Hypoxia Induces p53 Accumulation through MDM2 Down-Regulation and Inhibition of E6-mediated Degradation

Rodolfo Alarcon, Constantinos Koumenis, Rory K. Geyer, Carl G. Maki, and Amato J. Giaccia

Mayer Cancer Biology Research Laboratory, Department of Radiation Oncology, Stanford University School of Medicine, Stanford, California 94305-5468 [R. A., C. G. M.], and Harvard School of Public Health, Department of Cancer Cell Biology, Boston, Massachusetts 02115 [R. K. G., C. G. M.]

Abstract

Hypoxia, a result of DNA-damaging agents such as ionizing radiation, induces the nuclear accumulation of the p53 tumor suppressor protein. However, unlike the effect in ionizing radiation, hypoxia readily induces the nuclear accumulation of p53 in HPV E6-infected cells. In HPV-infected cells, a key regulator of p53 protein levels is the E6 oncoprotein. In association with the endogenous cellular protein E6-associated protein (E6AP), E6 can accelerate the degradation of p53 under aerobic conditions. To better define the mechanism of p53 induction in E6-infected cells by hypoxia, we studied the expression and association of E6 and E6AP with p53 in vivo. We found that hypoxia did not alter the protein levels of E6 or E6AP as compared with those found under aerobic growth conditions, indicating that protein inhibition of E6 or E6AP alone is not sufficient to explain the increased accumulation of p53 under hypoxic conditions. However, p53 did fail to coprecipitate with E6AP under hypoxia, indicating that hypoxia uncouples the interaction of p53 with E6 and E6AP. We also present evidence to indicate that hypoxia decreases the expression of the endogenous cellular regulator of p53 protein, the human MDM2 protein, resulting in an inhibition of p53 export from the nucleus to the cytoplasm for degradation. Taken together, these results suggest that the hypoxic induction of p53 is attributable to the down-regulation of MDM2 protein levels and uncoupling of p53 from its interaction with the E6/E6AP complex.

Introduction

Emerging evidence clearly indicates that the human tumor suppressor protein p53 is a key regulator of the response to cellular stresses such as DNA damage, nucleotide depletion, mitogenic oncoproteins, and the tumor microenvironmental stress of hypoxia (1, 2). In untransformed fibroblasts, wt p53 basal levels are low. However, in response to stress, latent p53 is activated, and its half-life is extended from 30 to >200 min (3). Thus, the increase in the half-life of p53 protein contributes to the apparent increase in p53 protein levels. An increase in the rate of p53 mRNA translation has also been shown to contribute to p53 steady-state levels (4).

In addition to protein accumulation, p53 is also posttranslationally modified and associates with accessory proteins to modulate critical downstream target gene expression. Of the many proposed biological activities attributed to p53, its roles in cell cycle regulation and apoptosis have been well established (5). For example, in response to DNA damage, p53 activation leads to a G1-phase cell cycle arrest that is mediated by the transcriptional up-regulation of the cyclin-depend-
lation in human colorectal carcinoma cell lines that express HPV E6 (30). Here we investigate the mechanism of p53 induction by hypoxia. We provide evidence indicating that hypoxia down-regulates MDM2 protein levels and thereby reduces p53 nuclear export, which leads to its degradation. Additionally, we demonstrate that hypoxia does not alter the protein levels of E6 or E6AP in E6-expressing cells but rather disassociates their interaction with p53, allowing for its nuclear accumulation. Finally, we show that hypoxic activation of p53 does not require the function of the p14ARF protein, indicating that oncogene-induced stabilization of p53 occurs through a separate pathway than that of hypoxia. Taken together, these results suggest a novel means of hypoxic induction of p53 protein that involves disruption of MDM2-mediated nuclear export and the concurrent inhibition of E6/E6AP-mediated degradation.

Materials and Methods

Cell Lines and Plasmids. The human colorectal carcinoma cell lines RKO and RC.10.2 (RKO.E6) were obtained from Dr. Kathleen Cho (Johns Hopkins University School of Medicine, Baltimore, MD) and were cultured in McCoy’s 5A medium with 10% fetal bovine serum. RC10.2 is a stable transfectant of RKO expressing the HPV-16 E6 gene (33). The p53/+/−/mdm2−/− MEFs were kindly provided by Dr. Guillermia Lozano (University of Texas M. D. Anderson Cancer Center, Houston, TX); MEFs were cultured in DMEM with 10% (34). Expression plasmids for wt p53 and hMDM2 were obtained from Dr. Arnold Levine (Rockefeller University, New York, NY; Ref. 35). The pE6GFP plasmid was constructed by using PCR to amplify the full-length HPV-16 E6 cDNA (kindly provided by Dr. Denise Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA) using the following oligonucleotides, 5′-GGATCCATATGGAGTTTCACTATTGTA-3′ and 5′-GGATCCTTGATGCTGTTCGAGTATT-3′. The PCR fragments were cloned into the BamHI restriction site of the jellyfish GFP expression plasmid, pEGFPN1 (Clonetech Laboratories, Palo Alto, CA).

