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Overexpression of Platelet-type 12-Lipoxygenase Promotes Tumor Cell Survival by Enhancing $\alpha_v\beta_3$ and $\alpha_v\beta_5$ Integrin Expression[1]

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ABSTRACT

Arachidonic acid metabolism leads to the generation of biologically active metabolites that regulate cell growth and proliferation, as well as survival and apoptosis. We have demonstrated previously that platelet-type 12-lipoxygenase (LOX) regulates the growth and survival of a number of cancer cells. In this study, we show that overexpression of platelet-type 12-LOX in prostate cancer PC3 cells or epithelial cancer A431 cells significantly extended their survival and delayed apoptosis when cultured under serum-free conditions. These effects were shown to be a result of enhanced surface integrin expression, resulting in a more spread morphology of the cells in culture. PC3 cells transfected with 12-LOX displayed increased $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin expression, whereas other integrins were unaltered. Transfected A431 cells did not express $\alpha_v\beta_3$; however, $\alpha_v\beta_5$ integrin expression was increased. Treatment of both transfected cell lines with monoclonal antibody to $\alpha_v\beta_5$ (and in the case of PC3 cells, anti-$\alpha_v\beta_3$) resulted in significant apoptosis. In addition, treatment with 100 nM 12(*S*)-hydroxy-eicosatetraenoic acid, the end product of platelet-type 12-LOX, but not other hydroxy-eicosatetraenoic acids, enhanced the survival of wild-type PC3 and A431 cells and resulted in increased expression of $\alpha_v\beta_5$. Furthermore, Baicalein or *N*-benzyl-*N*-hydroxy-5-phenylpentamide, specific 12-LOX inhibitors, significantly decreased $\alpha_v\beta_5$-mediated adhesion and survival in 12-LOX-overexpressing cells. The results show that 12-LOX regulates cell survival and apoptosis by affecting the expression and localization of the vitronectin receptors, $\alpha_v\beta_3$ and $\alpha_v\beta_5$, in two cancer cell lines.

INTRODUCTION

LOXs[3] constitute a family of lipid-peroxidizing enzymes that metabolize AA to biologically active metabolites, including hydroperoxy-eicosatetraenoic acids and HETEs, as well as leukotrienes (1). 12-LOX is one of at least three LOXs that is expressed as two main isoforms, a platelet type cloned from human platelets (2) and a leukocyte type from porcine leukocytes, which shares 65% homology to the platelet-type cDNA (3). Several lines of evidence implicate 12-LOX as a regulator of human cancer development. It is overexpressed in a variety of tumors including breast, colorectal, and prostate tumors (4–6) and has been shown to be present in a number of cancer cell lines (7–9). In addition, we have recently shown that inhibitors to 12-LOX block cell cycle progression by regulating the expression of proteins governing the transition from G_1 to S and induce apoptosis in prostate cancer cells (10), whereas overexpression of 12-LOX increases angiogenesis and metastatic growth in mice (6).

The ability of tumors to invade beyond hemostatic boundaries and form metastatic colonies requires the complex interplay of various cell surface-associated components regulating the proteolytic disruption of the ECM and the modification of cell adhesion properties (11). These cell-ECM interactions are mediated by integrins, a family of adhesive receptors that mediate the attachment of the cell to both structural and matrix-immobilized proteins to promote cell survival, proliferation, and migration (12, 13). Integrins perform a well-documented function in cellular invasion and metastasis (14, 15). Nonligated integrins are generally spread diffusely over the cell surface with no apparent linkage to the actin cytoskeleton. However, when ligated, integrins frequently cluster into specialized structures called focal adhesion complexes, thereby providing a convergence site for multiple signaling components (15, 16), while physically linking the receptors to actin microfilaments (17, 18). Ligand binding to integrins triggers a number of signaling pathways, some of which are primarily related to cell adhesion, whereas others provide survival signals to cells. For example, prevention of cell adhesion to the ECM will trigger apoptosis in various cells [in particular, epithelial cells (19–21)], suggesting that integrin-mediated attachment relays important survival signals to the cells. Conversely, attachment or adhesion to the basement membrane or individual ECM components has been shown to promote cell differentiation and extend cell survival under various experimental conditions (22–25).

Previous studies have indicated a role for AA metabolism in cell-matrix interactions and integrin signaling. For example, inhibition of cyclooxgenase-2 by nonsteroidal anti-inflammatory drugs blocks both platelet aggregation [by suppressing activation of integrin $\alpha_{IIb}\beta_3$ (26)] and endothelial cell migration [by suppressing activation of $\alpha_v\beta_3$ integrin (27)]. Other studies have reported the role of LOXs, in particular, 12-LOX, in the regulation of surface integrin expression. For example, adhesion of B16 murine melanoma cells to microvascular endothelial cells was enhanced by pretreatment of the endothelial cells with the 12-LOX product, 12(*S*)-HETE, via up-regulation of $\alpha_v\beta_3$ integrin expression (28). In the same cell line, ligation of $\alpha_{IIb}\beta_3$ integrin induced 12(*S*)-HETE production (29), implying coregulation of integrin expression and LOX activity in these cells. Similarly, 12(*S*)-HETE treatment of human endothelial cells enhanced monocyte adhesion through increased very late-acting antigen-4 integrin expression (30). Indeed, we have recently reported the interaction of 12-LOX with a number of cellular proteins, including the integrin β_4 subunit, as determined by yeast two-hybrid screening (31). The above observations establish a potential relationship between lipid-regulated adhesive functions and cellular responses to apoptosis induction.

In this study, we examine the involvement of 12-LOX in the survival of two tumor cell lines from different histological origins under growth-restrictive conditions. The results indicate that overexpression of platelet-type 12-LOX in PC3 or A431 cells (prostate and epidermoid cancer cell lines, respectively) enhances surface expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins in PC3 cells and $\alpha_v\beta_5$ in A431 cells.

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[3] The abbreviations used are: LOX, lipoxygenase; AA, arachidonic acid; HETE, hydroxy-eicosatetraenoic acid; ECM, extracellular matrix; BrdUrd, bromodeoxyuridine; FBS, fetal bovine serum; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; BHPP, *N*-benzyl-*N*-hydroxy-5-phenylpentamide; TBST, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween 20; Tdt, terminal deoxynucleotidyltransferase; HODE, hydroxyoctadeca-98, 11E-dienoic acid.

This increased integrin expression prolongs cell survival, delaying the induction of apoptosis in the absence of serum.

MATERIALS AND METHODS

Cell Lines. Two carcinoma cell lines, prostate carcinoma PC3 and epidermoid carcinoma A431, were obtained from the American Type Culture Collection (Manassas, VA) and maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. The cells were routinely cultured in RPMI 1640 or DMEN supplemented with 10% FBS (Life Technologies, Inc., Grand Island, NY), 2 mM L-glutamine, and 100 μg/ml penicillin-streptomycin. Experiments were performed when cells were approximately 80% confluent.

PC3 or A431 cells were transfected with the full-length cDNA encoding human platelet-type 12-LOX from pCMV-12-LOX (provided by Dr. Colin Funk, University of Pennsylvania) or empty vector and characterized as described in detail previously (6, 31). Transfected cells were taken from early passages and maintained in RPMI 1640 or DMEN containing 300 mg/ml Geneticin (G418; Life Technologies, Inc.) to prevent outgrowth of revertant cells.

Cell Proliferation/Survival Assay. Wild-type, mock-transfected, or 12-LOX-transfected PC3 or A431 cells were cultured in 96-well plates at a concentration of 5×10^3 cells/ml under serum-free medium (RPMI 1640 with 0.1% FBS) for 0–7 days. Assessment of cell survival/proliferation was carried out by means of a specific nonradioactive cell proliferation ELISA, based on the measurement of BrdUrd incorporation during DNA synthesis according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

Apoptosis Assays. Wild-type, mock-transfected, or 12-LOX-transfected PC3 or A431 cells were cultured in 75-cm^3 flasks at a concentration of 3×10^6 in serum-free medium (RPMI 1640 with 0.1% FBS) over time (0–7 days) to induce apoptosis. Apoptosis was quantitated on an Epics II flow cytometer (Coulter, Hialeah, FL), using the terminal incorporation of fluorescein-12-dUTP by Tdt into fragmented DNA according to the manufacturer's instructions (Roche Diagnostics), as reported previously (10).

In addition, fragmented DNA was extracted using the NP40/RNase/SDS/proteinase K method as described previously (32) and analyzed on a 1.2% agarose gel.

