Tumor Interstitial Fluid Promotes Malignant Phenotypes of Lung Cancer Independently of Angiogenesis

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Abstract

Angiogenesis is necessary for cancer progression, but antiangiogenic therapy actually promotes tumor recurrence, progression, and metastasis. This study focused on the contribution of the tumor interstitial fluid (TIF) to lung cancer progression. TIF was isolated and quantified for 10 µg protein/mL. Malignant driver characteristics of TIF were examined by tumor-initiating cells (TIC), self-renewal, epithelial–mesenchymal transition (EMT), autophagy, and apoptosis in vitro. In vivo tumor model was used to investigate the mechanistic roles of TIF in lung cancer progression. In vitro, TIF exhibited distinct malignant driver characteristics, which led to increased numbers of TICs, increased self-renewal and EMT, as well as decreased autophagy and apoptosis under cell starvation conditions. In vivo, the contribution of TIF was similar, as judged by increased TICs indicated by the cancer stem cell marker Nanog, the proliferation marker proliferating cell nuclear antigen, and the EMT marker N-cadherin; TIF also increased the formation of pulmonary tumors. Interestingly, the blockers of inflammation, Na-K-ATPase, and aldosterone receptor decreased TIF-induced tumor progression but increased angiogenesis. Further, we found that the water content of the tissue was positively correlated with the levels of plasma 5-hydroxyindoleacetic acid or tissue aquaporin-1 but not with CD31. However, vadimezan reduced angiogenesis but promoted TIF-induced tumor progression. Our results suggested that TIF could provide better nutrition to the tumor than angiogenesis and that it could promote the development of malignant phenotypes of lung cancer independently of angiogenesis. Cancer Prev Res; 8(11); 1120–9. ©2015 AACR.

Introduction

Because all human cells including cancer cells need oxygen and nutrients to survive, it is widely believed that the starvation of tumor cells via restriction of the blood supply to tumor is a promising strategy to combat cancer (1). Based on this idea, antiangiogenic drugs, in particular, anti-VEGF agents have entered the clinical armamentarium against cancer (2). However, the clinical efficacy of antiangiogenic treatments is transient but significant, and most patients soon develop resistance to therapy. They may also develop new and unexpected toxicities, including hemorrhage, hypertension, gastrointestinal perforation, impaired wound healing, and increased risk of thrombosis (3–5). The mechanisms of these unexpected toxicities that are induced by antiangiogenic agents are not yet fully understood. However, the main hypothesis is that the removal of angiogenic factors may cause the tumor (and the surrounding tissue) to become more hypoxic and may render the body less able to respond to the cancer (6). In addition, more recent work suggests that antiangiogenic agents could accelerate the growth of metastases both in an adjuvant setting and in patients with established metastatic disease (7). These contradictory results prompted us to consider whether it is indeed necessary that tumors obtain energy from angiogenesis to grow, and if not, what is necessary for a tumor to receive a full complement of nutrients so that it may grow. To improve antinutrient therapies in patients with cancer, it is necessary to identify additional nutrient pathways that are important for tumor growth and to gain mechanistic insight into their contribution to cancer progression. There are two critical components of tumor growth and vascularization in the tumor itself and in the surrounding tissues: TIF and the interstitial fluid pressure (IFP; ref. 8). Both experimental and theoretical studies have shown that IFP is elevated in the tumors of patients with cancer, and whereas little TIF is found in tumors of patients with cancer, it is primarily present near the tumor boundary (9). TIF originates from blood plasma that extravasates from the capillaries through pores and intercellular clefts in the vessel wall. TIF bathes and surrounds human cells and provides them with nutrients and a means of waste removal (10). It is generally believed that elevated tumor TIF is partly responsible for the poor penetration and distribution of therapeutic agents in solid tumors, but the presence of complex active molecules and nutrients in TIF indicates that it may also play an important role in cancer progression (11). Because tumor-associated edema is one of the important complications that affect the clinical prognosis (12), we propose that the TIF surrounding the tumor is not only a

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necessary nutrient source provided by catabolic host cells to fuel the growth of adjacent cancer cell, but is also a significant contributor to metastasis via the maintenance of unhindered fluid communication between the blood and interstitial spaces in tumors. Here, we show that TIF promotes malignant phenotypes of lung cancer independently of angiogenesis and that antiedema therapies can (a) improve survival in tumor-bearing mice, (b) decrease malignant phenotypes of lung cancer, and (c) alleviate hypoxia.

