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TNFα-induced activation of NFκB protects against UV-induced apoptosis specifically in p53-proficient cells

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The signaling pathways that depend on p53 or NFκB transcription factors are essential components of cellular responses to stress. In general, p53 is involved in either activation of cell cycle arrest or induction of apoptosis, while NFκB exerts mostly anti-apoptotic functions; both regulatory pathways apparently interfere with each other. Here we aimed to analyze the effects of NFκB activation on DNA damage-induced apoptosis, either p53-dependent or p53-independent, in a set of human cell lines. Four cell lines, HCT116 and RKO colon carcinoma, NCI-H1299 lung carcinoma and HL60 myeloblastoma, each of them in two congenic variants either containing or lacking transcriptionally competent p53, were used. Cells were incubated with TNFα cytokine to activate NFκB and then treated with ultraviolet or ionizing radiation to induce apoptosis, which was assessed by measurement of the sub-G1 cell fraction. We observed that treatment with TNFα resulted in a significant reduction in the frequency of apoptotic cells in UV-irradiated p53-proficient lines (with exception of the UV-resistant NCI-H1299 cells). This anti-apoptotic effect was lost when cells were pretreated with parthenolide, an inhibitor of NFκB activation. In marked contrast, TNFα-pretreatment of p53-deficient lines resulted in an increased frequency of apoptotic cells after UV irradiation (with exception of HL60 cells). Such anti- and pro-apoptotic influence of TNFα was less obvious in cells treated with ionizing radiation. The data clearly indicates functional interference of both signaling pathways upon the damage-induced apoptotic response, yet the observed effects are both cell type- and stimulus-specific.

Keywords: apoptosis, cytoprotection, signaling, NF-κB, *TP53*

INTRODUCTION

Mutational inactivation of the *TP53* gene is one of the most common genetic events that occur in human cancers, highlighting its central role as a tumor suppressor. The *TP53* gene encodes p53, a sequence-specific DNA-binding protein and transcription factor that controls the expression of a large panel of genes implicated in many aspects of cellular physiology including growth control, DNA repair, cell-cycle arrest, and apoptosis. The activity of p53 as a tumor suppressor is linked to its role as a major coordinator of DNA damage-induced cell-cycle checkpoint pathways and to its role in apoptosis of perturbed cells. Under normal conditions p53 is functionally inactive due to its rapid degradation after hallmarking by the ubiquitin ligase MDM2. However, upon infliction of cellular stress, MDM2-driven degradation is halted and then p53 accumulates and gains full competence in transcriptional activation. Although many different stress conditions can induce transcriptional activity of p53, it appears that two distinct signaling pathways play the major role in p53 activation. One of these is the damage-medi-

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Abbreviations: DMSO, dimethylsulfoxide; IAP, inhibitor of apoptosis protein; IKK, IκB-specific kinase; IκB, inhibitor of NFκB; IR, ionizing radiation; MDM2, p53-interacting protein; NFκB, transcription (nuclear) factor binding with kappa immunoglobulin gene in B-cells; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; TNF, tumor necrosis factor; UV, ultraviolet radiation.

ated activation that depends on several protein kinases, including but not restricted to ATM, ATR, CHK2, JNK, p38, c-Abl, and is initiated by different types of DNA lesions. Another regulatory mechanism is the growth factor/oncogene-mediated signaling pathway that depends on the tumor-suppressor protein p14ARF. Among the hundreds of elements that comprise the p53 pathway, a significant fraction create feedback control loops and communicate with other signal transduction pathways (reviewed in: Efeyan & Serrano, 2007; Levesque & Eastman, 2007; Rodier *et al*., 2007).

NFκB is a collective name for a family of transcription factors that are dimeric complexes formed by members of the multigene NFκB/Rel family, which in mammalian cells consists of five proteins: RelA (p65), RelB, c-Rel, NFκB1 (p50/p105) and NFκB2 (p52/p100). Generally, in resting cells the NFκB transcription factors are sequestered in the cytoplasm by association with members of another family of proteins called IκB. Importantly, most of the IκB-family's inhibitory potential is carried by IκBα whose synthesis is controlled by a highly NFκB-responsive promoter, which is an element of the autoregulation circuit of NFκB signaling. Activation of NFκB requires degradation of IκB, which allows nuclear translocation of NFκB and binding to *cis*-acting DNA regulatory elements. Regulation of NFκB depends on phosphorylation of the IκB inhibitor, which is catalyzed by IKK kinases activated by pro-inflammatory extracellular signals or cellular stress (reviewed in: Tian & Brasier, 2003). NFκB regulates numerous genes important for pathogen- or cytokine-induced inflammation, immune response, and cell proliferation. Importantly, because in stress conditions NFκB activates several genes that promote cell survival, including members of the IAP and anti-apoptotic Bcl-2 families, it can contribute to resistance of cancer cells to anti-cancer treatments. Various reports have shown that experimental activation of NFκB results in reduced apoptosis while its inhibition promotes apoptosis and suppresses tumor growth (reviewed in: Burstein & Ducket, 2003; Perkins, 2004).

