DLC1 Negatively Regulates Angiogenesis in a Paracrine Fashion

Yi-Ping Shih, Yi-Chun Liao, Yuan Lin, and Su Hao Lo

Abstract

The Rho GTPase-activating protein DLC1 is a tumor suppressor that is often deleted in liver cancer and downregulated in other cancers. DLC1 regulates the actin cytoskeleton, cell shape, adhesion, migration, and proliferation through its Rho GTPase-activating protein activity and focal adhesion localization. In this study, we silenced DLC1 in nonmalignant prostate epithelial cells to explore its tumor suppression functions. Small hairpin RNA-mediated silencing of DLC1 was insufficient to promote more aggressive phenotypes associated with tumor cell growth. In contrast, DLC1 silencing promoted pro-angiogenic responses through vascular endothelial growth factor (VEGF) upregulation, accompanied by the accumulation of hypoxia-inducible factor 1α and its nuclear localization. Notably, modulation of VEGF expression by DLC1 was dependent on epidermal growth factor receptor–MAP/ERK kinase–hypoxia-inducible factor 1 signaling but on RhoA pathways. Clinically, VEGF upregulation is a highly significant event in prostate cancers in which DLC1 is downregulated. Thus, our results strongly suggest that loss of DLC1 may serve as a "second hit" in promoting angiogenesis in a paracrine fashion during tumorigenesis. Cancer Res; 70(21); 8270-5. ©2010 AACR.

Introduction

Deleted in liver cancer 1 (DLC1) is a tumor suppressor that was originally identified in primary hepatocellular carcinoma (1). In addition to liver cancer, the loss or reduction of DLC1 expression due to gene deletion or promoter methylation has been reported in lung, prostate, breast, kidney, colon, uterus, ovary, and stomach cancers (2–4). Mutations that altered the expression and function of DLC1 were detected in pancreas (5), colon, and prostate cancers (6). DLC1 is shown to regulate actin cytoskeleton and focal adhesion organizations, cell shape, adhesion, migration, proliferation, and apoptosis (2–4). These functions may directly contribute to the suppressive activities of DLC1 in tumorigenicity and metastasis (2–4). The RhoA pathway negatively regulated through the Rho GTPase-activating protein domain of DLC1 is believed to be critical for these functions, which were mainly analyzed by ectopic expression approaches in cancer cell lines.

Angiogenesis is the formation of new blood vessels from the existing vasculature and is essential for the growth of the primary cancer and for the formation of metastasis (7). Vascular endothelial growth factor (VEGF) plays a major role in tumor angiogenesis. It can promote the proliferation, survival, and migration of endothelial cells and is essential for blood vessel formation (8). Hypoxia is the strongest stimulus for triggering VEGF expression in cancer cells. Nonetheless, many cancer cell lines express high levels of VEGF in normoxia (9).

Here, we have discovered a novel function of DLC1 in regulating angiogenesis. Silencing of DLC1 in nonmalignant prostate epithelial cells leads to the upregulation of VEGF which promotes angiogenesis in vivo and in vitro. This up-regulation of VEGF is mediated through the epidermal growth factor receptor (EGFR)–MAP/ERK kinase (MEK)–hypoxia-inducible factor 1 (HIF1) pathway. Clinically, upregulation of VEGF is highly associated with decreased DLC1 in prostate cancer.

Materials and Methods

Cell culture and reagents

MLC-SV40 kindly provided by Dr. Johng Rhim (Center for Prostate Disease Research, Bethesda, MD; ref. 10), and RWPE-1 cells purchased from the American Type Culture Collection (CRL-11609) were cultured in keratinocyte serum-free medium (Invitrogen). Human vascular endothelium cells (HUVEC) from American Type Culture Collection (CRL-1730) were cultured in endothelial cell growth medium (Genlantis). Cell lines were used within 3 months after receipt or resuscitation of frozen aliquots. The authenticity of these cell lines was assured by the provider by cytogenetic analysis. No additional test was done specifically for this study. Lipofectamine-2000 (Invitrogen) was used for transfections. Stable shGFP or shDLC1 cells were generated by infection with small hairpin RNA lentiviruses against...
with 500 μg Adenoviruses prior to experiments. Blocking antibody (R&D) at room temperature for 1 hour.

