Loss of Fibroblast HIF-1α Accelerates Tumorigenesis

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Abstract

Solid tumors consist of malignant cells and associated stromal components, including fibroblastic cells that contribute to tumor growth and progression. Although tumor fibrosis and aberrant vascularization contribute to the hypoxia often found in advanced tumors, the contribution of hypoxic signaling within tumor-associated fibroblasts to tumorigenesis remains unknown. In this study, we used a fibroblast-specific promoter to create mice in which key hypoxia regulatory genes, including VHL, HIF-1α, HIF-2α, and VEGF-A, were knocked out specifically in tumor stromal fibroblasts. We found that loss of HIF-1α and its target gene VEGF-A accelerated tumor growth in murine model of mammary cancer. HIF-1α and VEGF-A loss also led to a reduction in vascular density and myeloid cell infiltration, which correlated with improved tumor perfusion. Together, our findings indicate that the fibroblast HIF-1α response is a critical component of tumor vascularization.

Introduction

Tumor-associated stromal alterations influence the tumor microenvironment and significantly contribute to tumor growth and progression (1). Recently, it has also become clear that stroma coevolves with tumor cells (2, 3), and that stromal fibroblasts are a prominent cell type in that process (4, 5). Unlike fibroblasts residing in normal tissues, tumor-associated fibroblasts exhibit distinctive tumor-promoting features: they release growth and angiogenic factors, recruit inflammatory cells, and remodel extracellular matrix (6–11).

Hypoxia, or low oxygen tension, is a significant characteristic of the tumor microenvironment. An inadequate blood supply to tumor tissue frequently results in the formation of hypoxic and necrotic regions. This in turn triggers the expression of hypoxia-inducible factors (HIFs; refs. 12 and 13). HIFs are heterodimeric helix-loop-helix transcription factors that are composed of 2 subunits: a constitutively expressed HIF-β subunit, and an oxygen-dependent HIF-α subunit. The HIF-α proteins are posttranslationally regulated by oxygenation (14–16), through a complex containing the pVHL tumor suppressor (17).

Virtually all rapidly growing solid tumors contain multiple transient or chronic areas of severe hypoxia (oxygen tensions of less than 1%), and HIF-α levels are highly correlated with other prognostic markers of cancer, mortality, and metastasis (18–20). To determine how hypoxic signaling in tumor-associated fibroblasts affects tumorigenesis, a series of fibroblast-specific conditional knockout mice was created using an FSP-cre strain (6, 10, 21, 22). It was found that ablation of HIF-1α in stromal fibroblasts enhanced both tumor growth and perfusion while reducing vascular density. Fibroblast-specific deletion of VEGF-A had similar effects, indicating that hypoxic signaling and VEGF-A expression in stromal fibroblasts is an important aspect of solid tumor progression.

Materials and Methods

Cell culture

Mammary epithelial carcinoma cells from female, FVB strain mice carrying the MMTV-PyMT transgene (23, 24) were isolated by digestion of advanced mammary tumors with collagenase and hyaluronidase. Cells were cultured in DMEM with 10% FBS and 1% penicillin–streptomycin in a humidified incubator with 5% carbon dioxide. To isolate tumor-associated fibroblasts, digested cell suspensions from late stage tumors were plated on a Petri (bacterial culture) dish; cells adherent within an hour were harvested for genomic DNA analysis.

Transgenic mice

Mice in FVB background harboring an oncogenic Polyoma virus middle T transgene under the control of the promoter...
of an murine Moloney virus (MMTV) long terminal repeat (23, 24) were bred to mice carrying loxP recombinase recognition sites in the HIF-1α, HIF-2α, VHL, or VEGF-A genes (25–28). To induce fibroblast genetic ablation, mice with homozygous recombinase site-flanked alleles were bred to mice expressing Cre recombinase driven by the fsp1 promoter (6, 10, 21, 22). MMTV-PyMT transgenic mice in these backgrounds were then generated by breeding. All mice were backcrossed to a more than tenth-generation congenic FVB background.

Tissue processing, histology, immunohistochemistry, and immunofluorescence

Tumor-bearing mice were asphyxiated with CO₂ and perfused with PBS plus 5 mmol/L EDTA followed by 4% paraformaldehyde (PFA). Tumors were removed and further fixed with 4% PFA for 24 hours followed by embedding in paraffin. De-paraffinized and rehydrated 5-µm sections were stained for hematoxylin and eosin (H&E) and Mason’s trichrome, and immunostained with the following primary antibodies: monoclonal anti-HIF-1α (Cayman), biotinylated mouse anti-PCNA (BD Pharmingen), rat anti-CD34 (Abcam), rat anti-F4/80 (Serotec), mouse anti-αSMA (Chemicon), and rabbit anti-FSP1 (Millipore). Vectastain ABC (Vector Lab), EnVision kit, or CSA Kit (Dako) with DAB substrate were used to visualize primary antibody binding according to manufacturer’s instructions.