Western Blot Analysis of Integrin Expression. Total cell lysates were prepared for each of the wild-type, neo-transfected, or 12-LOX-transfected cell lines. Protein (30 μg) was fractionated on precast SDS-PAGE gels and then transferred to nitrocellulose membranes. After incubation for 1 h in blocking solution containing 5% skimmed milk dissolved in TBST, blots were probed overnight with primary antibodies against $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_v\beta3$, $\alpha_v\beta_5$, or α_v integrin (1:1500 dilution in 5% milk). After this, samples were washed three times in TBST, incubated for 1 h with horseradish peroxidase-conjugated goat antimouse IgG (1:1000 in TBST; Amersham Biosciences, Piscataway, NJ), and washed three times in TBST, and bound antibody complex was visualized using enhanced chemiluminescence (Pierce Chemical Co.). Equal loading of samples was illustrated by Western blotting for β-actin, a constitutively expressed protein.

For Western analysis of membrane-associated proteins, cells were taken at 80% confluence and suspended in homogenization buffer [25 mM Tris-HCl (pH 7.6) and 1 mM EGTA containing 5 mg/ml aprotinin, 10 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride]. Cells were then homogenized by sonication (15 s, ×3 on ice; Vibracell-Microtip) with intervals of 3 min. Homogenates were centrifuged at $10,000 \times g$ for 1 h at 4°C. The $10,000 \times g$ supernatant was regarded as cytosol, and this, along with the $10,000 \times g$ pellet, was rinsed once with homogenization buffer. Thereafter, the reconstituted pellet was centrifuged at $100,000 \times g$ (1 h, 4°C), and the pellet, termed the membrane fraction, was suspended in lysis buffer and used immediately for electrophoresis. Protein in each fraction was determined by the Bradford method using BSA as standard.

Flow Cytometric Analysis of Adhesion Molecule Expression. Flow cytometry was performed for the analysis of integrin receptors as described previously (33). Briefly, cultured PC3 or A431 cells (1×10^6) were dissociated with 0.2 mM EDTA, washed with PBS, and then fixed in 3.7% paraformaldehyde in PBS (pH 7.4). Cells were then incubated in 20% normal goat serum to block nonspecific binding. Subsequently, cells were incubated with primary antibodies [0.1 μg/ml mouse monoclonal antibody to $\alpha_2\beta_1$, $\alpha_3\beta_1$, β_1, $\alpha_v\beta_5$, $\alpha_v\beta_3$, or $\alpha_v\beta_6$ (Chemicon, Temecula, CA) or 1.0 μg/ml polyclonal antibody to α_v or $\alpha_5\beta_1$ (Chemicon)] followed by a 1:1000 dilution of FITC-labeled secondary antibodies (Invitrogen, Carlsbad, CA). Finally, cell surface fluorescence for individual integrin receptors was analyzed on an Epics Profile II flow cytometer.

$\alpha_v\beta_5$-Mediated Adhesion Assay. To support the results obtained by flow cytometric analysis, the expression of $\alpha_v\beta_5$ integrin on the surface of cells was determined using the integrin-mediated cell adhesion kit according to manufacturers instructions (Chemicon). Briefly, a single cell suspension was prepared nonenzymatically by incubating the cells in 5 mM EDTA in PBS for 15 min. Thereafter, cells were counted on a Coulter counter, and the concentration was adjusted to 3×10^5 cells/ml. The integrin-coated and control strips were rehydrated with 200 μl PBS/well, and 100 μl of the cell suspension were added to the strips. After 2 h of incubation at 37°C in a CO_2 incubator, the cell solution was discarded, and the plates were gently washed three times with PBS. Cell stain solution (100 μl) was added for 5 min, washed, and then extracted using 100 μl of extraction buffer. The concentration of bound cells was relative to the level of surface $\alpha_v\beta_5$ expression and was determined as absorbance at 570 nm by a spectrophotometer.

Antibody Blocking Studies. To establish a functional role for individual integrin receptors in 12-LOX-mediated apoptosis resistance, 12-LOX-transfected cells were serum-starved in the presence of various antibodies to integrin receptors (outlined in the Western blot section). Cell survival and apoptosis 48–72 h after starvation were evaluated by the BrdUrd cell proliferation assay and TUNEL staining, respectively. Morphological alterations were recorded on a phase-contrast microscope.

Treatment with 12(*S*)-HETE or 12-LOX Inhibitors. To prove that 12-LOX is responsible for the increase in survival and $\alpha_v\beta_5$ surface expression in both cell lines, wild-type cells were treated with 100 nM 5(*S*)-HETE, 12(*S*)-

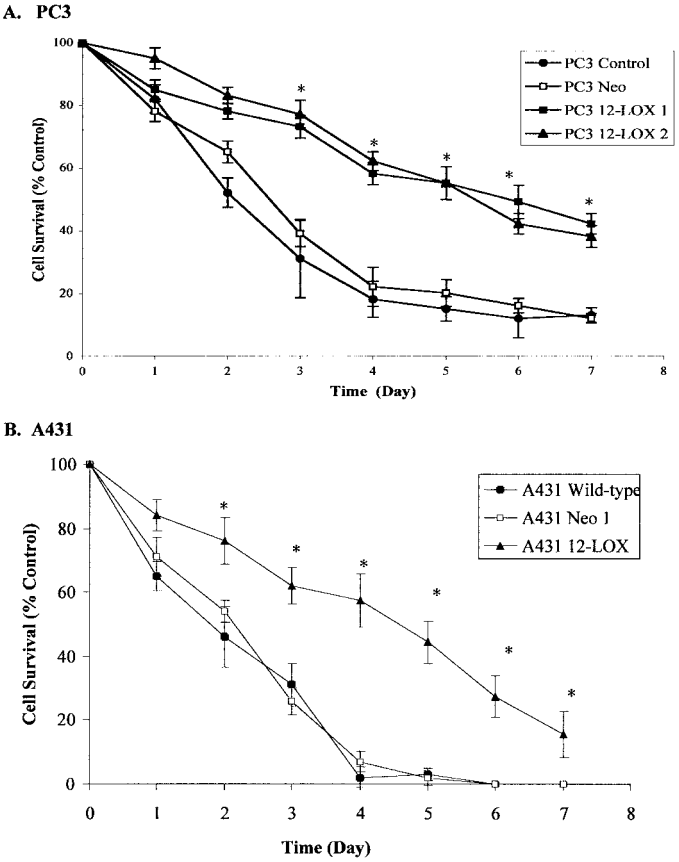

Fig. 1. Quantitation of the survival of PC3 (*A*) or A431 (*B*) transfected sublines cultured after serum withdrawl, as determined by BrdUrd incorporation assay. The values are expressed as the percentage of cell survival compared with day 0 (at which time the survival was considered to be 100%), and the *bars* represent the SE derived from three independent experiments. 12-LOX-transfected PC3 (*12-LOX 1* and *12-LOX 2*) and A431 cells (*12-LOX*) all survived significantly longer, under serum-starved conditions, compared with either wild-type or mock-transfected (*Neo 1*) controls, in each cell line. *, $P < 0.05$.

HETE, 15(*S*)-HETE, or 13(*S*)-HODE (Cayman Chemicals, Ann Arbor, MI) in serum-free conditions (0.1% FBS), and survival was assessed after 48 and 96 h by BrdUrd assay. In addition, 12-LOX-transfected cell lines were incubated with 10 μM Baicalein (Biomol, Plymouth, PA) or BHPP (Biomide Corp., Grosse Pointe Farms, MI), both of which are specific 12-LOX inhibitors, or the 5-LOX inhibitor Rev-5901 (Cayman Chemicals) for 48 h in serum-free conditions.

After treatment, cells were also assessed for their surface $\alpha_v\beta_5$ expression, using the integrin-mediated cell adhesion kit described previously. Wild-type PC3 or A431 cells were treated with 5(*S*)-HETE, 12(*S*)-HETE, 15(*S*)-HETE, or 13(*S*)-HODE 24 h before the adhesion assay.

RESULTS

Overexpression of Platelet-type 12-LOX in PC3 and A431 Cells Extends Their Survival and Delays Apoptosis in the Absence of Serum. Both of the cell lines used in this study demonstrated serum dependence for their continued growth. Several independent experiments revealed that the survival of both wild-type and neo-transfected PC3 cells declined steadily after 24 h of serum starvation (Fig. 1*A*). Within 3 days of serum withdrawal, only 35–40% of wild-type or neo-transfected cells were viable, compared with 75–80% of 12-LOX-transfected cells. Whereas 12-LOX-transfected PC3 cells cell did display a gradual decrease in cell viability over time, this decrease was much slower than that observed in either wild-type or neo-transfected cells. At all points after 3 days, there was a significantly extended survivability of 12-LOX-transfected PC3 cells ($P < 0.05$).