Conditioned medium was incubated with 1 μg anti-VEGF blocking antibody (R&D, clone 26503) for 30 minutes, followed by incubation at 37°C for 18 hours and then counted. For VEGF blockade, conditioned medium was incubated with 1 μg anti-VEGF blocking antibody (R&D) at room temperature for 1 hour.

Migration assay
Growth factor-reduced Matrigel (BD Biosciences) containing 60 units/mL of heparin (Sigma-Aldrich) and 1 mM reduced Matrigel (BD Biosciences) containing 60 units/mL of heparin (Sigma-Aldrich) was mixed with 2 × 10^6 cells, and s.c. injected into nude mice. After 5 days, cell plugs were harvested and embedded in optimal cutting temperature compound for immunohistochemical staining using CD31 antibody and VEGF antibody.

In vitro Matrigel angiogenesis assay
Growth factor-reduced Matrigel was used to coat a 96-well plate (50 μL/well) and HUVECs (20,000 cells/well) were seeded with conditioned medium (200 μL). After 4 hours of incubation, capillary-like structures were scored by measuring the lengths of tubes per field in each well at ×100 magnification with ImageJ software (NIH).

Aortic ring assay
Thoracic aortas from C57BL/6 mice were dissected and transferred to ice-cold PBS. The fat tissue was removed and 1-mm-long aortic rings were sectioned and embedded in growth factor-reduced Matrigel. Rings were cocultured with 500 μL of conditioned medium with or without 1 μg of anti-VEGF blocking antibody (R&D, clone 26503) for 8 days, and the outgrowth of endothelial tubes was counted.

Migration assay
HUVECs (80,000 cells) were added to the upper chamber in each transwell. Conditioned media (400 μL) were added to the lower chamber. Cells were fixed and stained 5 hours later. Cells migrated to the bottom surface were visualized microscopically and photographed. For VEGF blockade, conditioned medium was incubated with 1 μg of anti-VEGF blocking antibody (R&D) at room temperature for 1 hour prior to experiments.

Adenoviruses
Human DLC1 cdNA was subcloned into pENTRA1 vector and the DLC1/pENT vector was used in a site-directed recombination reaction to place DLC1 cDNA into the pAD/CMV/V5-DEST vector (Invitrogen). The adenoviral expression plasmid was transfected into 293A cells. After 10 to 12 days, the crude viral lysate was harvested and used for infection.

Immunohistochemical staining, scoring, and microvascular density counting
Prostate normal/cancer tissue arrays (Imgenex) were de-waxed and rehydrated. After antigen retrieval, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 20 minutes followed by normal serum blocking. Slides were incubated at 4°C overnight with anti-DLC1 (1:50, clone 3; BD Biosciences), anti-VEGF (1:50, SC152; Santa Cruz Biotechnology), or anti-CD31 (1:200, 01951D; PharMingen). Signals were detected with Vectastain ABC Elite Kit (Vector Laboratories) and diaminobenzidine substrate. Slides were counterstained with hematoxylin. Images were observed by Zeiss Axiosplan2 microscope. For microvascular density counting, the vessels were detected by CD31 immunostaining and mean values of the vessel count were calculated using the average of the three most intense vascularization areas at 200× magnification. Tissue array immunoreactivities were scored by the intensity of the staining (0, no staining; 1, weak; 2, moderate; 3, strong) and the percentage of stained cells (0, no staining; 1, 1–10%; 2, 10–50%; 3, 50–80%; 4, >80%). By multiplication of both values, a final score ranging between 0 and 12 was obtained.