Materials and Methods

Reagents

Proliferating cell nuclear antigen (PCNA), N-cadherin, Nanog, Vimentin, CD31, CD133, Aquaporin-1 (AQP1), E-cadherin, Cytokeratin 18, Aldosterone receptor, basic fibroblast growth factor (bFGF), EGF, heparin, trypsinization, acridine orange (AO), and ethidium bromide (EB) were obtained from Sigma. Paraformaldehyde, dexamethasone, digoxin, spirolactone, Evans blue, formamide, DAPI, and FBS were obtained from Sigma-Aldrich. Methylcellulose (MC), MTT, and dimethyl sulfoxide (DMSO) were obtained from Beijing Solarbio Science and Technology, Ltd. Enhanced Chemiluminescence Detection kit (ECL), 3',3'-diaminobenzidine (DAB), 3-aminomethylbenzylidene (ABC), and SABC-Rabbit-IgG-POD kit were obtained from Boster Biological Co. Ltd. RPMI1640 medium and glucose DMEM were obtained from Gibco Invitrogen. Mouse cytokeratin ROS kit, mouse cytokeratin TNE-6 kit, 5-HIAA kit, mouse cytokeratin h-CSR kit, and mouse cytokeratin 8-OHdG kit were obtained from Nanjing Jiancheng Bioengineering Institute.

Animals

The female C57BL/6J mice, 5 to 7 weeks of age, and experimental diets were obtained from Beijing Weitong Lihua Animal Co. All mice were housed in individual ventilated cages under a 12-hour light–dark cycle (lights on 7:00 AM to 7:00 PM). The animals were fed standard rodent chow and water and were maintained under pathogen-free conditions within the institutional animal facility. Food and tap water were given ad libitum. All procedures involving animals were approved by the Henan University Institutional Animal Care and Use Committee and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

Isolated tumor interstitial fluid

Lewis lung cancer (LLC) cells (1 × 10⁶) were injected subcutaneously into one flank of 8- to 12-week-old syngeneic C57BL/6 mice. When the tumors reached a size of 15 to 20 mm, the primary tumor was excised and the tumor interstitial fluid was immediately isolated by centrifugation at 1,000 rpm for 10 minutes as previously described (13). The fluid without erythrocytes was collected and stored at −80°C until further experimental processing after the protein concentration was quantified for 10 μg/mL by the Bradford protein assay.

Cell culture

LLC cells were grown in RPMI1640 medium supplemented with 10% (v/v) FBS. L929 cells (mouse embryonic lung fibroblast) were cultured in 4.5 g/L glucose DMEM supplemented with 10% FBS. All cells were grown in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Cells were tested every 6 months for mycoplasma using the PCR-based MycoAlert PLUS Mycoplasma Detection Kit (Lonza).

Cell proliferation assay

LLC cells (1 × 10⁴/well) were seeded in a 96-well plate and were incubated at 37°C for 48 hours in medium or in medium supplemented with 20% TIF; each condition was tested in six replicates. During the final 4 hours of the 48-hour incubation, the supernatants were discarded and 100 μL MITT (0.5 mg/mL) was added to each well. After 4 hours, the MITT was discarded and 100 μL of DMSO was added to each well. The absorbance of the samples at 570 nm was determined using a plate reader (Elx-800; Bio-Tek). The results of the cell proliferation assay are presented as a percentage of the control cells.

Cell migration assay

The migration assay was performed in a 24-well Transwell chamber with filter inserts (pore size, 8 μm). LLC cells were seeded into the upper compartment at a concentration of 5 × 10⁵ cells/mL in 200 μL serum-free medium, and 500 μL of complete medium or medium supplemented with 20% TIF was placed in the lower chamber. After incubation for 24 hours at 37°C, the adherent cells in the upper compartment were removed by a cotton swab, and the filter inserts were incubated in medium supplemented with 5 μg/mL DAPI for 30 minutes at 37°C. Cell migration was observed and analyzed using an inverted fluorescence microscope (Olympus).

