Opposing Effects of Hypoxia on Expression of the Angiogenic Inhibitor Thrombospondin 1 and the Angiogenic Inducer Vascular Endothelial Growth Factor

Keith R. Laderoute, Rodolfo M. Alarcon, Michael D. Brody, Joy M. Calaoagan, Eunice Y. Chen, A. Merrill Knapp, Zhong Yun, Nicholas C. Denko, and Amato J. Giaccia


ABSTRACT

Tumor angiogenesis, the development of new blood vessels during malignant progression, is a regulated process that has both genetic and physiological controls. Physiologically, angiogenesis is stimulated by decreases in tissue oxygenation (i.e., hypoxia). We investigated the effect of hypoxia on the expression of two angiogenic factors reported to be genetically regulated by the p53 tumor suppressor gene: (a) the angiogenic inhibitor thrombospondin 1 (TSP-1); and (b) the angiogenic inducer vascular endothelial growth factor (VEGF). Analysis of rodent cells that differ in their p53 genotype (p53×/+ or p53×−/−) indicated that in vitro exposure to hypoxia simultaneously suppressed TSP-1 and induced VEGF expression, regardless of the p53 genotype. On transformation of these cells with E1A and oncogenic H-ras, the basal level of TSP-1 expression was strongly diminished, whereas that of VEGF could still be induced by hypoxia. Consistent with these in vitro findings, sections of tumors derived from the transformed p53×+/+ and p53×−− cells showed that VEGF protein overlapped with regions of hypoxia, whereas TSP-1 protein was below the limits of detection in tumor tissue. Using a panel of normal/immortalized and transformed human cells, it was found that the ability of hypoxia to inhibit TSP-1 expression depends on the cell type and/or the degree of transformation. In contrast, VEGF expression was induced by hypoxia in all of the human cell types examined. Together, these findings suggest that hypoxic and oncogenic signals could interact in the tumor microenvironment to inhibit TSP-1 and induce VEGF expression, promoting the switch to the angiogenic phenotype.

INTRODUCTION

Decreased tissue oxygenation (hypoxia) is part of the natural history of solid tumors (reviewed in Refs. 1 and 2). Tumor hypoxia originates from the inability of neovasculature to provide an adequate blood supply to accommodate the metabolic demands of the tissue (2). Hypoxia contributes to processes involved in malignant progression, such as the elimination of p53 tumor suppressor gene activity (3), the induction of protooncogene expression (4–6), genetic instability (7, 8), and angiogenesis (9, 10). Angiogenesis, the recruitment of new blood vessels to regions of chronically low blood supply, is essential for the progression of solid tumors to malignancy (2, 9, 11). Increasing evidence supports the hypothesis that tumor angiogenesis is controlled by an “angiogenic switch,” a physiological mechanism involving a dynamic balance of angiogenic factors [i.e., inhibitors and inducers (9)]. Numerous angiogenic factors have been identified, including specific endothelial cell growth factors (e.g., VEGF3), cytokines and inflammatory agents (e.g., tumor necrosis factor α and interleukin 8), fragments of circulatory system proteins (e.g., angiotatin), and extracellular matrix components [e.g., TSPs (9, 12–16)]. Presumably, this diversity of angiogenic factors reflects a strict requirement for controlling angiogenesis under normal physiological conditions and in response to oncogenic events. Given that the switch to the angiogenic phenotype requires a positive net increase in inducing activity, we hypothesize that tumor hypoxia promotes angiogenesis by modulating the expression of both angiogenic inducers and inhibitors. The present study focuses on the influence of the hypoxic tumor microenvironment on the expression of two critical angiogenic factors, the inhibitor TSP-1 and the inducer VEGF.

VEGF is a secreted adhesive glycoprotein that has been shown to have antiangiogenic properties by an unknown mechanism (17–19). Although paradoxical effects of TSP-1 on malignant progression have been reported (20, 21), these observations may reflect the wide range of potential interactions of the complex domain structure of this molecule with its microenvironment, as well as the variable distribution of TSP-1 receptors in tissues (22, 23). Compelling evidence of an antiangiogenic
function for TSP-1 is provided by studies showing that activation of the angiogenic switch in Li-Fraumeni fibroblasts lacking wt p53 is associated with diminished TSP-1 expression (17, 24). Conversely, the introduction of wt p53 into BT549 human breast carcinoma cells generates an antiangiogenic activity involving up-regulated TSP-1 expression (15). In addition, overexpression of TSP-1 is antiangiogenic in tumor models (25, 26). Reports that endogenous TSP-1 expression can be induced by wt p53, repressed by oncogenic signals such as c-jun overexpression, and silenced by DNA methylation imply that down-regulation of TSP-1 contributes to oncogenesis (24, 27–30). Because both p53 and c-jun are inducible by hypoxia (4, 31), the regulation of TSP-1 expression by these proteins suggests that it is a hypoxia-responsive angiogenic inhibitor.

