Mutant p53 Enhances Nuclear Factor κB Activation by Tumor Necrosis Factor α in Cancer Cells

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Abstract

Mutations in the p53 tumor suppressor are very frequent in human cancer. Often, such mutations lead to the constitutive overproduction of mutant p53 proteins, which may exert a cancer-promoting gain of function. We now report that cancer-associated mutant p53 can augment the induction of nuclear factor κB (NFκB) transcriptional activity in response to the cytokine tumor necrosis factor α (TNFα). Conversely, down-regulation of endogenous mutant p53 sensitizes cancer cells to the apoptotic effects of TNFα. Analysis of human head and neck tumors and lung tumors reveals a close correlation between the presence of abundant mutant p53 proteins and the constitutive activation of NFκB. Together, these findings suggest that p53 mutations may promote cancer progression by augmenting NFκB activation in the context of chronic inflammation. [Cancer Res 2007;67(6):2396–401]

Introduction

The p53 tumor suppressor is a target for frequent mutations in human cancer. In its wild-type (wt) form, p53 functions as a transcriptional regulator and exerts potent antitumor effects. In response to a variety of stress signals, many of which are associated with cancer initiation or progression, p53 is activated; this promotes outcomes such as growth arrest, replicative senescence, or apoptosis (reviewed in refs. 1, 2).

Although the frequent p53 mutations serve primarily to abrogate the tumor suppressor function of wt p53, there is mounting evidence that the resultant mutant p53 proteins, often produced copiously in cancer cells, may also contribute actively to carcinogenesis through gain of function (3, 4). This notion was reinforced by recent studies employing mutant p53 "knock-in" mice, which display broader tumor spectrum and increased aggressiveness and metastatic potential (5, 6). In cultured cells, overexpression of tumor-associated p53 mutants was shown to attenuate apoptosis induced by a variety of agents (reviewed in refs. 3, 4). Furthermore, down-regulation of endogenous mutant p53 by RNA interference (RNAi) renders cancer cells more sensitive to killing by DNA-damaging chemotherapeutic agents *in vitro* (7) and *in vivo* (8).

Like p53, the transcription factor nuclear factor κB (NFκB) has also been extensively implicated in the regulation of apoptosis and modulation of cancer therapy responses. The NFκB family consists of several proteins, including RelA (commonly known as p65), RelB, c-Rel, p50, and p52, operating as heterodimers or sometimes homodimers (for recent reviews, see refs. 9, 10). By modulating the expression of its target genes, NFκB regulates critical biological processes, including immune and inflammatory responses. Moreover, aberrant NFκB activity has been implicated in carcinogenesis and in the control of the cellular response to anticancer agents (reviewed in refs. 9, 10). In many, although not all, cases, NFκB activation inhibits apoptosis and favors cell proliferation. Conversely, inhibition of NFκB can facilitate cell killing. Importantly, constitutive NFκB activation, associated with chronic inflammatory processes, can promote tumor development.

Notably, it was recently reported that excess mutant p53 can elevate NFκB activity via transactivation of the *NFκB2* gene, and that mutant p53 expression correlates positively with NFκB activity in cultured cancer cells (11, 12). Because the activation of NFκB by the cytokine tumor necrosis factor α (TNFα) can drive cancer progression in the context of chronic inflammation (13, 14), we investigated the effect of mutant p53 on the NFκB response to TNFα. We report that mutant p53 augments the activation of NFκB by TNFα, and that down-regulation of endogenous mutant p53 sensitizes cancer cells to killing by the cytokine. Remarkably, elevated mutant p53 protein is closely correlated with increased NFκB activation in human premalignant and malignant lesions. These findings suggest a role for mutant p53/NFκB cooperation in human cancer, especially under conditions of chronic exposure to inflammatory cytokines.

Materials and Methods

Cells and plasmids. H1299 p53R175H-inducible cells were maintained in RPMI-1640 (Sigma, St. Louis, MO) supplemented with 10% FCS (Sigma). To induce p53 expression from the metallothionein promoter, $ZnCl_2$ was added to a final concentration of 100 μmol/L. SKBR3 cells were maintained in McCoy's medium (Sigma) supplemented with 10% FCS. pSuper p53 (p53 RNAi) and pSuper LacZ (LacZi) plasmids were kindly provided by R. Agami (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Small interfering RNA (siRNA) oligonucleotides were purchased from Dharmacon (Chicago, IL). LacZi sense sequence: GUG ACC AGC GAA UAC CUG U dT dT; and p53i sense sequence: GCA UGA ACC GGA GGC CCA U dT were used. Recombinant human TNFα was purchased from R&D Systems (Minneapolis, MN).

Transfections. Transfections, including synthetic siRNA oligonucleotides (100 pmol/35-mm dish), were done with Dharmafect 3 (Dharmacon). Unless otherwise stated, transfections employing plasmid DNA only were done with Maxfect (Molecula, Columbia, MD).