A431 cells were more sensitive to serum deprivation, with cell survival reduced dramatically after only 24 h of serum starvation (Fig. 1*B*). Within 4 days of serum starvation, all wild-type and mock-transfected cells were dead. A431 cells overexpressing platelet-type 12-LOX demonstrated a significantly extended overall survival ($P < 0.05$) compared with either the wild-type or mock-transfected A431 cells. Cell numbers declined steadily after 2 days of serum starvation, and by day 7, approximately 20% of cells were viable. The slope of decline was much less than that of the wild-type cells. By day 9, all of the cells were dead (data not shown).

Consistent with these observations, apoptosis was significantly ($P < 0.05$) reduced in the 12-LOX-overexpressing clones (Fig. 2, *A* and *B*). Whereas wild-type and mock-transfected PC3 cells showed a rapid and steady induction of apoptosis after serum withdrawal, the 12-LOX-transfected cells displayed a delayed apoptotic response. Quantitation of apoptotic nuclei in both wild-type and neo-PC3 cells demonstrated that approximately 65% were apoptotic by day 4, 80% were apoptotic by day 6, and essentially 100% were apoptotic by day 8 after serum removal. Representative histograms showing the percentage of Tdt-FITC-positive PC3 cells at day 0 (2.67%) and day 8 (98.7%) are shown in Fig. 2, *C* and *D*, respectively. In sharp contrast, 12-LOX clones exhibited only 15% apoptosis by day 2, maintaining this low level until day 6, at which point a steady increase in apoptosis was observed (Fig. 2*A*). A431 cells displayed a similar response, with rapid induction of apoptosis after serum starvation in both wild-type and mock-transfected cells (60% and 50%, respectively, by day 2), whereas 12-LOX-transfected cells were significantly more resistant to apoptosis (14% by day 2). These results were confirmed by DNA fragmentation assays. 12-LOX-transfected PC3 (Fig. 2*E*) or A431

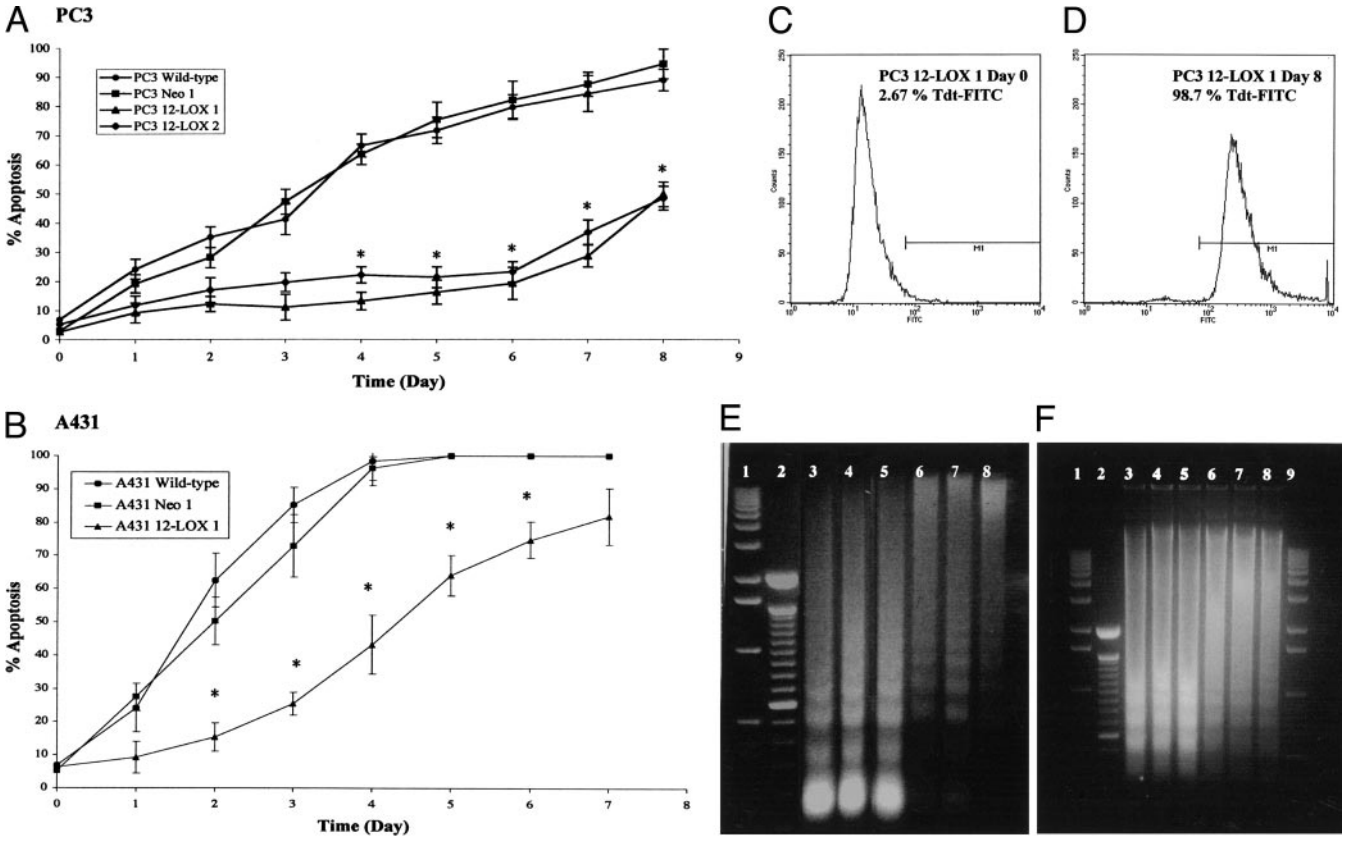

Fig. 2. 12-LOX-overexpressing cells demonstrated a delayed apoptotic response to serum starvation. Apoptosis was quantitated by TUNEL staining in PC3 (*A*) or A431 (*B*) cells serum-starved for 0–7 days. Significant apoptosis was observed after serum starvation in wild-type or mock transfectants (*Neo 1*) in both cell lines, compared with 12-LOX clones (*12-LOX 1* or *12-LOX-2*; *, $P < 0.05$). Representative Tdt-FITC% histograms are shown for PC3 wild-type cells at day 0 (*C*) and day 8 (*D*). Apoptosis was confirmed by DNA fragmentation assays in PC3 cells at day 4 after serum withdrawal (*E*) and in A431 cells at 2 days after serum withdrawal (*F*). *Lanes 1* and *9,* 1-kb ladder; *Lanes 2,* 100-kb ladder; *Lanes 3–5,* mock-transfected cells (*Neo 1*); *Lanes 6–8,* 12-LOX-transfected cells (*12-LOX 1*). It is obvious that the 12-LOX-transfected cells contain less fragmented DNA.

(Fig. 2*F*) cells displayed less fragmentation of DNA compared with mock-transfected controls after serum starvation for 2 (A431 cells) or 4 days (PC3).

Expression of Integrins in Tumor Cell Lines Determined by Flow Cytometry. Overexpression of platelet-type 12-LOX resulted in a more spread morphology under normal culture conditions compared with neo-transfected and wild-type cells, as observed after light microscopy (data not shown). Cell anchorage to the substratum is mediated by cell adhesion to the ECM proteins through integrin receptors; therefore, it is possible that 12-LOX overexpression may modulate the expression/function of integrin receptors to enhance cell spreading. We examined several potential integrins and adhesion molecules in wild-type PC3 and A431 cells to determine which ones are expressed endogenously. Wild-type PC3 cells were found to express high levels of $\alpha_2\beta_1$, $\alpha_3\beta_1$, β_1, and α_v on their surface (Fig. 3, *E, F, D,* and *H*, respectively), as indicated by a shift in the mean fluorescence relative to controls. A lower level of $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$ integrin expression was observed (Fig. 3, *G, I, J,* and *K*, respectively).