VEGF is a powerful angiogenic inducer that is activated by hypoxia at both the transcriptional and posttranscriptional levels (32–35). Inducible VEGF expression correlates with hypoxic regions present in tumor models in vitro and in experimental solid tumors in vivo (2, 36), suggesting a model of tumor angiogenesis in which VEGF isoforms generated in hypoxic regions stimulate host endothelial cells to assemble new vasculature by a feedback loop. VEGF expression in aerobic and hypoxic cells also appears to be enhanced by ras oncogenes, one of the most common oncogenic alterations in human cancer (10, 37). This response to oncogenic signals may explain, in part, why VEGF expression does not always correlate with evidence of tumor hypoxia, as reported for human cervical carcinomas exposed to the bioreductive hypoxia marker pimonidazole (38). Transcription of the VEGF gene in response to hypoxia is critically dependent on an enhancer site in its 5′-regulatory region for the hypoxia-inducible transcription factor HIF-1 (34, 39). Considering recent reports that wt p53 interacts with HIF-1 and inhibits its transactivation activity (40, 41), the dependence of VEGF transcription on HIF-1 suggests that p53 could antagonize VEGF-dependent angiogenesis stimulated by tumor hypoxia.

Current evidence indicates that the expression of TSP-1 and VEGF is genetically controlled by both tumor suppressor and proto-oncogene activity, providing molecular mechanisms that could contribute to the switch to the angiogenic phenotype when these controls are deregulated during oncogenesis (17). However, because tumor angiogenesis is also physiologically controlled by environmental signals such as hypoxia, it is important to determine the contribution of this stress to the expression of these angiogenic factors. In the present study, we investigated the influence of pathophysiological hypoxia on TSP-1 and VEGF expression by using normal and transformed rodent and human cells, including isoegenic rodent cells that are wt or null for p53.

**MATERIALS AND METHODS**

**Cell Culture and Hypoxic Treatments.** Primary p53+/+ and p53−/− MEFs were obtained from Dr. Scott Lowe (Department of Biology, Massachusetts Institute of Technology, Cambridge, MA). Transformed p53+/+ and p53−/− MEFs containing E1A and H-ras (EH-MEFs) have been described in detail elsewhere (3, 42). Rat-1 fibroblasts stably transfected with an expression vector for oncogenic H-ras (Rat-1R fibroblasts; T24 H-ras) have also been described elsewhere (10, 43). Both Rat-1 cell lines contain wt p53. Normal HCFs and HCEs were isolated from biopsies at Stanford University School of Medicine (44). Human NDKs were isolated by similar techniques from foreskin and cultured in keratinocyte-SFM medium (Life Technologies, Inc., Grand Island, NY). Immortalized derivatives of HCEs (HCE.E6E7) were obtained by retroviral infection of the HPV E6 and E7 genes, as described elsewhere (44). C33a and SiHa human cervical carcinoma and FaDu human squamous carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). Except for the NDKs, these human and rodent cells were cultured in DMEM containing 10% FCS, and all cells were incubated in a 5% CO2/air atmosphere at 37°C. HMVECs isolated from the dermis (HMVEC-d) were obtained from Clonetics Corp. (Walkersville, MD) and cultured on 60-mm-diameter plastic culture dishes in EndoPack growth medium according to the supplier’s instructions. HMVECs were incubated in a 5% CO2/air atmosphere at 37°C until they were 70–90% confluent before being exposed to hypoxia. Most of the in vitro hypoxia experiments described in this study were performed at pO2 = 0.01% (relative to air at pO2 = 21%). Rodent cells were incubated for 3 days after plating (3 × 103 to 1.5 × 106 cells/60-mm-diameter plastic culture dish) before exposure to hypoxia in aluminum gas-exchange chambers according to a standard protocol (6). The HCFs, HCEs, HCE.E6E7 cells, NDKs, and the C33a, SiHa, and FaDu cells were plated at 5 × 106 cells/100-mm-diameter plastic culture dish before exposure to hypoxia in aluminum gas-exchange chambers according to a standard protocol (6). The HCFs, HCEs, HCE.E6E7 cells, NDKs, and the C33a, SiHa, and FaDu cells were plated at 5 × 106 cells/100-mm-diameter plastic culture dish before exposure to hypoxia in an anaerobic box (Bactron X; Sheldon Manufacturing Inc., Cornelius, OR) containing an atmosphere of 90% CO2, 5% CO2, and 5% H2. Cells were harvested immediately in air to prepare total RNA for Northern analysis. Slightly different incubation conditions were used in the transient transfection studies described below.