The TNFα cytokine, a major activator of the NFκB pathway, initiates a broad range of cellular responses including growth stimulation as well as growth arrest or apoptosis, depending on dose and cell type. At the molecular level, TNFα binds the TNF receptor and induces its trimerization, which serves as a platform to recruit several signaling molecules. The active TNF receptor complexes can interact *via* TRADD and FADD adaptor proteins with initiator caspases to induce apoptosis or, *via* TRADD, with TRAF2 and RIP proteins which are involved in the activation of the NFκB and JNK/AP-1 pathways (reviewed in: Baker & Reddy, 1998). Importantly, NFκB plays a major anti-apoptotic role against TNFα cytotoxicity (Beg & Baltimore, 1996). Furthermore, TNFα can either repress (Saile *et al*., 2001) or stimulate (Drane *et al*., 2002) transcription of *TP53* gene, most possibly in a NFκB-dependent and cell type-specific fashion.

The NFκB-dependent and p53-dependent transcriptional pathways apparently interact with each other. It has been reported that activation of one may result in suppression of the other, possibly due to competition for transcriptional co-activators (Webster & Perkins, 1999). In fact, the NFκB regulons (sets of genes under regulation of this transcription factor) are different in cells differing in p53 status (Yan *et al*., 2008). On the other hand, the *TP53* gene promoter contains a putative NFκB-binding element, indicating a possible role of NFκB in its transcriptional regulation (Hellin *et al*., 1998). In addition, the p53 protein can be either stabilized (Fujioka *et al*., 2004) or destabilized (Tergaonkar *et al*., 2002) by NFκB-activated mechanisms, depending on the experimental model. The cross-talk between the NFκB and p53 pathways is further complicated by the fact that various p53-dependent genes respond differently to NFκB stimulation and various members of the NFκB/Rel family have different effects (Schumm *et al*., 2006).

It is generally accepted that in many types of cells genotoxic stress induces apoptosis, employing either p53-dependent or p53-independent mechanisms (reviewed in: Levesque & Eastman, 2007). On the other hand, in the majority of circumstances NFκB exerts an anti-apoptotic function (reviewed in: Burstein & Ducket, 2003; Perkins, 2004). Here we aimed to clarify whether TNFα-induced activation of NFκB interferes with induction of apoptosis in cells subjected to genotoxic stress. We analyzed the effects of treatment with TNFα on the frequency of apoptotic cells and cell cycle arrest in a panel of established tumor cell lines differing in their p53 status, and treated with UV or gamma radiation to induce DNA damage.

MATERIALS AND METHODS

Cell lines. Experiments were performed using the following cell lines: HCT116 and RKO colon carcinoma cells, NCI-H1299 non-small lung cancer cells, and HL60 acute myeloid leukemia cells. HCT116 and RKO colon carcinoma cells have a normal p53 status. HCT116 variant cells depleted of the *TP53* gene due to bi-allelic knock-out (Bunz *et al*., 1998) were a generous gift from Dr. B. Vogelstein. RKO variant cells stably transfected with human papillomavirus E6 protein gene (Kessis *et al*., 1993), a generous gift from Dr. M. B. Kastan, were also used. NCI-H1299

and HL60 have a negative p53 status (Chen *et al*., 1993; Wolf & Rotter, 1985); these cells were transiently transfected with the human *TP53* gene DNA using FuGENE transfection reagent (Roche) according to the manufacturer's protocol, using 1:3 and 1:6 DNA/FuGENE ratios in the case of NCI-H1299 and HL60 cells, respectively. The full coding sequence of the human *TP53* gene (NM_000546.2) under control of the human *EF1* gene promoter cloned into pEF1/V5-His-B vector (Invitrogen) was kindly provided by Mr. W. Pigłowski (the pEF1-p53 vector). NCI-H1299 and HL60 cells were grown in RPMI 1640 medium, HCT116 cells in McCoy's medium, and RKO cells in DMEM medium at 37°C and 5% CO_2; all media were supplemented with 10% heat-inactivated fetal bovine serum, and the medium for RKO cells with gentamycin (100 U/ml).