A431 cells expressed high levels of $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and β_1 on their surface (Fig. 4, *E, F, G,* and *D*, respectively), with lower levels of α_v, $\alpha_v\beta_5$, and $\alpha_v\beta_6$ integrins (Fig. 4, *H, J,* and *K*, respectively). A431 cells did not express integrin $\alpha_v\beta_3$ at all on their surface (Fig. 4*I*), as indicated by no shift in the mean log fluorescence compared with cells alone (Fig. 4*B*) or cells with secondary antibody only (Fig. 4*C*).

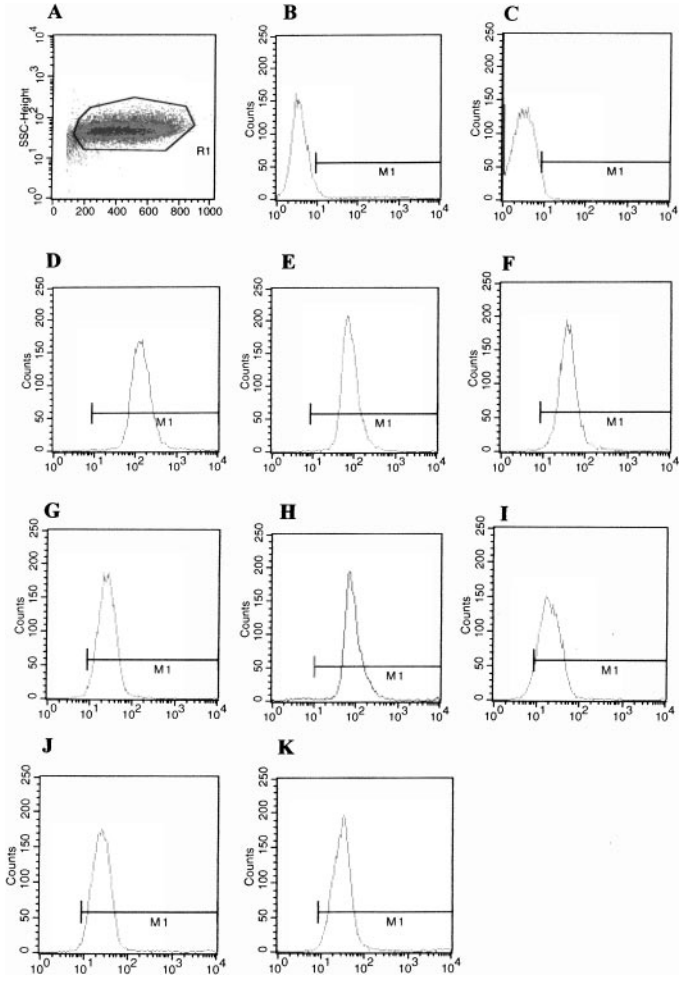

Fig. 3. Endogenous expression of integrins in PC3 carcinoma cells as determined by flow cytometry. Wild-type PC3 cells (*A*) were incubated with control (*B*; PBS) or rabbit IgG (*C*; 1.0 mg/ml) or various primary antibodies: β_1 (*D*; 0.1 mg/ml); $\alpha_2\beta_1$ (*E*; 1.0 mg/ml); $\alpha_3\beta_1$ (*F*; 0.1 mg/ml); $\alpha_5\beta_1$ (*G*; 0.1 mg/ml); α_v (*H*; 0.1 mg/ml); $\alpha_v\beta_3$ (*I*; 1.0 mg/ml); $\alpha_v\beta_5$ (*J*; 0.1 mg/ml); or $\alpha_v\beta_6$ (*K*; 0.1 mg/ml). Cells were then treated with FITC-labeled secondary antibodies, and analysis of cell surface fluorescence was performed by flow cytometry. PC3 cells express moderate levels of $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, and $\alpha_5\beta_1$ integrin receptors and high levels of α_v, $\alpha_2\beta_1$, $\alpha_3\beta_1$, and β_1, indicated by a prominent shift in the mean log fluorescence intensity compared with controls.

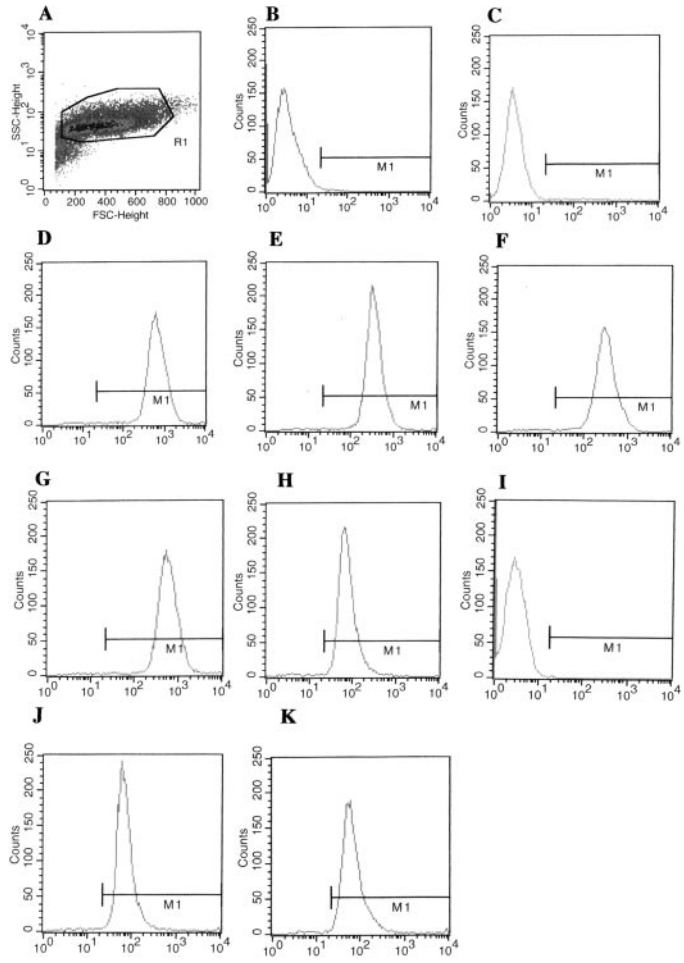

Fig. 4. Endogenous expression of integrins in A431 cells as determined by flow cytometry. Wild-type A431 cells (*A*) were incubated with control (*B*; PBS) or rabbit IgG (*C*; 1.0 mg/ml) or various primary antibodies: β_1 (*D*; 0.1 mg/ml); $\alpha_2\beta_1$ (*E*; 1.0 mg/ml); $\alpha_3\beta_1$ (*F*; 0.1 mg/ml); $\alpha_5\beta_1$ (*G*; 0.1 mg/ml); α_v (*H*; 0.1 mg/ml); $\alpha_v\beta_3$ (*I*; 1.0 mg/ml); $\alpha_v\beta_5$ (*J*; 0.1 mg/ml); or $\alpha_v\beta_6$ (*K*; 0.1 mg/ml). A431 cells did not express $\alpha_v\beta_3$ at all but expressed moderate levels of α_v, $\alpha_v\beta_5$, and $\alpha_v\beta_6$ integrin receptors. A431 cells highly expressed $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and β_1, indicated by a prominent shift in the mean log fluorescence intensity compared with controls.

Increased Surface Expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ Integrins in PC3 and $\alpha_v\beta_5$ in A431 12-LOX-transfected Tumor Cells. Under the same experimental conditions, flow cytometric studies demonstrated relatively consistent expression levels of several integrin receptors such as $\alpha_2\beta_1$, $\alpha_3\beta_1$, β_1, α_v, and $\alpha_v\beta_6$ between PC3 wild-type and 12-LOX-transfected cells (Table 1). However, 12-LOX-transfected cells demonstrated a consistent increase in the expression of $\alpha_v\beta_5$ (3.2-fold) and $\alpha_v\beta_3$ (3.0-fold) as compared with the wild-type cells. This increase is easily observed as a shift in the mean log fluorescence to the right in 12-LOX-transfected cells relative to wild-type cells.

