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Advances in Brief

Loss of Nuclear Factor-κB Is Tumor Promoting but Does Not Substitute for Loss of p53

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Abstract

Inactivation of apoptotic pathways is a common event in cancer. Two transcription factors that regulate apoptosis during tumorigenesis are p53 and nuclear factor (NF)-κB. Although NF-κB is generally considered a suppressor of cell death, we showed previously that NF-κB can contribute to p53-induced death. Here, we show that loss of p65, a critical subunit of NF-κB, can cause resistance to different agents that signal death through p53. Loss of p65 also enhances tumorigenesis induced by E1a and Ras. Unlike loss of p53, however, loss of p65 does not cause anchorage-independent growth or enable tumor development following expression of a single oncogene. These findings reaffirm the role of NF-κB in p53-induced death but show that its loss does not substitute for loss of p53 in tumor development. Moreover, this indicates that, although perhaps central to p53 function, loss of the ability to induce programmed cell death does not completely inactivate p53's tumor-suppressive effects.

Introduction

The importance of the p53 tumor suppressor in cancer is well established. In response to various forms of cellular stress such as DNA damage, oncogene activation, or hypoxia, the levels of p53 increase and inhibit the propagation of damaged cells, which may otherwise go on to form a tumor (1). A number of phenotypic effects have been associated with elevation of p53, but the ability to induce programmed cell death (apoptosis) has been considered primary to the protein's role in tumor suppression (2). As a result, the way in which p53 regulates apoptosis has been investigated extensively. The search for downstream components of the apoptotic response has received considerable attention because it is hoped that targeting these factors may prove therapeutically beneficial in tumors that have lost or mutated p53 (3). A large body of evidence has now accumulated to indicate that p53 can induce apoptosis by a variety of mechanisms (4). However, none of these are absolute, exemplifying the complex nature of this response and indicating that its mediation will be both cell-type and context specific. One intriguing factor that has been shown to be involved in death by p53 is the transcription factor nuclear factor (NF)-κB (5). The vast majority of studies, in particular those concerning apoptosis downstream of the proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin 1, have implicated NF-κB as a survival factor (6). Even in the context of p53-induced death, NF-κB has been shown to be antiapoptotic when studied in primary cells (7). When similar primary cells, however, are transformed with the oncogene, E1a, making them more characteristic of tumor cells, we found that during death induced by p53, the role of NF-κB changes and becomes proapoptotic (5). This apparent contradiction to the well-established antiapoptotic role of NF-κB was not without precedent, however, as NF-κB has also been reported to contribute to death induced by Fas or after infection with certain viruses (8, 9). In line with these conflicting roles in the regulation of apoptosis are a number of opposing studies as to the role of NF-κB during tumor development in animal models (10, 11). Until recently, however, NF-κB has not been considered as a tumor suppressor in human cancer because the only studies reporting its perturbation indicated elevated or enhanced expression instead of being lost (12). Although consistent with the model in which NF-κB can protect developing tumor cells from death and thereby contribute to tumor progression (13, 14), interpretation of these observations complicated by recent studies showing that NF-κB activity can be inhibited indirectly in some cancers. Two studies have now shown that two proteins, HSCO and β-catenin, which are elevated in cancer and have oncogenic roles, can bind to NF-κB and inhibit programmed cell death. Given this degree of uncertainty about the role of NF-κB in tumor progression, we decided to extend our previous work on the role of NF-κB during death induced by p53 to include *in vivo* tumor models. We show here that loss of NF-κB can cause resistance to other agents that are known to cause death through p53. In addition, we show that, consistent with a tumor-suppressive role, loss of NF-κB can contribute to tumor development but that its loss is not equivalent to loss of p53.

Materials and Methods

Plasmids. Adenovirus type 5 12S E1a sequences were expressed from the retroviral vector pLPC12SE1a, which has been described previously (15). V12Ha-Ras cDNA sequences were removed from the plasmid pLXSP3-V12Ras (from Martin McMahon) and cloned in to the retroviral vector pWZLHygro.