Cell treatments. Treatments started 24 h after inoculation or transfection of cells; all steps, with exception of irradiation, were performed in a humidified incubator at 37°C and 5% CO_2. Cells were incubated for 15 min in medium containing 10 ng/ml of TNFα cytokine (Roche), and then TNFα-containing medium was replaced with fresh TNFα-free medium for an additional 45 min before further treatment with radiation. The ionizing radiation (IR) was generated by a linear accelerator (Clinac 600; Varian); cells were exposed to a 4 Gy dose at a rate of 1 Gy/min. The UV radiation was generated by a 254 nm light source (UV Stratalinker 2400; Stratagene); cells were exposed to a 20 J/m^2 dose in dishes without lids after reducing the volume of medium. Irradiated cells were incubated for an additional 24 h and then collected and analyzed. Appropriate controls, either untreated or TNFα-treated only, were processed in parallel. Alternatively, cells were pre-incubated with 10 μM parthenolide (Sigma; dissolved in DMSO) for 4 h and then the medium was replaced with fresh medium containing TNFα. After 15 min of incubation with TNFα the medium was replaced with fresh medium and the cells were incubated for an additional 6 h before UV-irradiation (Fig. 4A).

Western blots. Cells were lysed in 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS supplemented with a protease inhibitor cocktail, centrifuged at 20000×*g* for 20 min, and the supernatants were collected and protein concentrations determined by Bradford assay (Bio-Rad). Equal amounts of lysates (25 μg protein) were separated using 12% SDS/PAGE and electrotransferred onto a nitrocellulose membrane (Millipore); membranes were transiently stained with Ponceau Red (Sigma) to monitor the amount of transferred proteins. The membranes were blocked with 5% nonfat milk in Tris-buffered saline and then incubated for 1 h at room temp. with polyclonal anti-IκBα antibody (C-15; Santa Cruz), monoclonal anti-p53 mAb (DO-1; Santa Cruz) or monoclonal anti-actin Ab (Oncogene Research Products). Proteins were visualized after incubation with alkaline phosphatase- or peroxidase-conjugated secondary antibody using BCIP/NBT alkaline phosphatase substrate solution (Sigma) or enhanced chemiluminescence (ECL) (Pierce), according to the manufacturers' protocols.

Flow cytometry. Cells were collected after a brief incubation with trypsin solution (Sigma), centrifuged for 2 min at 600×*g*, washed in PBS, fixed in 70% ethanol and stored at –20°C. Fixed cells were washed twice in PBS and re-suspended in PBS containing 50 μg/ml propidium iodide and 0.2 mg/ml RNase A for 15 min at room temp. and then immediately analyzed using a FACScan flow cytometer (Becton Dickinson). Ten thousand cells were counted in each sample for assessment of the cell-cycle phase distribution. The percentage of apoptotic cells was calculated from the sub-G1 peak of the DNA histograms using CellQuest software (Becton Dickinson). Relative frequencies of apoptotic cells were expressed as ratios of the percentage of sub-G1 cells in treated and in appropriate untreated control cultures.

RESULTS AND DISCUSSION

Experiments were performed with four established human cell lines. Two of these, HCT116 and RKO colon carcinomas, originally have wild-type p53 status, and were compared with a HCT116 variant depleted of the *TP53* gene due to bi-allelic knock-out constructed by the group of Vogelstein (Bunz *et al*., 1998), and a RKO variant that expresses the human papilloma virus E6 protein which binds to and inactivates p53 protein (Kessis *et al*., 1993). On the other hand, two other lines, NCI-H1299 non-small lung cancer and HL60 myeloblastoma, lack a functional p53 protein due to major deletions in the *TP53* gene (Wolf & Rotter, 1985; Chen *et al*., 1993). These cell lines were transiently transfected with the wild-type *TP53* gene under control of the constitutive promoter of the human *EF1* gene to produce a p53-proficient status.