A431 wild-type and 12-LOX-overexpressing cells displayed similar relative levels of $\alpha_2\beta_1$, $\alpha_3\beta_1$, β_1, α_v, and $\alpha_v\beta_6$ on their surface (Table 2). A431 cells did not express $\alpha_v\beta_3$ integrin; however, 12-LOX-transfected cells had increased surface $\alpha_v\beta_5$ integrin (1.8-fold) relative to wild-type or neo-transfected controls. Interestingly, there was a slight drop in the expression of both β_1 and $\alpha_2\beta_1$ integrins

Table 1 *Integrin expression between PC3 wild-type and 12-LOX-transfected cells*

Integrin	PC3 WT[a]	PC3 Neo	PC3 12-LOX C1	PC3 12-LOX C2	Fold increase 12-LOX *vs.* WT
β_1	148.08 ± 10.38	132.08 ± 20.11	245.9 ± 16.11	219.4 ± 18.16	2.1
$\alpha_2\beta_1$	83.78 ± 3.64	72.50 ± 3.35	172.5 ± 8.29		1.93
α_v	26.69 ± 2.02	29.02 ± 5.21	44.72 ± 8.21		1.62
$\alpha_v\beta_3$	25.09 ± 4.11	22.57 ± 2.15	**78.64 ± 4.03**[b]	**71.61 ± 2.06**[b]	**2.98**
$\alpha_v\beta_5$	41.71 ± 5.3	37.56 ± 4.15	**134.23 ± 6.03**[b]	**145.63 ± 7.16**[b]	**3.22**
$\alpha_v\beta_6$	50.97 ± 4.7	62.62 ± 8.3	87.60 ± 12.7	74.10 ± 16.4	1.64

[a] WT, wild-type.
[b] Integrins most significantly overexpressed.

Table 2 *Integrin expression between A431 wild-type and 12-LOX-transfected cells*

Integrin	A431 WT[a]	A431 Neo	A431 12-LOX C1	Fold increase 12-LOX *vs.* WT
β_1	118.45 ± 1.4	123.15 ± 2.43	106.23 ± 4.21	0.86
$\alpha_2\beta_1$	103.24 ± 2.23	108.97 ± 8.45	73.36 ± 10.29	0.87
$\alpha_3\beta_1$	78.45 ± 5.42	79.02 ± 3.21	74.72 ± 5.2	0.95
$\alpha_5\beta_1$	44.67 ± 3.76	44.97 ± 5.06	49.08 ± 2.49	1.09
α_v	58.2 ± 5.28	56.73 ± 5.15	63.08 ± 3.03	1.11
$\alpha_v\beta_5$	17.46 ± 3.93	18.86 ± 2.25	**32.09 ± 3.24**[b]	**1.70**
$\alpha_v\beta_6$	51.25 ± 6.41	48.17 ± 5.8	61.93 ± 7.6	1.28

[a] WT, wild-type.
[b] Integrin most significantly overexpressed.

(0.84- and 0.68-fold, respectively), suggesting that the expression of subsets of integrins may be lost in favor of other integrins.

Overexpression of $\alpha_v\beta_5$ in PC3 and A431 cells transfected with 12-LOX was confirmed by means of an integrin-mediated adhesion assay specific for $\alpha_v\beta_5$ (as outlined in "Materials and Methods"). Wild-type, neo-transfected, or 12-LOX-transfected clones were examined, and overexpression of 12-LOX resulted in significantly more adhesion to the plates as indicated by absorbance (Fig. 5). These results prove that overexpression of 12-LOX results in increased surface expression of $\alpha_v\beta_5$ in both PC3 and A431 cells.

Overexpression of 12-LOX in PC3 or A431 Tumor Increases Membrane Localization of Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in PC3 Cells and $\alpha_v\beta_5$ in A431 Cells. Having illustrated that overexpression of 12-LOX increases surface expression of $\alpha_v\beta_3$ or $\alpha_v\beta_5$ in each cell line, we next evaluated the protein expression of a variety of integrins by Western blot. $\alpha_2\beta_1$, α_v, and $\alpha_v\beta_5$ expression was examined in wild-type, neo-transfected, and 12-LOX-transfected cells, and no difference in expression was observed in either cell line (Fig. 6, *A* and *B*). This indicated that overexpression of 12-LOX did not alter translation of the protein but rather the distribution of the protein from intracellular stores to the membrane. This was confirmed by Western analysis of the membrane fractions of wild-type and 12-LOX-transfected PC3 cells for $\alpha_v\beta_3$ or $\alpha_v\beta_5$. 12-LOX-transfected PC3 cells expressed increased membrane levels of $\alpha_v\beta_3$ compared with either wild-type or neo-transfected cells (Fig. 6*C*). Similarly, membrane expression of $\alpha_v\beta_5$ was clearly increased in 12-LOX-transfected cells compared with either wild-type or neo-transfected cells (Fig. 6*D*). Clustering of integrins at the membrane and focal adhesion contacts provides a convergence site for multiple signaling components (15, 16), which could affect the survival of the cells. These studies suggest that 12-LOX-transfected PC3 cells may rely on the up-regulation of $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin or both to maintain their adherence to the substratum to sustain their cell survival. Similar results were observed in 12-LOX-transfected A431 cells compared with wild-type cells, where membrane expression of $\alpha_v\beta_5$ was increased (Fig. 6*E*).

Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ Are Important Factors for 12-LOX-transfected Tumor Cell Survival in the Absence of Serum. Having examined the expression of a number of integrins on both PC3 and A431 cells and shown that overexpression of 12-LOX increased surface expression of $\alpha_v\beta_3$ (PC3) and $\alpha_v\beta_5$ (A431 and PC3), we next examined which one(s) plays a causal role in sustaining cell survival during serum deprivation. 12-LOX-transfected cells were serum-starved in the presence of PBS (as control) or an antibody to $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, α_v, $\alpha_v\beta_3$, $\alpha_v\beta_5$, or $\alpha_v\beta_6$. After 2 days of serum withdrawal, control 12-LOX-transfected PC3 cells demonstrated typical spread morphology. In sharp contrast, the survival of 12-LOX-transfected PC3 cells was significantly reduced when cells were serum-starved in the presence of monoclonal anti-$\alpha_v\beta_3$ or anti-$\alpha_v\beta_5$ antibody, and a combination of both antibodies had an additive effect (Fig. 7*A*). In the presence of 0.01 μg/ml anti-$\alpha_v\beta_3$ or anti-$\alpha_v\beta_5$, pCMV-12-LOX PC3 cells underwent remarkable morphological changes consistent with apoptosis and were significantly ($P < 0.05$) more apoptotic than controls, as determined by TUNEL staining and fluorescence-activated cell-sorting analysis. In contrast, 12-LOX PC3 cells did not appear to be dependent on integrins $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, α_v, or $\alpha_v\beta_6$ for maintaining survival under serum-free conditions because pretreatment with the respective antibodies did not affect either the survival or apoptosis of the cells (Fig. 7*A*).

Similar results were observed in A431 cells overexpressing 12-LOX, with anti-$\alpha_v\beta_5$ dramatically reducing the survival of 12-LOX-transfected cells (Fig. 7*B*) and resulting in significant apoptosis of these cells relative to untreated controls, an effect that was not observed when any of the other integrins were blocked. These data indicate that integrin $\alpha_v\beta_5$ and, in the case of PC3 cells, integrin $\alpha_v\beta_3$ play a critical and functional role in supporting the survival of 12-LOX-transfected cells in the absence of serum.

12(*S*)-HETE Increases the Survival and Expression of $\alpha_v\beta_5$ in PC3 and A431 Cells. In a final subset of experiments, having shown that overexpression of 12-LOX in two different cell lines prolonged survival and enhanced surface $\alpha_v\beta_5$ integrin expression, we investigated whether the end product of 12-LOX metabolism, 12(*S*)-HETE, would have similar effects in wild-type cells. Wild-type PC3 or A431

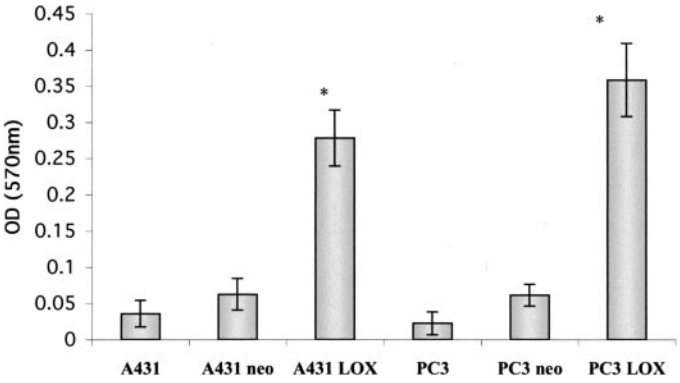

Fig. 5. 12-LOX-transfected A431 and PC3 cells exhibit increased $\alpha_v\beta_5$-mediated adhesion. PC3 or A431 wild-type, neo-transfected, or 12-LOX-transfected cells were seeded on $\alpha_v\beta_5$-specific adhesion plates as outlined in "Materials and Methods." In both PC3 and A431 cells, 12-LOX-transfected cells had significantly increased adhesion to the plates, confirming increased surface expression of the $\alpha_v\beta_5$ integrin. *, $P < 0.01$.

