bromide.

LOX antisense oligonucleotide transfection. Antisense oligonucleotides were synthesized by AntiVirals Inc (Corvallis, OR). Sequences of the LOX antisense and scrambled oligonucleotides were as follows: 12-LOX antisense: 5′-CGGCCCAUGGCGCCCCCCG-3′; 12-LOX scrambled: 5′-CGCGCCAUGGCCGCCCCACG-3′; 5-LOX antisense: 5′-GGAGGGGCAUGGCGCGGGCCG-3′; 5-LOX scrambled: 5′-GGAGGCGAUGCGGCGGCGCG-3′; 15-LOX antisense: 5′-GGAUGCGGGAGAGACCCAUC-3′ and 15-LOX scrambled: 5′-GGUAGCGGAUGAGACCCUAC-3′. These third generation, morpholino oligonucleotides, differ in structure from that of DNA and RNA by the replacement of the riboside moiety with a morpholino subunit containing the RNA bases and replacement of the negatively-charged phosphodiester linkage with a neutral phosphorodiamidate linkage. These oligonucleotides provide the best combination of chemical stability, aqueuous solubility and effective binding to nucleic acids (24). Antisense oligonucleotides were transfected using a modification of the scrape-loading procedure (25). Briefly, trypsinized pancreatic cancer cells were seeded into 25 cm^2 flasks in regular media for 48 hr then cultured in serum-free media. After 24 hr, 10 μM antisense, or scrambled oligonucleotide was added to the media and swirled briefly. The cells were then detached with a cell scraper ("rubber policeman") and dis-aggregated by repeatedly pipetting. Following cell culture for 72 h, DNA synthesis was measured by [^{3}H]-thymidine incorporation.

Cell proliferation assay. After reaching 70% confluence, pancreatic cancer cells were detached with trypsin-EDTA. The single cell suspension was seeded into 24-well plates at 30,000 cells/well. The cells were grown for 2 days to 80% confluence and then the media changed to serum-free medium for 24 hours. Media was replaced with fresh media, with or without appropriate inhibitors, and cellular DNA synthesis was assayed by adding 0.5 μCi/well of [^{3}H]-methyl-thymidine. After a 2 hour incubation, the cells were washed twice with PBS and solublized from each well with 0.4ml of 0.4M NaOH. Incorporation of [^{3}H]-thymidine into DNA was measured by adding scintillation fluid and counting the cpm in a scintillation counter (LKB BackBeta, Wallac, Turku, Finland).

Statistical analysis. Data was analyzed by analysis of variance (ANOVA) with Tukey's multiple comparison post-test for significance between individual groups. This analysis was performed with the Prism software package (GraphPad, San Diego, CA). Data are expressed as mean ± SEM.

RESULTS

Expression of 5-LOX and platelet-type 12-LOX expression in pancreatic cancer cells. Total RNA isolated from pancreatic cancer cells was subjected to reverse-transcription and nested-PCR amplification with primers specific for human 5-LOX and platelet 12-LOX. The specificity of the primers was confirmed

