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Increased metastatic potential in human prostate carcinoma cells by overexpression of arachidonate 12-lipoxygenase

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Abstract

Arachidonate 12-lipoxygenase (LOX) converts arachidonic acid to 12(S)-hydroxyeicosatetraenoic acid (HETE), a bioactive lipid implicated in tumor angiogenesis, growth, and metastasis. Alteration in 12-LOX expression or activity has been reported in various carcinomas including prostate carcinoma. However, little is known about the impact of the altered expression or activity of 12-LOX on tumor metastasis. In the present study, we examined whether or not an increase in 12-LOX expression in human prostate carcinoma cells can modulate their metastatic potential. We report that increased expression of 12-LOX in PC-3 cells caused a significant change in cell adhesiveness, spreading, motility, and invasiveness. Specifically 12-LOX transfected PC-3 cells were more adhesive toward vitronectin, type I and IV collagen, but not to fibronectin or laminin, than cells transfected with control vector. Increased spreading on vitronectin, fibronectin, collagen type I and IV also was observed in 12-LOX transfected PC-3 cells when compared to control PC-3 cells. The increased spreading of 12-LOX transfected PC-3 cells was blocked by treatment with 12-LOX inhibitors, baicalein and CDC. 12-LOX transfected PC-3 cells were more invasive through Matrigel than cells transfected with control vector. *In vivo*, tumor cell invasion to surrounding muscle or fat tissues was more frequent in nude mice bearing s.c. tumors from 12-LOX transfected PC-3 cells than in those from control vector transfected cells. When injected via the tail vein into SCID mice with implanted human bone fragments, there was an increase in tumor metastasis to human bone by 12-LOX transfected PC-3 cells in comparison to control vector transfected cells. Taken together, our data suggest that an increase in 12-LOX expression enhances the metastatic potential of human prostate cancer cells.

Introduction

Prostate cancer is the most commonly diagnosed cancer in American men. Mortality associated with prostate cancer is related to the spread of tumor cells beyond the prostate gland and metastasis of tumor cells to bone or other secondary sites. An understanding of how prostate cancer cells acquire the ability to invade, spread, and colonize at distant organs is essential for developing a targeted approach for effective management of prostate cancer. Arachidonic acid metabolism via cyclooxygenase (COX) or lipoxygenase (LOX) pathways can generate a number of bioactive eicosanoids. Among them, 12(S)-hydroxyeicosatetraenoic acid (HETE), the arachidonate product of 12-LOX has a plethora of biological activities including stimulating tumor cell adhesion, invasion, and metastasis [1]. The expression of 12-lipoxygenase has been detected in a growing list of tumors especially of epithelium origin such as prostate cancer [2], pancreatic cancer [3], breast cancer [4], lung cancer [5], gastric cancer [6], among others [7]. A clinical study involving over 120 prostate carcinoma specimens found that the elevation of 12-LOX mRNA expression occurred more frequently in advanced stage, high grade prostate cancer [2], suggesting that 12-lipoxygenase expression or activity may be associated with carcinoma progression and invasion *in vivo*. When over-expressed in prostate cancer PC-3 cells, 12-LOX was shown to stimulate tumor angiogenesis and growth *in vivo* [8]. A similar role of 12-LOX in tumor angiogenesis and growth was observed in breast cancer cells [4]. The level of endogenously produced 12(S)-HETE has been found as a determinant of metastatic potential of B16a melanoma cells and other tumor cells such as Clone A and Walker 256 [5, 9]. The aim of the present study was to investigate whether 12-LOX, when over-expressed, can modulate the metastatic potential of human prostate carcinoma cells. We present data suggesting that 12-LOX can effect complex changes in the metastatic phenotype such as adhesion, spreading, migration, and invasion *in vitro* and enhance metastasis to human bone implants *in vivo*.

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Materials and methods

Reagents

Low melting agarose, human vitronectin and fibronectin, and all culture reagents were purchased from Gibco-BRL (Gaithersburg, Maryland). Phamacological inhibitors of lipoxygenase, CDC, NDGA, and baicalein were purchased from Biomol (Plymouth Meeting, Philadelphia). A 12-LOX selective inhibitor N-benzyl-N-hydroxy-5-phenylpentanamide (BHPP) was a generous gift from Biomide Corp (Grosse Pointe Farms, Michigan). MTT and MTS/PMS reagents were obtained from Promega Corp. (Madison, Wiscousin). CytoMatrix cell adhesion strips pre-coated with various matrix proteins were purchased from Chemicon International (Temecula, California). Matrigel coated and non-coated modified Boyden invasion chambers were from Becton Dickinson (Bedford, Massachusetts). TRITC or FITC-phalloidin was purchased from Sigma (St. Louis, Missouri).

