Advances in Brief

p53 Does Not Repress Hypoxia-induced Transcription of the Vascular Endothelial Growth Factor Gene

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Abstract

Hypoxia-induced neovascularization mediated by vascular endothelial growth factor (VEGF) contributes to tumor progression. Based on its effects when overexpressed in transient transfection assays, p53 has been proposed to repress VEGF transcription. To investigate this hypothesis, we have analyzed endogenous VEGF mRNA levels in Hep3B cells stably expressing an inducible p53-estrogen receptor fusion protein and in irradiated RKO cells expressing endogenous wild-type p53. In both cell lines, VEGF mRNA levels increased in response to hypoxia, either in the presence or absence of functional p53. Our data provide no evidence for a causal relationship between the loss of p53 activity and increased VEGF expression that is observed during tumor progression. Studies that attribute repressor functions to p53 based on analysis of cells transiently overexpressing this protein should be interpreted cautiously.

Introduction

During tumor progression, cells acquire many mutations affecting the rates of cellular proliferation, cell death, and further mutation. An important mutational target is the gene encoding p53, a protein that regulates each of these processes (reviewed in Ref. 1). Tumor cells also adapt to adverse environmental conditions such as hypoxia that arise during dysregulated cellular proliferation. Transcriptional activation of the VEGF gene by HIF-1 in response to hypoxia (2, 3) leads to neovascularization that allows continued tumor growth (reviewed in Ref. 4). Hypoxia-mediated apoptosis of transfected cells was induced in the presence but not in the absence of wild-type p53 (5). Forced expression of p53 was associated with decreased VEGF mRNA levels and VEGF promoter activity in transfected 293 cells (6), suggesting that repression of VEGF transcription may represent another tumor-suppressing effect of p53. However, these conclusions were drawn from experiments in which p53 was transiently overexpressed, which also caused dose-dependent repression of transcription from a CMV promoter. In addition, 293 cells express adenovirus E1A and E1B proteins that affect p53 function (7–10). The experiments were also performed under nonhypoxic culture conditions in which VEGF transcription is low relative to hypoxic conditions (2, 3). We therefore analyzed the effect of wild-type p53 activity on VEGF expression under both hypoxic and nonhypoxic conditions in stably transfected and nontransfected cells.

Materials and Methods

Materials. Puromycin (Clontech) was used as a 1 mg/ml stock solution. 4HT (Research Biochemicals International, Natick, MA) was dissolved in DMSO at 1 mM stock solution. FBS and tissue culture media were purchased from Life Technologies, Inc. and Mediatech, respectively.

Tissue Culture. BT-4P and BT-2E subclones of Hep3B were described previously (11). To generate BT-2E, Hep3B cells were transfected with pBPuro alone. To generate BT-4P, cells were transfected with p53-mER-pBPuro containing human p53 coding sequences cloned in frame with and amino-terminal to a modified estrogen receptor with a Gly125Arg mutation (12) that renders the ligand binding domain responsive to 4HT and insensitive to estradiol. Hep3B, BT-4P, and BT-2E cells were maintained in MEM containing 10% FBS, 1% penicillin-streptomycin, and 2 μg/ml puromycin (BT-4P and BT-2E cells only). Human colorectal carcinoma RKO cells were maintained in DMEM with 10% FBS and 1% penicillin-streptomycin. Cells were exposed to hypoxia in a modular incubator chamber flushed with 1% O2/5% CO2/balance N2. When indicated, BT-4P or BT-2E cells were exposed to 500 nm 4HT. When indicated, RKO cells were exposed to 4 GY of γ-irradiation from a 137Cs source.

Transient Transfection Assays. Hep3B cells were electroporated with plasmid DNA and exposed to hypoxia, and cell lysates were prepared for luciferase and β-gal assays as described previously (2). Plasmid pCMV-p53 (13) was provided by B. Vogelstein (Johns Hopkins University). HIF-1α coding sequences were cloned 3′ to the CMV promoter in the expression vector pCPE4 (2).

Immunoblot Assays. Whole cell lysates were prepared in Laemmli buffer (14). Nuclear extracts were prepared as described previously (15). Protein concentration was determined by Bradford assay using a commercial kit (BioRad). Lysates and extracts were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The primary antibodies used were: Ab-6 (Oncogene Science) for detection of p53 in RKO cell lysates; a combination of Ab-2 and Ab-6 (Oncogene Science) for detection of p53ER in BT-4P and BT-2E extracts; anti-p21 (Pharmingen); and anti-topoisomerase I (Topogen).

RNA Blot Hybridization. Total RNA (15 μg) was fractionated by formaldehyde/agarose gel electrophoresis and transferred to nylon membranes. Probes were prepared by random primer labeling using [α-32P]dCTP and a commercial kit (Boehringer Mannheim) followed by spin column purification (Worthington Biochemical). Blots were incubated in Quick-Hyb (Stratagene) at 67°C for 2 h and washed in 0.1 X SSC and 0.1% SDS at 50°C. Probes for hybridization included a 0.8-kb murine p21 cDNA fragment excised from vector PCM35 (Ref. 14; provided by B. Vogelstein) and a 0.5-kb rat VEGF cDNA (provided by R. Wenger and M. Gassmann, University of Zurich, Zurich, Switzerland). Blots were stripped and hybridized with a 32P-labeled oligonucleotide complementary to 18S rRNA (provided by A. Choi, Johns Hopkins University).