**Growth of Tumors in SCID Mice.** The preparation of tumor xenografts in SCID mice from EH-MEFs has been described elsewhere (3, 43). Briefly, 8–10-week-old female SCID mice were injected with 8 × 106 to 2 × 107 cells intradermally in the midline of the back approximately 2 cm from the base of the tail. After 3–5 weeks, tumors were ready for excision and sectioning. To visualize hypoxic regions in the tumors, mice were injected i.p. with 0.2 ml of 10 mm EF5 (3) 1 h before tumor excision.

**Immunohistochemistry.** Tumors were embedded in OCT compound (Miles, Elkhart, IN) and frozen in liquid nitrogen. Cryostat sections were cut at 8–10 μm in thickness, placed on Fisher Plus slides (Fisher Scientific, Pittsburgh, PA), and stored in air-tight boxes at −80°C. The sections were returned to room temperature before opening the boxes. Sections were fixed in Streck Tissue Fixative (Streck Laboratories, Inc., Omaha, NE) for 30 min at room temperature. The fixed sections were washed three times in PBS (5 min/wash), incubated at room temperature for 30 min in a background suppressing solution [BRS; 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.25% (w/v) gelatin, and 0.05% (v/v) Tween 20], and washed as described above. To detect TSP-1 expression, sections were incubated with an anti-TSP-1 immune complex solution at 4°C overnight in a humidified chamber (45). The immune complex was formed by diluting the primary anti-TSP-1 monoclonal antibody (Clone TSP-B7; Sigma Immunochemicals, St. Louis, MO) 1:5000 with a solution of the sec-
ordary antibody (FluorX-conjugated antimonouse donkey serum; BDS, Inc., Pittsburgh, PA) diluted 1:100 in BRS. After rocking at 4°C overnight, heat-inactivated normal mouse serum (Sigma) was added to the immune complex solution to a final concentration of 0.1% (v/v), and the solution was rocked at 4°C for 2 h before applying 200 μl to each section. To detect VEGF, a rabbit antihuman polyclonal IgG antibody raised against a peptide fragment (amino acids 4-24) of human VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the primary antibody. This antibody was diluted to a final concentration of 5 μg/ml in PBS-HS and added to the sections (100 μl/section) for a 2-h incubation at room temperature. The slides were then washed three times in PBS-HS and incubated for 2 h at room temperature with FITC-conjugated antirabbit goat serum diluted in PBS-HS (200 μl/section). To visualize hypoxic regions of tumor sections by using EF5 immunostaining, an anti-EF5 monoclonal antibody (ELK3-51) conjugated with the indocarbocyanine dye Cy3 (obtained from Dr. Cameron Koch, University of Pennsylvania, Philadelphia, PA) was diluted to 30 μg/ml in PBS-HS and applied to each section (200 μl/section) for incubation at 4°C overnight. The slides were washed three times in PBS-HS (5 min/wash) and mounted in 2% n-propyl gallate in 70% glycerol-0.03 m Tris-HCl (pH 9.0). To quantitatively compare hypoxic regions on tumor sections with VEGF expression, fields containing both oxygenated (i.e., EF5-negative) and hypoxic (i.e., EF5-positive) areas were chosen randomly and photographed by using a Zeiss fluorescence microscope (Zeiss Axioskop). The number of immunofluorescent regions per unit area corresponding to EF5 binding and VEGF protein was then calculated to estimate the degree of overlap of VEGF expression with regions of hypoxia, as described previously for p53 protein expression (3).