Quantitative image analysis

Immunostained images were captured by Leica DMR microscope and SPOT RT camera (Diagnostic Instruments Inc.) and positive staining was quantified as described previously (29, 30).

Detection of hypoxia by pimonidazole

Pimonidazole hydrochloride (60 mg/kg body weight) was injected intraperitoneally 90 minutes before sacrifice. Pimonidazole adducts were detected by a 1MAb1 mouse monoclonal antibody.

Determination of vascular permeability by Evans blue dye

Evans blue dye (100 µL of 0.5%) was injected intravenously 30 minutes before sacrifice. Tumors (0.5 g) were incubated in formamide for 24 hours at 37°C to quantify vascular permeability. Absorbance of extracted Evans blue dye was measured using a spectrophotometer at 620 nm.

Immunoprecipitation and immunoblotting

PyMT mammary tumors were lysed in T-PER tissue protein extraction reagent (Thermo Scientific) according to manufacturer’s instructions. VEGFR2 was immunoprecipitated by incubating 1 mg of total lysate with rabbit anti-VEGFR2 (55B11; Cell Signaling). Immunoprecipitated VEGFR2 was probed by immunoblotting for HRP-conjugated anti-phosphotyrosine (4G10; Millipore) to detect phosphorylated VEGFR2 or for anti-VEGFR2 (Santa Cruz Biotechnology) to detect total VEGFR2. To quantify phosphorylated to total VEGFR2 ratios, immunoblot PVDF membranes were scanned with a Typhoon fluorescence scanner and the signals determined using ImageQuant software.

RNA, genomic DNA, and qPCR

Total RNA was isolated from mammary tumors using TRIzol reagent (Invitrogen) and subjected to 1-step quantitative RT-PCR (Applied Biosystems) to determine VEGF-A mRNA levels.

To determine the deletion efficiency of HIF-1α in stromal fibroblasts and gene expression in macrophages, genomic DNA, and total RNA were extracted from thioglycollate-driven...
peritoneal macrophages and F4/80-positive tumor-associated macrophages isolated by magnetic beads (Stem Cell Technology). Quantitative PCR (Applied Biosystems) was conducted using specific primers and probes (Supplementary Table S1 for sequences).

Subcutaneous tumor growth

Mammary epithelial carcinoma cells (1 × 10⁷ cells) were injected subcutaneously in accordance with institutional IACUC-approved protocols. Total tumor volume was calculated by the formula: volume = (width)² × length/2.

Figure 2. Deletion of stromal fibroblast HIF-1α promotes mammary tumor growth and progression. A, total mammary tumor mass of WT (PyMT;Hif-1αloxP/loxP) and fibroblast HIF-1α-null (PyMT;Fsp-cre;Hif-1αloxP/loxP) mice measured at the indicated age. B, total isograft tumor burden at 2 weeks postsubcutaneous injection of tumor cells into WT (Hif-1αloxP/loxP) and fibroblast HIF-1α–null (Fsp-cre;Hif-1αloxP/loxP) mice. C, immunostaining for PCNA in tumor sections from 15-week-old WT (PyMT;Hif-1αloxP/loxP) and fibroblast HIF-1α–null (PyMT;Fsp-cre;Hif-1αloxP/loxP) mice; scale bars, 100 μm. D, quantification of PCNA-positive areas in sections represented in C. E, representative images of H&E-stained mammary tumors collected from 15-week-old WT (PyMT;Hif-1αloxP/loxP) and fibroblast HIF-1α–null (PyMT;Fsp-cre;Hif-1αloxP/loxP) mice; scale bars, 100 μm. F, histologic stage distribution of mammary tumors from 15-week-old WT (PyMT;Hif-1αloxP/loxP) and fibroblast HIF-1α–null (PyMT;Fsp-cre;Hif-1αloxP/loxP) mice.
Statistical analysis

Unpaired Student t tests were used for statistical analyses. Data are expressed as mean ± SEM unless otherwise stated.

Results

Specificity of genetic ablation in fibroblasts

Deletion in fibroblasts used mice with various conditional alleles and mice that express Cre recombinase under the control of the fsp1 promoter (fsp1-cre; refs. 6, 10, 21, 22). These mice were bred into mice with the transgenic polyoma middle T gene (PyMT) under the control of the promoter of the mouse mammary tumor virus (MMTV) long terminal repeat: these develop advanced epithelial carcinomas in all mammary glands by the age of 15 weeks when on the FVB strain background (all mice in this study were maintained and evaluated in the FVB strain; refs. 23 and 24).