First, we verified whether the p53 protein could be synthesized in these cells and activated after exposure to a genotoxic stimulus. p53 protein was Western-blot detected in whole-cell lysates prepared from all cell lines, either untreated controls or 24 h after irradiation with UV (Fig. 1A). As expected, neither HCT116 p53-knocked-out cells nor NCI-H1299 and HL60 cells expressed any detectable p53 protein. RKO cells expressing the E6 viral protein synthesized low amounts of p53 protein, which, however, was not accumulated after UV irradiation. On the other hand, activation and accumulation of

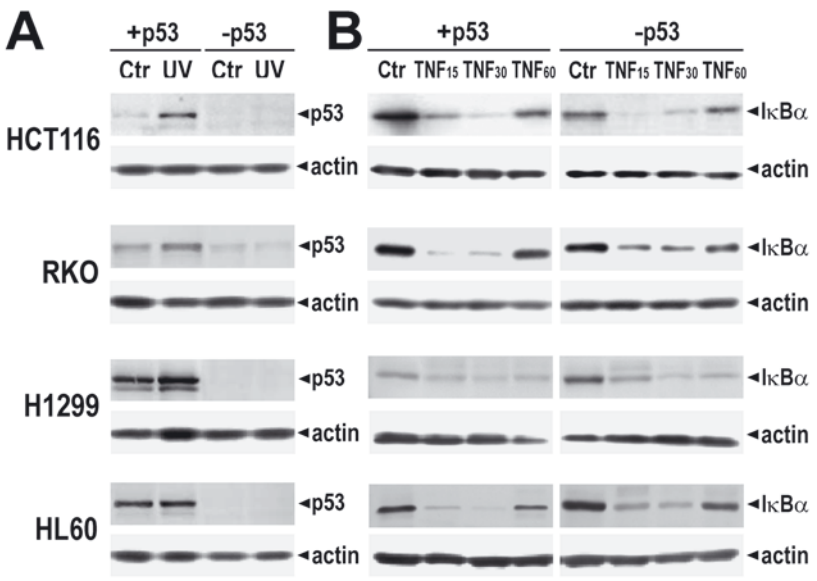

Figure 1. Activation of p53 and NFκB.
Western blots of whole-cell lysates. **A**. p53 levels in cells irradiated with UV and harvested 24 h later. **B**. IκBα levels in cells incubated for 15 min with TNFα and harvested after 15, 30 or 60 min. p53-proficiency (+p53) refers to wild-type HCT116 and RKO cells or *TP53*-transfected NCI-H1299 and HL60 cells. p53-deficiency (–p53) refers to wild-type NCI-H1299 and HL60 cells, HCT116 *TP53*-knock-out cells, and *E6*-transfected RKO cells. The levels of β-actin prove equal protein loading.

p53 was observed in UV-irradiated HCT116 and RKO cells. NCI-H1299 and HL60 cells transfected with the p53 gene clearly synthesized the p53 protein; however, its further accumulation in UV-irradiated cells was barely observed, possibly because of the abundant expression under control conditions. We then verified whether treatment with the TNFα cytokine activated NFκB in these cell lines. In general, treatment with TNFα induces a rapid IKK-dependent phosphorylation of IκBα, which results in its proteolytic degradation and subsequent activation of NFκB. This in turn leads to activation of the IκBα gene (which is one of the early NFκB-activated genes) and restoration of the IκBα level about one hour after initial treatment (reviewed in: Tian & Brasier, 2003). Here we performed a Western-blot detection of the total level of IκBα in lysates from cells, either p53-proficient or p53-deficient, at 15, 30 and 60 min after a pulse of TNFα (Fig. 1B). As expected, the treatment with TNFα resulted in rapid degradation of IκBα followed by subsequent restoration of its level in both p53-proficient and p53-deficient cells. The TNFα-dependent changes in the IκBα level were least pronounced in NCI-H1299 cells, especially in those transfected with *TP53*, where such changes were barely detected. We concluded that this experimental system with four cell lines and alternative p53 status for each of them was suitable to study the combined effects of genotoxic treatment and stimulation of NFκB.