Northern Analysis. Purification of total cellular RNA for Northern analysis was performed by using a standard guanidinium isothiocyanate method, and CsCl-purified RNA was resolved in 1% denaturing agarose gels and blotted onto nylon membranes, as described elsewhere (46). Alternatively, total RNA was purified by the RNeasy method (Qiagen Inc., Santa Clarita, CA). Probes included cDNA fragments of human TSP-1 (1.3 kb from the 5′ end; obtained from American Type Culture Collection; cross-reactive with rodent TSP-1 mRNA), and human VEGF165 (0.6 kb). The probes were labeled with [α-32P]dCTP by the random primer method (Amersham Pharmacia Biotech, Arlington Heights, IL). As controls for RNA loading/transfer, ethidium bromide fluorescence from the 28S rRNA band of total RNA was photographed, blotted rRNA bands were visualized by staining with methylene blue, or blots were probed with a labeled 2.0-kb cDNA probe for human β-actin that recognizes the corresponding mouse mRNA (Clontech Laboratories, Palo Alto, CA).

Message Stability. The half-life of TSP-1 mRNA was determined according to a protocol described elsewhere (44). Briefly, Rat-1 cells were exposed to hypoxia for 12 h, as described above. Hypoxic cells were removed from the aluminum hypoxia chambers in the anaerobic box held at 37°C. The hypoxic cells were given 5 μg/ml actinomycin D (Sigma) for 10 min (time 0), and then cells were harvested for total RNA at various times afterward. Total RNA was processed for Northern analysis, and TSP-1 mRNA signals on autoradiographs of the blots were measured by using a video densitometer system (Lynx Molecular Biology Work Station, Santa Clara, CA). The half-lives of TSP-1 mRNA were calculated from plots of the natural log (intensity) against the time of actinomycin D exposure starting at time 0.

Western Analysis. Cells were washed twice in ice-cold PBS and lysed by scraping in an ice-cold detergent buffer [50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 0.5% NP40, 50 mM NaF, 15 mM Na 3 VO 4, 1 mM Na 2VO 4, 20 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride]. After centrifugation at 12,000 × g for 20 min at 4°C, portions of the supernatants were diluted with equal volumes of 2× SDS sample buffer and boiled for 5 min. The protein concentrations of the supernatants were determined by a bicinchoninic acid assay (Pierce, Rockford, IL). Equal protein samples (5 μg) were used for gel electrophoresis. Proteins were resolved in a discontinuous 10% SDS-polyacrylamide gel and electrophorised in a buffer containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine, 0.1% SDS, and 15% methanol onto Immobilon P membranes (Millipore, Marlborough, MA) by using a TE 20 Mighty Small Transphor Tank Transfer Unit (Hoefer Pharmacia Biotech Inc., San Francisco, CA). Blots were blocked in 5% nonfat dried milk in PBS containing 0.1% Tween 20 at 4°C overnight. To detect HIF-1α protein, blots were washed once in PBS-0.1% Tween 20 and then incubated with rocking at room temperature for 2 h with a monoclonal antihuman HIF-1α antibody (Novus Biologicals, Inc., Littleton, CO) diluted 1:1,000 in PBS/0.1% Tween 20/5% nonfat dried milk. A secondary antirabbit IgG antibody conjugated with horse radish peroxidase (IgG-HRP; Santa Cruz Biotechnology) diluted 1:5,000 in PBS/0.1% Tween 20 was added, and the blot was incubated at room temperature for 2 h. After washing three times in PBS/0.1% Tween 20, primary antibody binding was detected and visualized by using the Enhanced Chemiluminescence Plus Western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

Plasmid Constructs and Transfections. Luciferase reporter plasmids containing fragments of the human TSP-1 gene were introduced into the nucleus of polyoma promoter region [TSP (−3203), (−548), and (−330)] were obtained from Dr. Paul Bornstein (Department of Biochemistry, University of Washington, Seattle, WA). They were constructed by using the pGL3-Enhancer plasmid (Promega Corp., Madison, WI) as described elsewhere (47). The constructs were verified by restriction digestion. The numerical designations of the plasmids are the same as those described previously (48). For the analysis of TSP-1 promoter activities, Rat-1 cells were plated in 6-well tissue culture dishes at 2.5 × 10 5 cells/well and incubated at 37°C for 24 h. Cells were then transfected with a reporter plasmid using the Lipofection Plus transfection agent (Promega) according to the procedure recommended by the manufacturer. Briefly, cells were incubated with plasmid DNA (0.5 μg/well) for 4 h in serum-free medium and allowed to recover for 24 h in complete medium. Before exposure to hypoxia, cells received fresh complete medium. After 10 h in the anaerobic box (pO 2 ≤ 0.02%), cells (and control aerobic cells) were placed on ice, and cell lysates were prepared by adding 150 μl Reporter Lysis Buffer (Promega)/well. Promoter activities were analyzed by measuring luciferase activity in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI).
RESULTS