Cell invasion assay

The invasion assay was performed in a 24-well Transwell chamber with filter inserts (pore size, 8 μm) coated with 50 μL Matrigel diluted 1:3. LLC cells were seeded into the upper compartment at a concentration of 5 × 10⁵ cells/mL in 200 μL serum-free RPMI1640 medium, and 500 μL of complete medium or medium supplemented with 20% TIF was placed in the lower chamber. After incubation for 24 hours at 37°C, the adherent cells in the upper compartment were removed by a cotton swab, and filter inserts were incubated in medium supplemented with 5 μg/mL DAPI for 30 minutes at 37°C. Invasion was observed and analyzed using an inverted fluorescence microscope.

In vitro wound-healing assay

L929 cells (3 × 10⁵ cells/well) were seeded into a 24-well plate and were cultured to confluence as a monolayer. The monolayer was then scratched with a 200 μL pipette tip to create an approximate 1-mm–wide wound area and was washed twice with PBS to remove floating cells. The cells were then treated with various concentrations of TIF or control serum for 48 hours. Images of the wounded cell monolayer were obtained using a microscope, and the repair rate of the wound was expressed as the percentage of the gap relative to the total area of the cell-free region immediately after the scratch was generated according to image analysis software (Olympus).

Cell self-renewal assay

A soft-agar–based cell transformation assay was performed in 6-well plates. The plates were precoated with 0.5% agar in culture medium that included various concentrations of TIF. Passage-controlled LLC cells (5 × 10³ cells/well) were mixed with RPMI1640 medium containing 0.5% agar to produce a final agar
concentration of 0.33%. This mixture was then transferred on top of the bottom layer of agar. The colonies were examined under a light microscope after 2 weeks in culture at 37 °C and 5% CO2. A tumor sphere assay was performed in ultra-low adherent 6-well plates under stem cell–selective conditions. Briefly, LLC cells (5 × 10^3 cells/well) were resuspended in 0.8% MC-based serum-free RPMI1640 medium supplemented with 20 ng/mL EGF, 20 ng/mL bFGF, 4 mg/mL heparin, and various concentrations of TIF. The cells were further allowed to grow for 14 days, and the number of spheres was counted by microscopy.

Tumor-initiating cell assay
LLC cells (1 × 10^4 cells/well) were seeded in a 6-well plate overnight at 37 °C. The cells were treated with various concentrations of TIF, and fresh medium was added every 2 days for 8 days. The cells were detached by trypsinization, and the tumor-initiating cells (TIC) were assessed according to the expression of CD133, Nanog, and PCNA by flow cytometry. Data were analyzed, and the percentage of TICs in each group was calculated using FlowJo 7.6 software (Beckman Coulter, Inc.).

Detection of apoptosis and autophagy
Apoptosis was evaluated by AO/EB fluorescence staining. Briefly, a suspension of TIF-treated LLC cells that were cultured under serum-free conditions for 12 hours was added to 4 μL/mL of dye mixture (100 μg/mL AO) and 100 μg/mL ethidium bromide (EB) in PBS and was washed once with PBS. After staining, the cells were visualized and analyzed by an IN Cell Analyzer 2000 (GE Healthcare). In regard to cell autophagy, TIF-treated LLC cells that were cultured under serum-free conditions for 12 hours were stained with AO (1 μg/mL) and were incubated at 37 °C for 30 minutes before observation. Red acidic vesicular organelles (AVO) that were stained by AO in autophagic cells were visualized and analyzed by an IN Cell Analyzer 2000.

In vivo tumor model
LLC cells (1 × 10^6) were suspended in 10 μL PBS or in 10 μL TIF and were administered into the posterior pharynx of C57BL/6 mice with the tongue retracted until aspiration was witnessed. Twenty-eight days after tumor incubation, orbital venous blood collection was performed for the plasma 5-HIAA assay using an ELISA kit; the tumors were also dissected and weighed. A portion of the lung with tumors was snap-frozen in liquid nitrogen and stored at −80 °C for Western blot analysis. Another portion of the lung with tumors was fixed in 4% paraformaldehyde solution overnight for immunohistochemical evaluation. The number of tumors on the surface of the lung was counted, and the entire lung was weighed. Ten mice in each group were used for survival analysis until they died.

To expose the mice to hyperoxic conditions, the mice were placed in a specially designed hermetic chamber with controlled O2 and CO2 levels; the temperature and humidity were monitored (Coy Laboratory Products) and were the same as the temperature and humidity used for the control animals. Oxygen concentrations were monitored continuously and were maintained at 15% ± 1% in the chamber during experiments.

