Platelet-Type 12-Lipoxygenase in a Human Prostate Carcinoma Stimulates Angiogenesis and Tumor Growth

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Materials and Methods

Abstract

Previously, we found a positive correlation between the expression of platelet-type 12-lipoxygenase (12-LOX) and the progression of human prostate adenocarcinoma (PCa; Gao et al., Urology, 46: 227–237, 1995). To determine the role of 12-LOX in PCa progression, we generated stable 12-LOX-transfected PC3 cells, which synthesize high levels of 12-LOX protein and 12(S)-hydroxyeicosatetraenoic acid metabolite. In vitro, 12-LOX-transfected PC3 cells demonstrated a proliferation rate similar to neo controls. However, following s.c. injection into athymic nude mice, 12-LOX-transfected PC3 cells formed larger tumors than did the controls. Decreased necrosis and increased vascularization were observed in the tumors from 12-LOX-transfected PC3 cells. Both endothelial cell migration and Matrigel implantation assays indicate 12-LOX-transfected PC3 cells were more angiogenic than their neo controls. These data indicate that 12-LOX stimulates human PCa tumor growth by a novel angiogenic mechanism.

Introduction

The growth and metastasis of solid tumors are dependent upon the ability of tumor cells to induce angiogenesis (1). Angiogenesis, the formation of new blood vessels from preexisting ones, involves endothelial cell proliferation, motility, and differentiation. Tumor cells can secrete a variety of angiogenic factors, such as basic fibroblast growth factor and vascular endothelial growth factor, to stimulate angiogenesis (2). Tumor cells also produce angiogenesis inhibitors such as thrombospondin and angiostatin to control angiogenesis (2). The balance between angiogenesis stimulators and inhibitors determines the angiogenicity of tumor cells (2). In human PCa, the level of vascularization positively correlates with tumor stage (3–5). Inhibition of angiogenesis by linomide or TNP-470 potently inhibits PCa growth and metastasis by causing necrosis and apoptosis in tumors (6, 7). Although various potential angiogenesis factors have been identified in prostate cancer (8), it is still unclear by which process PCa cells become angiogenic. We have previously detected the expression of platelet-type 12-LOX in human PCa and demonstrated a correlation between 12-LOX mRNA expression and pathological stage (9). Platelet-type 12-LOX uses only arachidonic acid as substrate and forms 12(S)-HETE exclusively (10). Here, we have examined the function of 12-LOX on PCa tumor growth. Our data demonstrate that 12-LOX has no detectable effect on PCa cell growth in vitro but stimulates PCa tumor growth in vivo. This effect of 12-LOX on tumor growth is closely related to increased angiogenesis. Both in vitro and in vivo angiogenesis assays suggest that PCa cells expressing high levels of 12-LOX are more angiogenic than those expressing no or low levels of 12-LOX. Our results provide a novel function for platelet-type 12-LOX in PCa progression.

Materials and Methods

Cell Culture. Rat angiogenic endothelial cell line RV-ECT (a gift from Dr. Collin Funk, Center for Experimental Therapeutics, University of Pennsylvania; Ref. 10), and pCMV-neo, which encodes a neomycin-resistant protein. PC3 cells transfected with pCMV-neo were used as controls. Transfectants were selected using 1 mg/ml geneticin (G418) in RPMI with 10% FBS and then cloned using a limiting dilution method in 96-well plates. The cloned transfectants were propagated and characterized for 12-LOX mRNA expression by Northern blot and 12-LOX protein expression by Western blot. Human epidermoid carcinoma A431 cells that express 12-LOX (12) were used as a positive control. The probe used in Northern blot was the 12-LOX cDNA from pCMV-12-LOX construct. Rabbit 12-LOX polyclonal antibody used in Western blot was purchased from Oxford Biomedical Inc. (Oxford, MI). Actin antibody was from Amersham (Arlington Heights, IL). The synthesis of 12(S)-HETE by 12-LOX transfectants was determined using a RIA kit from Perspective Diagnostics (Cambridge, MA) according to the manufacturer’s instructions.