Pimonidazole staining showed regions of hypoxia (oxygen tension of less than 10 mmHg or ~2%) in stromal and...
malignant epithelial areas in this tumor type (Fig. 1A). To determine the efficacy of deletion in fibroblasts, tumors from wild-type (WT) mammary tumor-bearing mice were isolated and cell suspensions from were placed on a nontreated sterile polystyrene surface, followed by harvesting of adherent cells within an hour. This procedure enriches for fibroblasts, and quantitative PCR analysis of genomic DNA from this population showed an approximate rate of deletion of 50% (Fig. 1B). This was likely an underrepresentation of the total deletion efficiency, because of the relative impurity of this population of cells. As other transgenic alleles of fsp-cre have been reported to induce deletion in myeloid cells, an analysis of peritoneal macrophages from mutant mice was carried out; this showed no evidence of deletion of conditional alleles (Supplementary Fig. S1A–S1C), indicating that this population of macrophages is not affected by the expression of this allele.

Deletion of HIF-1α alone in fibroblasts accelerates mammary tumor growth and progression

To determine how mammary tumor progression is influenced by fibroblast-specific deletion of HIF pathway factors, total mammary tumor mass was analyzed from mice-bearing mutant alleles of HIF-1α, HIF-2α, and a negative regulator of HIF-α’s, the VHL tumor suppressor gene (25–28). These analyses found no difference in rate of tumor progression in the VHL deletion and HIF-2α deletion animals; however, tumor mass was found to be significantly greater in HIF-1α mutant mice relative to control mice carrying the MMTV-PyMT transgene at 15 weeks of age (Fig. 2A; Supplementary Figs. S2A, S3A, and S3B).

To confirm these latter findings in a subcutaneous tumor model, syngeneic mammary epithelial tumor cells were injected into WT and fibroblast HIF-1α-null mice. Tumor growth was again significantly accelerated in fibroblast HIF-1α-null mice compared with WT controls (Fig. 2B; Supplementary Fig. S2B), suggesting that the protumorigenic effect of HIF-1α deletion in fibroblasts is not restricted to the transgenic mammary tumor model.

Enhanced tumor growth in fibroblast HIF-1α-null mice was associated with greater tumor cell proliferation, as determined by proliferating cell nuclear antigen (PCNA) staining (Fig. 2C and D). Histologic tumor stage analysis
was done as previously described (23), and revealed a greater level of advanced carcinoma in fibroblast HIF-1α-null tumors, and a greater level of premalignant hyperplasia and adenoma in WT tumors (Fig. 2E and F). These data suggest that deletion of HIF-1α in fibroblasts promotes tumorigenic progression.

**Deletion of HIF-1α in fibroblasts reduces tumor vascular density**

As shown in many studies (31), advanced malignancy is typically accompanied by high vessel density and a distinctive and generally abnormal vessel structure, characterized by spatially disorganized, highly dilated, and tortuous blood vessels (Fig. 3A). In transgenic mammary tumors from animals lacking fibroblast HIF-1α, vascular structures have a reduced density (Fig. 3B–D). This relatively diminished microvasculature was also seen in subcutaneous tumors created via injection of mammary tumor cell lines into fibroblast HIF-1α-null mutants relative to WT mice (Fig. 3E and F).

Although levels of VEGF-A mRNA, as determined by qPCR, were similar in WT and fibroblast HIF-1α-null mammary tumors (Fig. 3G), activation of the key VEGF receptor, VEGFR2, was decreased in fibroblast HIF-1α-null mammary tumors when compared with WT controls (Fig. 3H). Quantitative analysis showed a reduced ratio of phosphorylated VEGFR2 to total VEGFR2 in fibroblast HIF-1α-null mammary tumors versus tumor extract from WT control mice (Fig. 3I). These data suggest that deletion of fibroblast HIF-1α suppresses VEGFR2 signaling, without altering the...
Deletion of HIF-1α levels in mammary tumors, perhaps acting through localized alterations in VEGF-A signaling. Tumor vasculature often has reduced levels of pericytes, which provide structural and functional support for vessel-lining endothelial cells. Representative double immunofluorescence staining for CD34 and the pericyte marker α-smooth muscle actin (αSMA) indicates that fibroblast HIF-1α-null tumors contain more αSMA-positive blood vessels than WT control tumors (Supplementary Fig. S4A). Consistently, mRNA expression of the pericyte markers, PDGFR and αSMA, is elevated in total lysates of fibroblast HIF-1α-null tumors relative to levels seen in WT control tumors (Supplementary Fig. S4B).