Twenty-four hours after combined treatment with TNFα and genotoxic irradiation, cells were harvested and their distribution in cell cycle phases was assessed by flow cytometry. The percentage of cells having an inappropriately low content of DNA (so-called sub-G1 cells) reflects cells at terminal stages of apoptosis. For better visualization, the frequencies of cells undergoing apoptosis were expressed as the ratio between the percentage of apoptotic cells induced by the treatment and the percentage undergoing spontaneous apoptosis in untreated controls (Fig. 2). We observed that treatment with TNFα alone induced only a minor increase in the frequency of apoptotic cells in all tested lines, irrespective of their p53 status. Irradiation with UV induced massive apoptosis in RKO, HCT116 and HL60 cells, in both p53-proficient and p53-deficient lines. Notably, the p53-dependent pathways seem most essential in UV-irradiated HCT116 and RKO cells whereas the p53-independent pathways seem most essential in HL60 cells. UV radiation induced practically no sub-G1 cell fraction in the NCI-H1299 line, irrespectively of its p53 status. Combined treatment with TNFα and UV radiation allowed us to test for possible interference of NFκB activation and apoptotic responses to UV-induced damage. It is important to note that in all p53-proficient lines where apoptosis could be induced by UV (i.e., HCT116, RKO and HL60), pre-treatment with TNFα markedly (about 2-fold) reduced the frequency of UV-induced sub-G1 cells. Furthermore, such protection was not observed in p53-deficient cell lines. In the TNFα-pretreated UV-irradiated HCT116 and RKO p53-deficient cells the levels of the sub-G1 fraction were even higher compared to cells only irradiated with UV. Similar results of TNFα-pretreatment, in both p53-proficient and p53-deficient HCT116 and RKO cells, were observed if the sub-G1 cell fraction was assessed 12 and 48 h after irradiation with UV (not shown). An increased sub-G1 fraction could be also detected in TNFα-pretreated and UV-irradiated p53-deficient NCI-H1299 cells. In a marked contrast to UV radiation, ionizing radiation (at least at the dose used) did not result in an increased sub-G1 cell