Hypoxia Suppresses TSP-1 and Induces VEGF mRNA Expression Independently of Cellular p53 Status. As mentioned above, wt p53 has been reported to positively regulate the expression of TSP-1 (24). However, despite the presence of wt p53 in both primary p53<sup>+/+</sup> MEFs and Rat-1 fibroblasts, in vitro exposure of these cells to hypoxia suppressed TSP-1 mRNA expression (Figs. 1A and 2). In addition, exposure of p53<sup>−/−</sup> primary MEFs to identical hypoxic conditions suppressed TSP-1 mRNA expression with essentially the same kinetics as those for the primary p53<sup>+/+</sup> MEFs (Fig. 1A). Together, these findings demonstrate that prolonged hypoxia can physiologically override the signal for the induction or sustained expression of TSP-1 mRNA in primary or immortalized rodent fibroblasts containing wt p53. In contrast to primary MEFs, TSP-1 mRNA was barely detectable in total RNA from the transformed p53<sup>−/−</sup> and p53<sup>−/−</sup> MEFs (EH-MEFs) under either aerobic or hypoxic conditions (data not shown). This low level of TSP-1 mRNA could be an effect of oncogenic ras activity on TSP-1 expression (49). Two lines of evidence support this possibility: (a) TSP-1 mRNA levels in primary MEFs (p53<sup>+/+</sup> and p53<sup>−/−</sup>) are abundant (Fig. 1A); and (b) stable transfection of Rat-1 fibroblasts with oncogenic H-ras (Rat-1R cells) almost eliminated detectable TSP-1 mRNA expression under aerobic conditions (Fig. 2). The potential interaction between hypoxia and transformation on TSP-1 expression is considered in more detail below.