Cell Culture and Retroviral Infections. Primary wild-type, p65−/−, and p53−/− mouse fibroblasts (MEFs) obtained from E13.5 embryos were maintained in DMEM containing 10% fetal bovine serum at 37°C in an atmosphere of air containing 10% CO_2. Retroviral infections were undertaken using Phoenix-Eco retroviral-packaging cells, which were maintained as described above. One million Phoenix cells were plated in a 9-cm dish for each retroviral infection. The following day after plating, cells were transfected with 15 μg of retroviral vector DNA for 16 h, washed, and then three harvests of infectious supernatant were collected in DMEM containing 20% FBS at 12-h intervals. MEFs were seeded at density of 7.5×10^5/9-cm dish, 24 h before infection. Retroviral supernatants were purified through a 0.45-μm filter and then added to MEF cultures together with Polybrene (hexadimethrine bromide; Sigma) at a final concentration of 5 μg/ml. Three rounds of infection at 12-h intervals were undertaken firstly with pLPC12SE1a. After a 24 h recovery period in standard growth medium, cells were selected for 4 days in 2.25 μg/ml puromycin (Sigma). Where indicated, cells were subsequently infected with pWZLHygo-V12Ras and then selected for 6 days in 85 μg/ml hygromycin B (Roche).

Apoptosis Assays. MEFs were treated for 24 h with either 1 μg/ml Adriamycin (doxorubicin; Sigma), 50 ng/ml TNF-α (Sigma), 150 μM deferoxamine mesylate (Sigma) or 280 μM H_2O_2 as indicated. Total populations of

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cells, including adherent and floating cells, were then harvested and fixed in methanol for at least 24 h. After centrifugation, cells were then stained with 0.5 mg/ml propidium iodide in PBS containing 125 μg/ml RNase A for 30 min. Samples were then analyzed by flow cytometry (FACScalibur; Becton Dickinson) as described previously. Data acquired was analyzed using CellQuest analysis software (Becton Dickinson), and the apoptosis induced in each treatment was taken as being indicated by the percentage of cells with a sub-G_1 DNA content relative to the rest of the population.

Anchorage-Independent Growth Assays. Bacterial culture dishes (60 mm) were coated in 0.5% base agar containing standard growth medium as described above. Cell suspensions of MEFs containing 2×10^5 cells were mixed with an equal volume of top agar containing 0.6% agar and double concentration growth medium and then layered on top of the base agar plates described above. Dishes were cultured at 37°C. After 2 weeks, the dishes were visualized by microscopy for the appearance of colonies.

Tumorigenicity Assays. To measure the tumorigenic potential of the different MEF cultures, cells were injected in to athymic mice. Four- to 6-week-old male nude mice were obtained from Charles River Laboratories and maintained in the building 571 animal facility at National Cancer Institute-Frederick in accordance with the institutional guidelines. Animals were kept in numbers of no greater than 5/cage and had unlimited access to food and water. A total of 2×10^6 cells from each of the MEF cultures were suspended in PBS and injected s.c. into each flank of an animal, with 10 animals being used for each cell line. After injection, mice were monitored three times/week for tumor formation. Once apparent, the dimensions of the tumor were measured three times weekly in three dimensions with calipers. Animals with tumor measuring in excess of 1200 mm^3 were sacrificed. The whole study was terminated after 1 year.

Results

Our previous studies had shown that blockade of NF-κB activation using a constitutively active mutant (IκBSR) of its endogenous regulator, IκB, could inhibit cell death induced by p53. Furthermore, using mouse embryo fibroblasts (MEFs) expressing E1a, which undergo apoptosis in a p53-dependent manner, we found that loss of p65 (a subunit of NF-κB) could cause resistance to the chemotherapeutic drug, Adriamycin (Ref. 5; Fig. 1). We decided to investigate this observation additionally to see if loss of p65 could cause resistance to other agents that are known to activate p53. To do this, we treated

