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p53-Dependent and p53-Independent Induction of Insulin-Like Growth Factor Binding Protein-3 by Deoxyribonucleic Acid Damage and Hypoxia

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Abstract

IGF binding protein (IGFBP)-3, the principal carrier of IGFs in the circulation, contributes to both endocrine and autocrine/paracrine growth control; it can be induced by GH, cytokines, retinoic acid, and tumor suppressors. Induction of IGFBP-3 by the tumor suppressor p53 has been shown in various models that directly manipulate p53 activity. However, the physiologic settings under which this induction occurs have not been established. DNA damage and hypoxia are two important physiologic activators of p53. We have demonstrated for the first time that IGFBP-3 is an *in vivo* target of p53 in response to ionizing radiation. This effect was tissue specific.

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Furthermore, we demonstrated that genotoxic drugs could increase IGFBP-3 protein levels and secretion in tumor cell lines in a p53-independent manner. Finally, we have established that IGFBP-3 induction under hypoxic conditions is independent of p53 in tumor cell lines derived form multiple tissue types. Thus, IGFBP-3 is induced by physiologic conditions that also induce p53, although p53 is not always required. Because IGFBP-3 can inhibit growth and induce apoptosis in IGF-dependent and IGF-independent manners, its induction by DNA damage and hypoxia suggest IGFBP-3 plays a role in the physiologic protection against aberrant cell growth.

Mutations in P53 are the most frequently occurring genetic lesions among human cancers, and many of the cancers that do not harbor p53 mutations have mutations in regulators of p53 function (1, 2). p53 normally resides in low concentrations in a latent form but is activated via protein accumulation, posttranslational modifications (3), and subcellular translocation (4, 5) to induce cell cycle arrest or apoptosis, thereby preventing the propagation of damaged cells. The stimuli for p53 activation include DNA damage, chromosomal defects, telomere shortening, cellular hypoxia, and oncogene overexpression, and their diversity contributes to the importance of p53 as a tumor suppressor (6). Once activated, p53 causes cell cycle arrest or apoptosis primarily through sequence-specific transcriptional transactivation of target genes (7) and, to a lesser extent, transcriptional repression, protein-protein interactions, and direct mitochondrial activities (8).

IGF binding protein (IGFBP)-3 is one of the genes transcriptionally activated by p53 (9, 10). Originally identified as the principal carrier of IGFs in the circulation and as such, an important component of the GH-IGF axis, IGFBP-3 also plays a role in autocrine and paracrine growth control and apoptosis (11). Multiple p53 mutants with selective loss of the ability to transactivate IGFBP-3 and Bcl-2-associated X protein could not induce apoptosis, whereas mutants without $p21^{WAF1/CIP1}$ transactivation could not induce cell cycle arrest (12–14). We have previously demonstrated that IGFBP-3 mediates p53-induced apoptosis during serum starvation using two contrasting neoplastic cell models (15). p53-null prostate cancer cells were stably transfected with a doxycycline-inducible p53 construct to turn p53 on, and lung cancer cells containing fully functional wild-type p53 were stably transfected with human papillomavirus E6, which targets p53 for ubiquitin-mediated degradation (16,17), to turn p53 off. Whereas these two models enabled us to directly manipulate p53 levels to study the effects on IGFBP-3 and apoptosis, they are artificial constructs. The question remains, what is the physiologic context of IGFBP-3 induction by p53? If IGFBP-3 were a physiologic target of p53, then it should be induced by conditions that physiologically induce p53.

Materials and Methods

Mice and treatments

Healthy 6- to 7-wk-old female $p53^{-/-}$ and $p53^{+/+}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME). Two animals in each experimental group received a single ip injection of either 0.5 mg dexamethasone (Elkins-Sinn Inc., Cherry Hill, NJ) or sterile 1× PBS. Two animals of each genotype received 5 Gy total body irradiation from a ^{137}Cesium γ-source at a dose rate of 1.532 Gy/min. The mice were killed at 6 and 24 h using an

approved protocol (University of Pennsylvania Institutional Animal Care and Use Committee), which followed recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Tissues were harvested, snap frozen in liquid nitrogen, and kept at −80 C until used for the Taqman RT-PCR experiments.