Cell culture

The generation and characterization of 12-LOX transfected PC-3 cells, labeled PC-3/12-LOX (nL-2, nL-8, and nL-12), and the control vector transfected PC-3 cells (PC-3/neo; neo-α and neo-σ) were previously described [8]. Cells were maintained in RPMI 1640 with 10% FBS in the presence of 0.2 mg/ml of geneticin. Prior to experiments, the expression of 12-LOX in the transfected cells was confirmed by immunoblot analysis as previously described [8].

Anchorage-independent growth on soft agarose

PC-3 cells were suspended in 0.3% low-melting agarose with complete RPMI medium, plated at a density of 400 cells per 35-mm wells, pre-coated with 0.5% agarose and maintained at 37 °C. Inhibitors of LOX, BHPP [10] and NDGA, were added at the final concentration of 10 and 20 μmol/l, respectively. Three weeks later, plates were stained with vital stain MTT/PMS reagents. Colonies with more than 50 cells were counted under an SP SZ-4060 stereomicroscope (Olympus America, Melville, New York).

Cell adhesion and spreading assay

To assay for cell adhesion on different matrices, CytoMatrix cell adhesion strips were used according to manufacturer's instruction. Cells were harvested using versene and resuspended in SF-RPMI at a density of 0.5×10^6 cells per ml. Cells were held in suspension for 5 min and added into the wells pre-coated with various extracellular matrix proteins as well as into a 96-well culture plate as a control for the total cell number. After 1 h of incubation at 37 °C, non-adherent cells in adhesion strips were removed and the attached cells were washed twice with serum free RPMI. Adherent cells in adhesion strips as well as total cells in 96-well culture plates were quantified by MTS/PMS reagents. To calculate the percentage of adherent cells, the readings from adhesion strips (adhered cells) were divided by the corresponding readings from the 96-well culture plate (total cells).

For cell spreading assay, cells were harvested using versene, resuspended in SF-RPMI, and added into wells pre-coated with various ECM proteins as indicated. After 1 h, cells were fixed and the number of spread cells was counted under phase contrast microscope in a double blind approach. An individual cell was counted as spread when its diameter was at least twice that of nucleus [21]. The spreading factor was calculated as the percentage of spread cells among total adherent cells [21].

Cytochemistry

Cells were grown on glass coverslips in RPMI-10%FBS for 16 h and then treated with baicalein (10 μmol/l) or CDC (10 μmol/l) for 1 h. Then cells were fixed for 10 min in 3.7% para-formaldehyde solution and stained with 0.1 μg/ml TRITC-phalloidin for 45 min at room temperature. The staining was observed under an epifluorescence microscope and images were acquired through a Zeiss LSM 310 laser confocal microscope.

Cell invasion assays

Matrigel coated and non-coated modified Boyden invasion chambers were used in the assay for invasion and migration, respectively. Cells were harvested using Versene, re-suspended in SF-RPMI 1640, and 5×10^5 cells in 0.5 ml were plated on the top chamber. Then 1 ml of RPMI with or without 10% NIH3T3 conditioned media, used as chemotactic stimulant, was added in triplicates into the lower chamber. After 16–18 h incubation, cells on the top side of the transwell membrane were removed using cotton swabs. The membrane was then removed, fixed in a quick-fix solution, double stained, and mounted for observation and counting as previously described [8]. Usually 12 fields (X100) representing two perpendicular cross-lines of each membrane were counted. Invasion index was calculated as the number of invading cells through Matrigel divided by the number of migrating cells in the absence of Matrigel (uncoated).