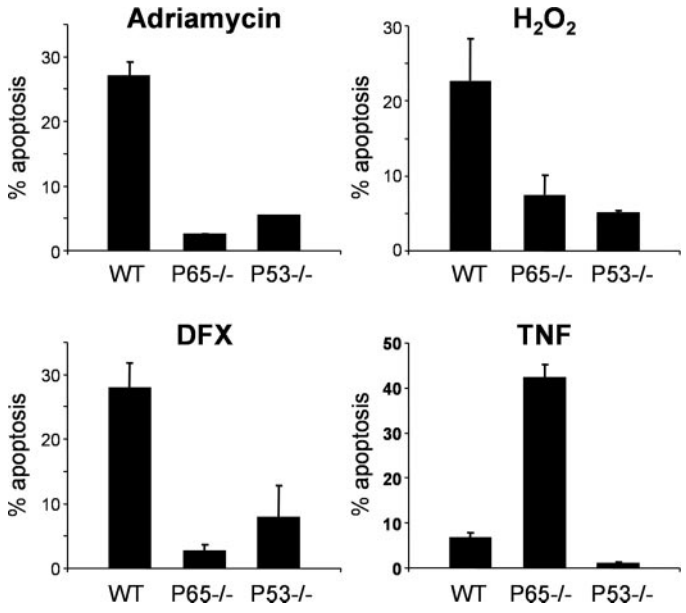

Fig. 1. Cultures of E1a-expressing, wild-type (WT), p53−/−, and p65−/− mouse fibroblasts were treated with the compounds indicated for 24 h. Cells, both adherent and floating, were then harvested and analyzed by flow cytometry. The percentage of cells with a sub-G_1 content was taken as a measurement of apoptotic rate.

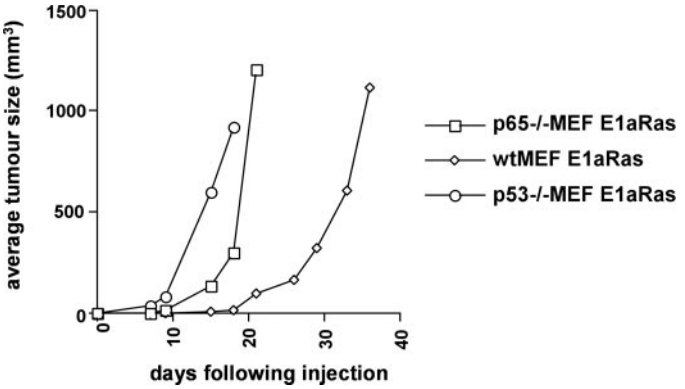

Fig. 2. Cultures of mouse fibroblasts (MEFs) expressing both E1a and Ras were injected in to the flanks of athymic mice. Animals were checked periodically for tumor formation, and the dimensions of tumors were measured in three dimensions with calipers.

cells with H_2O_2 to generate reactive oxygen species and with deferoxamine mesylate to mimic the effects of hypoxia. As can be seen in Fig. 1, whereas p65-null, E1a-expressing MEFs are different to p53-null MEFs in that they are hypersensitive to treatment with TNF-α, they are very similar in that they are essentially equally resistant to the effects of Adriamycin, H_2O_2 and deferoxamine mesylate (Fig. 1).

The ability to induce apoptosis has been considered to be the primary mechanism of p53-mediated tumor suppression *in vivo* (16). In our assays, NF-κB is important for this effect *in vitro*, so we decided to test if loss of NF-κB could contribute to tumorigenicity and also whether its loss is sufficient to substitute for the loss of p53. In the first instance, we generated wild-type-, p53-null-, and p65-null-transformed MEFs containing E1a and an activated Ha-Ras (V12Ras). These cells were then injected s.c. in to the flanks of nude mice, and the animals were monitored for tumor formation. Consistent with previous studies that E1a and Ras are sufficient for tumorigenic conversion of wild-type MEFs, tumors formed in animals injected with these cells within 20–40 days. E1a and Ras were also sufficient to cause tumorigenic conversion of p65-null and p53-null MEFs. Interestingly, loss of p65 enhanced this effect compared with wild-type cells in a similar manner to loss of p53, causing tumors to form rapidly in 10–20 days (Fig. 2).