Taqman real-time quantitative RT-PCR

Total RNA was isolated from radiation-sensitive tissues as previously described (18), and 1 μg was used for reverse transcription (RT) and amplification using TaqMan reverse transcription reagents according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Parallel samples were similarly treated but without adding reverse transcriptase (−RT) to provide a control for genomic contamination. Taqman RT-PCR assay was conducted according to the manufacturer's specifications (Applied Biosystems) and as previously reported for other p53 target genes (19). A master mix of TaqMan reagents was prepared, and 10 ng of each RT (−RT) sample were used in the Taqman PCR. Each tube contained a probe and primer set for both IGFBP-3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control; the IGFBP-3 probe was labeled with FAM (6-carboxyfluorescin), whereas that for GAPDH was labeled with VIC (Applied Biosystems). Using Primer Express version 1.0 (Applied Biosystems), the following primer and probe sequences were designed to span IGFBP-3 intron/exon boundaries to prevent amplification of genomic DNA: 5′-CAAAGCACAGACACCCAGAACTT-3′, 5′-TGCGGCAGGGACCGT-3′, and probe 6FAM-TCCTCCGAGTCTAAGCGGGAGACAGAA. GAPDH primers and VIC-labeled probe were obtained from Applied Biosystems.

RT-PCRs were carried out in 96-well plates using the ABI PRISM 7700 sequence detection system (Applied Biosystems). Each sample was analyzed in quadruplicate, and Taqman master mix with no template served as negative control. Because the increase in fluorescence was proportional to the concentration of template in the PCR, the standard curve method was used to quantitate the amount of IGFBP-3 in each reaction according to manufacturer's protocol (Applied Biosystems). Any contribution of genomic contamination to the threshold cycle value for a sample was ignored because the threshold cycle values of the −RT reactions were at least 10 cycles less than for the corresponding samples in which reverse transcriptase was added. All IGFBP-3 measurements were normalized to GAPDH concentrations to control for any loading differences.

Cell culture treatment with genotoxic drugs

As previously reported (15, 20), two sister cell lines created from the $p53^{+/+}$ human lung carcinoma cell line (H460) differ in their p53 content; H460 cells stably transfected with a plasmid containing the gene for E6 (H460-E6) contain far less p53 than H460 cells stably transfected with the empty plasmid as control (H460-neo). E6, a protein produced by tumor-associated human papillomaviruses (HPV types 16 and 18), binds p53 and targets it for ubiquitin-dependent degradation (16, 17). E6 has more recently been shown to also inhibit the acetylation, and hence activation, of p53 (21). H460 cells were maintained at all times in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) with 500 μg/ml G418 (Mediatech, Inc., Herndon, VA) for continued transfectant selection pressure. $p53^{-/-}$ PC-3

cells (American Type Culture Collection, Manassas, VA) were maintained in F-12K nutrient mixture (Kaighn's modification) (Life Technologies).

Cells were plated in equal cell densities onto 6-well plates. After adhering overnight in complete medium, duplicate wells were changed to serum-free medium with or without a genotoxic drug at concentrations previously shown to induce wild-type p53 expression (20). Etoposide (Bristol Laboratories, Princeton, NJ) was added for a final concentration of 2.4 μm. Adriamycin (Pharmacia & Upjohn Co., Kalamazoo, MI) was added at 0.2 μg/ml, as published, or 0.8 μg/ml to test the hypothesis that higher concentrations may be needed in p53-null cells. Conditioned medium was harvested at the times shown, and IGFBP-3 immunoblot was performed by established techniques (22) using an affinity purified, polyclonal goat antihuman IGFBP-3 antibody (Diagnostic Systems Laboratories, Webster, TX). Densitometry analysis was performed (Alpha imager 2000 4.0, Alpha Innotech, San Leandro, CA) to quantify the relative amounts of IGFBP-3 protein in the conditioned media.

To control for possible loading differences, aliquots of each conditioned medium were analyzed by the modified Lowry technique for total protein content (23). Serial dilutions of a standard protein solution of 0.1 mg/ml BSA (Sigma, St. Louis, MO) were used to generate the standard curve. For each experimental conditioned medium, one aliquot was diluted 20-fold and another 40-fold, to get readings within the range of the BSA standards. Then 0.1ml of a 5-ml carbonate solution/0.1ml copper sulfate solution mixture was added to each cuvette, and 1 min later, 0.1 ml Folin reagent (diluted 1:1 with water) was also added. After 30 min, the spectrophometric absorbances were read at 710 nm (λ 2 UV/Vis spectrophotometer, PerkinElmer Life and Analytical Sciences, Inc., Boston, MA) and converted into protein concentrations using the standard curve. The protein concentrations of the two aliquots for each condition were averaged and used to normalize the quantities of IGFBP-3 protein as determined by the densitometry readings.