Immunofluorescent Staining. For E6GFP detection, 10 μg of plasmid DNA were electroporated into exponentially growing RKO cells at 0.25 kV and 960 nF using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA) and plated on Lab-Tek slides (Nunc, Naperville, Ill.). Cells were treated and fixed as described (30), and fluorescence was observed 24 h after transfection by fluorescence microscopy. All hypoxia treatments were performed in a hypoxic chamber (<0.2% O2; Shelldon Corp., Cornelius, OR) or in specially designed aluminum hypoxia chambers.

Immunoblotting. After treatment, cell extracts were prepared in 200 μl of lysis buffer [10 mm Tris (pH 7.5), 1 mM EDTA, 400 mM NaCl, 10% glycerol, 0.5% NP-40, 5 mM NaF, 0.5 mM sodium orthovanadate, 1 mM DTT, and 0.1 mM PMSF]. Protein concentrations for each sample were determined using the DC protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer’s specifications. Equivalent amounts of protein were analyzed on NUPAGE 10% or 4–12% SDS-polyacrylamide gels (Novex, San Diego, CA). Primary antibodies used for immunoblotting were: DO-1 mouse monoclonal for human p53, SMP-14 mouse monoclonal antibody for human MDM-2 (Oncogene Science); and the M-19 rabbit polyclonal for human p21 (Santa Cruz Biotechnology) for 24 h at 4°C. The immunoprecipitates were then washed four times with 10X buffer [50 mM HEPES (pH 7.9), 250 mM KCl, 0.2% NP40, 0.1% Triton X-100, 0.01% SDS, and 1 mM DTT], resuspended in 30 μl of SDS gel-loading buffer [0.5 M Tris (pH 6.8), 2 M β-mercaptoethanol, 12% SDS, 30% glycerol, and 0.1% bromphenol blue], and analyzed on 4–12% NUPAGE SDS-polyacrylamide gels as above. For p53, MDM2, and E6AP detection, blots were probed as above.

Results

Hypoxia Induces p53 Protein Accumulation in RKO and RKO.E6 Cells. To study the molecular mechanism of p53 induction by hypoxia, we first characterized the induction of p53 levels in the human colorectal carcinoma cell line, RKO (33). We compared the induction of p53 by hypoxia to that of IR in the E6-expressing cell line, RKO.E6. When RKO or RKO.E6 cells were exposed to hypoxia (0.02%) for 24 h, p53 protein increased to a similar degree as cells treated with the proteasome inhibitor, MG132 (Fig. 1A). Hypoxia induced p53 protein accumulation in the RKO.E6 cell line, indicating that E6-mediated degradation of p53 in RKO.E6 cells was somehow attenuated under low oxygen conditions. In contrast, treatment with 4 Gy of IR did not affect the ability of E6 to mediate the degradation of p53. Similarly, UV irradiation failed to induce p53 protein accumulation in RKO.E6 cells, also suggesting that targeted degradation of p53 was unaffected by this form of damage. Although hypoxia induces p53 protein accumulation in a variety of cell lines, p53 protein is transcriptionally latent but functional in inducing apoptosis.4 Because most established human cell lines exhibit low levels of apoptosis, we did not detect a significant increase in apoptosis in the RKO or RKO.E6 cell lines under the stress-inducing conditions used in this study. To determine whether the p53 protein induced by hypoxia in the RKO.E6 cell line was functional, we irradiated cells under a low oxygen environment with 4 Gy of ionizing radiation, continued to incubate the cells for an additional 4 h under low oxygen conditions, and then probed for p21 induction (Fig. 1A). p21 protein was detected in cells irradiated under hypoxia or after treatment with the proteasome inhibitor but not in cells treated with hypoxia or UV alone. As hypothesized, the p53-dependent induction of p21 in response to IR was intact in the RKO cell line; however, only a minimal induction of p21 occurred in RKO.E6 cells. The increase in p21 protein induced by MG132 in RKO cells did not require functional p53 (39). These results demonstrate that hypoxia can induce p53 protein accumulation that can be transcriptionally activated by DNA damage in an E6-expressing cell.

Many stresses have been shown to induce p53 accumulation, at least in part, by extending the protein’s half-life (40). To determine whether hypoxia also increases p53 stability, we measured the half-life of p53 exposed to either hypoxia or IR. Six h after treatment, cells were incubated with cycloheximide to inhibit de novo p53 synthesis, and the steady-state levels of p53 were determined at the indicated times (Fig. 1B). The half-life of p53 in nonirradiated RKO cells was between 30 and 60 min. In RKO.E6 cells, p53 protein was undetectable because of E6-mediated degradation (Fig. 1C). In contrast, hypoxia increased the half-life of p53 up to 120 min in both RKO and RKO.E6 cells, demonstrating that hypoxia extends the half-life of p53 in both an E6-positive and -negative background. We only observed an increase in p53 half-life in RKO cells in response to IR, whereas levels remained undetectable in RKO.E6-treated cells.