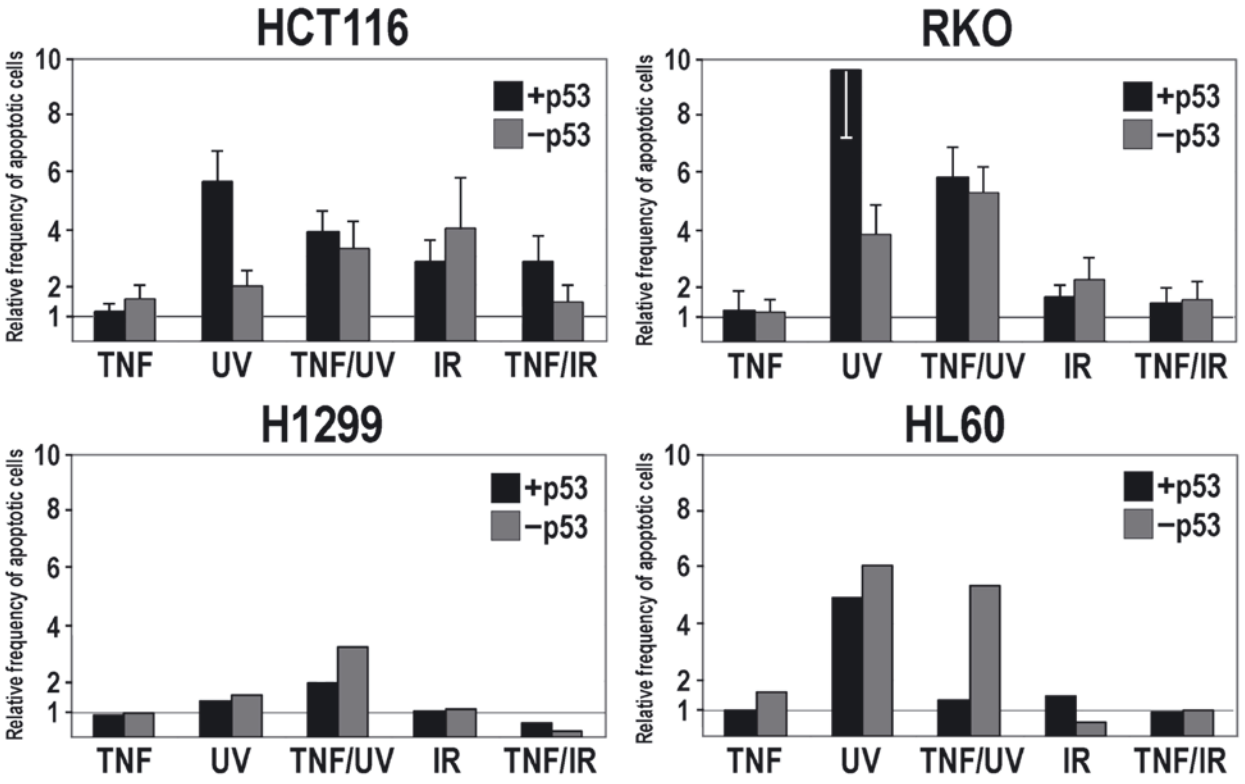

Figure 2. Induction of apoptosis.
Cells were incubated with TNFα (TNF) or irradiated with either ultraviolet (UV) or ionizing (IR) radiation as described in Methods. Alternatively, cells were irradiated 45 min after the end of TNF treatment (TNF/UV and TNF/IR). Cells were harvested 24 h after irradiation (or corresponding culture time), then apoptotic (sub-G1) cells were assessed by flow cytometry. Relative frequencies of apoptotic cells were expressed as ratios of the percentage of sub-G1 cells in treated and in appropriate untreated controls. Experiments with HCT116 and RKO cells were repeated 2 or 3 times; shown are the average values ±S.D.

fraction, with the exception of HCT116 lines where increased levels of apoptotic cells could be detected after exposure (especially in the p53-deficient line). Surprisingly, however, in the latter case the results of pretreatment with TNFα were opposite to those observed with the scheme including UV radiation; pretreatment with TNFα protected against IR-induced apoptosis in p53-deficient but not in p53-proficient HCT116 cells.

Flow cytometry measurement of cell fractions differing in their DNA content allows determination of the distribution of different phases of the cell cycle and to detect induction of cycle arrest if it occurs. Here we determined the ratio between the fraction of cells in the G2/M phase and in the G0/G1 phase in all experimental schemes; Figure 3 shows the results of such analysis performed 24 hours after the treatment. Treatment with TNFα alone did not perturb the G2:G1 ratio, as compared to untreated controls, in any of the cell lines analyzed. UV irradiation, either alone or in combination with TNFα, induced G2 arrest in HCT116 cells, both p53-proficient and p53-deficient. The same treatment did not perturb the G2:G1 ratio in RKO and NCI-H1299 lines. In contrast, UV irradiation alone induced G1 arrest in p53-proficient HL60 cells, whereas the combination of UV and TNFα induced G2 arrest in p53-deficient HL60 cells. Importantly, G2 arrest was observed in all lines except NCI-H1299 exposed to ionizing radiation, independently of TNFα pretreatment. Interestingly, 24 h after this treatment more cells were arrested in the G2 phase in p53-deficient lines than in p53-proficient ones. However, this was apparently a delayed effect because 12 h after exposure to IR, more cells were arrested in the G2 phase in the p53-proficient lines as compared to the p53-deficient ones (not shown). In conclusion, the data obtained indicate that the protection against p53-dependent UV-induced apoptosis exerted by TNFα-activated NFκB is independent of the cell cycle effects: protection could be exerted without additionally perturbing the UV-affected cell cycle, both in cells where the treatment induced G2 arrest (i.e., HCT116) and in cells without such arrest (i.e., RKO). However, the ability to induce cell cy-