Hypoxia

Hypoxic conditions for thin film cell cultures were achieved by the gas exchange procedure (24) using an MS2200V manifold (Animas Corp., Philadelphia, PA). Cells were plated at 2 $\times 10^6$ cells per 4 ml/dish on 60-mm Permanox dishes that had been previously degassed for 48 h (25). After 24 h in standard medium, cells were changed to thin-layer medium supplemented with 1 $_M$ HEPES and 75 mg glucose to prevent acidosis and placed in the MS2200V Manifold chambers (24). Sequential gas exchanges to stepwise replace the chamber's air content with nitrogen mixed with 5% CO_2 were undertaken; serial exchanges were required to avoid large vacuum differentials that could impair cell adherence and viability. Once the desired hypoxic condition was created, the chambers were incubated in a reciprocal shaker for the experimental duration to maximize equilibration. Afterward, the cells were harvested, and an aliquot of the medium was examined for pH and glucose content. Per established techniques, total RNA was isolated from the cells, and IGFBP-3 mRNA content was analyzed by Northern blotting. RPL-32 mRNA was used to control for loading differences in the Northern blots.

Results

Irradiation

We examined whether IGFBP-3 would be induced by p53 in multiple tissues (thymus, spleen, small intestine) that have been previously shown to undergo p53-dependent apoptosis after treatment with ionizing radiation. As shown in Fig. 1, IGFBP-3 mRNA increased in the thymus of $p53^{+/+}$ mice by almost 8-fold at 6 h and 22-fold at 24 h after total body irradiation. Similar induction was not observed in the $p53^{-/-}$ mice. Dexamethasone treatment, which induces apoptosis by a p53-independent pathway, served as a positive control in the experimental design. In similar experiments (data not shown), irradiation led to a p53-dependent doubling of IGFBP-3 mRNA in the small intestine and no change in IGFBP-3 mRNA in the spleen.

Genotoxic drugs

We then examined whether other DNA damaging agents could induce IGFBP-3 in a p53-dependent manner. IGFBP-3 Western immunoblots were performed on the conditioned media of H460 cells after 3 h of serum starvation with or without exposure to etoposide (Fig. 2). Similar to prior report (15), H460 cells stably transfected with E6 (H460-E6) have less p53 and also less IGFBP-3 than H460 cells transfected with empty plasmid (H460-neo). Nonetheless, etoposide treatment increased IGFBP-3 secretion by both H460-E6 and H460-neo cells. To further assess the p53-independence of IGFBP-3 induction by genotoxic drugs, p53-null PC-3 cells were exposed to serum starvation and adriamycin for 6 h. As shown in Fig. 3, adriamycin more than doubled the IGFBP-3 secretion by PC-3 cells at 0.2 μg/ml, the concentration of adriamycin previously reported in studies of $p53^{+/+}$ cell lines (20).

Hypoxia

Thin film cell cultures were subjected to hypoxia by the gas exchange procedure. Hypoxic conditions induce a metabolic switch to glycolysis, which results in accelerated glucose deprivation and cellular acidosis (26). Distinguishing the effects of hypoxia from those of the resultant acidosis becomes an important feature of the experimental design. To this end, the thin layer medium was supplemented with 1 M HEPES and 75 mg glucose, and the adequacy of this approach in preventing acidosis was confirmed (Fig. 4). Figure 5 shows the time course and dose response of IGFBP-3 induction by hypoxia in $p53^{+/+}$ H460 cells. IGFBP-3 mRNA increased progressively with the duration of exposure to oxygen partial pressure of 1%, and at 24 h, IGFBP-3 mRNA was significantly greater in cells kept in oxygen partial pressures of 1% or less. A similar dose response of IGFBP-3 mRNA was seen in $p53^{-/-}$ PC-3 cells (Fig. 6). In fact, 24 h of hypoxia at oxygen partial pressure of 1% induced IGFBP-3 mRNA across multiple neoplastic cell lines irrespective of their p53 status (Fig. 7); these included cell lines derived from osteogenic sarcoma (Saos-2), breast adenocarcinoma (MCF7), cervical carcinoma (HeLa), prostate carcinoma (PC-3 and DU145), and lung large cell carcinoma (H460-E6 and H460-neo).