4 C. Koumenis et al., submitted for publication.
Hypoxia Regulates ARF and MDM2 Protein Levels. Recent studies have proposed that the regulation of p53 protein stability can be mediated by the p14ARF protein and through an autoregulatory loop with the MDM2 protein (37, 41). To determine a role for ARF or MDM2 in regulating p53 stability in E6-expressing cells, we immunoblotted for each protein in cells treated with hypoxia for 0, 6, 12, or 24 h (Fig. 2A). In RKO cells, we observed p53 protein accumulation by 6 h, being maximal at 24 h. Under these same conditions, we found that hypoxia induced a rapid decrease of both ARF and MDM2 levels, which was maximal at 24 h. Under these conditions, we found that ARF and MDM2 levels were detectable and remained unchanged in both cell lines, even after 24 h of treatment. Because reliable E6 antibodies that would detect endogenous E6 protein in RKO cells do not exist, we fused the E6 gene to the NH2-terminus of the GFP and measured GFP expression in transiently transfected RKO cells (Fig. 2, B and C). In this system, we observed no change in the expression of GFP fluorescence or protein in aerated or hypoxia-treated cells.

Previous studies have established that neither E6 nor E6AP by itself stably associates with p53 and that E6AP can form a stable complex with E6 in the absence of p53 (27, 42), indicating that only a ternary complex is stable. Because hypoxia did not affect E6GFP or E6AP levels, we determined whether endogenous E6AP could still interact with p53 under hypoxia. We found that E6AP would only coprecipitate with p53 under aerobic growth conditions and after proteasome inhibitor treatment. We observed a substantially reduced interaction of E6AP with p53 under hypoxia (Fig. 2C). Taken together, these results suggest that the pathway leading to stabilization of p53 protein by hypoxia in E6-expressing cells does not require the down-regulation of E6 or E6AP protein levels but the inhibition of the formation of E6/E6AP/p53 complexes.

Hypoxia Inhibits MDM2-mediated Degradation of p53. Recent studies have described a novel nuclear export signal domain in MDM2 that allows it to shuttle from the nucleus to the cytoplasm (43, 44). Given this finding and our observation that hypoxia down-regulates MDM2 expression, we investigated whether ectopic expression of hMDM2 and wt p53 in p53/MDM2 null MEFs would still lead to accelerated p53 degradation under hypoxia (Fig. 3A). Cotransfection of hMDM2 and p53 lead to a decrease in the amount of p53 detected in these cells under both aerobic and hypoxic conditions. Cotransfection of p53 with E6GFP also led to degradation under both aerobic and hypoxic conditions. Thus, transient overexpression of MDM2 or E6 in cells was sufficient to mediate the degradation of p53 under hypoxia. In contrast, a MDM2 nuclear shuttling mutant, MDM2NES, was unable to degrade p53 under both aerobic or hypoxic conditions, suggesting the ability of MDM2 to export p53 from the nucleus was sufficient to mediate the degradation of p53 under hypoxia. These observations also suggest that the enforced expression of E6 in a cell can overcome hypoxia-mediated uncoupling of p53 from MDM2/MDM2AP complexes. We suggest that hypoxia does not alter the ability of the ub/proteasome degradation machinery responsible for the destruction of p53 within a cell, but that hypoxia-induced p53 protein can be attributed in large part to MDM2 down-regulation or destabilization of p53/E6AP complexes.
conjugates under hypoxic conditions and after reoxygenation (Fig. 3B). After hypoxia treatment, we did not observe a decline in the extent of p53-ub conjugates, most likely resulting from undetectable MDM2 activity, but there was a substantial induction in the amount of p53-ub conjugates after reoxygenation. At the same time, p53 levels decreased while MDM2 protein returned to normal levels. Thus, upon reoxygenation MDM2 expression returned with a concurrent increase in p53-ub conjugates and a decrease in total p53 levels. As expected, p53-ub conjugates were present after IR but not after UV treatment, although p53 levels only increased after IR (3). These results confirm our observation that simply inhibiting the expression of MDM2 could result in p53 accumulation, whereas increasing the levels of MDM2 results in accelerated p53 degradation.