Results

Effect of p53 on VEGF Reporter Gene Expression in Transiently Transfected Cells. Transient transfection experiments were performed in Hep3B human hepatoblastoma cells. These cells were chosen because they are p53 null, expressing neither mutant nor wild-type p53. When indicated, BT-4P or BT-2E cells were transfected with p53-mER-pBPuro containing human p53 coding sequences cloned in frame with and amino-terminal to a modified estrogen receptor with a Gly125Arg mutation (12) that renders the ligand binding domain responsive to 4HT and insensitive to estradiol. Hep3B, BT-4P, and BT-2E cells were maintained in MEM containing 10% FBS, 1% penicillin-streptomycin, and 2 μg/ml puromycin (BT-4P and BT-2E cells only). Human colorectal carcinoma RKO cells were maintained in DMEM with 10% FBS and 1% penicillin-streptomycin. Cells were exposed to hypoxia in a modular incubator chamber flushed with 1% O2/5% CO2/balance N2. When indicated, BT-4P or BT-2E cells were exposed to 500 nm 4HT. When indicated, RKO cells were exposed to 4 GY of γ-irradiation from a 137Cs source.

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wild-type forms of p53 protein (16). Hep3B cells were transfected with the reporter plasmid P11-VEGF, which contains a 47-bp hypoxia response element from the human VEGF promoter cloned 5' to a SV40 promoter/luciferase transcription unit (2) and reporter plasmid pSVβgal, which contains a SV40 promoter/β-gal transcription unit (Fig. 1A). The cells were also transfected with 1 µg of an expression vector encoding HIF-1α (pCEP4/HIF-1α), the limiting and hypoxia-inducible subunit of the transactivator HIF-1 (17), to provide higher basal transcription levels for analysis of repression by p53. Finally, 1 µg of an expression vector containing either no insert (pCEP4) or the p53 coding sequence (pCMV-p53) was included to determine the effect of p53 on reporter transcription.

Transfected cells were split on to duplicate plates and exposed to 20 or 1% O2 for 24 h, and cell lysates were analyzed for luciferase and β-gal activity. Expression of HIF-1α increased transcription of P11-VEGF but not pSVβgal (data not shown), and in response to hypoxia, P11-VEGF transcription increased 4.6-fold, whereas transcription of pSVβgal was unaffected (Fig. 1B), as reported previously (2). In the presence of p53, transcription of VEGF-P11 decreased by more than 80% in cells exposed to 20 or 1% O2. p53 also repressed VEGF-P11 transcription in the absence of HIF-1α expression vector (data not shown). Transcription of a 2.6-kb VEGF promoter/luciferase reporter was also repressed by p53 in nonhypoxic 293 cells (6). Taken together, these results suggest that if p53 represses VEGF transcription in a sequence-specific manner, it acts via the 47-bp hypoxia response element.

Although less markedly affected, pSVβgal transcription was repressed by as much as 65% in cells transfected with 1 µg of p53 expression vector. Transfection of 1 µg of a p53 expression vector had no effect on transcription of a CMV promoter/βgal reporter in 293 cells, although transcription was repressed in a dose-dependent manner by ≥2 µg of p53 expression vector (6). The slightly different results of these two studies may be due to higher p53 expression in electroporated Hep3B cells compared to that in calcium phosphate-transfected 293 cells. Taken together, these data suggest that the repressive effect of p53 may not be highly specific for the VEGF promoter.

**Effect of a p53-Estrogen Receptor Fusion Protein on VEGF Transcription.** To eliminate potential artifacts associated with transient transfection, the effect of p53 on endogenous VEGF transcription was analyzed in a Hep3B subclone stably transfected with an inducible form of p53. BT-4P cells express puromycin resistance and a p53-modified estrogen receptor fusion protein (p53mER) that is active only in the presence of the synthetic ligand 4HT (11). Twenty-four h after the addition of 4HT, p53mER was detectable by immunoblot assay within nuclear extracts of BT-4P cells (Fig. 2A). p53mER protein levels in whole cell lysates were unaffected by the addition of 4HT (data not shown), suggesting that ligand binding induces nuclear translocation of the fusion protein.