Discussion

In summary, irradiation, genotoxic drugs, and hypoxia, three conditions that normally induce p53, all induced IGFBP-3, although p53 was not always required. Irradiation significantly induced IGFBP-3 in the thymus and, to a lesser extent, the small intestine, in a p53-dependent and tissue-specific manner. p53 is a determinant of tissue-specific radiosensitivity, and the most radiosensitive tissues (hematopoietic and lymphoid tissues, spermatogonia, small intestine, hair follicles, and embryos) show the greatest differences in radiosensitivity between $p53^{+/+}$ and $p53^{-/-}$ mice (27). Irradiation causes DNA damage, which is detected by sensor proteins and transduced through ataxia telangiectasia mutated gene (ATM)/ataxia-telangiectasia and rad3-related (ATR) to effector kinases; ATM, both directly and through its targets, leads to p53 stabilization and activation, which ultimately contributes to the cell's fate of DNA repair, cell cycle arrest, or apoptosis (28). *In situ* hybridization experiments of irradiated mouse thymus, spleen, and various intestinal segments showed tissue-specific induction patterns of the p53 targets KILLER/DR5, Bid, Noxa, and Puma, that correlated with caspase-3 activation, a marker of apoptosis. Caspase-3 was not activated in irradiated $p53^{-/-}$ mice or the livers of $p53^{+/+}$ mice; $p21^{WAF1}$, the principal mediator of p53-induced cell cycle arrest, was the major p53 target induced in the liver (29). Thus, it is not surprising for IGFBP-3 induction by irradiation to be p53 dependent and tissue specific. The magnitude of IGFBP-3 induction in the thymus was on par with that seen, under similar experimental conditions, for the p53 targets KILLER/DR5, Bcl-2-associated X protein, E128/PIG8, and Fas/APO1 (19).

p53 is induced by DNA damage, whether it is caused by irradiation or genotoxic drugs. In fact, most clinically used chemotherapeutic agents kill $p53^{+/+}$ tumors more effectively than tumors with mutant p53 (30), although their use is not limited to $p53^{+/+}$ tumors. Cellular responses to DNA damage are specific and relate to the type and extent of DNA damage, degree of p53 induction, and cell type (6). Etoposide causes DNA strand breakage, via interactions with DNA topoisomerase II or formation of free radicals, whereas adriamycin intercalates nucleotide bases, thereby inhibiting DNA replication and DNA and RNA polymerases (31). Etoposide increased IGFBP-3 secretion by H460 lung cancer cells with or without E6, and adriamycin increased IGFBP-3 in the p53-null prostate cancer cells, PC-3. The effects were rapid (evident within 3–6 h), p53-independent, and specific to the combination of cell type and damaging agent.

How can genotoxic drugs induce IGFBP-3 in the absence of p53? One possibility is p73, a p53 family member that is rarely mutated (and frequently increased) in cancer. p73 and the third family member, p63, were required for adriamycin-induced apoptosis despite normal p53 status (32). Recently p73 was found to be induced in SW480 colon carcinoma cells by multiple chemotherapeutic agents, including etoposide and adriamycin and in turn led to apoptosis, even though these tumor cells lacked functional p53 (33). Created by different promoters and alternative splicing, isoforms of p73 and p63 that contain the N-terminal transactivation domain can activate p53 target genes and cause apoptosis; the isoforms lacking this domain function as dominant inhibitors of p53 (34). Thus, p53-independent induction of IGFBP-3 by genotoxic drugs may be mediated by p73 or perhaps by p63 or other p53-unrelated signals.

IGFBP-3 was significantly induced by hypoxia at oxygen partial pressures of 1% or less. Acidosis, due to cellular hypoxia, was prevented (Fig. 4). It is important to distinguish the effects of hypoxia itself *vs.* those of the ensuing acidosis. This technical question is particularly relevant to studies of IGFBP-3 biology because multiple IGFBP-3 regulators are themselves regulated by acidosis. For example, cathepsins, lysosomal enzymes implicated in malignant processes, are acid activated (35), whereas prostate-specific antigen is functional at neutral pH (36). Cathepsins and prostate-specific antigen appear to be secreted into seminal plasma, but their role there is not yet known. They can both function as IGFBP-3 proteases, and increased IGFBP-3 proteolysis has been found in prostate carcinogenesis (35, 37).