In Vitro Proliferation Assay. To study the growth kinetics of PC3 transfectants in culture, 2 × 10^5 cells per well were seeded in 96-well culture plate. The number of viable cells at intervals of 48 h was assessed using an MTS cell reagent (Promega Corp., Madison, WI). The A_490 nm readings 2–3 h after plating were used as baselines. The number of cells was expressed as the percentage of increase from the A_490 nm baselines.

Animal Model and Histochemical Studies. A total of 4 × 10^6 12-LOX-transfected PC3 cells or neo control cells in 200 µl of HBSS were injected s.c. into the right flank of 4–6-week-old male BALB/c nude mice (obtained from University of South Florida, Tampa, FL). The resulting tumors were measured using a vernier caliper, and tumor volume was calculated using the formula: (width^2) × length × 0.5 (7). Six to 7 weeks after injection, mice were sacrificed, and the tumors were resected and photographed under an SP SZ-4060 stereomicroscope (Olympus America, Melville, NY). Tumors were fixed in 10% neutral buffered formalin and embedded in paraffin, and sections (5 µm) were prepared for histology staining. Sections were stained with H&E to examine the presence of necrosis. The assessment of tumor necrotic area was performed for a total of 10 HPFs per tumor using a double-blind approach.

CD31 staining was used to assess tumor vascularization. Immunohistochemical staining for CD31 (Dako Corp.; dilution, 1:20) was performed.
using a standard avidin-biotin complex-immunoperoxidase procedure. The slides were counterstained with hematoxylin. The vascularity was assessed qualitatively on the basis of overall vessel organization and quantitatively by microvessel density. A total of 10 fields per tumor were evaluated for both microvessel density and vessel organization. The microvessel density was indicated by the average number of vessels crossing an arbitrary line across one HPF field. The rating of vessel organization was performed according to the following scale: 0, disorganized, staining randomly distributed; 1, intermediate, vessel-like structures formed; and 2, highly organized, vessels structured and organized.

**Endothelial Cell Migration Assay.** For the cell migration assay, RV-ECT endothelial cells were harvested by trypsinization and resuspended in RPMI with 10% FBS, and 5 × 10^5 cells in 0.5 ml were plated on the top chamber of a modified Boyden chamber (Becton Dickinson, Bedford, MA). Then, 1 ml of RPMI-10% FBS medium conditioned from PC3 or various transfectant cultures or fresh medium with 12(S)-HETE was added in triplicate into the lower chamber. After 4 h of incubation, the cells on the top side of the transwell membrane were removed with cotton swabs. The membrane was then cut out, fixed in a quick-fix solution, double-stained, and mounted for observation and counting. Usually, 12 fields (×100) representing two perpendicular cross-lines of each membrane were counted.

**Matrigel Implantation Assay for Tumor Cell-induced Angiogenesis.** The Matrigel implantation assay was performed as described by Ito et al. (13) with the following modifications. Matrigel (Becton Dickinson, Bedford, MA; 0.4 ml premixed with 2 × 10^6 PC3 12-LOX transfectant or neo control cells) was injected s.c. into nude mice (four mice per group). Mice were sacrificed 12 days after injection and dissected to expose the implants for recording.