Animal models and histochemical studies

All experimental animals were maintained according to the NIH standards established in the 'Guidelines for the Care and Use of Experimental Animals'. Experimental protocols were approved by the Animal Investigation Committee of Wayne State University. The athymic nude mice tumor xenograft model was described previously [8]. Briefly, 4×10^6 PC-3/12-LOX or PC-3/neo cells in 200 μl of HBSS were injected subcutaneously into the right flank of 4–6-week-old male Balb/c nude mice (obtained from University of South Florida, Tampa, Florida). Six to seven weeks after injection, tumors were resected, fixed in 10% neutral buffered formalin, paraffin embedded and sections (5 μm) were prepared for histology staining. Sections were stained with hematoxylin and eosin (H & E) to examine local invasion

into surrounding muscle or fat tissue. The assessment of the presence of local tissue invasion was performed for a total of 10 high power fields (HPF) in a double-blind approach and recorded either as present or absent.

The SCID-human bone orthotopic model for human prostate carcinoma was previously described [11]. Briefly, human fetal femurs and humeri of 16–22 weeks gestation were divided in half longitudinally and then in half transversely into 4 fragments of approximately 0.3 × 1.0 cm. These bone fragments were implanted subcutaneously in the flank through a small skin incision with the opened marrow cavity placed against the mouse muscle. Three weeks after bone implantation, 1×10^6 PC-3/neo or PC-3/12-LOX cells were injected into mice via the tail vein. Eight weeks after injection, the mice were sacrificed and the presence of tumor metastasis in human bone implants was recorded as previously described [11].

Results

Stimulation of anchorage-independent growth of PC-3 cells by 12-LOX expression

Anchorage-independent growth is a key parameter for cells to acquire a metastatic phenotype. It has been found that lipoxygenase-like activity is required for ras-stimulated anchorage-independent growth of tumor cells [12] and that overexpression of 15-LOX-1 in PC-3 cells stimulated anchorage-independent growth [13]. We examined whether 12-LOX modulates anchorage-independent growth by monitoring colony formation. Figure 1A showed the levels of 12-LOX expression in stable 12-LOX transfected PC-3 cells or their vector controls selected for this study [8]. As shown in Figure 1B, 12-LOX transfected PC-3 cells (nL8 and nL12) had increased anchorage-independent growth when compared to PC-3/neo cells (neo-α) ($P < 0.01$). The increased anchorage-independent growth of PC-3/12-LOX cells was inhibited by BHPP (10 μmol/l), a specific 12-LOX inhibitor [10], or by NDGA (20 μmol/l), a general LOX inhibitor, suggesting that the activity of 12-LOX is involved the anchorage-independent growth of tumor cells.

Modulation of cell adhesion, spreading, and actin microfilaments by 12-LOX in PC-3 cells

Adhesion of tumor cells to ECM proteins or endothelium is an integral part of metastasis. 12(S)-HETE has been shown to promote adhesion of tumor cells and endothelial cells to various ECM proteins as well as tumor cell adhesion to subendothelial matrix [14, 15]. To study whether an elevation of 12-LOX in prostate cancer cells can influence cell adhesion, we assessed the adhesion of 12-LOX transfected PC-3 cells on fibronectin, vitronectin, laminin and collagens type I and type IV. As shown in Figure 2, PC-3/12-LOX cells (nL8) have significant increased adhesion on vitronectin ($P < 0.05$), collagen type I ($P < 0.05$), and collagen type IV ($P < 0.05$), when compared to PC-3/neo (neo-σ). In