Hypoxia inhibits cell growth yet can lead to a more malignant phenotype in tumors by inducing genes whose products are involved in angiogenesis and energy metabolism; IGFBP-3 was one of multiple hypoxia-induced genes identified by DNA array analysis (38). The hypoxic response is primarily mediated by the transcription factor hypoxia-inducible factor (HIF-1), which is frequently overexpressed in human cancers (39, 40). Hypoxia was shown to induce IGFBP-3 mRNA in wild-type embryonic stem cells, whereas stem cells homozygous for a targeted loss of the HIF-1α subunit had undetectable levels of IGFBP-3 mRNA (41). HIF-1α was required for induction of p53 by anoxia/hypoglycemia (42); HIF-1α overexpression protected p53 from mouse double minute-2-homolog (Mdm2) mediated proteasomal degradation and inhibited nuclear export of p53 by mouse double minute-2 homolog (Mdm2) (43). It is tempting to assume that hypoxic induction of IGFBP-3 is mediated by increased IGFBP-3 transcription as a p53 target gene. However, our results clearly indicate that hypoxia can increase IGFBP-3 mRNA levels, even in the absence of p53. A recent paper (44) demonstrated uncoupling of HIF-1α and p53 expression during the cellular response to hypoxia; p53 induction by hypoxia occurred not at reduced oxygen tensions alone but when the ensuing acidosis and nutrient deprivation were allowed as well. Hypoxia-induced growth arrest was abolished by deletion of HIF-1α, also in a p53-independent manner (45). More experiments are needed to determine whether this p53-independent, hypoxia- and HIF1α-induced growth arrest is due to IGFBP-3 activity.

IGFBP-3 is not the only member of the IGF axis regulated by hypoxia. HIF-1 activates transcription of numerous target genes through DNA sequence-specific binding in a hypoxia response element (HRE) that can be located in the 5′ or 3′ flanking regions or in the intronic sequences of the target gene (38, 39). Giudice and colleagues (46, 47) described a potent response of IGFBP-1 to hypoxia via canonic HREs and proposed that this may contribute to the intrauterine growth retardation of hypoxic fetuses. Insulin, IGF-I, and IGF-II were shown to induce expression of HIF-1α, which in turn was required for hypoxic induction of IGF-II, IGFBP-2, and IGFBP-3 but not IGFBP-4 (41). This may represent a negative feedback autocrine growth factor loop. IGFBP-1, -2, and -3 all contain two to three canonic HREs at various locations of their genomic sequence (data not shown) and may have coevolved to maintain their role as regulatory molecules for hypoxia-induced proliferation.

IGFBP-3 is also not the only member of the IGF axis regulated by p53. Like HIF-1, p53 activates transcription of numerous target genes through DNA sequence-specific binding (48). For IGFBP-3, the p53 binding sites are located in its first two introns (9) and promoter

(10,49). In addition, p53 can repress transcription of another set of target genes. p53 was shown to transcriptionally repress, by inhibiting DNA binding of the TATA-box-binding protein subunit of the transcription factor TFIID, to the initiator region of the type 1 IGF receptor gene (50) and the third promoter (P3) of the IGF-II gene (51). By inducing IGFBP-3 and repressing the IGF receptor and IGF-II, p53 effectively inhibits the cell survival and proliferation signaling of the IGF axis. For example, tetracycline-inducible p53 expression in an osteosarcoma cell line reduced IGF-I-induced tyrosine phosphorylation of the IGF receptor and insulin receptor substrate-1 (52), and over-expression of wild-type but not mutant p53 in murine hemopoietic cells decreased the number of IGF receptors and increased cellular sensitivity to apoptosis caused by IL-3 withdrawal (53).

In conclusion, we have shown that IGFBP-3 is induced by physiologic conditions that also induce p53, although p53 is not always required. IGFBP-3 induction by DNA damage and hypoxia suggest IGFBP-3 plays a role in the physiologic protection against aberrant cell growth. This may be accomplished through two principal mechanisms (11). First, because the IGF binding affinity for IGFBP-3 exceeds that for the type 1 IGF receptor, IGFBP-3 competitively inhibits IGF signaling. Current evidence supports a permissive, but not causal, role for IGF-I in cancer development (54). Second, IGFBP-3 can inhibit growth and induce apoptosis in an IGF-independent fashion that involves IGFBP-3-specific association proteins, including putative IGFBP-3 cell surface receptors (55, 56) and retinoid X receptor in the nucleus (57). Accumulating evidence, from epidemiologic studies of cancer risk to *in vivo* tumor models to *in vitro* cell growth experiments, suggests IGFBP-3 contributes to cancer protection (58). Unlike classic tumor suppressors, IGFBP-3 knockout mice do not develop spontaneous tumors, and there is no identified IGFBP-3-related familial cancer syndrome. Thus, IGFBP-3 more likely serves as one of the multiple, low-penetrance tumor susceptibility and resistance genes that determine cancer incidence and therapeutic responsiveness (59).