Results
Silencing of DLC1 in nonmalignant prostate epithelial cells does not promote tumorigenicity but does enhance angiogenesis

To investigate the loss of function of DLC1 in normal prostate epithelial cells, we have generated DLC1 knockdown (shDLC1) by small hairpin RNA in the nonmalignant prostate epithelial cell line, MLC-SV40. DLC1 protein level was significantly reduced in shDLC1 than control shGFP cells, and as expected, RhoA activity was enhanced due to the reduction of the negative regulator DLC1 (Fig. 1A). To examine whether the loss of DLC1 enhanced their tumorigenicity, we injected shDLC1 or shGFP cells into nude mice. None of them developed tumors during 3 months of observation (data not shown). Interestingly, we detected increased small blood vessels (CD31 positive) around the injected shDLC1 cells at 5 days postinjection, and this was confirmed by significantly higher microvascular density in shDLC1 (Fig. 1B and C). We have generated an additional pair of shDLC1 and shGFP in another nonmalignant prostate cell line, RWPE-1. Similar results were observed using this pair of RWPE-1 cells throughout this project (Fig. 1A) and only data from MLC-SV40 cells are shown. These results suggest that silencing of DLC1 did not promote the proliferation of prostate epithelial cells, but somehow, it enhanced the angiogenic responses of endothelial cells.

DLC1 negatively regulates VEGF expression
Because VEGF is the major proangiogenic factor, we examined VEGF levels in xenografts by immunohistochemical staining and found significantly elevated VEGF staining in injected shDLC1 cells, which were accompanied by CD31-positive endothelial cells (Fig. 1B and C). In cultured cells, VEGF mRNA and protein levels were upregulated in shDLC1 cells and the conditioned medium from shDLC1 also contained higher levels of VEGF (Fig. 2A), confirming that lack of DLC1 promotes VEGF expression and secretion. Furthermore, shDLC1 conditioned medium enhanced angiogenic-related responses of endothelial cells by forming more tube-like networks, faster cell migration, and more sprouting of capillaries (Fig. 2B). These effects were significantly reduced when anti-VEGF blocking antibody was applied...
However, adding a similar amount of recombinant VEGF (500 pg/mL) to fresh medium did not have the same effects as detected with shDLC1-conditioned medium, suggesting that either recombinant VEGF was not as potent as endogenous VEGF or that there might be additional factor(s) in the conditioned medium that also contribute to the observed effects. Re-expression of small hairpin RNA-resistant DLC1 in shDLC1 markedly suppressed VEGF expression (Fig. 2C). A similar effect was detected when re-expression of DLC1 in prostate cancer cell lines, including LnCap, DU145, and even VEGF low-expressing PC3 cells (Fig. 2C), confirming that DLC1 negatively regulates VEGF expression.

**DLC1-regulated VEGF expression is mediated through the EGFR-MEK-HIF1 pathway but not the RhoA pathway**

Because Rho GTPase-activating protein activity is critical for a variety of regulatory functions of DLC1, including cell shape, migration, and tumorigenicity, we test whether VEGF upregulation in shDLC1 cells is regulated through the RhoA pathway. By overexpression of RhoA wild-type, constitutive active and dominant negative mutants in MLC-SV40 cells, or silencing of RhoA expression in shDLC1 cells, VEGF levels were not affected (Supplementary Fig. S1), suggesting that DLC1-mediated VEGF expression is not regulated by the RhoA pathway.

To investigate the potential pathway(s) involved, several pharmacologic inhibitors were used. Although Jak (AG490) inhibitor had no effect on VEGF expression, inhibitors to EGFR (AG1478, AG1517) and MEK (PD98059, U0126) markedly reduced VEGF expression in shDLC1 cells in a dose-dependent manner (Fig. 3A). These inhibitor dosages have no effect on cell proliferation (Supplementary Fig. S2). Because HIF1α is a major transcription factor that mediates VEGF expression, we measured the protein and subcellular localization of HIF1α. Indeed, HIF1α protein level was increased and accumulated in the nuclei of shDLC1 cells (Fig. 3B and C). Concomitantly, increased EGFR (Tyr1068), MEK (Ser217/221), and ERK (Thr202/Tyr204) phosphorylation levels were detected (Fig. 3B). In addition, HIF1α protein levels and nuclear localization were markedly reduced by EGFR or MEK inhibitors (Fig. 3B–D). Furthermore, silencing of EGFR significantly decreased MEK and ERK phosphorylation, as well as VEGF expression in shDLC1 (Fig. 3D). These data suggest that DLC1-mediated VEGF expression is modulated through the EGFR-MEK-HIF1 pathway.
Downregulation of DLC1 is associated with upregulation of VEGF in prostate cancer