**Fig. 1** The effect of hypoxia on *in vitro* expression of VEGF and TSP-1 in isogenic primary and transformed p53<sup>+/+</sup> and p53<sup>−/−</sup> MEFs. Autoradiographs of Northern blots of total RNA from aerobic MEFs (5% CO<sub>2</sub>/air) and MEFs exposed to hypoxia (PO<sub>2</sub> ≤ 0.01%) for the indicated times are shown. The blots were probed sequentially for the mRNAs for VEGF and TSP-1. A, primary MEFs. B, transformed MEFs (EH-MEFs). No significant signal for TSP-1 mRNA was detected on the blots for the EH-MEFs. The bottom panels show ethidium bromide fluorescence from the 28S rRNA band in the original gel and an autoradiograph of the β-actin mRNA signal from the total RNA samples used for each Northern analysis. These findings are representative of at least two independent experiments. The slight depression of the β-actin signal for the p53<sup>+/+</sup> primary MEFs subjected to hypoxia for 24 h may be a consequence of their response to prolonged stress (3). C, image of a Western blot of total protein showing the accumulation of HIF-1α in p53<sup>+/+</sup> primary MEFs exposed to hypoxia (pO<sub>2</sub> ≤ 0.01%) for the indicated times.
Unlike TSP-1 expression, VEGF expression was induced in vitro in response to prolonged hypoxia in both p53<sup>+/+</sup> and p53<sup>−−</sup> MEFs and EH-MEFs, although different kinetics were seen in the primary and transformed cells (Fig. 1, A and B). Thus, the induction of VEGF mRNA in primary or transformed MEFs exposed to stringent hypoxia was not significantly influenced by the presence or absence of wt p53. Because it has been proposed that HIF-1α stabilizes wt p53 in hypoxic cells (40, 41), we also determined whether HIF-1α protein was induced in primary p53<sup>+/+</sup> MEFs under the same low oxygen conditions. Fig. 1C shows that HIF-1α protein accumulated well above aerobic levels in the primary p53<sup>+/+</sup> MEFs and that this expression was sustained even during prolonged stress (50). Therefore, the presence of both HIF-1α protein and wt p53 did not prevent or inhibit the hypoxic induction of VEGF mRNA in these primary rodent fibroblasts. This dominant effect of hypoxia on VEGF expression was also confirmed in vivo using tumor-bearing mice injected before sacrifice with the hypoxia-specific marker EF5. EF5 immunofluorescence from tumor sections or hypoxic cells has been shown to correlate well with other established indicators of tumor oxygenation, such as in situ pO<sub>2</sub> measurements (51) and the determination of radiobiological hypoxia (52, 53). Fig. 3 shows that VEGF immunofluorescence overlapped with tumor hypoxia (as detected by EF5 immunofluorescence) on the same sections of tumor xenografts derived from p53<sup>+/+</sup> and p53<sup>−−</sup> EH-MEFs. Quantitative analysis of the EF5 and VEGF signals on randomly chosen sections demonstrated that the ratio of EF5:VEGF immunofluorescence was not significantly different in tumors derived from either p53 genotype (Table 1). Because the activation and binding of EF5 under low oxygen conditions and the hypoxic induction of VEGF expression have different dependencies, an exact overlap is not expected. These findings indicate that hypoxia can induce VEGF protein expression in tumor cells independently of p53 status, similar to its effect on VEGF mRNA accumulation observed in vitro (Fig. 1B). Finally, in accord with the low level of TSP-1 expression found in the p53<sup>+/+</sup> and p53<sup>−−</sup> EH-MEFs in vitro, TSP-1 immunofluorescence was low or undetectable on sections of either of the corresponding EH-MEF tumors (data not shown).
The Effect of Hypoxia on TSP-1 Expression Could Involve Both Transcriptional and Posttranscriptional Contributions. To investigate potential mechanisms by which hypoxia could inhibit or suppress TSP-1 expression in rodent fibroblasts, we performed transient transfection studies with reporter gene constructs and mRNA stability studies. Rat-1 cells were transiently transfected with reporter constructs containing fragments of the proximal promoter region of the human TSP-1 gene. These fragments included putative p53 binding sites in the first intron of the gene (24). Fig. 4A shows that compared with aerobic controls, hypoxia (PO2 ≤ 0.02% for 10 h) weakly inhibited but did not suppress the basal activity of the proximal TSP-1 promoter region in Rat-1 cells. Because these differences are statistically significant, this finding indicates that the decline of TSP-1 mRNA accumulation observed in hypoxic Rat-1 cells (Fig. 2) could involve cis-acting elements within the proximal promoter region. Moreover, these studies indicate that the intronic p53 binding sites are not sufficient to transactivate the TSP-1 proximal promoter region in hypoxic rodent cells containing wt p53. The decreased basal response of the truncated TSP-1 promoter constructs TSP (−548) and TSP (−330) relative to that of TSP (−2033) may be a consequence of the elimination of serum-responsive cis-acting elements from these constructs (47, 54). To determine whether hypoxia also affects the half-life of TSP-1 mRNA, we used actinomycin D to block transcription in aerobic Rat-1 cells and in Rat-1 cells exposed to hypoxia for 12 h. Fig. 4B shows a representative Northern blot of total RNA obtained from aerobic and hypoxic Rat-1 cells at 0, 15, 30, 45, 60, and 75 min after a 10-min incubation with actinomycin D. Analysis of plots of the natural log (signal intensity) versus time from two independent Northern blotting experiments gave a value of 87 ± 4 and 108 ± 4 min for the half-life of TSP-1 mRNA in hypoxic and aerobic Rat-1 cells, respectively. Taken together, these findings involving the effect of hypoxia on reporter gene expression regulated by the human TSP-1 proximal promoter region and on the half-life of endogenous TSP-1 mRNA suggest that both transcriptional and posttranscriptional effects contribute to the decline of steady-state TSP-1 mRNA levels in Rat-1 cells exposed to prolonged stress. However, it should be recognized that the relative contribution of these mechanisms to the maintenance of TSP-1 mRNA levels in response to hypoxia could depend on several variables, including the degree of stress and the cell type. The effect of cell type is considered in more detail below.