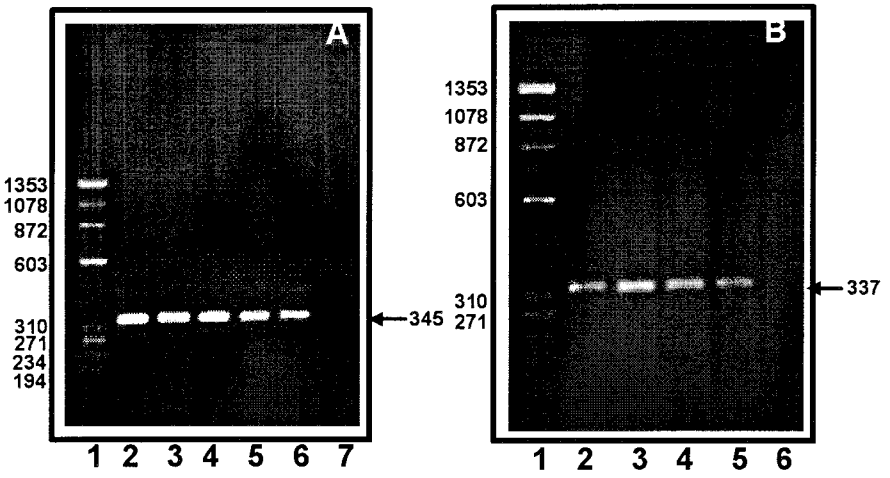

FIG. 1. Expression of 5-LOX and platelet-type 12-LOX mRNA in pancreatic cancer cells. Total RNA was isolated from pancreatic cancer cells, reverse transcribed, and then amplified by nested PCR. The PCR product was separated on 2% agarose gel and visualized by ethidium bromide staining. (A) 12-LOX expression: lane 1, DNA marker; lane 2, MiaPaCa2; lane 3, PANC-1; lane 4, ASPC-1; lane 5, Capan2; lane 6, human platelet RNA control; lane 7, non-RT control of PANC-1 RNA. (B) 5-LOX expression: lane 1, DNA marker; lane 2, MiaPaCa2; lane 3, PANC-1; lane 4, ASPC-1; lane 5, human platelet RNA control; lane 6, non-RT control of PANC-1 RNA.

by the parallel amplification of RNA extracted from human leukocytes and platelets as controls. All human pancreatic cancer cell lines, MiaPaCa2, PANC-1, ASPC-1 and Capan2 expressed 12-LOX mRNA identical to that from human platelet RNA (Fig. 1A). The 5-LOX mRNA was also expressed in the four human pancreatic cancer cell lines identical to that from human leukocyte RNA (Fig. 1B). In contrast, both 5-LOX and 12-LOX mRNA were undetectable in normal human pancreatic ductal cells indicating an upregulation of these gene in pancreatic cancer cells (not shown here, normal human pancreatic ductal cells were from cadaver donors and provided by Dr. Pour at Epply Cancer Institute, Omaha, NE, USA).

Lipoxygenase inhibitor-induced growth inhibition in pancreatic cancer cells. The general LOX inhibitor NDGA [$F(5,24) = 94$, $P < 0.0001$], the 5-LOX inhibitor Rev5901 [$F(5,24) = 203$, $P < 0.0001$], the 12-LOX inhibitor baicalein [$F(5,24) = 84$, $P < 0.0001$], and the FLAP inhibitor MK886 [$F(5,24) = 6.3$, $P < 0.004$] all caused marked inhibition of thymidine incorporation in MiaPaCa2 cells in a concentration-dependent (Fig. 2) and time-dependent [$F(3,15) = 357$, 591, 27.9, and 37.6 respectively, all $P < 0.0001$] (Fig. 3) manner. Similar results were obtained with LOX-inhibitor treatment of another poorly-differentiated pancreatic cancer cell line, PANC-1 and two well-differentiated pancreatic cancer cell lines, Capan2 and ASPC-1 (Table 1). With the exception of high concentrations of NDGA (20 and 100 μM) which caused some cell necrosis, no toxicity was seen following treatment with baicalein, Rev5901, MK886 or with NDGA (8 μM or less). The inhibition of proliferation was paralelled by a decrease in cell number over a three day period with all four inhibitors. Our results also showed that a combination of MK886 and baicalein produced an inhibition of thymidine incorporation even greater than the effect of the individual inhibitor. The combination of 5-LOX and 12-LOX inhibitors was as effective as the non-specific LOX inhibitor, NDGA suggesting that metabolites of 5-LOX and 12-LOX may act independently to increase proliferation of pancreatic cancer cells.