Because this data indicated that loss of p65 has similar effects on tumor formation as loss of p53, we next sought to determine whether loss of p65 was essentially equivalent to loss of p53 in this assay. We generated wild-type, p53 null, and p65 null MEF cultures expressing E1a alone. Previous studies have shown that the expression of E1a alone, without the cooperation of a second oncogene such as V12Ras, is insufficient for tumor development in nude mice. The loss of p53, however, complements for this second oncogene, and expression of E1a in p53-null fibroblasts can result in tumorigenic cells (15). As expected, tumors formed rapidly in animals injected with p53-null-E1a cells (within 10–12 days), whereas no tumors formed in animals injected with E1a-expressing wild-type MEFs (Fig. 3). Surprisingly, no tumors formed from the E1A-expressing p65 null cells either, and in this assay, p65-null MEFs were indistinguishable from E1a-expressing wild-type MEFs, with no noticeable tumor formation even up to a year after injection of the cells (Fig. 3).

We considered therefore that the difference in the tumorigenic potential between the E1a-expressing p65-null and p53-null cells may not reflect differences in sensitivity to programmed cell death but could be the result of another tumor-promoting characteristic gained by loss of p53, but not by loss of p65. Firstly, we tested if there was simply a difference in the growth rate between the two cells types. As can be seen in Fig. 4*A*, the p53-null cells did grow faster *in vitro* than

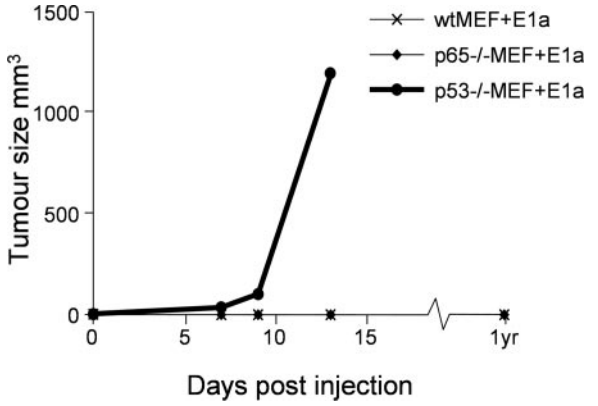

Fig. 3. Cultures of E1a-expressing mouse fibroblasts (MEFs) were injected in to the flanks of athymic mice. Animals were checked periodically for tumor formation and scored. Animals exhibiting tumors in excess of 1200 mm^3 were sacrificed.

the p65-null cells. However, this difference is unlikely to fully explain the difference in the tumorigenic potential of the cells because there was a comparable difference in the growth rate between the E1a-expressing wild-type and p65-null cells without any difference in the ability of these two cell types to form tumors in nude mice (Fig. 3). We next tested whether the p65-null and the p53-null cells exhibited differences in their ability to grow in semisolid agar—an *in vitro* indicator of anchorage-independent growth and a characteristic that has been suggested to contribute to tumor development. This revealed that loss of p53, but not loss of p65, was sufficient to render E1a-expressing cells capable of growth in semisolid agar (Fig. 4*B*).

Discussion

During malignant progression, tumor cells need to acquire a number of characteristics to allow them to proliferate and survive outside their normal environment (17). The p53 protein has a clear function in preventing tumor development, although exactly how each of the numerous activities shown by p53 contribute to this function is not clear. There is compelling evidence to suggest that the apoptotic function of p53 is critical in controlling tumor development, but a recent study of mice expressing a p53 mutant that shows loss of the apoptotic function, while retaining cell cycle arrest activity, suggests that failure to induce apoptosis is not sufficient for loss of tumor suppressor function (18). These studies indicate that other activities of p53 such as the cell cycle arrest function or maintenance of genomic stability may also play an important role in preventing tumor progression.