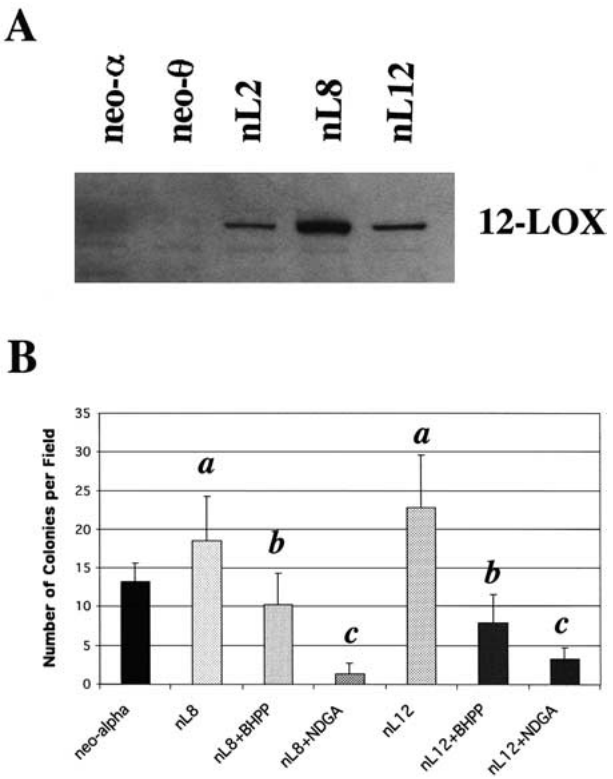

Figure 1. Increased anchorage-independent growth in PC-3 cells as a result of 12-LOX expression. (A) The levels of 12-LOX expression in various 12-LOX transfected PC-3 cells and their vector controls as shown by immunoblot. (B) Anchorage-independent growth assay and drug treatment were conducted as described in Materials and methods. Columns, average number of colonies per field; bars, standard deviation (*a*, $P < 0.01$ when compared to neo-α. *b*, $P < 0.05$ when compared to their solvent controls, nL8 and nL12, respectively. *c*, $P < 0.01$ when compared to their solvent controls, nL8 and nL12, respectively. Student's *t*-test). The experiments were repeated twice.

contrast, there was no significant difference between neo-σ and nL-12 in adhesion on fibronectin and laminin. Similar observations were also made using other clones such as nL2, nL8, and neo-α.

Once cells attach to ECM proteins, they undergo spreading. As shown in Figure 3, in comparison with PC-3/neo, PC-3/12-LOX cells have increased spreading on fibronectin ($P < 0.01$), and collagen type I ($P < 0.01$) and collagen type IV ($P < 0.05$). It is interesting to note that while there was no significant difference between PC-3/12-LOX and PC-3/neo in adhesion on fibronectin, there was an approximate four-fold increase in spreading on fibronectin by PC-3/12-LOX cells. No significant difference in spreading on laminin was found between PC-3/12-LOX and PC-3/neo cells.

Next we examined whether 12-LOX modulates the formation and distribution of actin microfilaments. As shown in Figure 4, 12-LOX transfected PC-3 cells (nL2) presented a spindle, more spread-out morphology while their vector control cells (neo-σ) possessed more round and refractile phenotype. Staining with TRITC-phalloidin revealed that actin microfilaments were mainly located at the cortical region in a focalized manner in 12-LOX transfected PC-3 cells (nL2, Figure 4, top panel, right). In contrast, actin mi-

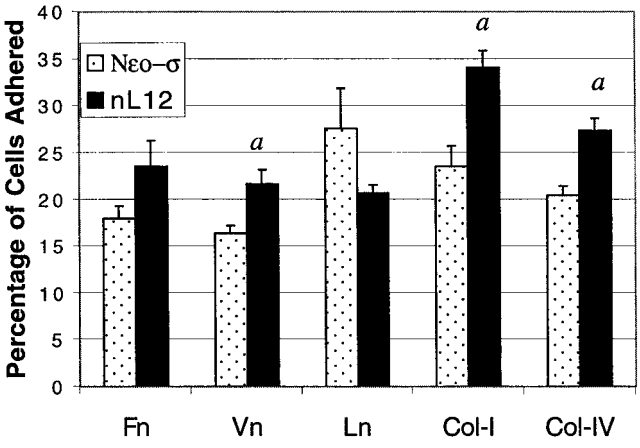

Figure 2. Altered cell adhesiveness to extracellular matrix proteins by 12-LOX over-expression. Cells (neo-α and nL12) were harvested and the adhesion of cells toward various ECM proteins was analyzed as described in Materials and Methods. Fn, fibronectin; Vn, vitronectin; Ln, laminin; Col-I, collagen type I; and Col-IV, collagen type IV. Columns, the average percentage of cells adhered. Bars, standard deviation (a, $P < 0.05$ when compared to their respective control, neo-α. Student's t-test). Shown here are typical observations from four independent experiments.