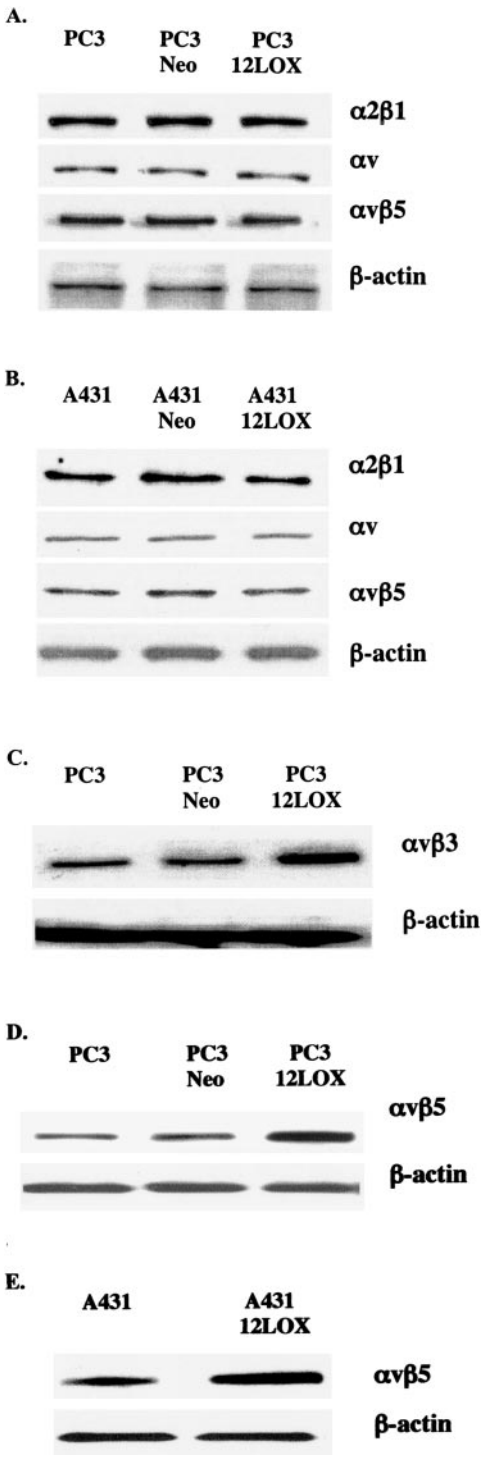

Fig. 6. Western blot analysis of integrin expression in PC3 (*A*) or A431 cells (*B*). Total cell lysates were prepared from wild-type, neo-transfected (*Neo*), and 12-LOX-transfected clones. Expression of $\alpha_2\beta_1$, α_v, and $\alpha_v\beta_5$ was determined in both PC3 and A431 cell lines. Expression of β-actin was determined as a control on cellular loading. There was no difference in the expression of any of the analyzed integrins, indicating that 12-LOX did not alter integrin expression at the translational level. *C,* increased membrane expression of $\alpha_v\beta_3$ integrin in 12-LOX-transfected PC3 cells. Membrane fractions were isolated and examined by Western analysis. *D,* increased membrane expression of $\alpha_v\beta_5$ in PC3 cells after transfection with 12-LOX. *E,* increased membrane expression of $\alpha_v\beta_5$ in A431 cells after transfection with 12-LOX.

cells were grown in 96-well plates in the absence of serum treated with 100 nM 5(*S*)-HETE, 12(*S*)-HETE, 15(*S*)-HETE, or 13(*S*)-HODE every 12 h for 48 h. Treatment with 12(*S*)-HETE resulted in significantly greater survival of both PC3 (85% compared to 58%; Fig. 8*A*) and A431 cells (60% compared with 41%; Fig. 8*B*) under serum-starved conditions. At similar concentrations, 5(*S*)-HETE, 15(*S*)-HETE, or 13(*S*)-HODE had no effect on the survival of either cell line. Separately, PC3 and A431 cells overexpressing 12-LOX were treated with 10 μM of the specific 12-LOX inhibitors Baicalein or BHPP or a specific 5-LOX inhibitor, Rev-5901, for 48 h. Inhibition of 12-LOX with either inhibitor resulted in a significant decrease in the survival of both PC3 (Fig. 8*C*) and A431 (Fig. 8*D*) cells overexpressing 12-LOX. Most importantly, inhibition of 5-LOX did not alter the survival of either cell line. The two 12-LOX inhibitors used were structurally different, and the same results were observed with either inhibitor, eliminating the possibility of separate effects unrelated to 12-LOX inhibition.

In a separate experiment, we examined the effect of each treatment on surface $\alpha_v\beta_5$ expression by examining the attachment of wild-type PC3 or A431 on $\alpha_v\beta_5$-mediated adhesion kits after treatment. 12(*S*)-HETE (100 nM) resulted in a significant increase in the number of cells attached to the plates in both PC3 (Fig. 9*A*) and A431 cells (Fig. 9*B*). In contrast, treatment with 5(*S*)-HETE had no effect, and 15(*S*)-HETE or 13(*S*)-HODE appeared to slightly reduce attachment to the plates, although this was not significant. These results confirm conclusively that the end product of 12-LOX metabolism, 12(*S*)-HETE, regulates both the survival and surface expression of $\alpha_v\beta_5$ integrin on PC3 and A431 cells.

DISCUSSION

The AA-metabolizing enzyme 12-LOX is a key regulator of tumor growth. This pathway has been implicated in tumor cell proliferation and motility, the regulation of apoptosis, and tumor angiogenesis (7–10). Platelet-type 12-LOX is actively expressed in the prostate cancer PC3 cell line (34) and is actively expressed at higher levels in the epidermoid A431 cell line (31). In this study, we report that overexpression of platelet-type 12-LOX decreases cell apoptosis induced by serum starvation and extends the survival of these two characterized tumor cell lines. The prolonged survival of the cells was not a result of increased cell proliferation (data not shown) but was due to an intrinsic property, which rendered them less susceptible to apoptosis. Transfection with 12-LOX in either cell line resulted in a more spread morphology compared with either wild-type or mock-transfected cells, even under normal culture conditions.

As late as 4 days after serum withdrawal, when 70% of wild-type and mock-transfected PC3 cells were apoptotic, the majority (80%) of the pCMV-12-LOX-transfected PC3 cells were still adherent and viable. Similar results were observed in A431 cells, with significant apoptosis as early as 2 days after serum deprivation compared with 12-LOX-transfected cells. Decreased apoptosis in PC3 and A431 cells overexpressing 12-LOX relative to mock-transfected controls was confirmed by DNA laddering. Invariably, cells would first round up and detach from the substratum (*i.e.,* culture flasks) before becoming morphologically and biochemically apoptotic. Indeed, even under normal growth conditions, 12-LOX-transfected PC3 and A431 cells exhibited a more spread morphology in culture, an effect that could be seen to a greater extent when the cells were serum-starved for 4 days. In each case, whereas the majority of wild-type or mock-transfected cells would detach from the substratum, the 12-LOX-transfected cells remained attached and more extended in shape. This observation suggests that in both cell lines examined, the more spread 12-LOX-transfected cells may possess a stronger adherence to the ECM, which would allow them to survive longer in the absence of trophic factors. This would be consistent with previous studies proposing that cells which extend themselves over a large surface area survive better and proliferate faster than cells with a more rounded shape (35).

