Nuclear Factor-κB Protects the Liver against Genotoxic Stress and Functions Independently of p53

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Abstract

p53 and NF-κB are two key effectors in the chemotherapy-induced genotoxic response. Although p53 is a universal inducer of apoptosis in many stress responses, including the genotoxic response, the role of nuclear factor (NF) κ-B is not consistent and was reported to both counteract and mediate apoptosis. Although the reason for the apparent contradictory effects of NF-κB is not understood, it may partly be related to the reported cross-regulation of NF-κB and p53. Thus far, all studies exploring the cross-talk between p53 and NF-κB in conjunction with apoptosis have been performed in tissue-cultured cells and may therefore not faithfully represent conditions that prevail within a chemotherapy-subjected organism. To address this concern, we examined the respective roles of NF-κB and p53 in a liver model of doxorubicin-induced DNA damage. Using this animal model, we report that NF-κB is activated in response to doxorubicin-induced genotoxic stress and exerts a pronounced protective effect in opposing chemotherapy-induced tissue damage. Importantly, the activation of NF-κB occurs independently of p53 status. Furthermore, although p53 is also induced in this in vivo system, its induction is independent of NF-κB and does not contribute to the extent of tissue damage. These findings may have important implications with respect to the potential use of NF-κB modulators in cancer therapy.

Introduction

The success of cancer chemotherapy is critically dependent on the ability of the chemotherapeutic agent to induce effective apoptosis in the target cancer cells (1, 2). This ability varies dramatically among different cell types and is regulated positively and negatively by numerous signal transduction pathways (3). Of pivotal importance in this regard are the p53 tumor suppressor protein and the transcription factor NF-κB.1 p53 is the most commonly mutated gene in human cancer. In response to genotoxic stress, the normally very minute levels of wt p53 are increased, thereby contributing to genome stabilization or, alternatively, commitment of the cell-to-cell cycle exit or apoptosis (for general reviews on p53, see Refs. 4–8). Conversely, loss of normal p53 function results in enhanced frequency of genomic instability and compromises the induction of growth arrest and apoptosis by DNA-damaging genotoxic insults. p53 can induce apoptosis via the transcriptional activation of an array of pro-apoptotic genes and probably also through transcription-independent mechanisms (9, 10). It is possible that different cell types and underlying stress conditions would preferentially mobilize different apoptotic pathways in response to p53 activation.

NF-κB has been implicated in both negative and positive regulation of cell death. In most cell types, activation of NF-κB endows the cells with a survival advantage. This is mediated largely through the induction of antiapoptotic genes such as IEX-1 and XIAP and the Bcl-2 homologues A1/Bfl-1, BCL-XL, and Nr13 (11–15). There are, however, also some conditions where NF-κB has in fact been shown to facilitate apoptosis rather than prevent it; this has been reported particularly in cells of neuronal origin (16). Because apoptosis induction is a mechanism of action of radiotherapy and most chemotherapeutic agents (1, 2), the antiapoptotic properties of NF-κB are likely to abrogate the effectiveness of these major modes of treatment. Many malignant cells possess an inherent chemotherapy protection mechanism through their constitutive NF-κB activity (reviewed in Refs. 17–19), yet radiation and various chemotherapy drugs may induce NF-κB in other cells, thereby conferring treatment resistance both in vitro (20–25, 26) and in various xenograft mouse models (27–31). Furthermore, inhibition of NF-κB was also found to augment the cytotoxic effect of chemotherapy in p53-mutated cells (27).

Numerous studies support the existence of a cross-talk between p53 and NF-κB. Depending on the particular experimental system and assays used, this interrelationship can be either antagonistic or synergistic, giving rise to a complex picture, e.g., NF-κB has been reported to augment p53 expression, largely through activation of the p53 gene promotor (32–36), although this may not be common to all cell types and activating signals. In apparent contrast to its ability to increase p53 expression, NF-κB can actually block the activity of p53 as a sequence-specific transcription factor (37–42). This has been attributed, at least in part, to competition of p53 and NF-κB for limited pools of coactivators. Furthermore, Twist, a recently recognized oncogene that counteracts both myc- and p53-induced apoptosis, is a prominent target of NF-κB (43). It was shown that Twist inhibits the expression of p53-dependent genes in response to DNA damage, possibly by interfering with the ADP ribosylation factor/MDM2 pathway (43). Conversely, excess p53 activity often leads to inactivation of NF-κB in tumor cells, through competition for common coactivators as well as through down-regulation of NF-κB/p65 expression (37, 39–41).