Deletion of HIF-1α in fibroblasts improves blood perfusion
To determine whether the vasculature in fibroblast HIF-1α-null tumors was functionally altered, tumors in fibroblast HIF-1α-null tumors and those derived from WT controls were stained with the hypoxia marker pimonidazole: levels of this indicator were decreased in fibroblast HIF-1α-null tumors compared with WT controls (Fig. 4A and B), indicating improved perfusion and decreased tumor hypoxia.

Tumor blood vessel permeability was also reduced in fibroblast HIF-1α-null tumors; intravenous injection of Evans blue dye, which can be used to evaluate vascular permeability, showed significantly reduced levels of the dye had leaked from subcutaneous tumors in fibroblast HIF-1α-null mice, relative to the levels seen in tumor-bearing WT control mice (Fig. 4C).

Deletion of HIF-1α in fibroblasts attenuates tumor-associated macrophage infiltration
Numerous studies have previously showed that infiltration of macrophages is a characteristic of tumor progression in many cancers, especially during the malignant transition that accompanies the "angiogenic switch" (32, 33). To determine the effect of fibroblast-specific HIF-1α deletion on tumor-associated macrophage recruitment, immunostaining for the macrophage marker F4/80 was carried out (Fig. 5A–D) on transgenically induced mammary tumors from fibroblast HIF-1α-null mice and those from WT controls. This assay showed a significant decrease in tumor-associated macrophages in fibroblast HIF-1α-null mutant tumors, and suggests that loss of stromal fibroblast HIF-1α attenuates tumor-associated macrophage infiltration.

Deletion of VEGF-A in fibroblasts results in increased subcutaneous tumor growth
Given that VEGF-A is chemotactic for macrophages in mammary tumors, and that blocking VEGF-A activity normalizes tumor vasculature (29, 34–37), it was important to determine whether fibroblastic VEGF-A expression is also linked to acceleration of tumorigenesis. After injection of mammary epithelial tumor cells, it was found that subcutaneous tumor masses were significantly greater in fibroblast VEGF-A-null mice compared with WT controls (Fig. 6A and B). It was also found that when fibroblast VEGF-A-null mice carried the MMTV-PyMT transgene,
mammary tumor blood vessels exhibited decreased micro-vessel density compared with WT controls. This was associated with reduced vessel diameters (Fig. 6C, top, and D).

Targeted loss of VEGF-A in fibroblasts also reduced tumor-associated macrophage recruitment into transgenically induced mammary tumors (Fig. 6C, bottom and D) but did not affect total tumor mass at 15 weeks of age (Supplementary Fig. S3C).

Discussion

Tumor–stromal cell interactions have been intensively investigated in the past several years, but are complex and remain undefined to a significant degree (3). The data presented here identifies stromal fibroblast HIF-1α signaling as a significant negative regulator of tumor development.

HIF-1α is generally regarded as a tumor-promoting transcription factor and targeted deletion of HIF-1α in malignant epithelial cells, endothelial cells or myeloid cells accordingly has been shown to suppress tumor growth (38–40). We show here, however, that deletion of HIF-1α or VEGF-A in fibroblasts enhances tumor growth.

Overexpression of angiogenic factors can lead to a deterioration of tumor vessels and result in a hyper-permeable and nonfunctional state (31). Blood vessels in fibroblast-null HIF-1α- or VEGF-A–null tumors showed evidence of decreased permeability and density, and tumors had reduced hypoxia and an increased level of growth and progression.

Tumor-associated macrophage infiltration is attenuated in stromal fibroblast-specific HIF-1α and VEGF-A–null mammary tumors in the current study, suggesting that stromal fibroblast-derived HIF-1α or VEGF-A mediates at least in part the proangiogenic macrophage infiltration of tumors. We have previously shown that myeloid cell-specific deletion of VEGF-A normalizes tumor vasculature and enhances tumor growth (29), and earlier studies have showed that infiltration of tumor-associated macrophages is required for tumor angiogenesis and metastatic dissemination (33, 41). It will be important to determine how the process of HIF-1α response in the fibroblast lineages targeted here influences more advanced aspects of tumorigenic progression, including those of invasion and metastasis. Further analysis may still reveal important roles for the HIF-α factors expressed by fibroblasts in processes such as premetastatic bone marrow recruitment of inflammatory cells.

In summary, this study shows the importance of the HIF-1 response in fibroblasts for both macrophage recruitment and infiltration, and for induction of alterations in tumor vasculature, with resulting effects on tumor perfusion and progression. There are key links between fibroblast function and inflammatory response that in turn affect tumor vascularization. Thus, fibroblast HIF-1α response is likely a critical factor in the link between malignancy, inflammation, and tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Evans, C. Stockmann, M.S. Ostrowski, R.S. Johnson

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