To evaluate the correlation of abnormal angiogenesis and TIF-induced tumor progression, TIF-irradiated mice received dexamethasone (an inhibitor of inflammation) 5 mg/kg, digoxin (a Na^+–K^+-ATPase inhibitor) 2 mg/kg, and spironolactone (an aldosterone receptor antagonist) 20 mg/kg, and vadimezan (an antiangiogenic agent) 25 mg/kg by intragastric administration once daily for 4 weeks.

The integrity assay of the tumor epithelium
The integrity of the tumor epithelium was assayed with the Evans blue dye extra-barrier technique. Thirty minutes before they were sacrificed, Evans blue was injected into the mice via the tail vein at a dose of 50 mg/kg. The mice were then sacrificed, and the lung with tumors was removed. Each sample was weighed and homogenized in 5 mL of 25% formamide solution. The samples were then incubated at 37 °C for 24 hours and centrifuged at 5,000 rpm for 30 minutes to obtain the supernatant. Subsequently, the optical density of Evans blue in the supernatant was determined spectrophotometrically at 620 nm. The amount of Evans blue was expressed as mg/g of tumor tissue calculated according to the Evans blue standard curve.

The tumor water content measurement
At the end of the experiments, the mice were euthanized and the lung with tumors was immediately dissected and weighed to determine the wet tumor weight. Next, the tissue was immediately wrapped in preweighed, labeled aluminum foil and placed in an oven for 72 hours at 100 °C. The specimens were then removed from the oven and reweighed to obtain the dry tumor water weight. The tumor water content in each sample was then calculated using the wet-dry method formula, as follows:

Water content (TIF) = (wet weight–dry weight)/wet weight × 100.

Measurement of intratumoral inflammatory markers
The lung with tumors (0.2 g) was homogenized in 2 mL of PBS using a tissue homogenizer. The homogenate was centrifuged at 3,000 rpm for 5 minutes. The supernatant was then harvested for a cytokine assay, which was conducted with a mouse ELISA kit (TNF-α, hs-CRP, and 8-OHdG).

Measurement of lactate and pyruvate levels and reactive oxygen species
Tumor samples were homogenized and sonicated in RIPA buffer (20 mmol/L Tris–HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L Na2EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L beta-glycerophosphate, 1 mmol/L Na3VO4, 1 mg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride) on ice. Next, the lysates were centrifuged at 12,000 rpm for 15 minutes at 4 °C, and, then, the lysates were diluted to 2 mg/mL in PBS. After passage through a 10-kDa cut-off membrane (VWR, 82031-348), the lysates were deproteinized. In regard to the lysates from the TIF-treated cells, the same process was performed. The levels of lactate and pyruvate were determined with a Lactate kit and a Pyruvate Assay kit according to the manufacturer’s instructions. The levels of reactive oxygen species (ROS) in the tumor lysates were measured by a quantitative sandwich enzyme immunoassay technique according to the manufacturer’s instructions.

Immunohistochemical analysis
The sections were deparaffinized, rehydrated, and washed in PBS. Antigen retrieval was performed in 10 mmol/L sodium citrate buffer at pH 6.0 for 20 minutes in a microwave oven. After blocking with 3% hydrogen peroxide for 10 minutes, the sections
were incubated with 0.1% TritonX-100 in PBS for 20 minutes at room temperature and next immersed in a blocking solution of 5% BSA for 20 minutes at room temperature. The primary antibodies (1:100) were added in an IHC Antibody Diluent, and the sections were incubated with PBS and incubated for 1 hour at room temperature with a horseradish peroxidase (HRP)–conjugated Goat anti Rabbit IgG polyclonal antibody, and the sections were rinsed with PBS and incubated for 1 hour at room temperature with SABC (strept avidin–biotin complex). The sections were rinsed with PBS, and the signal was developed with the peroxidase substrate DAB, which appears as a brown reaction product, or with AEC, which appears as a red reaction product. All sections were counterstained with hematoxylin and were imaged under a microscope. A semi-quantitative histologic score (HSCORE) was used and calculated with the following formula:

$$HSCORE = \sum \pi(1 + 1),$$

where "\(\pi\)" represents the percentage of stained cells for each intensity (0, no staining; 1, < 10% stained cells; 2, 10%–60% stained cells; 3, >60% stained cells) and "1 + 1" represents the staining intensity (0, no labeling; 1, weak; 2, moderate; 3, strong; ref. 14).