To examine DLC1 and VEGF expression patterns in clinical samples, we have stained DLC1 and VEGF in consecutive prostate tissue sections. By immunohistochemical analysis, DLC1 is highly expressed in normal prostate epithelial cells, whereas VEGF expression is very weak, if present (Supplementary Fig. S3). In prostate cancer specimens,
DLC1 expression was reduced in 86.3% (107 of 124), whereas VEGF protein level was increased in 75.8% (94 of 124) of tumor samples (Table 1). When comparing the expression of VEGF in DLC1 downregulated versus no change prostate cancer samples, increased VEGF expression is a statistically significant event in DLC1 downregulated prostate cancer.

**Discussion**

Our current studies show that loss of DLC1 tumor suppressor alone is not sufficient for tumorigenesis. This is in agreement with the finding that DLC1 knockdown had little effect on the colony formation of liver progenitor cells in vitro but had efficiently promoted tumor development in Myc expressing and lacking p53 liver progenitor cells engrafted into mouse livers (11). Although lack of DLC1 does not increase cell tumorigenicity, it enhances angiogenesis, which plays an essential role in cancer progression. Tumor growth and metastasis are dependent on angiogenesis, and the preventions of tumor angiogenesis and/or metastasis are perceived as attractive approaches in the regulation of tumor progression. Because DLC1 also prevents cancer cell migration and metastasis (12, 13), understanding various DLC1-mediated pathways might offer potential targets for both angiogenesis and metastasis.

**Table 1.** DLC1 and VEGF expressions in prostate cancers

<table>
<thead>
<tr>
<th>Total, 124 cases</th>
<th>VEGF up (94)</th>
<th>VEGF down or no change (30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLC1 down (107)</td>
<td>78/124 (62.9%)</td>
<td>29/124 (23.4%)</td>
</tr>
<tr>
<td>DLC1 up or no change (17)</td>
<td>16/124 (12.9%)</td>
<td>1/124 (0.8%)</td>
</tr>
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*NOTE: Immunohistochemical analysis comparing prostate cancer to normal tissues indicated that increased VEGF expression in DLC1 downregulated prostate cancer was statistically significant. Fisher’s exact test (P = 0.046).*
metastasis by enhancing the function of DLC1 or by suppressing its negatively regulated downstream molecules. Interestingly, our recent finding (14) showed that DLC2 knockout mice display enhanced angiogenic responses induced by Matrigel or tumor cells. These data suggest that the DLC family may play various roles in angiogenesis.

Other focal adhesion molecules may regulate VEGF expression. For example, overexpression of integrin-linked kinase stimulates VEGF expression, whereas silencing of integrin-linked kinase reduces VEGF production in prostate cancer cells (15). TM45FS, a transmembrane protein that interacts with integrins, regulates VEGF expression through an integrin α5-Src-STAT3 pathway (16). Both integrin-linked kinase and TM45FS positively regulate VEGF expression, whereas DLC1 negatively controls VEGF production. This negative regulation of DLC1 seems to be mediated through-out EGFR signaling, which is known to regulate the synthesis and secretion of VEGF in cancer cells. Maity and colleagues (17) showed that EGFR activation regulates VEGF in glioblastoma cells through the phosphoinositide-3-kinase pathway but not the mitogen-activated protein kinase pathway. In

head and neck squamous carcinoma, it is dependent on MEK/mitogen-activated protein kinase but not phosphoinositide-3-kinase pathways (18). These findings suggest that different mechanisms are involved in the ability of EGFR signaling in modulating VEGF expression in various types of cancer cells. Our data indicate that lack of DLC1 upregulates VEGF in an EGFR-MEK-HIF1-dependent fashion. Currently, we are investigating how and whether DLC1 directly or indirectly regulates the activity of EGFR.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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