To analyze the effect of p53mER induction on the expression of p53-mediated transcription, BT-4P or BT-2E cells (which only express puromycin resistance; Ref. 11) were treated with 4HT or vehicle (DMSO) for 24 h. Under these conditions, increased expression of the p53-inducible p21 gene was detected at the level of protein (Fig. 2B, A).
Fig. 2. Effect of 4HT-induced p53mER on VEGF mRNA expression in Hep3B cells. A, p53mER levels in BT-4P nuclear extracts. BT-4P cells were treated with 500 nM 4HT (Lane 2) or DMSO (Lane 1) for 24 h before nuclear extract preparation and immunoblot assay. Migration of molecular mass markers (in kDa) and p53mER are indicated at left. B, p21 protein expression in BT-4P and BT-2E cells. BT-4P cells were treated with 500 nM 4HT or DMSO for 24 h (Lanes 1 and 2) or 48 h (Lanes 3–6) under hypoxic (Lanes 4 and 6) or nonhypoxic (Lanes 1, 2, 3, and 5) conditions. BT-2E cells were treated with 500 nM 4HT under hypoxic (Lane 8) or nonhypoxic (Lane 7) conditions for 48 h. Whole cell lysates were prepared for p21 immunoblot assay. C, p21 mRNA expression. Cells were treated as described above, and total cellular RNA was isolated for blot hybridization using a p21 cDNA probe. D, VEGF mRNA expression. Aliquots of total cellular RNA prepared as described above were analyzed by blot hybridization using a VEGF cDNA probe (top panel). The blot was then stripped of radioactivity and hybridized with an 18S rRNA oligonucleotide probe (bottom panel).

Discussion
We draw the following conclusions from the data presented in this paper: (a) in transient expression assays, p53 repressed transcription of the endogenous VEGF gene in Hep3B cells was not affected by p53 expressed from genomically integrated sequences.

Induction of p53 by Irradiation. Even though p53mER activated transcription of p21 as well as cyclin G and mdm2 (11), it was possible that this chimeric protein retained some but not all of the biological functions of endogenous wild-type p53. Therefore, as a second independent means of inducing p53 activity, RKO cells that express only endogenous wild-type p53 were exposed to 20 or 1% O2, either untreated or after exposure to γ-irradiation. After γ-irradiation, p53 and p21 levels were increased in cells exposed to 20 or 1% O2, whereas levels of the control protein topoisomerase were unchanged (Fig. 3A). Compared to control cells exposed to 20% O2, VEGF mRNA levels increased 3.9-fold in cells exposed to 1% O2 and 3.6-fold in cells exposed to γ-irradiation followed by 1% O2 (Fig. 3B). Thus, in a second independent assay system, induction of p53 had no significant effect on the hypoxia-mediated expression of VEGF mRNA.

Discussion
We draw the following conclusions from the data presented in this paper: (a) in transient expression assays, p53 repressed transcription of reporter genes in a relatively nonspecific manner, because the
probes specific for VEGF mRNA (top panel) or 18S rRNA (bottom panel). Whole cell lysates were subjected to immunoblot assay (lanes 1 and 4) or RT (lanes 2 and 3) for 10 h after γ-irradiation (lanes 3 and 4) or no treatment (lanes 1 and 2). Cells were treated as described above, total cellular RNA was prepared, and aliquots were analyzed by blot hybridization using probes specific for VEGF mRNA (top panel) or 18S rRNA (bottom panel).

The presence of the VEGF hypoxia response element resulted in only a moderate increase in susceptibility to p53-mediated repression; and (b) levels of p53mER or endogenous wild-type p53 that were sufficient to activate p21 expression were not sufficient to repress basal or hypoxia-induced VEGF expression.

These results indicate that conclusions regarding repressor functions of p53 based on analysis of cells transiently overexpressing this protein should be interpreted cautiously. Transfection assays involve the transcription of chimeric reporter genes contained on bacterial plasmids, in contrast to the transcription of endogenous genes present on human chromosomes. Transient transfection of expression vectors is likely to result in very high concentrations of p53 in the transfected cells. We used an inducible form of p53 because of the ability to titrate its activity based on ligand concentration. However, even at high concentrations of 4HT (500 nm) that resulted in marked induction of nuclear p53mER and of p21 gene expression, we observed no significant repression of VEGF expression, suggesting that p53 may repress the transcription of genes present on plasmid but not on chromosomal DNA.

In a previous report, transient expression of p53 repressed endogenous VEGF mRNA levels in 293 cells (6). This effect may have been related to the transient transfection of p53 expression vector by calcium phosphate precipitation, the presence of adenovirus E1A and E1B proteins in 293 cells, or some other confounding factor, as suggested by the observation that the reported inhibition of VEGF expression by transfectected p53 (80%) exceeded the stated transfection efficiency (50–70%; Ref. 6). When wild-type p53 is overexpressed in cells containing mutant forms of p53 or proteins such as E1A and E1B that affect p53 function, interpretation is particularly problematic. In this regard, a mutant (Ala135-Val) form of murine p53 has been shown to induce expression of VEGF mRNA (18). Thus, overexpression of wild-type p53 may counteract the effect of mutant p53, leading to an apparent repression of VEGF transcript. Repression was not observed in Hep3B and RKO cells that only expressed wild-type p53.

Although tumor progression is associated with loss of p53 activity and increased VEGF expression, the data presented in this paper provide no evidence for a causal relationship between these phenomena. The many effects of p53 on cellular function do not include repression of VEGF transcription, at least in the Hep3B and RKO cell lines that we have analyzed. p53 may nevertheless have important effects on vascularization through its transcriptional activation of the gene(s) encoding thrombospondin 1 and possibly other angiogenesis inhibitors (19, 20) such that the loss of wild-type p53 in tumor cells may lead to increased angiogenesis due to decreased levels of inhibitors.

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References

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