Apoptosis assays. Analysis of apoptosis by fluorescence-activated cell sorting was as described (7). WST1 kit was purchased from Roche

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Diagnostics (Basel, Switzerland). Caspase 3 kit was purchased from Promega (Madison, WI).

Luciferase assays. Luciferase assays, using the dual luciferase system (Promega), were essentially as described (7).

Immunohistochemistry. Immunohistochemical staining of tumor sections used the indirect streptavidin-biotin-peroxidase protocol (15). Protein expression in cultured cells was visualized by indirect immunofluorescence.

Results

Mutant p53 enhances induction of NFκB activity by TNFα. To assess the impact of tumor-associated mutant p53 proteins on the transcriptional activity of NFκB upon exposure to TNFα, we used p53-null human large cell lung cancer H1299 cells, stably expressing the hotspot mutant p53R175H under the metallothionein promoter. Upon exposure to zinc, expression of mutant p53 is readily induced (see Fig. 3*A*). Treatment with TNFα in the absence of zinc stimulated NFκB activity, recorded with a luciferase reporter plasmid driven by five consecutive NFκB binding sites (Fig. 1*A*). NFκB activity peaked early and did not increase any further beyond 6 h of TNFα exposure. In contrast, induction of mutant p53 expression by addition of zinc led to a continuous increase in NFκB activity in response to TNFα treatment. Induction of mutant p53 alone did not increase NFκB activity (Supplementary Fig. S1, *top*), nor did zinc affect NFκB