DISCUSSION

Although tumor hypoxia is clearly an important determinant of malignant progression (2, 57–59), it is not known how many pathways for tumor development are influenced by this stress. Recognition that pathophysiological hypoxia can drive the evolution of malignant phenotypes such as tumor angiogenesis has stimulated studies of the transcriptional control of hypoxia-inducible gene expression (e.g., see Refs. 4, 6, 34, 60, and 61). Current evidence indicates that the hypoxia-responsive transcription factor HIF-1 (i.e., HIF-1α: HIF-1β; reviewed in Refs. 62 and 63) is a critical regulator of tumor angiogenesis and the associated growth of a tumor mass (61, 64–66). It has been proposed that these pathological effects of HIF-1 are mediated in part by the direct transcriptional activation of VEGF and components of glucose metabolism (64), similar to its function in normal tissues exposed to hypoxia (62, 63). Recent reports that wt p53 interacts directly with HIF-1α at the protein level (40, 41) suggest a more complex model for HIF-1 in tumorigenesis. In this model, loss of wt p53 would remove a check on HIF-1 activation in response to hypoxia (41), favoring VEGF expression but discriminating against the expression of angiogenic inhibitors such as TSP-1 (67). However, analysis of the mRNA levels in a panel of human cells obtained from different normal tissues and relevant tumors. Potentially, these normal cells could also be exposed to hypoxia in a heterogeneous tumor. Fig. 5A indicates that the inhibition of TSP-1 expression by stringent hypoxia can be influenced by cell type. For example, hypoxia strongly decreased the level of TSP-1 mRNA in HCEs, had less of an inhibitory effect on the basal level in NDKs, and had no detectable effect on the level of TSP-1 mRNA in HCFs. Interestingly, it has been reported that TSP-1 expression is posttranscriptionally induced in hypoxic endothelial cells, supporting a cell type-specific effect on the regulation of this gene by hypoxia (55). Fig. 5B shows that although there appeared to be a small induction of TSP-1 mRNA accumulation in normal HMVECs by 6 h of hypoxia, in general there was no detectable decrease in this message, even after 24 h of prolonged stress. Among the immortalized/tumor cells, hypoxia strongly decreased TSP-1 mRNA levels in HCE.E6E7, C33a, and SiHa cells but had little or no effect on TSP-1 mRNA levels in FaDu cells. However, the basal level of TSP-1 expression was already relatively low in FaDu cells. In this context, examination of aerobic TSP-1 mRNA levels in the panel of cells in Fig. 5A suggests that basal TSP-1 expression decreases in response to transformation: relative aerobic TSP-1 mRNA levels in either HCEs or their immortalized/minimally transformed counterparts (HCE.E6E7 cells) were found to be substantially higher than those in the human cervical carcinoma cell lines (C33a and SiHa). Similarly, aerobic NDKs were found to have higher basal TSP-1 expression than aerobic FaDu cells, which were obtained from an epidermoid carcinoma (56). Because the SiHa cell line is HPV positive (containing E6 and E7), whereas the C33a and FaDu cell line lines are HPV negative, the relative basal TSP-1 expression in these cells does not seem to depend critically on how transformation was achieved. Together, these findings suggest that hypoxia could interact with oncogenic signals to further inhibit or suppress TSP-1 expression in the tumor microenvironment.

### Table 1: Overlap of EF5 and VEGF immunofluorescence in vivo

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<tr>
<th>Tumor</th>
<th>EF5</th>
<th>VEGF</th>
<th>EF5/VEGF</th>
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<tr>
<td>p53&lt;sup&gt;+/+&lt;/sup&gt; EH-MEF</td>
<td>10 ± 4</td>
<td>3 ± 1</td>
<td>3.3 ± 1.7</td>
</tr>
<tr>
<td>p53&lt;sup&gt;−/−&lt;/sup&gt; EH-MEF</td>
<td>10 ± 3</td>
<td>4 ± 2</td>
<td>2.5 ± 1.5</td>
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<sup>a</sup> Number of EF5 or VEGF immunofluorescent regions per 0.01 mm<sup>2</sup>.<br>
<sup>b</sup> Values are the means ± SD of measurements from at least three tumor sections.
effect of wt p53 on the induction of VEGF expression has produced conflicting results (68, 69), and the generality of the conclusion that p53 and HIF-1 interact in hypoxic cells is not established (70). As part of this study, we investigated the relationship between wt p53 and its effect on the simultaneous expression of TSP-1 and VEGF in response to pathophysiological hypoxia in both rodent and human cells. There are three major findings of the present study. First, hypoxia acted in a dominant manner to suppress TSP-1 expression in normal/immortalized rodent fibroblasts independently of their p53 genotype. In normal human cells, hypoxia also inhibited TSP-1 expression, but this effect was dependent on the cell type. Second, in both rodent and human cells, the level of basal/aerobic TSP-1 expression was decreased in response to transformation or to the degree of transformation. In general, where basal TSP-1 expression was detectable by Northern analysis of the transformed cells, it could be inhibited by stringent hypoxia. Third, in contrast to TSP-1, hypoxia induced VEGF expression in both normal/immortalized and transformed rodent and human cells, again independent of the presence of wt p53.