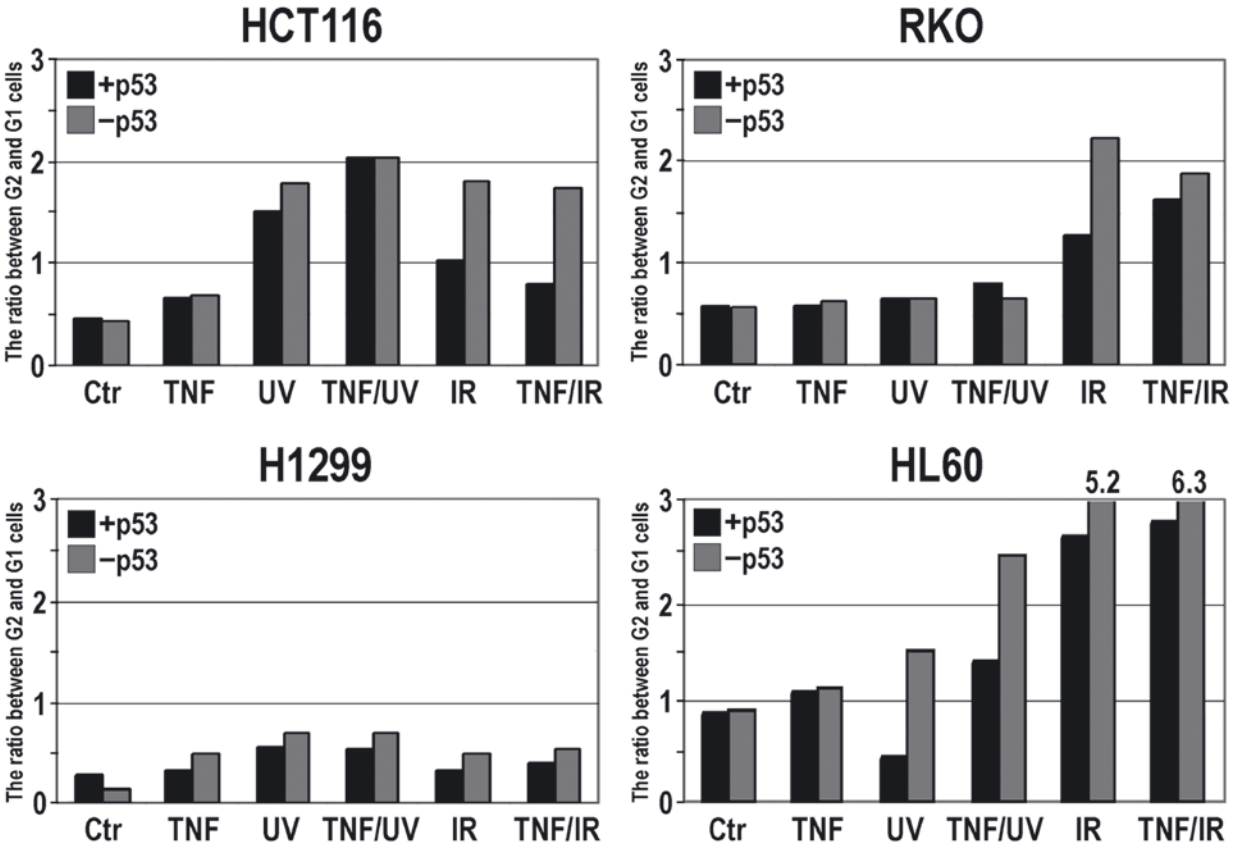

Figure 3. Cell cycle arrest.
Cells were treated with the indicated combination of TNFα and/or radiation and the ratio of the percentage of cells in G2/M and in G1/G0 was assessed by flow cytometry after 24 h.

cle arrest may contribute to the overall resistance to UV and explain the relatively higher frequency of apoptotic cells in the RKO lines as compared to the HCT116 lines. Similarly, the low frequency of apoptotic cells after exposure to ionizing radiation could be related to induction of G2 arrest in such cells.

TNFα-mediated activation of NFκB-dependent genes is the major cytoprotective mechanism that counterbalances apoptotic pathways potentially triggered by this cytokine (Delhalle *et al*., 2002). Parthenolide is a sesquiterpene lactone that enables disruption of recruitment of IKK to the TNF receptor, which results in blockade of IKK-dependent activation of NFκB (Zhang *et al*., 2004). In order to verify whether the observed protection against UV-induced apoptosis was dependent on NFκB-activation, HCT116 cells were pre-incubated with parthenolide before treatment with TNFα. We observed that such pre-incubation resulted in the absence of activation of NFκB after TNFα-treatment, in both p53-proficient and p53-deficient cells, which was evidenced by unaffected levels of IκBα in such cells (Fig. 4B). Pre-treatment with parthenolide increased the frequency of apoptosis in p53-proficient cells, especially in TNFα-treated ones. Most importantly, however, in cells pretreated with parthenolide TNFα-treatment exerted no protection against UV-induced p53-dependent apoptosis (Fig. 4C), indicating that protection against UV-induced apoptosis in p53-proficient cells depends on TNFα-dependent activation of NFκB.

Here we have reported that TNFα-induced activation of NFκB exerts a cytoprotective anti-apoptotic effect in cells exposed to UV radiation specifically in p53-proficient cells. In marked contrast, TNFα does not protect UV-irradiated p53-deficient cells and may further stimulate apoptosis in such cells. Taking into account the complexity of possible interactions among the p53-, NFκB- and TNFα-dependent pathways, several mechanism could contribute to this phenomenon. First, NFκB-activated pathways could differently interfere with p53-dependent and p53-independent apoptotic pathways (e.g., NFκB-dependent pathways might suppress only p53-dependent apoptosis). TNFα-mediated activation of NFκB could result in suppression of p53-regulated genes, including pro-apoptotic ones, possibly due to a gene-nonspecific mechanism of competition for common tran-