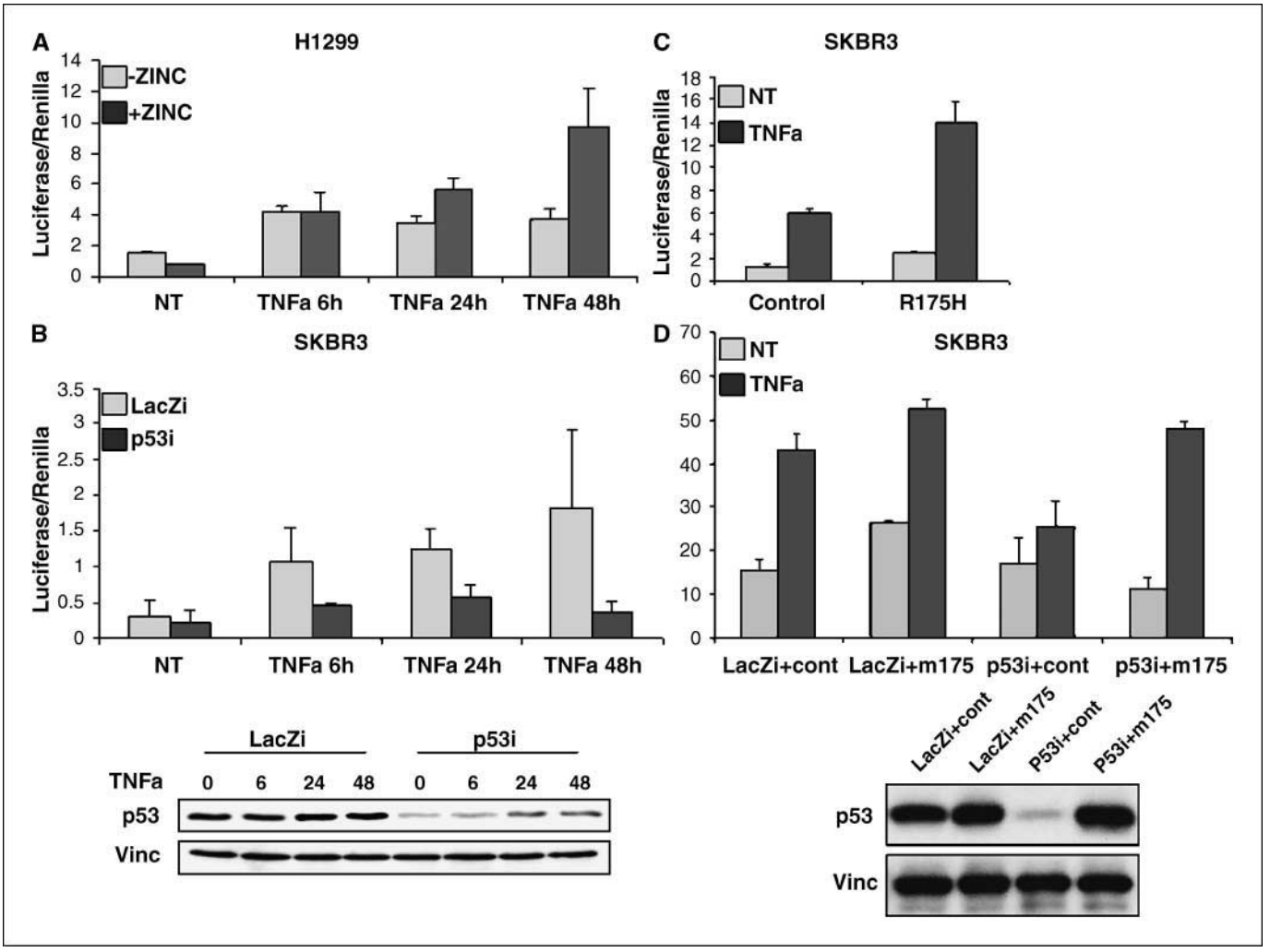

Figure 1. Mutant p53 enhances the activation of NFκB by TNFα in cancer cells. *A,* H1299 cells expressing mutant p53R175H under transcriptional control of the metallothionein promoter were transiently transfected with 200 ng firefly luciferase reporter plasmid driven by five consecutive artificial NFκB binding sites (NFκB-luc), as well as 5 ng cytomegalovirus Renilla luciferase. Twenty-four hours later, $ZnCl_2$ was added where indicated to induce mutant p53 expression. Four hours later, 50 ng/mL TNFα was added for the indicated time periods, after which cells were extracted and subjected to dual luciferase analysis. Firefly luciferase readings were normalized for *Renilla* luciferase. NT, nontreated. *Columns,* means; *bars,* SD. *B,* SKBR3 cells were transiently transfected with NFκB-luc (200 ng) and CMV *Renilla* luciferase (5 ng), together with plasmids (400 ng) expressing p53 shRNA (p53i) or LacZ shRNA (LacZi). Twenty-four hours later, cells were treated with 50 ng/mL TNFα for the indicated time periods and subjected to luciferase analysis as in *A* (*top*). *Columns,* means; *bars,* SD. Western blot analysis for p53, with vinculin (*Vinc*) as a loading control, is shown in the bottom. *C,* SKBR3 cells were transiently transfected with a plasmid encoding mutant p53R175H or control vector, each at 250 ng/35 mm dish, together with 100 ng NFκB-luc and 5 ng CMV-*Renilla* luciferase. Transfection employed the JetPEI reagent (Polyplus). Twenty-four hours later, TNFα was added to 50 ng/mL where indicated. Luciferase analysis was done after an additional 48 h. *Columns,* means; *bars,* SD. *D,* SKBR3 cells were transiently transfected with synthetic siRNA specific for p53 (p53i) or LacZ (LacZi) as a control. One day later, cells were transfected with NFκB-luc (200 ng) and CMV *Renilla* luciferase (5 ng), together with a plasmid encoding p53R175H, made substantially siRNA resistant by the introduction of multiple mismatches within the siRNA-complementary sequence (m175), or pCDNA3 as a control. Forty-eight hours later, cells were treated with 50 ng/mL TNFα for 48 h and subjected to luciferase analysis as in *A* (*top*). The *bottom* shows Western blot analysis for p53, with vinculin as a loading control, done on samples not treated with TNFα. The sequence of the siRNA was GCAUGAACCGGAGGCCCAUdT, derived from positions 733 to 751 of the p53 open reading frame, whereas in the sequence of the corresponding p53 region, the m175 plasmid was GCATGAATCGAAGACCGAT.

activation in parental p53-null H1299 cells (Supplementary Fig. S1, *bottom*). Hence, mutant p53 overexpression selectively augments cytokine-triggered NFκB activation.

To explore this notion in a more relevant context, we employed SKBR3 human breast cancer cells, harboring endogenous p53R175H. SKBR3 cells were transiently transfected with a plasmid expressing p53-specific small hairpin RNA (shRNA; ref. 16) to knockdown endogenous p53 and then challenged with TNFα. Cells transfected with a control lacZ shRNA plasmid mounted a robust and long-lasting NFκB response when treated with TNFα (Fig. 1*B*). In contrast, knockdown of endogenous mutant p53 severely blunted the extent and duration of NFκB activation. Increasing the levels of p53R175H further augmented NFκB activation (Fig. 1*C*). Similar results were obtained when endogenous mutant p53 was knocked down with a synthetic siRNA (Fig. 1*D*). Importantly, transfection of a plasmid encoding p53R175H, made substantially siRNA resistant by the introduction of multiple mismatches within the siRNA-complementary sequence (m175, Fig. 1*D*), overcame efficiently the inhibitory effect of the siRNA. Thus, endogenous mutant p53 in cultured human cancer cells enables stronger and longer NFκB activation by TNFα.

Mutant p53 enhances nuclear accumulation of NFκB in response to TNFα. Exposure of cells to TNFα triggers rapid nuclear translocation of the p50/p65 NFκB dimer, enabling its transcriptional effects. We therefore employed a synthetic p53 siRNA to knock down endogenous SKBR3 mutant p53 and monitored p65 localization in response to TNFα. As seen in Fig. 2*A* (*red*), the extent of p53 knockdown was not uniform throughout the culture: whereas many cells hardly exhibited any nuclear p53, consistent with an efficient knockdown, a minority retained easily detectable residual nuclear p53.