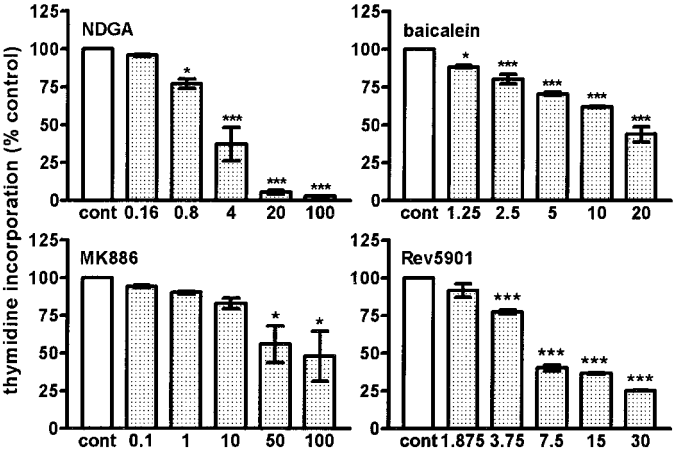

FIG. 2. Effects of different concentrations of NDGA, baicalein, MK886 and Rev5901 on cell proliferation in the pancreatic cancer cell line, MiaPaCa2. Cells were seeded into 24 well plate at a density of 3×10^4 cells/well and grown for two days in DMEM media containing 10% FBS. The cells were then cultured in serum-free condition for 24 hours, then treated with different concentrations of of NDGA, baicalein, MK886 and Rev5901 for 24 hours. Results are expressed as % of control of thymidine incorporation. * = $P < 0.5$; *** = $P < 0.001$ compared to control.

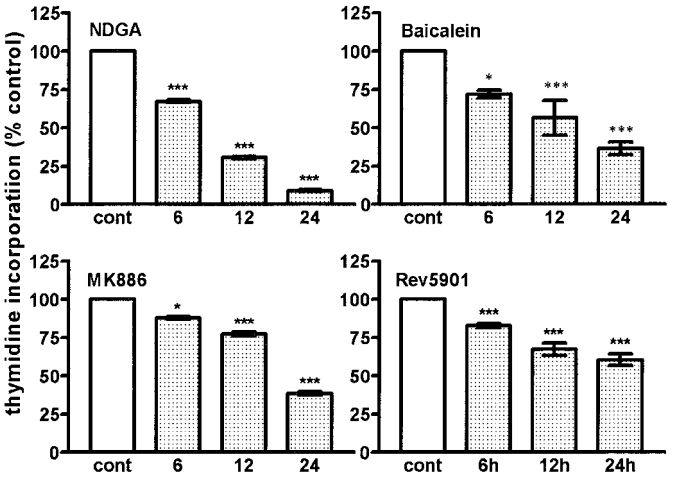

FIG. 3. Time-course of NDGA, baicalein, MK886 and Rev5901-induced growth inhibition in the pancreatic cancer cell line, MiaPaCa2. Cells were seeded into 24 well plates at a density of 3×10^4 cells/well and grown for two days in DMEM media containing 10% FBS. The cells were then cultured in serum-free condition for 24 hours, then treated with 20 μM NDGA or 20 μM baicalein, 100 μM MK886 or 15 μM Rev5901 for 6, 12 and 24 hours. The results are expressed as % of control of thymidine incorporation. * = $P < 0.05$; *** = $P < 0.001$ compared to control.

LOX antisense oligonucleotide-induced growth inhibition in pancreatic cancer cells. Proliferation of both PANC-1 and ASPC-1 was significantly inhibited by both 10 μM 5-LOX ($P < 0.05$) and 12-LOX ($P < 0.05$) antisense oligonucleotides but not the control scrambled oligonucleotides or the 15-LOX antisense oligonucleotide (Fig. 4).