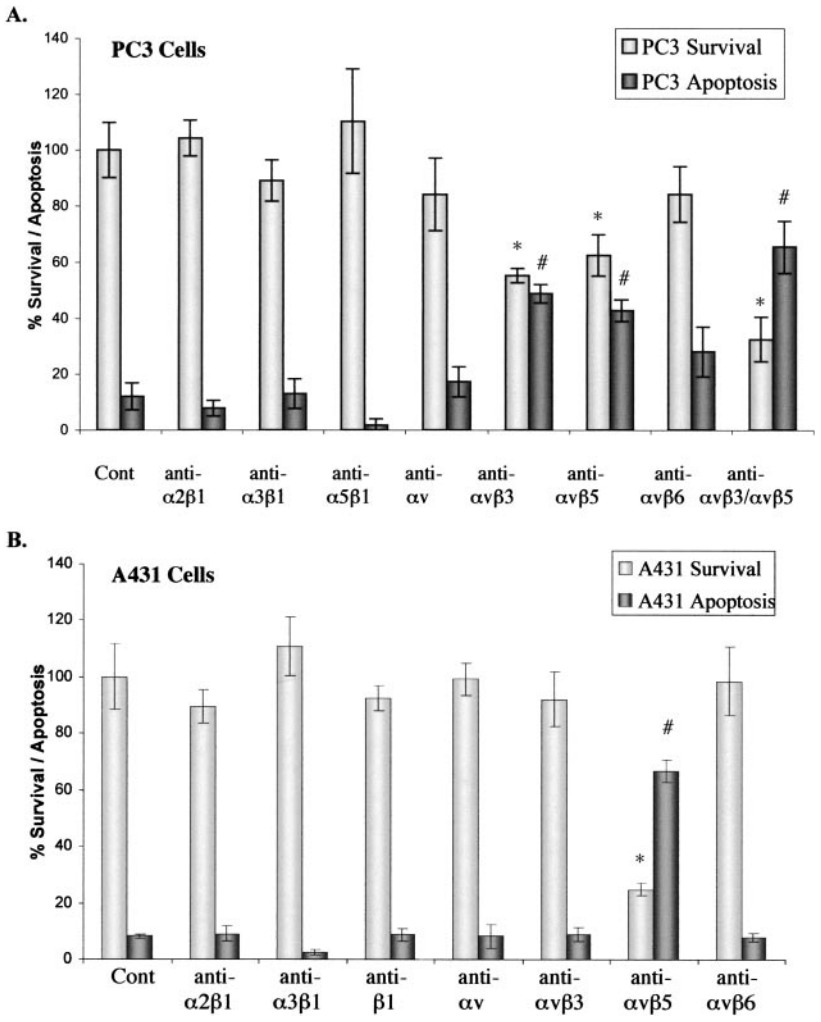

Fig. 7. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are important for the survival of 12-LOX-transfected cancer cells in the absence of serum. Survival of 12-LOX-transfected PC3 or A431 cells was examined by BrdUrd incorporation, and apoptosis was determined by TUNEL staining. 12-LOX-transfected PC3 (*A*) or A431 cells (*B*) were serum-starved for 2 days in the presence of various antibodies directed against different integrins. Results are expressed as the percentage of cell survival or apoptosis, and the mean ± SD was derived from three independent experiments. Treatment with anti-$\alpha_v\beta_3$ or anti-$\alpha_v\beta_5$ resulted in a significant decrease in PC3 (12-LOX) cell survival (*, $P < 0.05$) and increased apoptosis (#, $P < 0.05$) compared with controls, and this effect was greater when a combination of the two antibodies was used ($P < 0.02$; $P < 0.01$). Anti-$\alpha_v\beta_5$ significantly decreased survival (*, $P < 0.02$) and increased apoptosis (#, $P < 0.02$) of A431 (12-LOX) cells compared with untreated controls.

Cell shape is maintained mostly by cell-cell and cell-matrix interactions, as well as by intracellular cytoskeletal structures that are physically linked to cell-matrix interaction sites at subcellular structures termed focal adhesions. Integrins are the major cell surface receptors mediating cell-substrate adhesions. The disruption of the integrin-mediated adhesion has consistently been shown to induce anoikis (apoptosis as a result of loss of anchorage), and various integrin molecules play an important role in supporting cell survival. Integrin $\alpha_v\beta_3$ is one of the most studied and has been implicated in the survival of human vascular endothelial (36) and embryonic kidney cells (21), as well as in the survival of melanoma (37) and lymphoid tumor cells (38). Similarly, several β_1 integrins (*e.g.,* $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$) have been shown to mediate the survival of a variety of cell types (19, 39–41), whereas the integrin α_5 subunit has been shown to suppress apoptosis of colon carcinoma cells induced by serum deprivation (42).

Wild-type PC3 cells expressed α_v, $\alpha_2\beta_1$, $\alpha_3\beta_1$, or β_1 as their predominant integrin receptors. These cells also expressed lower levels of integrin $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$ on their surface. PC3 cells overexpressing 12-LOX demonstrated a significant increase in their surface expression of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, whereas the expression of other integrin receptors examined by flow cytometry was unaltered. These observations were confirmed by immunofluorescence staining and by means of integrin-specific adhesion assays. These results suggested that $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are potential survival factors for pCMV-12-LOX PC3 cells cultured in the absence of serum. Two subsequent lines of experimental evidence support this hypothesis. First, in the 12-LOX-overexpressing PC3 cells, membrane expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ was increased relative to that in wild-type or neo-transfected cells. This increased expression at the cell surface would presumably mediate tighter cell-matrix adhesions and contribute to a more spread morphology in culture. Second, and more importantly, a definite cause and effect relationship was established between the increased surface integrin expression and survival. 12-LOX-transfected PC3 cells, when serum-starved in the presence of a monoclonal antibody to $\alpha_v\beta_3$ or $\alpha_v\beta_5$, demonstrated a significantly reduced survivability, which was similar to that of wild-type and mock-transfected PC3 cells. Dramatically, the 12-LOX-overexpressing PC3 cells treated with anti-$\alpha_v\beta_5$ antibody formed large clusters and aggregates, in which the majority of cells rounded up and underwent apoptosis by day 2. This effect was greater when a combination of anti-$\alpha_v\beta_3$ and anti-$\alpha_v\beta_5$ was used. In addition the effect was shown to be receptor specific because antibodies to several other integrin receptors were ineffective in altering survival. Our results suggest a common characteristic of these integrins, *i.e.,* regulating the survival of PC3 cells under serum-deprived conditions.

In the epidermoid A431 tumor cell line, a high basal expression of β_1-associated integrins (namely, β_1, $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$) was observed. Lower expression of α_v, $\alpha_v\beta_5$, and $\alpha_v\beta_6$ was observed. This cell line did not express the $\alpha_v\beta_3$ integrin. Overexpression of 12-LOX resulted in increased surface expression of $\alpha_v\beta_5$ integrin, with little effect on any of the other integrins examined. Interestingly, we did