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Abbreviations

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HIF	hypoxia-inducible factor
HRE	hypoxia response element
IGFBP	IGF binding protein
RT	reverse transcription
−RT	samples treated similarly to RT but without adding reverse transcriptase

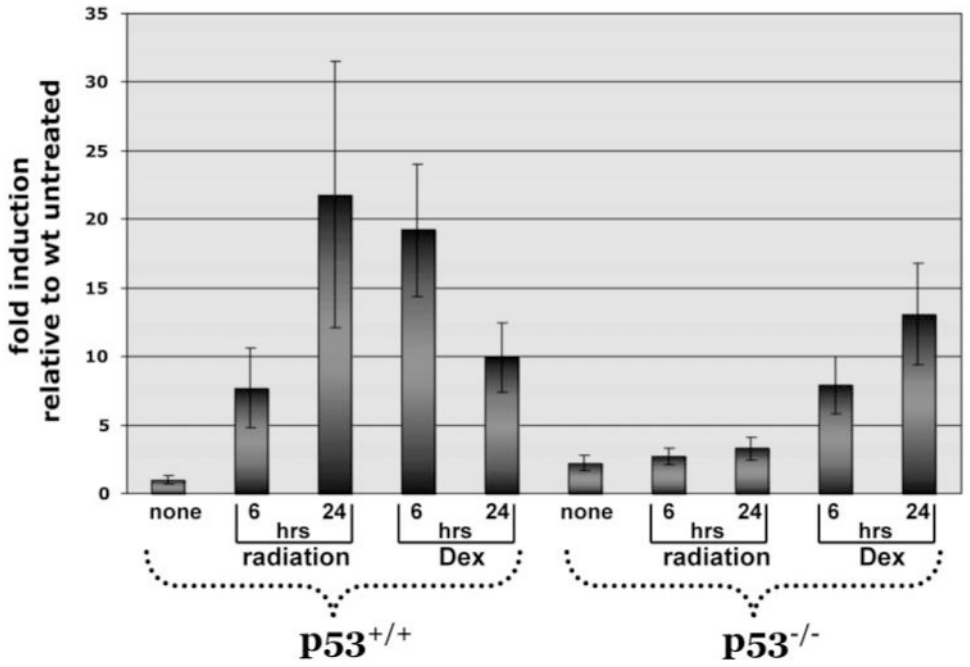

Fig. 1.

IGFBP-3 mRNA expression in the thymus after irradiation or dexamethasone (Dex) treatment. Pairs of $p53^{+/+}$ and $p53^{-/-}$ mice were treated with 5 Gy total body irradiation or a single ip injection of either 0.5 mg dexamethasone or sterile 1× PBS and killed 6 or 24 h later. Radiation-sensitive tissues were harvested (thymus shown here), total RNA isolated, and RT-PCR performed to quantify IGFBP-3 mRNA levels, normalized to GAPDH. RT-PCR results presented as fold increase relative to untreated $p53^{+/+}$ mice (mean of quadruplicate replications ± sd).

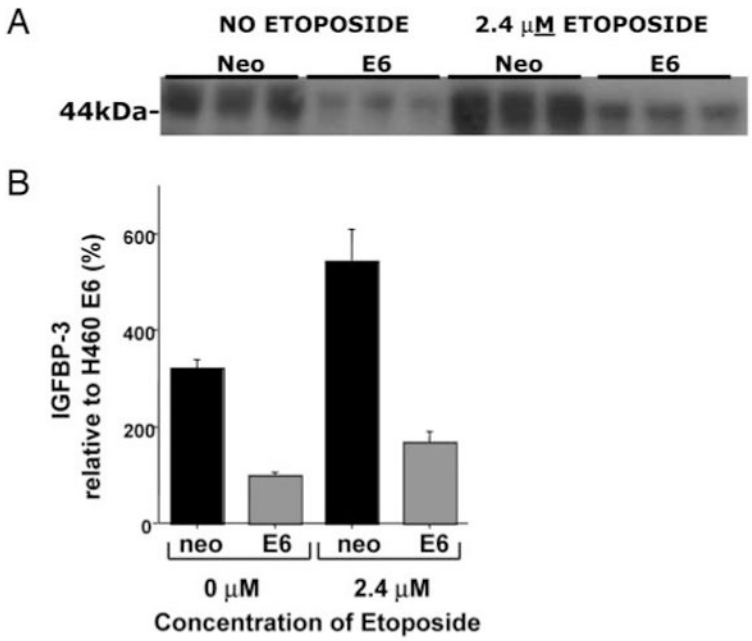

Fig. 2.