5-HETE and 12-HETE reversed lipoxygenase inhibitor-induced growth inhibition of pancreatic cancer cells. To provide further evidence for the role of LOX in regulating pancreatic cancer cell proliferation, we examined whether the metabolites of 5-LOX and 12-LOX could reverse NDGA-induced inhibition in pancreatic cancer cell growth indicated by [^{3}H]- thymidine incorporation. 5-HETE or 12-HETE (100 nM) not only directly stimulated cell proliferation ($P < 0.05$), but also significantly reversed 4 μM NDGA-induced growth inhibition ($P < 0.05$) in PANC-1 cells (Fig. 5).

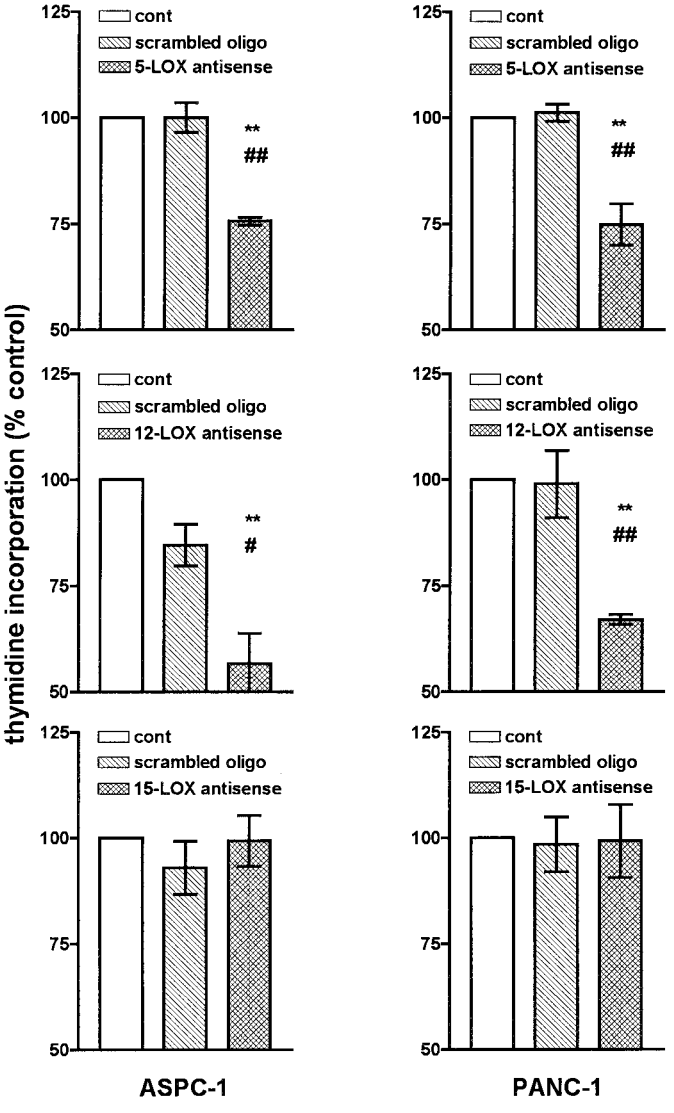

FIG. 4. LOX antisense oligonucleotide-induced growth inhibition in PANC-1 and ASPC-1 cells. Cells grown in 25cm^2 flasks were serum-starved for 24 hours. Then 10 μM 5-LOX, 12-LOX or 15-LOX antisense oligonucleotide, or control scrambled oligonucleotide was added to the media. After culture for 72 hours DNA synthesis was measured as [^{3}H]-methyl-thymidine incorporation. Results are expressed as % of control. ** = $P < 0.01$ compared to untreated control; # = $P < 0.05$ compared to control scrambled oligonucleotide; ## = $P < 0.01$ compared to scrambled oligonucleotide.