Western blot analysis

For Western blotting in vivo, lungs with tumors were homogenized and sonicated in RIPA buffer (20 mmol/L Tris–HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L Na2EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L beta-glycerophosphate, 1 mmol/L Na3VO4, 1 mg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride) on ice. Next, the lysates were centrifuged at 12,000 rpm for 15 minutes at 4°C, and the protein concentration was determined using the Bradford protein assay.

A total of 40 μg of protein was separated on a 12% SDS-PAGE gel and electroblotted onto a polyvinylidene fluoride membrane. The immunoblot was incubated with blocking solution (5% skim milk) at room temperature for 1 hour, followed by incubation overnight with primary antibodies against E-cadherin, cytokeratin 18, N-cadherin, or vimentin at 4°C. The blots were washed and incubated with a 1:1,000 dilution of HRP-conjugated secondary antibody for 1 hour at room temperature. The protein bands were detected with an Enhanced Chemiluminescence Detection kit (ECL).

Statistical analysis

All data are expressed as the mean ± SDs and were analyzed with SPSS (Statistical Package for the Social Sciences) 17.0 software (SPSS Inc.). The differences among the groups were evaluated with a one-way ANOVA followed by Dunnett adjustment. \(P < 0.05\) was used as the criterion for statistical significance.

Results

TIF increased the malignancy of LLC cells in vitro and the number of TICs in vitro

Tumor metastasis is the main cause of cancer mortality, and to observe the effects of TIF on malignant phenotypes of cancer, we used isolated TIF (10 μg protein/ml) to treat LLC cells and examined cell proliferation, migration, and invasion. SDS-PAGE gel electrophoresis showed that protein molecular in TIF focused on 10 to 20 KD (date not shown). We found that TIF promoted LLC cell proliferation, migration, and invasion in a dose-dependent manner compared with control (standard nutrition supply of standard medium supplemented with 10% FBS; Fig. 1A). Simultaneously, TIF sustained LLC cell survival via prevention of apoptosis and autophagy under serum-free conditions (Fig. 1B). On the contrary, TIF impaired fibroblast wound healing (Fig. 1C), which indicates inhibition of normal wound healing. In additional experiment, vadimezan below cytotoxic concentration (<20 μmol/L) was added to the TIF and had no effect on the tumor-promoting effect of TIF (data not shown).

TICs, which are known as cancer stem cells, are the main contributors to tumor progression (15). The immunohistochemical analysis demonstrated that exposure of LLC cells to TIF increased the percentage of TICs in vivo, as indicated by the expression of the cancer stem cell marker Nanog and the proliferation marker PCNA (Fig. 1D). These results verified that TIF promoted tumor malignancy.

TIF promoted LLC cell self-renewal in vitro and tumor progression in vivo

To further observe the contribution of TIF to tumor malignancy, the expression levels of CD133, Nanog, and PCNA were examined in TIF-treated LLC cells by flow cytometry. We found that chronic TIF exposure of LLC cells led to increased CD133+ , Nanog+, and PCNA+ cell populations in a dose-dependent manner, whereas these cell populations were less frequent in LLC control cells (Fig. 2A). The ability for self-renewal is a feature of TICs (16), and in this study, chronic TIF-exposed LLC cells formed more colonies and tumor spheres in a soft-agar colony formation assay and a tumor sphere formation assay, which are the most stringent indicators of TIC transformation. In contrast, the passage-controlled LLC cells exhibited a minimal number of small colonies and spheres (Fig. 2B).

Next, we confirmed the tumor-promoting effects of TIF in situ lung cancer model. Consistent with the in vitro results, the exposure of LLC cells to TIF promoted tumor progression. The weight of the lung and the number of lung tumors were 0.415 ± 0.061 g and 31.5 ± 5.6, respectively, in the TIF-exposure group, whereas these values were 0.318 ± 0.052 g and 15.3 ± 4.4, respectively, in the control group (Fig. 2C). In this model, TIF also decreased the integrity of the tumor epithelium indicated by increased Evans blue dye (Fig. 3A) and increased tumor edema indicated by the water content of the tissue (Fig. 3B).