IGFBP-3 secretion by H460 cells after 3 h treatment with etoposide A, Western immunoblot. B, Densitometry analysis. $p53^{+/+}$ H460 cells had been stably transfected with E6 or empty plasmid as control (neo); E6 leads to p53 degradation and at 3 h significantly reduced IGFBP-3 secretion (15). Conditioned media were collected after 3 h in serum-free medium with or without etoposide. IGFBP-3 Western immunoblot was performed as was densitometry analysis. An aliquot of each sample underwent total protein quantification by the modified Lowry technique to control for loading differences. Densitometry results, normalized to total protein, are presented as percent relative to untreated H460-E6 cells. Each *bar* represents mean ± SD of triplicate replications.

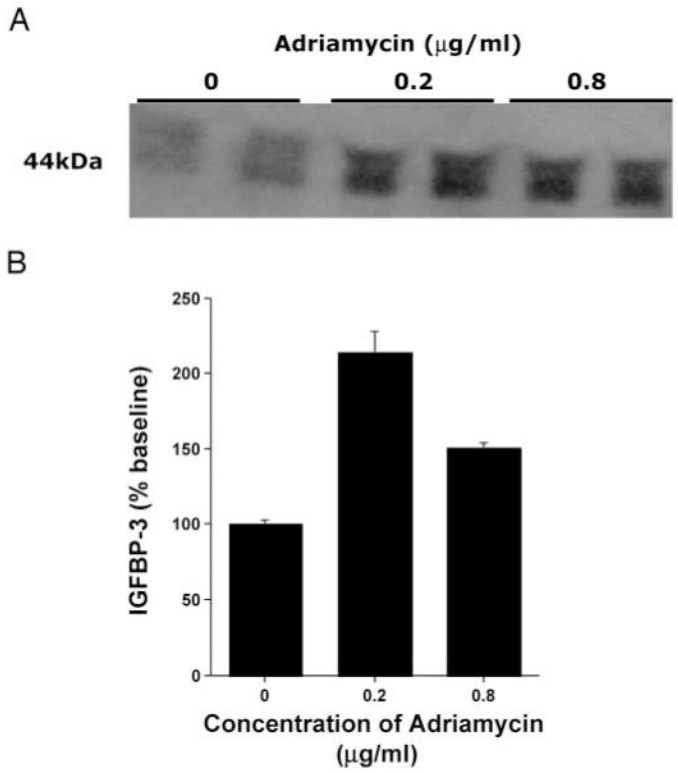

Fig. 3.

IGFBP-3 secretion by $p53^{-/-}$ PC-3 cells after 6 h treatment with different concentrations of adriamycin. A, Western immunoblot. B, Densitometry analysis. Conditioned media were collected after $p53^{-/-}$ PC-3 cells were treated with serum-free medium with or without adriamycin at different concentrations. IGFBP-3 Western immunoblot and densitometry analysis were performed. An aliquot of each sample underwent total protein quantification by the modified Lowry technique to control for loading differences. Densitometry results, normalized to total protein, are presented as percent relative to untreated PC-3 cells. Each *bar* represents mean ± SD of duplicate replications.

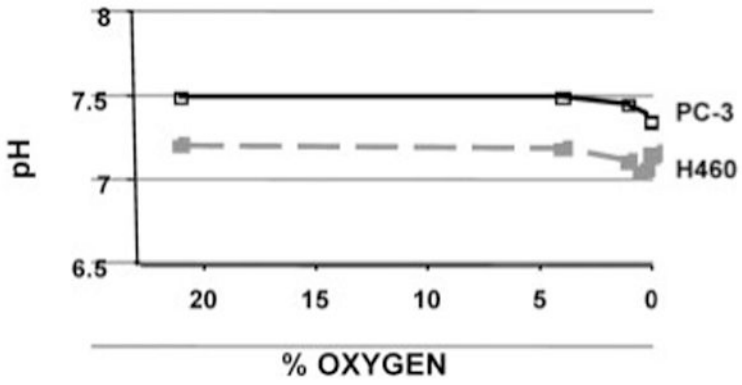

Fig. 4.

pH of conditioned medium after 24 h at different O_2 partial pressures. PC-3 cells are represented by *open black squares/solid line*, H460 cells by *closed gray boxes/dashed line*. Thin film cell cultures were subjected to increasing degrees of hypoxia by the gas exchange procedure using an MS2200V manifold and incubated in a reciprocal shaker for 24 h. Afterward, the cells were harvested for Northern blot analysis (Figs. 5B and 6), and an aliquot of the medium was examined for pH and glucose content. Before any changes can be attributed to hypoxia, it is important to ensure that cellular acidosis, which frequently results from hypoxia, is prevented.