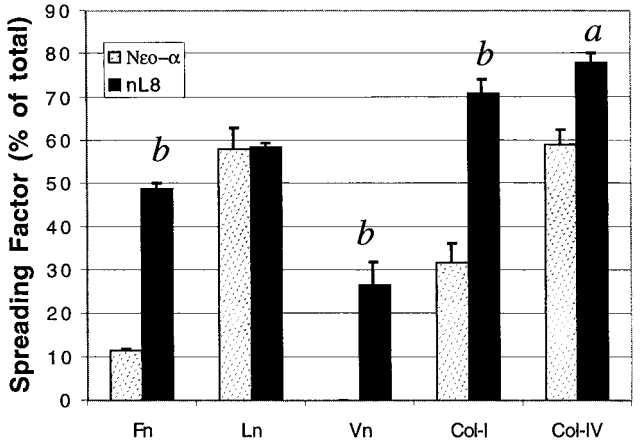

Figure 3. Modulation of tumor cell spreading by 12-LOX expression. Cell spreading on various ECM proteins were counted and calculated as the percentage of total cells adhered as detailed in 'Materials and methods'. Fn, fibronectin; Vn, vitronectin; Ln, laminin; Col-I, collagen type I; and Col-IV, collagen type IV. Columns, the average percentage of cells spread from the total cells adhered; Bars, standard deviation (a, $P < 0.05$ and b, $P < 0.01$ when compared to their respective control, neo-α. Student's t-test). Shown here are typical observations from three independent studies on the spreading of tumor cells.

crofilaments were more evenly distributed in vector control cells (neo-σ) cells (Figure 4, top panel, left). The increased distribution of actin microfilaments at the cortical region as observed in 12-LOX transfected PC-3 cells was inhibited by treatment of cells with 12-LOX inhibitors baicalein (Figure 4, middle panel, right) or CDC (Figure 4, bottom panel, right). Similar observations were also made with other clones of 12-LOX transfected PC-3 cells (nL2 and nL8) or with another 12-LOX inhibitor i.e., BHPP (data not shown). The results suggest that 12-LOX can modulate cell adhesion and spreading through regulating the formation or temporal distribution of actin microfilaments to cortical regions.

Overexpression of 12-LOX in PC-3 cells enhances invasiveness

Cell invasion is essential for tumor expansion and metastasis. To study whether 12-LOX can enhance PC-3 cell motility, we measured the invasiveness of PC-3/12-LOX and PC-3/neo through Matrigel *in vitro* using the conditioned media from NIH-3T3 cells as chemotactants. As shown in Figure 5A, PC-3 cells/12-LOX cells (nL12) were more invasive in response to chemotactical sources, as indicated by invasion index, than PC-3/neo. We further assessed the incidence of local tissue invasion in s.c. tumors *in vivo*. As shown in Figure 5B, there was an increased incidence of surrounding tissue invasion in tumors derived from PC-3/12-LOX cells (nL-12), as compared to those from PC-3/neo (neo-σ). The results collectively suggest an increased invasiveness of 12-LOX transfected PC-3 cells, when compared to control vector transfected cells, both *in vivo* and *in vitro*.

Experimental metastasis and 12-LOX expression

To study whether 12-LOX can modulate prostate cancer metastasis *in vivo*, we injected PC-3/12-LOX and PC-3/neo cells into athymic mice via the tail vein. Experimental metastasis in mouse lung and other organs was not detected even three months later. A SCID-hu bone model was recently developed to study bone metastasis of prostate cancer cells [11]. This model was used to assess the ability of 12-LOX transfected PC-3 cells and PC-3/neo to form experimental metastasis in this model. An increased incidence of homing to human bone implants was observed in the group injected with 12-LOX transfected PC-3 cells (nL12), when compared to that of vector control cells (neo-σ) (Figure 5C).

Discussion

Mortality from prostate cancer is largely due to complications from tumor metastasis. Tumor metastasis is a complex, multi-step process involving the constant interaction among tumor cells and host tissues or organs. It remains largely unknown which genetic and phenotypic changes in human prostate cancer cells enable them to metastasize to distant organs such as bone. An understanding of how tumor cells acquire the capability to metastasize is essential for developing a targeted approach for effective management of prostate cancer. Platelet-type 12-LOX metabolizes arachidonic acid to form 12(S)-HETE, an eicosanoid widely implicated in tumor growth and metastasis [9]. In many experimental systems there is a positive correlation between biosynthesis of 12(S)-HETE and the metastatic potential of tumor cells. For example, in murine B16a cells, subpopulations with high metastatic potential produced approximately four-fold more 12(S)-HETE than the low metastatic cells [9]. The high metastatic cells demonstrated a 5- to 10-fold increase in lung colonizing ability that was inhibited by pretreatment of cells with a selective 12-LOX inhibitor, BHPP [5]. In the rat Dunning model system, 12(S)-HETE was found to enhance the invasiveness of tumor cells via activation of PKC-α [16]. In