As expected, p65 in untreated cells was predominantly cytoplasmic (Fig. 2*A, NT, top*), indicating that NFκB was largely inactive. In contrast, 30 min of TNFα treatment resulted in nuclear translocation of p65, indicative of NFκB activation (Fig. 2*A, bottom*). Remarkably, although some p65 nuclear translocation was evident in all cells, those harboring higher residual nuclear mutant p53 levels consistently displayed also more intense nuclear p65 staining (Fig. 2*A, bottom*). Visual quantification of multiple cells confirmed a nonrandom association between higher mutant p53 concentration and more pronounced nuclear p65 accumulation (Fig. 2*B*).

After 3 h of TNFα treatment, some cells already displayed very little nuclear p65, indicative of shutoff of the transcriptional activity of NFκB, whereas others still retained varying amounts of nuclear p65 (Fig. 2*C*). Remarkably, cells with higher nuclear mutant p53 retained preferentially also nuclear p65, which was rarely seen in cells with low p53 (Fig. 2*C*; quantified in Fig. 2*D*).

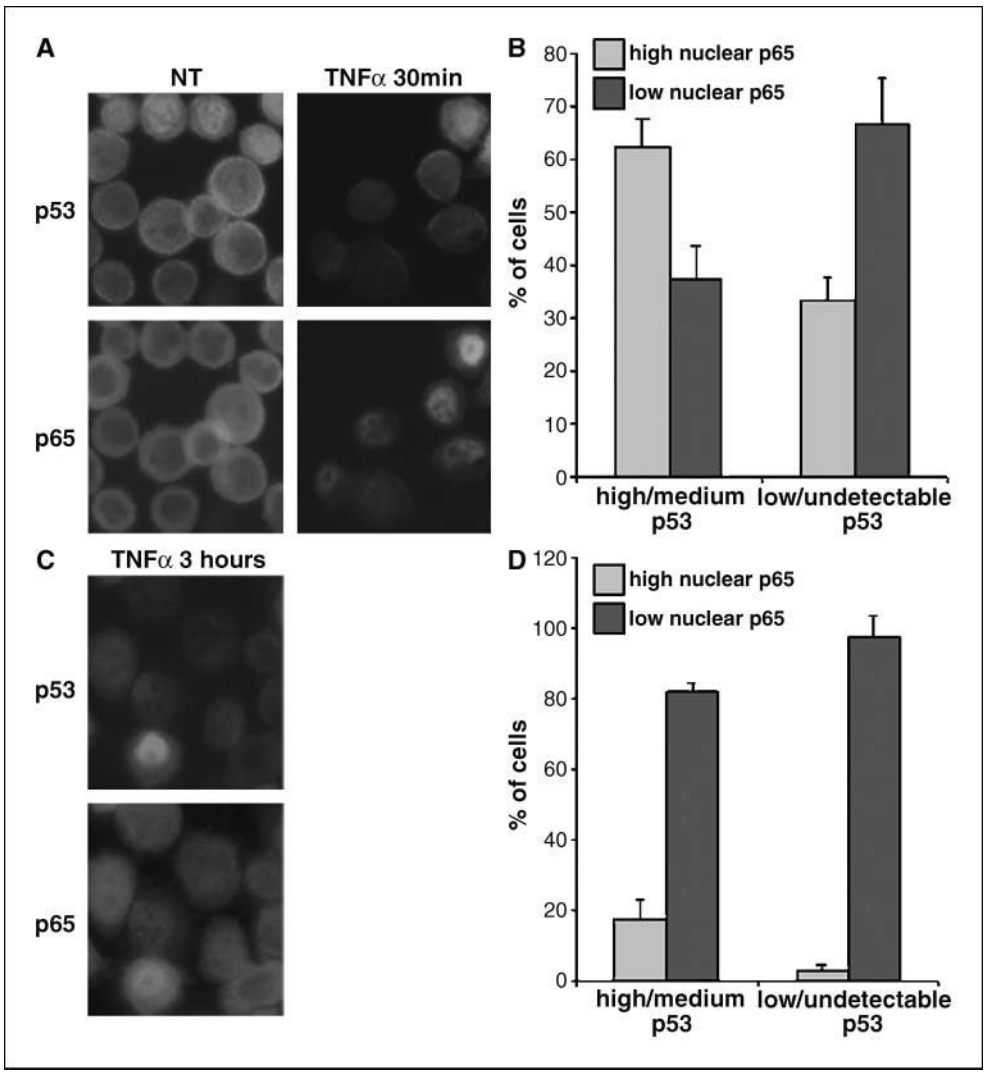

Figure 2. Nuclear p65 accumulation upon TNFa treatment is enhanced in the presence of mutant p53. *A,* SKBR3 cells were seeded on glass coverslips and transfected with synthetic p53 siRNA. Twenty-four hours later, cells were treated with 50 ng/mL TNFα for 30 min (*TNF*) or left untreated (*NT*). Slides were then fixed in 4% paraformaldehyde in PBS for 10 min, rinsed 3× with PBS, blocked for 30 min with PBS containing 0.1% Triton X-100, 5% goat serum, and 0.2% bovine serum albumin, and incubated overnight with either anti-p65 C-20 (Santa Cruz Biotechnology, Santa Cruz, CA) or a mixture of the p53-specific monoclonal antibodies PAb1801 and DO-1. Slides were washed 3× with PBS, incubated with secondary antibodies conjugated to Alexa 466 (Molecular Probes, Eugene, OR) or Cy3 (Chemicon, Temecula, CA) for 1 h, and visualized under a fluorescence microscope. *B,* SKBR3 cells were treated as in (*A*). Multiple fields were observed by fluorescence microscopy, and relative p53 and nuclear p65 levels were scored in parallel for each individual cell. Cells were segregated into two categories, based on residual p53 levels after knockdown. The distribution of relative nuclear p65 levels among cells of each category is shown. The data are based on counting a total of 324 cells, in four groups. *Columns,* means; *bars,* SD. *C,* cells from the same experiment as in (*A*) were fixed and stained after 3 h of treatment with TNFα. *D,* SKBR3 cells were treated as in (*C*). Multiple fields were observed by fluorescence microscopy, and relative p53 and nuclear p65 levels were scored in parallel for each individual cell. Cells were segregated in two categories, based on residual p53 levels after knockdown. The distribution of relative nuclear p65 levels among cells of each category is shown. The data are based on counting a total of 242 cells in four groups. *Columns,* means; *bars,* SD.