The finding that hypoxia can suppress TSP-1 expression in normal/immortalized rodent fibroblasts containing wt p53 contrasts with reports indicating that p53 positively regulates this gene in angiogenic human fibroblasts (17, 24). One explanation for this finding is that prolonged or stringent hypoxia may override normal physiological controls on the TSP-1 gene. For example, the effect of hypoxia on TSP-1 expression could be the outcome of a general metabolic response, the inhibition of macromolecule synthesis during prolonged hypoxic stress (71, 72). Alternatively, because hypoxia did not suppress basal transcriptional activity of the TSP-1 proximal promoter region in this study, it may affect TSP-1 expression by a specific mechanism such as the inhibition of mRNA stabilization. Although mRNA stabilization was postulated to account for the accumulation rather than the decline of TSP-1 mRNA in hypoxic endothelial cells (55), it is possible that the posttranscriptional control of TSP-1 expression in normal cells is cell type specific. In accord with this possibility, we observed that the same in vitro hypoxic conditions that suppressed TSP-1 expression in rodent fibroblasts had little or no effect on the level of TSP-1 mRNA in HCFs and microvascular endothelial cells but suppressed or inhibited TSP-1 mRNA levels in HCEs and NDKs. Interestingly, the expression of TSP-2 mRNA in NIH 3T3 cells appears to involve message stability (73). Further research is needed to understand the diverse regulation of TSP-1 and TSP-2 expression in hypoxic cells.

**Fig. 4** Effect of prolonged hypoxia on basal activity of the proximal promoter region of the human TSP-1 gene and on TSP-1 mRNA half-life in Rat-1 cells. A, Rat-1 fibroblasts were transiently transfected with 0.5 μg of the indicated luciferase reporter plasmids containing fragments of the human TSP-1 proximal promoter region (see “Materials and Methods”) and exposed to hypoxia (pO₂ ≤ 0.02%) for 10 h before harvesting for protein. The ordinate values are proportional to the luciferase activities in lysates of transfected and control cells. Error bars, the SD for replicate readings. B, panels shown are from an autoradiograph of a Northern blot of total RNA from Rat-1 cells exposed to prolonged hypoxia (pO₂ ≤ 0.01% for 12 h; top two panels) and from aerobic Rat-1 cells (5% CO₂/air; bottom two panels) harvested at the indicated times after exposure to the transcriptional inhibitor actinomycin D (5 μg/ml). Time 0 was defined as 10 min after the addition of actinomycin D at 37°C. Hypoxic cells were incubated with actinomycin D and harvested under anaerobic conditions at 37°C. The bottom panels in each set are from an autoradiograph showing the β-actin signal from the same Northern blot.
necessary to determine the degree to which transcriptional and posttranscriptional controls regulate TSP-1 mRNA expression in response to tumor hypoxia. The generally low levels of basal TSP-1 expression found in transformed rodent and human cells in this study may be the consequence of oncogenic changes leading to TSP-1 silencing or down-regulation (17, 30). Inhibition of basal TSP-1 expression has been reported for various oncogenes or oncogenic signals, including oncogenic ras, v-src, v-myc, polyoma middle T antigen, and overexpressed c-jun (27–29, 49, 74–76). As mentioned above, the finding that oncogenic ras almost eliminated detectable TSP-1 expression in Rat-1 fibroblasts supports this idea. We hypothesize that tumor hypoxia can synergize with oncogenic signals to suppress TSP-1 expression in the tumor microenvironment, analogous to its cooperation with ras to induce VEGF expression in transformed cells (10).

In summary, this study demonstrates that hypoxia can act in a dominant manner to inhibit the expression of TSP-1 and induce that of VEGF in diverse rodent and human cells independently of any p53-associated controls on these genes. Because hypoxia can select against transformed cells containing wt p53 in vivo (3), these findings indicate that tumor hypoxia could activate the angiogenic switch in a population of apoptotically resistant tumor cells (11), providing a powerful stimulus for malignant progression. Establishing the relative contributions of hypoxia-dependent and -independent mechanisms to the expression of angiogenic factors such as TSP-1 and VEGF in human tumors is an important area for further research.

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