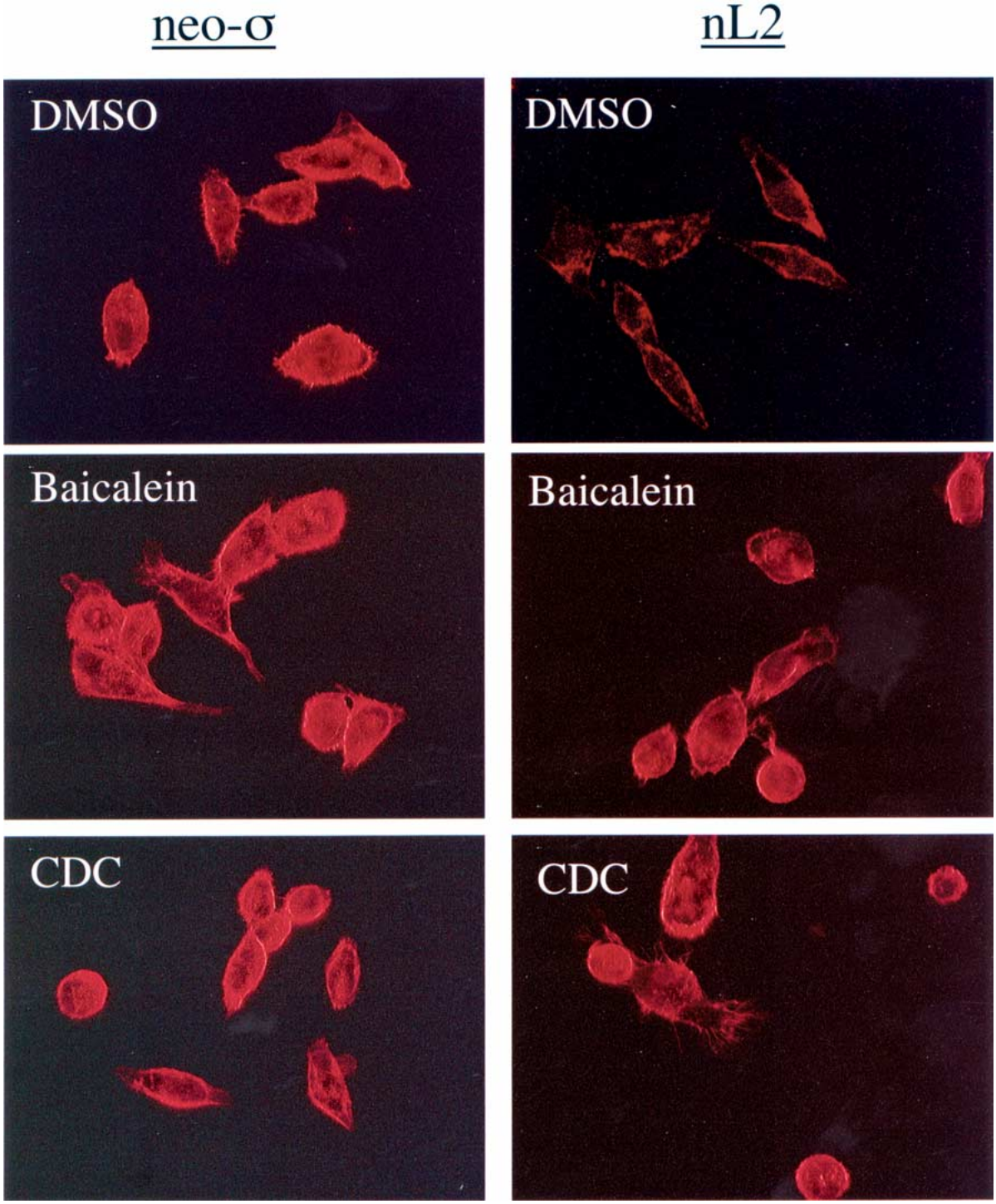

Figure 4. Modulation of actin microfilaments by 12-LOX. Cells were grown on glass coverslips, and treated with inhibitors of 12-LOX, baicalein or CDC, for 1 h. Afterwards, cells were processed for staining for actin filaments with TRITC-phalloidin. Note the morphological difference between 12-LOX transfected PC-3 cells (nL2) and their vector control, neo-σ. In nL2, cells presented a spindle shape with actin microfilaments mainly localized in the cortical regions and focal adhesions. In contrast, neo-σ or nL12 cells treated with baicalein or CDC, presented a round shape with actin filaments more evenly distributed across the cells. Shown here are typical observations from two-independent experiments.