Discussion

The data presented in this communication indicate that the hypoxic accumulation of p53 protein may result from MDM2 protein down-regulation and the uncoupling of p53 from E6/E6AP complexes. These results provide a possible mechanistic explanation for our previous observations that hypoxia, but not IR, induced p53 levels in primary cervical epithelial cells immortalized with the HPV E6 and E7 genes (32). Recent studies have found that inactivation of MDM2 by p14ARF mediates p53 accumulation in oncogenically transformed MEFs (46). However, our findings indicate that hypoxia uses an ARF-independent pathway for signaling up-regulation of p53 (Fig. 2B). Because IR-induced p53 is also ARF independent, we propose that the induction of p53 by hypoxia is separable from that of DNA damage agents and mitogenic oncogenes such as E1A or c-Myc (46, 47). Nevertheless, ARF down-regulation is an intriguing observation, and it is conceivable that hypoxia may regulate tumor progression by both down-regulating the ARF tumor suppressor pathway and by selecting for apoptosis-resistant mutants.

We have found that hypoxia is a potent inducer of Ser-15 phosphorylation in both E6-positive and -negative cell lines. Recent studies have suggested that this modification disrupts the p53/MDM2

Discussion

The data presented in this communication indicate that the hypoxic accumulation of p53 protein may result from MDM2 protein down-regulation and the uncoupling of p53 from E6/E6AP complexes. These results provide a possible mechanistic explanation for our previous observations that hypoxia, but not IR, induced p53 levels in primary cervical epithelial cells immortalized with the HPV E6 and E7 genes (32). Recent studies have found that inactivation of MDM2 by p14ARF mediates p53 accumulation in oncogenically transformed MEFs (46). However, our findings indicate that hypoxia uses an ARF-independent pathway for signaling up-regulation of p53 (Fig. 2A). Because IR-induced p53 is also ARF independent, we propose that the induction of p53 by hypoxia is separable from that of DNA damage agents and mitogenic oncogenes such as E1A or c-Myc (46, 47). Nevertheless, ARF down-regulation is an intriguing observation, and it is conceivable that hypoxia may regulate tumor progression by both down-regulating the ARF tumor suppressor pathway and by selecting for apoptosis-resistant mutants.

We have found that hypoxia is a potent inducer of Ser-15 phosphorylation in both E6-positive and -negative cell lines. Recent studies have suggested that this modification disrupts the p53/MDM2...
interaction, thus leading to p53 stabilization after IR (48). The decrease in MDM2 levels after hypoxic treatment indicates that phosphorylation of Ser-15 may not be necessary for hypoxia-induced p53 accumulation. However, we did detect a low level of p53-ub conjugate formation, even after 24 h of hypoxia, suggesting that there remains residual MDM2 activity capable of mediating the ubiquitination of p53. Thus, p53 phosphorylation may inhibit the efficient ubiquitination of p53 by residual MDM2 protein.

Ectopic expression of MDM2 under both aerated or hypoxic conditions was sufficient to mediate the degradation of p53 in p53/ MDM2 null MEFs (Fig. 3A), indicating that the cellular machinery necessary for p53 degradation is still intact under hypoxic conditions. Because the overexpression of a MDM2 export mutant failed to degrade p53, degradation of p53 under hypoxic conditions requires nuclear export. Recently, it has been suggested that E6-mediated degradation of p53 requires the nuclear export function of MDM2 (49). However, we found that enforced expression of an E6GFp fusion protein was sufficient to mediate the degradation of p53 in cells lacking MDM2. Because the E6GFp fusion protein was both nuclear and cytoplasmic in these overexpression studies and to date no export function has been attributed to E6 or GFP, we believe that E6-mediated degradation of p53 can precede nuclear export of p53. That is, E6 may first interact with E6AP and then in the cytoplasm; before p53 has translocated into the nucleus, E6 may direct E6AP-dependent ubiquitination and degradation of p53. Thus, under normal physiologic conditions were MDM2 and E6 protein are functional, hypoxia is able to uncouple p53 from E6-dependent degradation in the cytoplasm and inhibit MDM2 mediated export of p53, which results in p53 accumulation. On the basis of these studies, we propose that the mechanism by which hypoxia induces p53 protein levels involves both the disruption of MDM2-mediated nuclear export and the concurrent inhibition of E6/E6AP-mediated degradation.

Acknowledgments

We thank Dr. Kathleen Cho for the RKO and RKO.E6 cell lines, Dr. Guillermima Lozano for the p53/Mdm2 null MEFs, Dr. Moshe Oren for the human p53 expression plasmid, Dr. Arnold Levine for the MDM2 expression plasmids, and Dr. Denise Galloway for the HPV 16 E6 cDNA.

References


Hypoxia Induces p53 Accumulation through MDM2 Down-Regulation and Inhibition of E6-mediated Degradation


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/59/24/6046

Cited articles This article cites 49 articles, 29 of which you can access for free at: http://cancerres.aacrjournals.org/content/59/24/6046.full.html#ref-list-1

Citing articles This article has been cited by 35 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/59/24/6046.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.