**Results**

**Generation of PC3 Transfectants That Constitutively Synthesize 12-LOX and 12(S)-HETE.** To determine the function of 12-LOX in PCa progression, PC3 cells were transfected with a platelet-type 12-LOX cDNA construct. Stable transfecants were cloned and named the nL series. Several stable transfecants (neo series) isolated from PC3 cells transfected with pCMV-neo were used as controls. Northern blot analyses of transfecant clones show that the levels of 12-LOX mRNA were increased in various nL clones, compared to the neo controls or wild-type PC3 (Fig. 1A). The 12-LOX mRNA levels in various nL clones were higher than in A431, a cell line that constitutively expresses 12-LOX (12). 12-LOX-transfected PC3 cells also had higher levels of 12-LOX protein than neo controls or wild-type PC3, as revealed by Western blot analysis (Fig. 1B). Among the various clones analyzed, nL-2, nL-8, nL-11, and nL-12 expressed 12-LOX at the highest levels. We also found that 12-LOX-transfected PC3 clones nL-2, nL-8, and nL-12 synthesized 6–10-fold more 12(S)-HETE than the neo control or wild-type PC3 cells (Fig. 1C), indicating that 12(S)-HETE biosynthesis was greatly enhanced in 12-LOX-transfected PC3 cells.

**12-LOX Transfectants Have an In Vivo but not an In Vitro Growth Advantage.** In vitro, the growth rates of several 12-LOX transfecant clones were similar to those of neo controls and wild-type PC3 cells (Fig. 2A), with an approximate doubling time of 36 h. However, following s.c. injection into nude mice, 12-LOX-transfected PC3 cells grew faster and formed larger tumors than did neo controls (neo-α and neo-α; Fig. 2B). As shown in Fig. 2C, tumors derived from 12-LOX-transfected PC3 cells were larger than those obtained from neo controls, indicating that 12-LOX-transfected PC3 cells had an in vivo growth advantage compared to neo controls or wild-type PC3 cells. Similar results were obtained with an additional 12-LOX transfecant clone tested (nL-8; data not shown). Assessment of tumor necrosis from PC3 cells transfected with 12-LOX cDNA revealed that tumor necrosis was significantly reduced in the tumors derived from 12-LOX-transfected PC3 cells (P < 0.05 by Student’s t test), whereas 12.1% of tumor area of neo-α tumors were necrotic (n = 7; range, 5–35%), only 1.9% of tumor area in the nL-12 clone was necrotic (n = 8; range, 0–10%). A significant decrease in tumor necrosis was also observed in the tumors derived from 12-LOX transfecants nL-2 and nL-8, compared to neo-α (data not shown), suggesting that the increased tumor growth by 12-LOX transfecants is mainly due to the reduction of tumor necrosis.

**Increased Angiogenesis in the Tumors from 12-LOX Transfectants.** Because angiogenesis plays an important role in tumor growth by influencing tumor necrosis and apoptosis (2), we studied whether the increased tumor growth by 12-LOX transfecant is angiogenesis

![Fig. 1. Generation of PC3 transfecant synthesizing high levels of 12-LOX and 12(S)-HETE. The transfecant of PC3 cells and the cloning of stable transfecants were performed as described in "Materials and Methods." A, Northern blot analysis of 12-LOX mRNA levels in various clones of PC3 12-LOX transfecants. Top, blot probed with 12-LOX cDNA; bottom, blot probed with actin cDNA as the loading control. B, Western blot analysis of 12-LOX protein expression in various clones of PC3 12-LOX transfecants. The blot was probed with a 12-LOX polyclonal antibody and actin antibody. C, 12(S)-HETE levels in various 12-LOX transfecants. The levels of 12(S)-HETE in total cell lysates were measured using RIA and were normalized to cell number and expressed as pg of 12(S)-HETE/1 × 10^6 cells.](image-url)
12-LOXYGENASE, ANGIOGENESIS, AND TUMOR GROWTH

Fig. 2. 12-LOX transfectants have an in vivo but not in vitro growth advantage. A, growth kinetics of PC3 transfectants in culture. Cell proliferation of various transfectants was measured as described in “Materials and Methods.” Shown here are the growth curves of PC3 wild type (C), neo-α (Θ), nL-8 (V), and nL-12 (A). Data points, means of six determinations; bars, SE. Other clones such as nL-2 and neo-α also had similar growth kinetics (data not shown). B, growth kinetics of the tumors derived from 12-LOX transfectants and neo controls. Data points, mean volumes of eight tumors for nL-12 (Θ) and neo-α (C), five tumors for nL-2 (□) and neo-α (V), and six tumors for PC3 wild type (○); bars, SE. C, mice with tumors from 12-LOX transfectants or from neo control. Left, three mice with tumors from neo-α (arrow); right, three mice bearing tumors from 12-LOX-transfected PC3 cells (nL-12; arrows).