Because the stemness of cancer cells is associated with epithelial–mesenchymal transition (EMT; ref. 17), we also examined EMT according to the expression of E-cadherin, cytokeratin 18, N-cadherin, and vimentin. As expected, chronic exposure of LLC cells to TIF led to decreased expression of the epithelial markers E-cadherin and cytokeratin 18 and elevated expression of the mesenchymal markers N-cadherin and Vimentin, compared with LLC control cells (Fig. 3C).

TIF promoted tumor progression and induced tissue inflammation similarly to hypoxia exposure in vivo

Accumulating evidence suggests that the tumor microenvironment decisively contributes not only to the survival of primary neoplastic cells but also to subsequent key events that occur during neoplastic disease progression, including tumor growth, invasion, and metastasis (18). Of all of the characteristics of the tumor microenvironment, hypoxia and inflammation have been...
Figure 1.

TIF increased the malignancy of LLC cells in vitro and the number of TICs in vivo. A, TIF promoted LLC cell proliferation, as tested by MTT, as well as migration and invasion, as tested by DAPI staining in Transwell chambers. B, TIF sustained LLC cell survival through the prevention of apoptosis, as tested by AO/EB staining (the cytoplasm stained red) and autophagy, as tested by AO staining, (red acidic vesicles) under serum-free conditions. C, TIF impaired fibroblast wound healing. D, TIF increased the TICs as demonstrated by the expression of the cancer stem cell marker Nanog and the proliferative marker PCNA according to an immunohistochemical analysis (n = 10). The data shown (mean ± SD) are representative of triplicate assays. Significant differences (*, P < 0.05; **, P < 0.01) compared with control by one-way ANOVA with Dunnett adjustment.
Figure 2.
TIF promoted LLC cell self-renewal in vitro and tumor progression in vivo. A, TIF increased the CD133+, Nanog+, and PCNA+ cell population, as tested by flow cytometry of LLC cells (n = 3). B, TIF promoted LLC cell self-renewal, which was shown by soft-agar colony formation and tumor sphere formation assays (n = 3). C, TIF promoted tumor progression in situ in a mouse lung cancer model, as demonstrated by the number of lung tumors and the weight of the lungs (n = 10). The data shown (mean ± SD) are representative of replicate experiments. Significant differences (∗, P < 0.01) compared with control by one-way ANOVA with Dunnett adjustment.

shown to be particularly important for cancer progression (19). Therefore, we evaluated the possible correlation of TIF-induced tumor progression with hypoxia and inflammation. We found that TIF promoted tumor progression similarly to hypoxia exposure in situ mouse lung cancer model, as demonstrated by the number of lung tumors and the weight of the lungs (Fig. 4A). The tumor number and lung weight were 35.6 ± 5.3 mm³ and 0.436 ± 0.065 g, respectively, in the chronic hypoxia group, which had no difference compared with TIF group but had a statistically significant difference compared with control group (the tumor number and lung weight were 15.3 ± 4.4 mm³ and 0.318 ± 0.052 g, respectively). The median survival was 35.5 days and 34.5 days in the TIF and chronic hypoxia exposure groups, respectively, whereas it was 44.5 days in the control group (Fig. 4B). Moreover, higher levels of inflammatory markers, including TNFα, hs-CRP, and 8-OHdG, were observed in the groups that were exposed to TIF and to chronic hypoxia compared with the control group (Fig. 4C).

A metabolic shift from a positive energy balance to negative energy equilibrium is an important characteristic of tumor progression due to the rapid proliferation of the tumor. This activates glucose anaerobic glycolysis and results in increased levels of lactate, pyruvate, and ROS (20), and thus we also examined whether TIF promoted a metabolic shift in cancer cells. We found that exposure of LLC cells to TIF increased the levels of ROS, lactate, and pyruvate, whereas mice that were chronically exposed to hypoxia exhibited tumor-promoting characteristics similar to those induced by TIF (Fig. 5A). In addition, TIF promoted the water content of tissue similarly to hypoxia exposure (Fig. 5B), and when LLC cells were exposed to TIF, this resulted in more tumor hypoxia, as indicated by an immunohistochemical analysis of hypoxyprobe (pimonidazole hydrochloride) and carbonic anhydrase IX (Fig. 6A). These results suggested that TIF-induced tumor progression was associated with hypoxia and inflammation.