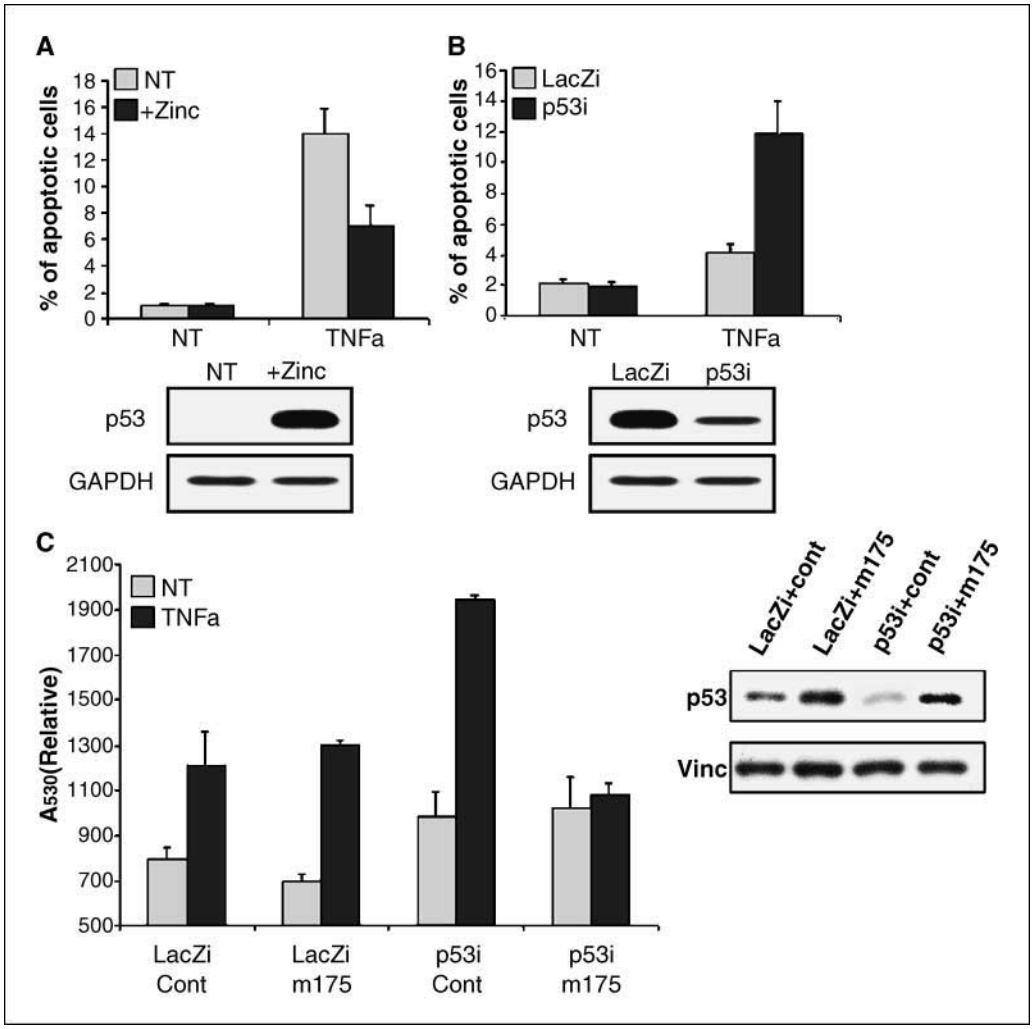

Figure 3. Mutant p53 protects cancer cells against killing by TNFα. *A,* H1299 cells stably expressing mutant p53R175H under the metallothionein promoter were treated with $ZnCl_2$. Four hours later, cells were treated with 50 ng/mL TNFα for 48 h or left untreated. Cell death was assessed by staining of fixed, permeabilized cells with propidium iodide, followed by fluorescence-activated cell sorting analysis. Numbers indicate the percentage of cells with sub-G_1 DNA content, indicative of apoptosis (*top*). Mutant p53 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were assessed by Western blot analysis (*bottom*) employing sequential probing with a mixture of the p53-specific monoclonal antibodies PAb1801 and DO-1 followed by anti-GAPDH antibody (Chemicon). *B,* SKBR3 cells were transfected with synthetic p53 siRNA or LacZ siRNA (*LacZi*) as a control. Twenty-four hours later, cells were treated with 50 ng/mL TNFα or left untreated. Cell death and protein analysis were done as in (*A*). *C,* SKBR3 cells were transiently transfected with synthetic siRNA specific for p53 (p53i) or LacZ (LacZi) as a control. One day later, cells were transfected with an siRNA-resistant plasmid encoding p53R175H (m175) or pCDNA3 as a control. Forty-eight hours later, cells were treated with 200 ng/mL TNFα for an additional 48 h, at which time the cultures were trypsinized and the cells counted. A total of 20,000 cells of each sample were subjected to analysis of caspase-3/7 activity (Apo-ONE kit, Promega). *Right,* Western blot analysis for p53, with vinculin as a loading control, done on samples not treated with TNFα.

Collectively, these observations confirm that the amount of mutant p53 correlates closely with the extent of NFκB activation, arguing that mutant p53 can promote more efficient and more persistent NFκB activation by TNFα in cancer cells.

Down-regulation of mutant p53 sensitizes tumor cells to killing by TNFα. By engaging the extrinsic death pathway, TNFα can trigger apoptosis in target cells, which may be prevented by concurrent activation of the NFκB pathway. We therefore determined whether the ability of mutant p53 to augment NFκB activation could counteract TNFα-induced apoptosis. Indeed, expression of Zn^{2+}-inducible p53R175H in H1299 cells, which elevates NFκB activity in cytokine-treated cells (see Fig. 1*A*), partially compromised apoptotic death upon TNFα treatment (Fig. 3*A*); Zn^{2+} alone did not protect parental H1299 cells (Supplementary Fig. S2).

To assess the impact of endogenous mutant p53 on TNFα-induced apoptosis, synthetic siRNA was employed to knock down p53 in SKBR3 cells. As seen in Fig. 3*B*, down-regulation of endogenous mutant p53 resulted in significantly enhanced apoptosis. A similar effect was seen when a p53 shRNA expression plasmid was used instead of synthetic RNA (Supplementary Fig. S3*A*). Furthermore, mutant p53 knockdown resulted in elevated caspase activation (Supplementary Fig. S3*B*), indicative of increased apoptosis. Sensitization of mutant p53-depleted cells to the adverse effects of TNFα was also seen with the WST1 assay, which monitors viable, metabolically active cells (Supplementary Fig. S3*C*). Interestingly, in control cells (LacZi), TNFα sometimes stimulated proliferation mildly. Importantly, transfection of a siRNA-resistant plasmid encoding p53R175H overcame efficiently the proapoptotic effect of p53 siRNA, as assessed by caspase activity or annexin V staining (Fig. 3*C* and Supplementary Fig. S3*D*, respectively; compare p53i-Cont to p53i-m175).

Hence, mutant p53 can offer increased protection against TNFα-induced cell death. Acquisition of p53 mutations is therefore