In this study, we have compared cells that lack p53, with cells defective for NF-κB activity through loss of p65. Previous studies have shown a clear role for NF-κB in protection of cells from TNF-induced apoptosis, and many studies have demonstrated that inhibition of NF-κB function in tumor cells can result in enhanced sensitivity to apoptosis (19, 20). NF-κB expression may, therefore, enhance tumor progression in some situations (10). However, NF-κB can also show proapoptotic activities and, in particular, can be required for p53-induced apoptosis (5, 14). The E1A-expressing p65 null cells used for our study retain enhanced sensitivity to TNF-induced apoptosis but are defective in the p53-induced apoptotic response. However, unlike E1A-transformed p53 null cells, they are not tumorigenic. These studies show that loss of p65 does not affect p53's activity completely, only p53's ability to induce apoptosis and that loss of this response alone is not sufficient to drive tumor progression. In these cells, it is possible that the loss of tumor suppression that accompanies a failure of p53-induced apoptosis is counterbalanced by an enhanced sensitivity to TNF-induced apoptosis. However, because this effect of TNF-α does not manifest itself in E1a/Ras tumors, and in light of recent results showing that a non-apoptotic p53 mutant retains some tumor suppressor activity (18), it also seems possible that the failure of the E1A p65-null cells to form tumors in mice reflects the retention of additional p53 functions that are not impacted by loss of p65.

Interestingly, expression of activated Ras in the E1A-expressing cells revealed a difference in the tumorigenic potential of p65 null cells. As shown previously, wild-type cells expressing E1A and Ras are tumorigenic, although the tumor-suppressive role of p53 in these cells is revealed by the greatly accelerated rate of tumor development in the absence of p53. In this context, loss of p65 has a similar effect as loss of p53 in accelerating tumor development. It is easy to speculate that the presence of V12Ras allows tumor formation in p65-null cells, which do not form tumors with E1a alone, because it overcomes a cell-cycle arrest checkpoint, which is already absent in p53-null cells, thereby making p65-null and p53-null cells equivalent with respect to apoptosis and growth. The true reason for this effect, however, remains unknown.

Taken together, our findings underscore the complexity of the contribution of NF-κB and p53 to tumor development. The prosurvival activity of NF-κB in protection from TNF-induced apoptosis appears to be balanced with the contribution of NF-κB to other pathways of cell death such as that driven by p53. Furthermore, activities of p53 in addition to induction of apoptosis are important contributors to tumor suppression. Both of these considerations probably contribute to the observation that loss of p65 does not substitute for loss of p53. Ultimately, these results underscore the complex nature of both p53 and NF-κB function, where the cellular response is

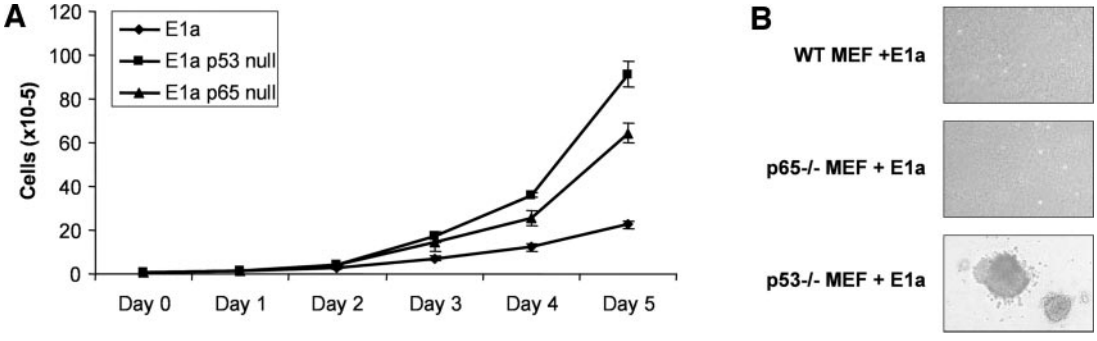

Fig. 4. *In vitro* growth characteristics of E1a-expressing cells. *A*, cultures of E1a-expressing wild-type, p53-null, and p65-null cells were plated in 6-cm dishes at a density of 1×10^5/dish. Cells were then counted every day for 5 days. The results shown are derived from at least three independent experiments for each time point. *B*, cells were plated in soft agar as a test for anchorage-independent growth. After incubation for 2 weeks, the cultures were examined and photographs taken at ×20 magnification. The pictures shown are representative of what was seen in two independent experiments.

dependent not only on the stimulus but also on the cellular and genetic context of the cell involved.

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