TIF-induced tumor progression was independent of angiogenesis

Tumor edema, a frequently encountered phenomenon, causes cavity effusion and affects the clinical outcome in patients with cancer (21). It is widely accepted that tumor edema is associated with dysfunctional angiogenesis (22), and AQPl is a candidate oncogene that is epigenetically modified in many types of carcinomas (23). To evaluate the correlation of abnormal angiogenesis with TIF-induced tumor progression, we examined the vascular disruption marker (serotonin metabolite 5-HIAA) in the plasma in mice as well as AQPl and CD31 expression. We found that TIF significantly increased the plasma levels of 5-HIAA in tumor-bearing mice (Fig. 5C). The inhibition of inflammation by dexamethasone (an inhibitor of inflammation), the inhibition of Na-K-ATPase by digoxin (an Na-K-ATPase inhibitor), and the inhibition of aldosterone receptors by spironolactone (an aldosterone receptor antagonist) could decrease the plasma level of 5-HIAA (Fig. 5C). Inhibition of these processes also prevented tumor growth indicated by the tumor number and lung weight (Fig. 5D and E and Fig. 6B). An immunohistochemical analysis showed that TIF significantly increased tumor AQPl expression.
(the scores were 32 and 45 in control group and TIF group, respectively, \( P < 0.05 \)) but not CD31 expression (the scores were 18 and 21 in control group and TIF group, respectively, \( P > 0.05 \); Fig. 6C). Dexamethasone, digoxin, and spirolactone decreased the expression of AQP1 in the tumor but increased CD31 expression. On the contrary, vadimezan, a conventional antiangiogenic agent, reduced CD31 expression, but led to increase in plasma 5-HIAA and tumor AQP1 expression, and thus promoted tumor progression (Fig. 5C, D, E and Fig. 6B and C). These results suggested that TIF-induced tumor progression was associated with abnormal angiogenesis, which is associated with AQP1 upregulation, inflammatory effusion, and the activation of E-cadherin.

Figure 3.
TIF promoted tumor permeability and EMT. A, TIF increased the tumor barrier permeability as indicated by staining with Evans blue dye (\( n = 5 \)). B, TIF increased the water content of tissue (\( n = 5 \)). C, TIF promoted EMT as indicated by a reduction in the expression of E-cadherin and cytokeratin 18, and an increase in N-cadherin and vimentin (\( n = 5 \)). The data shown (mean ± SD) are representative of replicate experiments. Significant differences (\( **, P < 0.01 \)) compared with control by one-way ANOVA with Dunnett adjustment.

Figure 4.
TIF promoted tumor progression and induced tissue inflammation similarly to hypoxia exposure in vivo. A, TIF promoted tumor progression similarly to hypoxia exposure in situ mouse lung cancer model, as demonstrated by the number of lung tumors and the weight of the lungs (\( n = 10 \)). B, TIF decreased the survival of tumor-bearing mice similarly to hypoxia exposure (\( n = 10 \)). C, TIF induced tissue inflammation as indicated by TNFα, hs-CRP, and 8-OHdG similarly to hypoxia exposure (\( n = 5 \)). The data shown (mean ± SD) are representative of replicate experiments. Significant differences (\( **, P < 0.01 \)) compared with control by one-way ANOVA with Dunnett adjustment.
Na-K-ATPases and aldosterone receptors; however, this process was determined to be independent of angiogenesis.

**Discussion**

TIF, a solution that bathes and surrounds tumor cells and provides them with nutrients and a means of waste removal, is a significant contributor to tumor edema and cavity effusion (24). Despite the link among tumor edema, cavity effusion, and tumor-related mortality, TIF has garnered little attention with respect to its role in the development of novel therapies for cancer; therefore, its relative contribution to cancer progression remains unclear (25). Interstitial fluid flow is a nearly ubiquitous and physiologically relevant biophysical force that is elevated in tumors because of tumor-associated angiogenesis and lymphangiogenesis, as well as changes in the tumor stroma. Not only does it apply physical forces to cells directly, but interstitial flow also creates gradients of soluble signals in the tumor microenvironment, thus influencing cell behavior and modulating cell–cell interactions (26). This study showed that TIF promoted LLC cell proliferation, migration, and invasion in a dose-dependent manner. In addition, TIF was associated with resistance to autophagy and apoptosis under cell starvation conditions. These results suggested that TIF is not only one of the major drivers of tumor progression but that it is also one of the most important resources of nutrients.