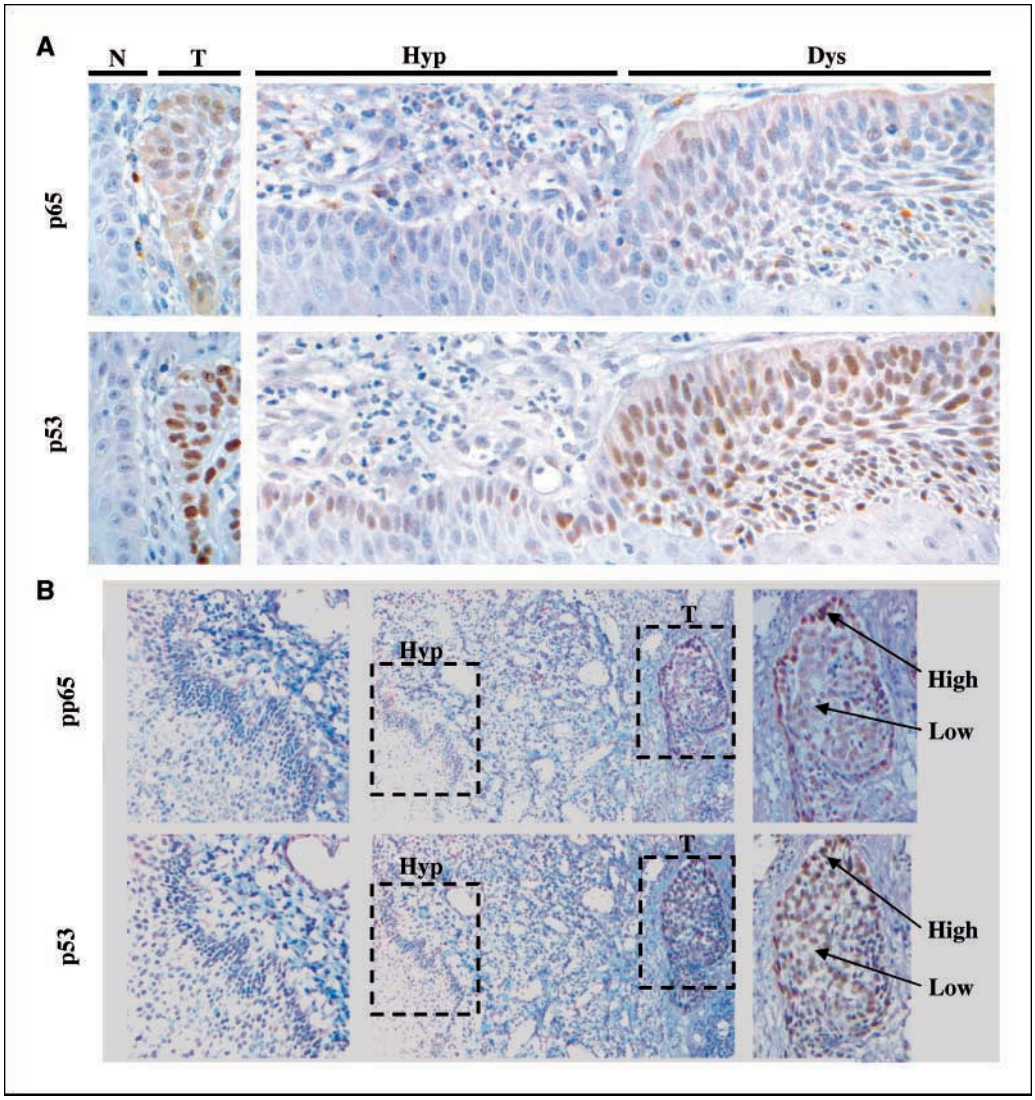

Figure 4. Mutant p53 overexpression coincides with NFκB activation in human malignant and premalignant head and neck lesions. *A,* representative immunohistochemical results of NFκB p65 and p53 staining, on serial sections of paraffin-embedded specimens derived from human head and neck premalignant and malignant lesions. Formalin-fixed paraffin-embedded material, surgically resected from 15 patients with head and neck squamous cell carcinomas, some also containing adjacent hyperplastic and dysplastic lesions of the same patient on a single slide, was from a previously published series (20); the p53 status of these cases has been previously described (20). This group was supplemented with 10 new tumor specimens. The presence of mutant p53 was concluded on the basis of positive immunohistochemical staining for p53. In 12 of those cases, the presence of p53 mutations was confirmed by single-strand conformational polymorphism analysis (SCCP) and in three of those cases also by direct sequencing. Five of the immunohistochemistry-negative tumors were also subjected to SCCP analysis, and all five generated a pattern consistent with the presence of wt p53 only. Anti-p53 (DO-7; DAKO, Carpinteria, CA) and anti-NFκB p65 (F-6; Santa Cruz Biotechnology) monoclonal antibodies were employed on 5-μm paraffin sections using the indirect streptavidin-biotin-peroxidase protocol (19). Sections were incubated with the primary antibody at 1:100 dilution, at 4°C overnight. Haematoxylin was used as counterstain. *N*, normal tissue; *T*, tumor. *Hyp* and *Dys*, hyperplastic and dysplastic lesions, respectively. *B,* immunohistochemical analysis of Ser^{536}-phosphorylated p65 (pp65) and p53 on serial sections of a head and neck squamous cell carcinomas harboring hyperplastic and tumor (*T*) tissue in the same section (*middle*). Direct DNA sequencing identified the p53 mutation in this tumor as $Val^{175}Phe$ (data not shown). Staining was essentially as in (*A*), except that a polyclonal anti-phospho-p65 NFκB (Ser^{536}) antibody (Cell Signaling Technology, Danvers, MA) was employed to monitor NFκB activation. Sections were incubated with the primary antibody at 1:10 (phospho-p65) or 1:100 dilution (p53) at 4°C overnight. Higher magnification images of the dashed boxes are also shown. Arrows indicate areas of higher and lower phospho-p65 staining intensity within the tumor. Greater detail is provided in Supplementary Fig. S2.

expected to provide incipient tumor cells with a selective survival advantage in a microenvironment involving chronic exposure to this proinflammatory cytokine.