the present study, overexpression of 12-LOX in PC-3 cells increased a number of phenotypic changes in PC-3 cells such as cell adhesiveness, spreading, migration, and invasion *in vitro*. We also found that there was an increase in the incidence of local tissue invasion and metastasis *in vivo* by 12-LOX transfected PC-3 cells, when compared to cells transfected with control vector. These observations suggest a potential stimulatory role for 12-LOX in human prostate tumor metastasis.

The increased metastatic potential in 12-LOX-expressing tumor cells may be, at least in part, due to the biological activities elicited by 12(S)-HETE. It has been found that 12(S)-HETE modulates several parameters related to the metastatic potential of tumor cells such as motility [17], se-

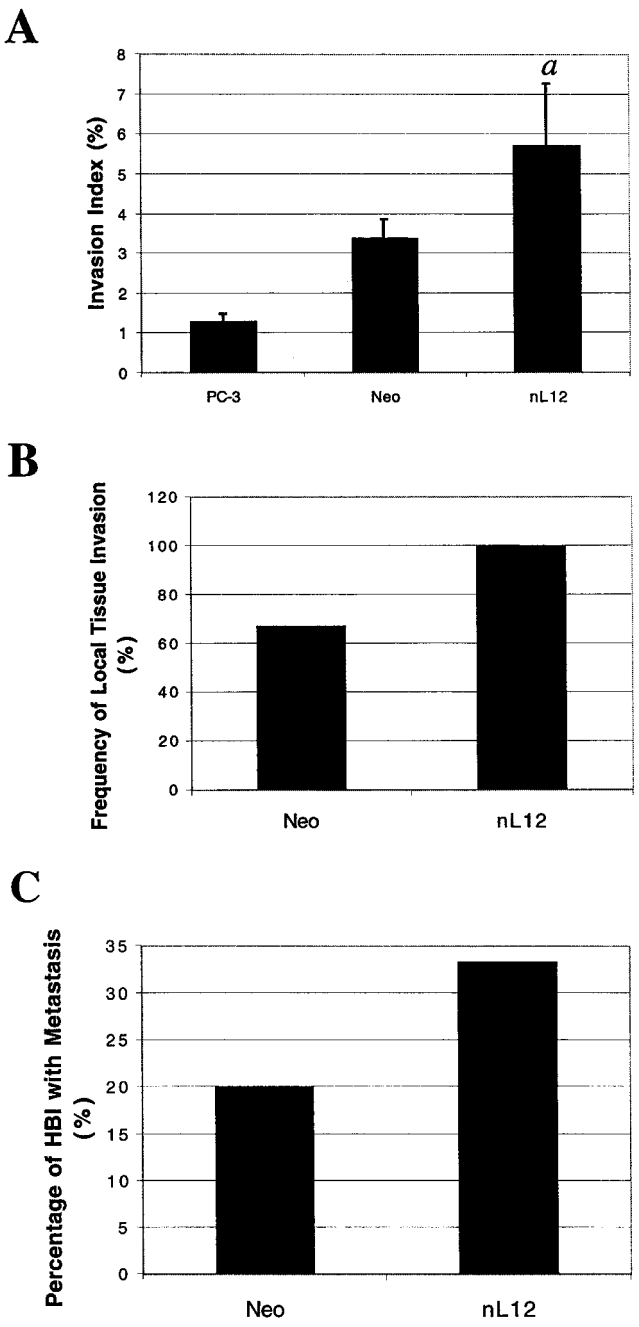

Figure 5. Increased invasiveness and metastasis in 12-LOX transfected PC-3 cells. (A) Invasion through Matrigel. The number of invading cells through Matrigel were normalized with the number of migrating cells in the absence of Matrigel and expressed as invasion index (percentage of invading cells among migrating cells). Columns, the average invasion index from 3 chambers; Bars, standard deviation (a, $P < 0.05$ when compared to control, neo-σ. Student's t-test). (B) Frequency of tissue invasion by PC-3 tumors in athymic nu/nu mice. The presence or lack of invasion into surrounding tissues was assessed in H&E stained tumor sections in a double-blind approach and recorded either present or absent. Shown here is the frequency of incidence of tissue invasion in tumors derived from PC-3/12-LOX (nL12, $n = 8$) or PC-3/neo (neo-σ, $n = 6$). (C) Tumor metastasis to bone. The SCID-hu bone model was used to assess the ability of PC-3/12-LOX (nL12) and PC-3/neo cells (neo-σ) to metastasize to implanted human bone fragment. Shown here is the percentage of human bone implants with metastasis in mice injected with PC-3/12-LOX (nL12, number of mice, $n = 6$) or those with PC-3/neo (neo-σ, number of mice, $n = 5$).