Cancer stem cells, as TICs, play a key role in all stages of carcinogenesis (27). To confirm the contribution of TIF to cancer progression, we examined effect of TIF on the percentage of cancer stem cells in vitro and in vivo. We found that TIF increased the percentage of cancer stem cells and the self-renewal ability of cancer cells, and, simultaneously, TIF also promoted EMT and a metabolic shift in cancer cells. In addition, in this study, we found that increased TIF was associated with chronic hypoxia and inflammation, which may be independent of angiogenic densities. Interestingly, we found that the inhibition of inflammation, Na-K-ATPase, and aldosterone receptors was able to decrease AQP1 and 5-HIAA and prevent tumor growth, whereas it promoted angiogenesis. In contrast, vadimezan, a conventional antiangiogenic agent, reduced angiogenesis, but led to an increase in AQP1 and 5-HIAA, and thus promoted tumor growth. This results in an alternative concept that posits that therapies that block TIF may significantly starve cancer cells and cause these cells to lose their ability to self-renew; this in...
The development of cancer is a complex process during which a normal cell undergoes a progressive series of alterations that result in the acquisition of an altered proliferative capacity, invasiveness, and metastatic potential (28). However, nutrient acquisition and utilization govern tumor progression (29). A competitive balance exists between normal cells and tumor cells in terms of nutrient acquisition and utilization (30). We hypothesized that to obtain nutrients, TICs mainly excrete cytotoxic components into the interstitial fluid, which induces cytotoxic edema and normal cell death. The systemic reduction of interstitial fluid prevents the excretion of cytotoxic components by cancer cells and prevents the use of recycled nutrients, which would result in starvation of the cancer cells. Indeed, abundant ROS and catabolic enzymes are present, which on the one hand may cause cell death and degradation and thus produce recycled nutrients; on the other hand, these enzymes may lead to the induction of EMT and stemness (31–33). It is not surprising that in this study, TIF impaired the proliferation and migration of fibroblasts in a wound-healing assay, whereas it promoted the stemness of cancer cells. Our findings show that cancer cells become less reliant on angiogenesis and instead rely on TIF for nutrient acquisition. At the same time, TIF grants cancer cells the capacity to maintain access to available nutrients even when they are scarce.

One major cause of morbidity and death from cancer is the augmentation of uncontrolled TIF (34). Previous studies have demonstrated the significant correlation between high microvascular densities, elevated IFP, and metastasis in cancers (35). Our results have provided direct evidence that TIF is the driving force for lung cancer cells to survive under conditions of nutrient scarcity. We provide further evidence that a reduction in TIF via a decrease in the tumor permeability, which occurs independently of angiogenesis, may improve lung cancer morbidity and mortality without the strong side effects that are associated with conventional chemotherapy and without the stromal hypoxia that is associated with vascular disruption therapy. Therefore, any therapeutic strategy that can lower TIF represents a promising strategy for cancer treatment and requires further clinical investigation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: H. Li, G. Li, G. Du
Development of methodology: H. Li, G. Li, L. Liu, Z. Guo, X. Ma, N. Cao, H. Lin, G. Du
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Li, G. Li, G. Du

Figure 6.
TIF induced tissue hypoxia, and TIF-induced tumor progression was independent of angiogenesis. A, TIF promoted tissue hypoxia, which was demonstrated by immunohistochemical analysis of hypoxyprobe (pimonidazole hydrochloride) and carbonic anhydrase IX just as in hypoxia exposure (n = 5). B, vadimezan promoted TIF-induced tumor progression, whereas dexamethasone, spirolactone, and digoxin prevented it (n = 10). C, TIF increased tissue AQP1 (B), which was promoted by vadimezan and inhibited by dexamethasone, spirolactone, and digoxin; however, TIF had no effect on CD31 expression, which was decreased by vadimezan and increased by dexamethasone, spirolactone, and digoxin in tumor-bearing mice (n = 5), according to an immunohistochemical analysis (n = 5).
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): H. Li, G. Li, L. Liu, G. Du
Writing, review, and/or revision of the manuscript: H. Li, G. Li, G. Du
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Han, Y. Duan, G. Du
Study supervision: G. Du

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