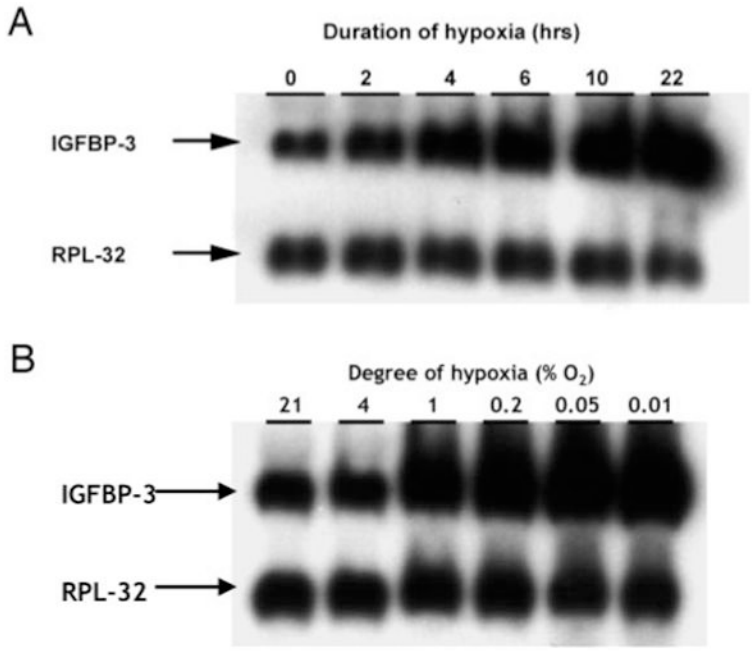

Fig. 5.

Time course (A) and dose response (B) of IGFBP-3 mRNA levels in $p53^{+/+}$ H460 cells treated with hypoxia. Thin film cultures of H460 cells were subjected to 1% oxygen partial pressure for different durations (A) or 24 h of progressively decreasing oxygen partial pressures (B). Cells were harvested, total RNA isolated, and IGFBP-3 mRNA analyzed by Northern blotting. RPL-32 is shown as loading control.

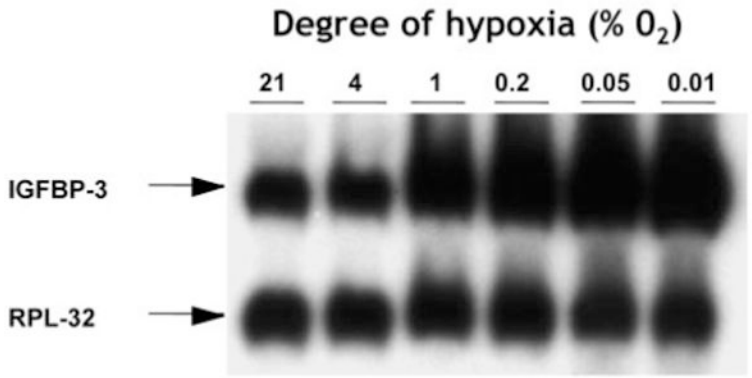

Fig. 6.

Dose response of IGFBP-3 mRNA levels in p53-null PC-3 cells after 24 h of varying degrees of hypoxia. The experiment of Fig. 5B was performed with PC-3 cells. Northern blotting was used to evaluate IGFBP-3 mRNA levels, and RPL-32 served as loading control.

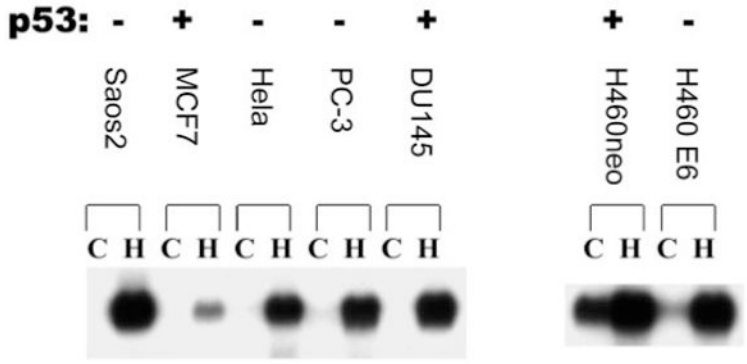

Fig. 7.

Northern blots of IGFBP-3 mRNA in multiple human cancer cell lines. Using the thin film cell culture technique, each cell type was exposed to 24 h of hypoxia (H; $P0_2$ = 1%) or normoxia for control (C). After the 24-h incubation in a reciprocal shaker, cells were harvested, total RNA isolated, and IGFBP-3 mRNA evaluated by Northern blot analysis. The p53 status of each cell type is indicated above its doublet.