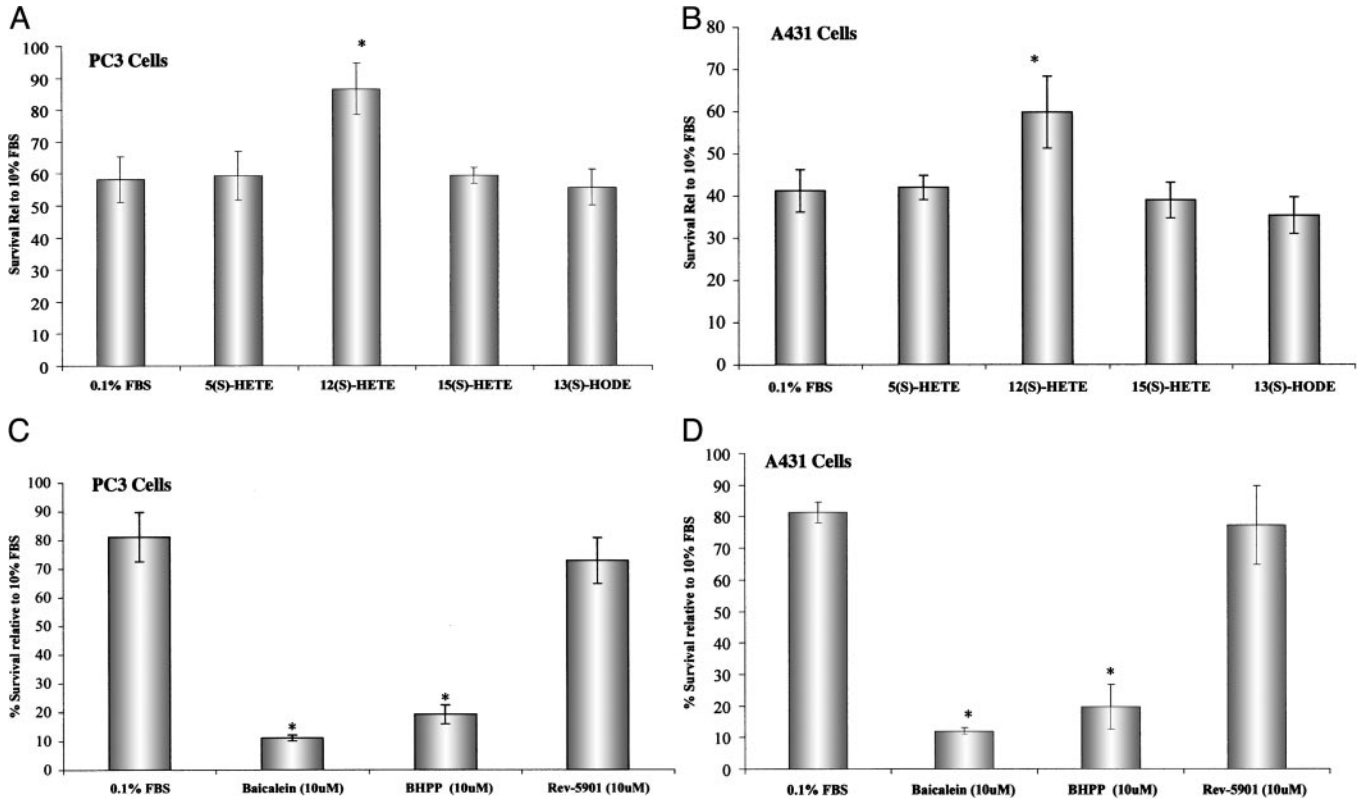

Fig. 8. The end product of 12-LOX metabolism, 12(*S*)-HETE, increased the survival of wild-type PC3 (*A*) or A431 (*B*) cells under serum-starved conditions. Wild-type cells were grown for 48 h in the absence of serum and treated with 100 nM 5(*S*)-HETE, 12(*S*)-HETE, 15(*S*)-HETE, or 13(*S*)-HODE at 12-h intervals. Survival was assessed by BrdUrd assay ("Materials and Methods"). Treatment with 12(*S*)-HETE significantly increased the survival of either PC3 or A431 cells, whereas other treatments did not. *, $P < 0.05$. In addition, 12-LOX-transfected PC3 (*C*) or A431 (*D*) cells were grown for 48 h under serum-starved conditions and treated separately with 10 μM Baicalein or BHPP, two specific 12-LOX inhibitors, or with a specific 5-LOX inhibitor, Rev-5901. Both 12-LOX inhibitors dramatically reduced the survival of 12-LOX-transfected cells, whereas the 5-LOX inhibitor had no effect. *, $P < 0.05$.

observe a slight drop in the expression of both β_1 and $\alpha_2\beta_1$ integrins in A431 cells, which suggests that the expression of subsets of integrins may be lost in favor of other integrins. This has been reported previously in the case of breast cancer, where $\alpha_2\beta_1$ and $\alpha_6\beta_1$ expression was lost in favor of α_v and $\alpha_v\beta_3$ expression in neoplastic tissue compared with normal epithelium (43).

Consistent with the previous results observed in PC3 cells, treatment of A431 cells overexpressing 12-LOX with neutralizing antibody to $\alpha_v\beta_5$ resulted in significant apoptosis and dramatically reduced survival of these cells under serum-starved conditions. Neutralizing antibodies to other integrins did not affect the survival of the 12-LOX-transfected cells. Interestingly, an antibody to $\alpha_5\beta_1$, which was abundantly expressed on A431 cells, also did not reduce the survival of pCMV-12-LOX A431 cells, although this receptor has frequently been implicated in the survival of many other cell types (19, 44–46). It is possible that $\alpha_5\beta_1$ mediates basal cell adhesion instead of providing a survival signal in that cell system. In either case, the fact that overexpression of platelet-type 12-LOX in two tumor cell lines of different histological origin results in enhanced expression of the same integrin implies a general phenomenon of 12-LOX regulating a specific subset of integrins. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ have been shown to regulate cell migration; however, unlike $\alpha_v\beta_3$, $\alpha_v\beta_5$-mediated cell spreading and migration require activation of protein kinase C (46).

The stable end product of platelet-type 12-LOX metabolism, 12(*S*)-HETE, is a well-established protein kinase C activator (47) and, more importantly, has been shown to increase the surface expression of $\alpha_v\beta_3$ integrin on CD clone 3 endothelial cells (28). Interestingly, we have demonstrated previously (48) that 12(*S*)-HETE extended the survival of rat Walker W256 cells cultured in the absence of serum. In this study, we confirmed the effects of 12-LOX overexpression in both cell lines by treating wild-type PC3 or A431 cells with 12(*S*)-HETE to examine whether this provided a survival advantage in both cell lines. As expected, treatment with 12(*S*)-HETE, and not other LOX metabolites, *i.e.,* 5(*S*)-HETE, 15(*S*)-HETE, or 13(*S*)-HODE, resulted in significantly more viable cells compared with controls. In addition, 12(*S*)-HETE increased the surface expression of $\alpha_v\beta_5$ in wild-type cells, as illustrated by an integrin-mediated adhesion assay, an effect that was not observed when cells were treated with the other LOX metabolites. Also, treatment of PC3 or A431 cells overexpressing platelet-type 12-LOX with inhibitors of 12-LOX (Baicalein or BHPP) resulted in a significant reduction in the survival of either cell line, whereas the selective 5-LOX inhibitor Rev-5901 had no effect. We have demonstrated previously (10) that inhibition of 12-LOX with either of these structurally different inhibitors induces cell cycle arrest and apoptosis in two prostate cancer cell lines. Our results suggest that a more general phenomenon may be observed because survival and apoptosis of an epidermal tumor cell line, A431, was also regulated by 12-LOX and its end product, 12(*S*)-HETE.

In summary, these results demonstrate that the 12-LOX pathway of AA metabolism is a critical regulator of cell survival and apoptosis in both prostate PC3 and epidermal A431 tumor cell lines. Enforced expression of this enzyme specifically increased the expression and membrane localization of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin in PC3 cells and $\alpha_v\beta_5$ in A431 cells. Enhanced expression of these integrins was shown to confer a survival advantage on these cells, delaying apoptosis as a result of serum deprivation. Therefore, the inhibition of 12-LOX is a potential therapeutic approach in the treatment of met-

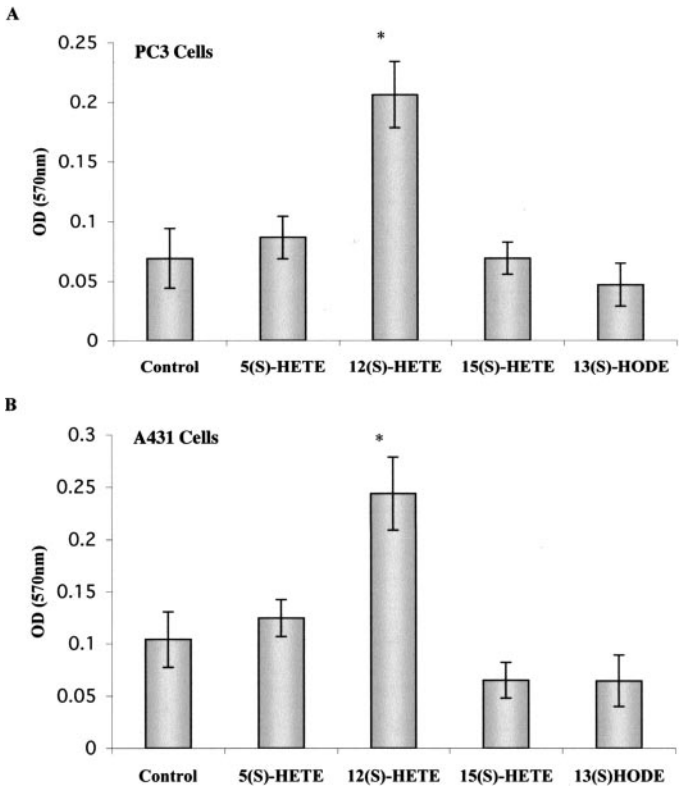

Fig. 9. 12(*S*)-HETE increases the $\alpha_v\beta_5$-mediated adhesion of wild-type PC3 (*A*) or A431 (*B*) cells. Wild-type cells were treated with 100 nM 5(*S*)-HETE, 12(*S*)-HETE, 15(*S*)-HETE, or 13(*S*)-HODE for 24 h (at 12-h intervals). Adhesion via $\alpha_v\beta_5$ was assessed by means of a commercial adhesion assay ("Materials and Methods"), and results were expressed as absorbance. Treatment with 12(*S*)-HETE significantly increased the adhesion of either PC3 or A431 cells to the plates, whereas other treatments did not. *, $P < 0.05$.

astatic disease. It is likely that treatments aimed at inhibiting 12-LOX activity should improve the efficacy of conventional treatments aimed at inducing tumor cell apoptosis, such as chemotherapy and/or radiation therapy.

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