Nuclear NFκB and reduced apoptosis correlate with mutant p53 in premalignant and malignant lesions. To assess the relevance of our *in vitro* observations to human cancer, we asked whether the presence of mutant p53 correlated with NFκB activity in tumors. To that end, we analyzed the expression of p65 and mutant p53 in head and neck squamous cell carcinoma. In all tumors with mutant p53, increased nuclear p65 staining was present in areas of intense p53 staining (Fig. 4*A*). Moreover, when comparing individual tumors across the series, a clear correlation was found between p65 staining intensity and presence of p53 mutations (Supplementary Fig. S4). Interestingly, this was also evident in dysplastic epithelium (Fig. 4*A*), suggesting that this correlation is established already very early in cancer development. Nuclear p65 was not present in immediately adjacent hyperplastic epithelium.

NFκB activation in head and neck squamous cell carcinomas was also assessed with an antibody specific for Ser^{536}-phosphorylated p65 (Fig. 4*B*). Strong NFκB activation (*pp65*) was observable in tumorous (*T*), but not adjacent hyperplastic regions. Of note, pp65 was most prominent in a subset of mutant p53-overexpressing cells, positioned in the periphery of the growing tumor. Staining for leukocyte common antigen confirmed the presence of infiltrating inflammatory cells in close proximity to the tumor (Supplementary Fig. S5). It is tempting to speculate that cytokines secreted by those cells drive the chronic activation of NFκB in adjacent tumor cells.

Essentially, similar observations were made in non–small cell lung cancers. All 20 cases with p53 mutations displayed moderate to intense cytoplasmic and often nuclear p65 staining (Supplementary Fig. S6*C* and *D*), whereas normal bronchial epithelium stained negative for both proteins (*A* and *B*). In contrast, only faint cytoplasmic p65 was observed in 15 tumors harboring wt p53 (*E* and *F*). Notably, the apoptotic index of cases with mutant p53 was significantly lower than in those with wt p53 (Supplementary Fig. S7).

Thus, within human tumors, mutant p53 overexpression correlates closely with increased NFκB activity and reduced apoptosis. Moreover, at least in some tumors, this correlation is already established early in tumor progression.

Discussion

This study reveals that mutant p53 can promote NFκB activation in response to cytokine stimulation in cultured cancer cells and probably also within actual tumors. The molecular mechanism whereby this is achieved awaits further elucidation, although the data suggest that mutant p53 may affect both the strength and duration of NFκB activation. Moreover, it remains to be determined whether all, or only some, tumor-associated p53 mutations exert a similar effect.

Mutant p53 expression was found to correlate positively with NFκB activity in cultured cancer cells (11, 12), even without external triggers. Thus, mutant p53 may maintain higher basal NFκB activity, which is further elevated when a proper activation signal such as TNFα is delivered. In particular, basal NFκB activity can be augmented through the ability of mutant p53 to transactivate the *NFKB2* gene, encoding the p100/p52 subunit of NFκB (12), as we also observed (data not shown). However, this feature of mutant p53 is unlikely to account for its effect on the TNFα response, which is mediated by p50/p65 heterodimers rather than by p52. Accordingly, we observed a pronounced effect of mutant p53 on nuclear accumulation and retention of p65 upon cytokine exposure, as well as a strong correlation between mutant p53 overexpression and nuclear p65 staining in tumors.

Unlike mutant p53, much more is known about the interrelationship between wt p53 and NFκB. Whereas the two transcription factors often antagonize each other biochemically and biologically (e.g., ref. 17), they can sometimes cooperate or otherwise exhibit an interdependence (e.g., refs. 18, 19). The factors that determine which way the wt p53-NFκB crosstalk goes remain unknown; however, it is conceivable that wt p53 may sometimes acquire properties resembling those exhibited constitutively by mutant p53. Such aberrant behavior of wt p53, favoring survival rather than apoptosis, may be particularly advantageous in tumor cells emerging in a stressful environment.

Our findings suggest that chronic exposure to inflammatory cytokines will enforce a selective pressure for p53 mutations. In addition to abrogating the proapoptotic function of wt p53, those mutations might allow incipient cancer cells to benefit, rather than suffer, from the effects of TNFα and similar cytokines. Consequently, the down-regulation of mutant p53 in tumors exhibiting constitutive NFκB activation may attenuate the latter's antiapoptotic effect, rendering the tumors more sensitive to killing by cytotoxic anticancer agents.

Acknowledgments

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