dependent. We found significant vascularization in tumors derived from 12-LOX-transfected PC3 cells, whereas the neo control tumors showed little vessel penetration (Fig. 3A). Immunostaining with CD31 antibody, which detects the presence of endothelial cells, showed that the vascular networks in tumors derived from nL-12 were sinusoidal in pattern and well developed in structure (Fig. 3B, right). In contrast, in neo control tumors, endothelial cells were present but were randomly distributed and did not form an organized vascular network (Fig. 3B, left). There were fewer vessels in neo-α tumors than in nL-12, as suggested by microvessel density (Fig. 3C). The assessment of the vessel organization demonstrated that the majority of vessels in the tumors derived from 12-LOX-transfected PC3 cells were highly organized, whereas in those from neo-α, they showed a disorganized to intermediate pattern (Fig. 3D). In tumors derived from nL-2 and nL-8, we also observed a similar increase in angiogenesis when compared to neo-α (data not shown).

Increased Angiogenicity of 12-LOX Transfectants. The increased angiogenesis in the tumors generated from 12-LOX-transfected PC3 cells raises the question of whether the observed increase in angiogenesis is the cause or a consequence of the increased tumor growth. To address this issue, we first assayed the conditioned culture medium of PC3 12-LOX-transfected PC3 cells or neo controls for their ability to stimulate endothelial cell migration. As shown in Fig. 4A, the medium from the 12-LOX-transfected PC3 cells induced more RV-ECT migration than did the medium from neo controls. Under similar assay conditions, 12(S)-HETE itself also stimulated RV-ECT migration at nanomolar levels (Fig. 4B). The increased angiogenicity of 12-LOX transfectants was confirmed by the Matrigel implantation assay. As shown in Fig. 4C, within 12 days, 12-LOX-transfected PC3 cells (nL-12) in Matrigel induced massive angiogenesis, indicated by the accumulation of blood in the gel, compared to the neo control (neo-α). The results clearly illustrate that the 12-LOX-transfected PC3 cells are more angiogenic than their neo controls.

Discussion

Here, we found that the increased expression of 12-LOX in human PCa cells stimulates prostate tumor growth by enhancing their angiogenicity. The findings have significant bearing on the regulation of PCa progression because, in patients diagnosed with prostate carcinoma, some tumors are extremely malignant, with rapid progression, whereas others are localized and dormant for many years. Exploration of the mechanism underlying the transition from latent to rapidly growing PCa will provide useful information for PCa management.
Fig. 3. Increased angiogenesis in the tumors from 12-LOX-transfected PC3 cells. A, tumor morphology. Left, two tumors from neo-α; right, two tumors from 12-LOX-transfected PC3 cells (nL-2). ×8. B, CD31 immunostaining. Brown, positive staining. Left, control tumor. Note the scattered vascular spaces which are randomly distributed and do not form a structured vascular network. Right, tumor from 12-LOX-transfected PC3 cells. Note the numerous vascular channels showing a highly organized sinusoidal pattern surrounding small nests of tumor cells. ×250. C, microvessel density. Columns, microvessel densities, expressed as the average number of vessel-like structures crossing an arbitrary line in one HPF; bars, SE. Note the significant increase in microvessel density in the tumors derived from nL-12 (n = 7) as compared in those of neo-α (n = 7; *, P < 0.05 by Student’s t test). D, organization of intratumoral blood vessels. The vessel organization was scored as described in “Materials and Methods.” Columns, mean scores of tumors derived from nL-12 (n = 7) and neo-α (n = 7); bars, SE (**, P < 0.01 by Student’s t test).