TABLE 1

Effect of LOX Inhibitors on Thymidine Incorporation in Different Pancreatic Cancer Cells, Expressed as % of Control

Cell lines	NDGA 20 μM	Baicalein 20 μM	MK886 100 μM	Rev5901 30 μM
PANC-1	12 ± 2***	55 ± 8***	20 ± 6***	30 ± 1***
ASPC-1	11 ± 6***	27 ± 3***	50 ± 10*	8 ± 1***
Capan 2	5 ± 1***	49 ± 3***	57 ± 4*	25 ± 3***
MiaPaCa2	5 ± 1***	44 ± 5***	48 ± 16*	25 ± 1***

Note. Cells were treated with LOX inhibitors for 24 hours in 24 well plates in serum-free condition. * = $P < 0.05$; *** = $P < 0.001$.

Arachidonic acid and linoleic acid-induced pancreatic cancer cell proliferation. Both PANC-1 and MiaPaCa2 were treated with either 100 nM arachidonic acid or linoleic acid and cell proliferation was measured by [^{3}H]-methyl-thymidine incorporation. Proliferation of both PANC-1 and MiaPaCa2 cells was significantly enhanced by both arachidonic acid and linoleic acid following 48 ($P < 0.05$) and 72 ($P < 0.01$) hours of treatment (Fig. 5).

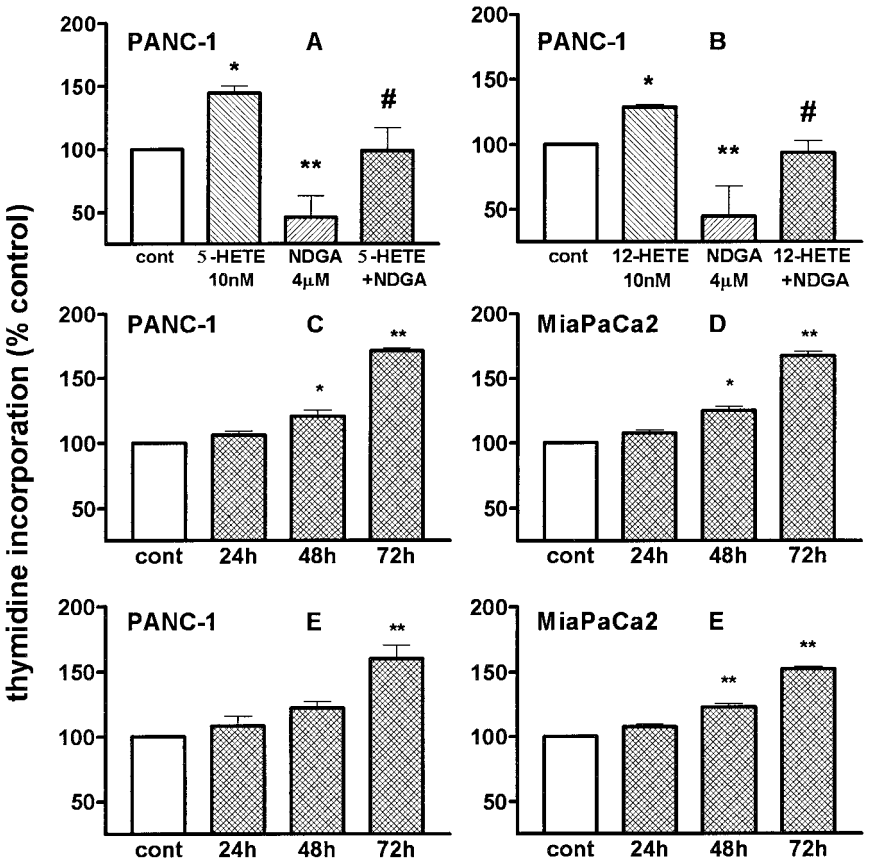

FIG. 5. Effect of A: 5-HETE and B: 12-HETE on NDGA-induced growth inhibition in PANC-1 cells. Cells seeded in 24 well plates were treated with 4 μM NDGA, 100 nM of 5-HETE or 12-HETE or a combination of NDGA with 5-HETE or 12-HETE for 3 days. Arachidonic acid-induced proliferation in C: PANC-1 and D: MiaPaCa2 cells. Linoleic acid-induced proliferation in E: PANC-1 and F: MiaPaCa2 cells. Cells were seeded into 24 well plates and grown for two days in DMEM media containing 10% FBS. Cells were then cultured in serum-free conditions for 24 hours, then treated with 100 nM arachidonic acid or linoleic acid for 24, 48 or 72 hours. Results are expressed as % control of thymidine incorporation. * = $P < 0.05$; ** = $P < 0.01$, compared to control, # = $P < 0.05$ compared to NDGA alone.