cretion of lysosomal proteinases cathepsin B and L [18, 19], expression of integrin receptor $\alpha_{IIb}\beta 3$ [20, 21], tumor cell adhesion to endothelium and spreading on subendothelial matrix [15], as well as lung colonizing ability *in vivo* [9]. 12(S)-HETE was also found to act as a mediator of linoleic acid stimulated invasion and MMP-9 production in MDA-MB-435 cells [22]. We have investigated whether expression of 12-LOX stimulate MMP expression in PC-3 cells using zymogram [23] and did not detect any expression of MMP-2 or MMP-9 in either 12-LOX transfected PC-3 cells or control vector transfected cells (unpublished observation). It is known that PC-3 cells express MMP-2 or MMP-9 at minimal levels, but upon interaction with stromal cells or collagen, MMP-9 expression was stimulated [24]. In the present study, we found that 12-LOX transfected PC-3 cells have increased adhesiveness and spreading on collagen type I and IV. It is unknown, however, whether 12-LOX can stimulate the expression of proteases in PC-3 cells upon interaction with collagen or other extracellular matrix proteins.

In addition to increased adhesion and spreading to collagen type I and IV, 12-LOX transfected PC-3 cells also had increased adhesion onto vitronectin and increased spreading on vitronectin and fibronectin. The increased adhesion and spreading to select extracellular proteins may be related to a difference in surface expression or avidity of integrins in PC-3 cells as a result of 12-LOX expression. It has been documented extensively that 12(S)-HETE increases the surface expression of $\alpha_v\beta_5$ and $\alpha_{IIb}\beta_3$ in tumor cells as well as in endothelial cells [20, 21]. Studies are ongoing to test whether there is a change in the surface expression or avidity of integrin receptors in PC-3 cells as a result of 12-LOX expression.

The modulation of cell adhesion, spreading, and invasion by 12-LOX may be related to the altered formation and temporal distribution of actin microfilaments as a result of increased 12-LOX expression. It has been shown that 12(S)-HETE can increase stress fiber formation in B16a melanoma cells through a protein kinase C-dependent process [25]. In this study we found that actin microfilaments were mainly located at cortical regions as well as focal adhesions in 12-LOX transfected PC-3 cells, in comparison to control vector transfected cells. Further, the increased distribution of actin microfilaments to cortical sites in 12-LOX transfected PC-3 cells was inhibited by treatment of cells with 12-LOX inhibitors, CDC or baicalein. Changes in the actin microfilament network has long been related to cell adhesion, spreading, motility, and morphology. However, the exact role of actin microfilaments in response to 12-LOX expression in modulation of the metastatic phenotype awaits further study.

Research on prostate cancer metastasis is hampered by the limited ability of established human PCa cell lines to form experimental metastasis in animal models and also by the lack of appropriate animal models to reflect the metastatic behavior of human prostate cancer cells. The SCID-hu bone model was developed to study the ability of human prostate cancer to metastasize to bones [11]. Using this model, we found an increase in metastasis to human bone

implants by 12-LOX transfected PC-3 cells when compared to control vector transfected cells. The formation of metastasis in human bone implants by 12-LOX transfected PC-3 cells may be related to their increased ability of anchorage-independent growth and selective adhesion to collagen type I, an extracellular matrix protein commonly found in bone [26].

In summary, we found that an increase in 12-LOX expression in PC-3 cells elicited several phenotypic changes and increased the invasive and metastatic potential of tumor cells. These observations, together with our previous studies regarding the expression pattern of 12-LOX in clinical prostate tumor tissues [2] and the role of 12-LOX in tumor angiogenesis and growth [8], suggest an enabling role of 12-LOX in the progression of human prostate carcinoma.

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