Our observations here, together with our previous demonstration of the correlation between 12-LOX expression and PCa progression in clinical samples (9), suggest that 12-LOX may play a critical role in the progression of human PCa.

The increased tumor growth observed with 12-LOX-transfected PC3 cells is due to the reduction in tumor necrosis as a result of increased angiogenesis. The increased 12-LOX levels in PC3 cells did not confer a growth advantage in vitro, suggesting that 12-LOX overexpression does not have direct effect on PC3 cell growth and that the growth advantage of 12-LOX transfectants in vivo is due to the host environment. This tumor-host interaction based mechanism is supported by the observed increase in angiogenesis in the tumors from 12-LOX-transfected PC3 cells. Because angiogenesis is required for tumor expansion, the lack of or inhibition of angiogenesis has been demonstrated to induce tumor cell necrosis and apoptosis, thereby limiting tumor growth in PCa (2, 6–7). Indeed, histological analysis revealed that the tumors derived from neo controls had increased necrosis, suggesting that it is the insufficient vascularization that limited the growth of the neo control tumors.

The increased angiogenesis in the tumors from 12-LOX-transfected PC3 cells is at least partly due to their increased angiogenicity. 12-LOX-transfected PC3 cells have increased ability to stimulate endothelial cell migration in vitro and neovascularization of Matrigel in vivo, compared to their neo controls. The angiogenicity of tumor cells is controlled by the balance between stimulators and inhibitors of angiogenesis (2). Therefore, it will be interesting to determine how 12-LOX up-regulates the angiogenicity of PCa cells. One explanation is that 12-LOX or 12(S)-HETE may increase the angiogenicity of tumor cells by influencing the expression of angiogenic or angiostatic molecules. An alternative interpretation is that 12(S)-HETE may directly alter the balance in favor of angiogenic factors due to its proangiogenic nature. This is supported by our finding here that 12(S)-HETE stimulated endothelial cell migration at nanomolar levels and previous reports showing that 12(S)-HETE stimulated endothelial cell proliferation (14), retraction (15), and adhesion and that it increased the surface expression of integrin α5β3 in both macro- and microvascular endothelial cells (16). It is noteworthy that integrin α5β3 is predominantly associated with angiogenic blood vessels (17) and plays an essential role in human cancer angiogenesis (18). Thus, 12(S)-HETE may directly increase the angiogenicity of PCa cells by stimulating angiogenesis or by eliciting several proangiogenic responses that can be additive or synergistic to effects from other angiogenic factors produced by PCa cells because different factors have their own distinct effects on the process of angiogenesis (19). Studies are ongoing to determine whether increased 12-LOX expression in PCa cells influences the gene expression of angiogenic factors and whether 12(S)-HETE can stimulate angiogenesis alone or by its additive or synergistic interaction with other putative angiogenic factors.
Fig. 4. Increased angiogenicity of 12-LOX-transfected PC3 cells. A, stimulation of endothelial cell migration by the conditioned medium from 12-LOX transfectants. The conditioned media were harvested after 24 h of culture and used for migration assay as described in "Materials and Methods." Columns, average numbers of cells migrated per field; bars, SE (***, P < 0.01 by Student’s t test). B. 12(S)-HETE stimulates endothelial cell migration. The migration assay was performed essentially as described in A except that media with various levels of 12(S)-HETE, instead of the conditioned media, were placed into the lower chamber. Columns, means; bars, SE (**, P < 0.01 by Student’s t test). C, induction of angiogenesis in Matrigel by 12-LOX transfectants. Top, three Matrigel implants premixed with 2 x 10^6 neo-α cells. Note the vessel penetration into the gel; bars, SE (**, P < 0.01 by Student’s t test). Bottom, in contrast, the Matrigel premixed with 2 x 10^6 12-LOX transfectant (nl-12) demonstrates considerable blood accumulation.

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References

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