DISCUSSION

Epidemiological studies and animal experiments support the hypothesis that dietary fats, particularly essential fatty acids (EFA), play an important role in pancreatic cancer growth and development (4–6). Arachidonic acid, a major component of EFA, is metabolized through three pathways, catalyzed by cyclooxygenases, lipoxygenases and epoxygenase respectively (11). Cyclooxygenase leads to the biosynthesis of prostaglandins, prostacyclin and thromboxanes, while lipoxygenases convert arachidonic acid into (S)-hydroxyeicosatetraenoic acid (5-HETE, 12-HETE and 15-HETE) and a series of other products (11). Both 5-LOX and 12-LOX can influence growth of cancer cells such as human erythroleukemia, rat Walker carcinoma, mouse melanoma, colon, lung, prostate and breast cancer (12–18, 26, 27). Expression and function of 5-LOX and 12-LOX in pancreatic cancer have not been previously reported, except for a single paper describing the effect of the FLAP inhibitor, MK886 in PANC-1 cells (19). Using human leukocyte and platelet RNA as a positive control in this study, we demonstrated substantial expression of both 5-LOX and platelet-type 12-LOX in human pancreatic cancer cell lines but not in normal human pancreatic ductal cells.

The results with LOX inhibitors demonstrate that blocking either 5-LOX or 12-LOX activity substantially inhibits pancreatic cancer cell proliferation *in vitro*, suggesting that both 5-LOX and 12-LOX are required for rapid pancreatic cancer cell proliferation. The importance of 5-LOX and 12-LOX in growth regulation was confirmed by transfecting specific 5-LOX or 12-LOX antisense oligonucleotides into pancreatic cancer cells.

Several lines of evidence have implicated the involvement of 12-HETE in cancer cell proliferation, motility and cytoskeleton. 12-HETE stimulates both colon and lung cancer cell proliferation *in vitro* (12–15). 12-HETE also enhances tumor cell motility and upregulates cell surface expression of cellular adhesion

molecules by a protein kinase C-dependent pathway (13, 14, 26, 29, 30). Our current results show that arachidonic and linoleic acids as well as the LOX products, 5-HETE and 12-HETE, all enhance pancreatic cancer cell proliferation, indicating that both 5-LOX and 12-LOX play a critical role in the regulation of pancreatic cancer cell growth. However, whether other downstream metabolites of LOX such as leukotrienes are involved in this regulation of pancreatic cancer cell proliferation and survival need to be further investigated.

It is well known that pancreatic cancer cells themselves produce several growth factors such as TGFα, amphiregulin and heparin-binding EGF, which autoactivate the EGF receptor tyrosine kinase and increase cell proliferation (31–33). Pancreatic tumors are surrounded by pancreatic islets and their growth is strongly stimulated by high levels of endogenous insulin (34). Whether LOX participates in the exogenous or endogenous growth factor-stimulated pancreatic cancer cell proliferation needs to be further elucidated. If pancreatic cancer cell 5-LOX or 12-LOX is upregulated by growth factors and growth stimulation induced by these autocrine growth factors involves 5- or 12-LOX, then blockade of 5- or 12-LOX may be therapeutically valuable to pancreatic cancer.

In summary, this study clearly supports that pancreatic cancer cell growth and apoptosis were regulated by both 5-LOX and platelet type 12-LOX. Growth inhibition of pancreatic cancer cells by LOX inhibitors was associated with induction of apoptosis and differentiation.

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