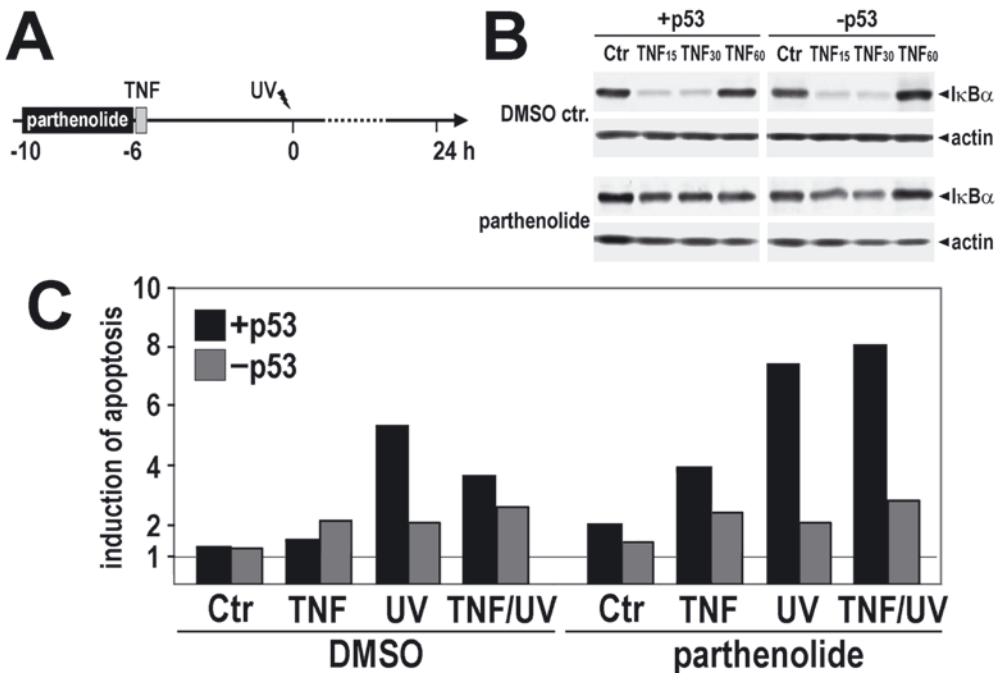

Figure 4. Effect of inhibition of NFκB activation by parthenolide pretreatment of HCT116 cells.
A. Experimental scheme. **B**. Level of IκBα in lysates from cells harvested 15, 30 or 60 min after TNFα-treatment of HCT116 cells pretreated with either parthenolide or DMSO as a solvent control. **C**. Relative frequencies of apoptotic cells after pretreatment with either parthenolide or DMSO and then with the indicated combination of TNFα and UV radiation; sub-G1 cell fractions were assessed by flow cytometry 24 h after UV.

scriptional co-activators (Webster & Perkins, 1999). Notably, however, in some cancer cells (including HCT116) NFκB could specifically repress expression of the pro-apoptotic *BAX* gene involved in p53-dependent apoptosis (Bentires-Alj *et al*., 2001). Second, p53 could interfere with activation of specific NFκB-dependent pathways (e.g., p53 might be required for activation of certain NFκB-dependent anti-apoptotic genes). In fact, it is known that p53 affects expression of several NFκB-regulated genes (Yan *et al*., 2008), possibly due to modulation of different components of the NFκB regulatory network (Rocha *et al*., 2003), although a p53-dependent stimulation of NFκB-activated anti-apoptotic genes has not been reported so far. Additionally, p53 might affect the balance between the TNFα-induced NFκB-dependent and NFκB-independent mechanisms, possibly *via* regulation of the TRADD adaptor protein (Morgan *et al*., 2002). However, it should also be noted that in some experimental models NFκB could exert pro-apoptotic functions due to stabilization and activation of p53 (Hellin *et al*., 1998; Fujioka *et al*., 2004; Aleyasin *et al*., 2004). In addition, the potency of p53 to sensitize cells to TNFα-induced apoptosis has been reported in certain cell lines (Cai *et al*., 1997; Ameyar *et al*., 1999; Rokhlin *et al*., 2001; Ameyar-Zazoua *et al*., 2002). These observations combined with our data clearly indicate that functional interactions between p53, NFκB, and TNFα are specific for the apoptotic stimulus and cell type as well.

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