In agreement with the broad antiapoptotic activity of NF-κB and its capacity to counteract the transactivator function of p53, NF-κB has also been shown to inhibit p53-mediated apoptosis (37, 40, 41, 42, 44). A possible mechanism for the p53-antagonizing effect of NF-κB in response to chemotherapy has been proposed recently, involving IKK2-dependent up-regulation of MDM2, a major physiological inhibitor of p53. This results in the destabilization of p53, thus attenuating chemotherapy-induced apoptosis (44). However, there also exist situations where NF-κB and p53 appear to cooperate in apoptosis, rather than inhibit each other’s activity. Of note is the finding that under conditions where p53-dependent apoptosis is triggered by...
chemotherapy, p53 induces NF-κB, and this activation is required for the apoptotic effect of p53 (45). Thus far, all studies on a cross-talk between p53 and NF-κB and the resulting effect on apoptosis were performed in tissue-cultured cells (cell lines and mouse embryo fibroblasts), mostly involving DNA transfections and protein overexpression. This may not represent faithfully the conditions that prevail within a whole organism exposed to chemotherapy. To address this concern, we examined the respective roles of NF-κB and p53 in a liver model of doxorubicin-induced DNA damage. Using this animal model, we report that NF-κB is induced in response to genotoxic stress independently of p53 and exerts a pronounced protective effect in counteracting chemotherapy-induced tissue damage. These findings may have important implications regarding the potential use of NF-κB modulators in cancer therapy.

Materials and Methods

Mice. Generation of mice with specific liver-inhibited expression of NF-κB has been described before (46). p53 KO mice (47) were obtained from The Jackson Laboratory (Bar Harbor, ME). All mouse experiments were authorized by the Animal Research Authority of the Hebrew University.

Doxorubicin Treatment. Animals were injected i.p. with 25 mg/kg doxorubicin and 25 mg/kg verapamil (Teva) dissolved in saline. At the indicated time points, animals were sacrificed, and liver and spleen samples were fixed in 4% buffered formaldehyde at 25°C for 24 h.

Histology and Immunohistochemistry. Formalin-fixed, paraffin-embedded liver samples were sectioned (4 μm), deparaffinized in xylene, and rehydrated through graded ethanol. Sections were stained with PAS and reticulin stains or with antibodies to p53 and P65. For monitoring NF-κB or p53 activation, slides were exposed to heat-induced antigen retrieval in 10 mM citrate buffer (pH 6.0), using a pressure cooker. The slides were quenched with 1% BSA in 0.05 M Tris/HCl (pH 7.6) for 30 min, then incubated for 60 min at 37°C with anti-p65 goat polyclonal antibody for NF-κB (sc-109-G diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-p53 rabbit polyclonal antibody (CM5 diluted 1:100; Novocastra, Newcastle, United Kingdom). After washing, the sections were incubated for 60 min with biotin-conjugated rabbit anti-goat antibody for NF-κB and biotin-conjugated mouse anti-rabbit antibodies (1:200; Chemicon International, Temecula, CA) and biotin-conjugated mouse antirabbit antibodies (1:200; Chemicon International) for p53, washed again, incubated with streptavidin-conjugated peroxidase, and examined after incubation with 3-amino-9-ethyl carbazole and counterstaining with hematoxylin.

Results

To study the in vivo relationship between p53 and NF-κB under conditions of DNA damage, we used a mouse strain harboring a liver-specific dlrBκ, which allows to control hepatocyte NF-κB activity through the Tet-off regulation system (46). DNA damage was elicited by i.p. injection of doxorubicin, an anthracycline-type agent (48). As a first step, we tested the accumulation of doxorubicin in the liver. The distribution of the drug in the liver was assessed by its
auto-fluorescence under confocal microscopy in liver section taken between 2–24 h postinjection. Doxorubicin fluorescence was detectable both in hepatocytes and Kupffer cells when the drug was injected alone but accumulated to a larger extent and for prolonged periods in the hepatocytes after coinjection with verapamil, an antagonist of the MDR1 drug-expulsion system (Ref. 49; Fig. 1). Hepatocyte fluorescence was evident at 4–12 h postinjection, whereas at later intervals, fluorescence was mainly restricted to Kupffer cells, indicating rapid liver clearance of the drug.

Next, we studied the kinetics of NF-κB and p53 induction in the

![Fig. 2. Sequential activation of NF-κB (p65) and p53 in the liver in response to doxorubicin. Mice were injected with doxorubicin/verapamil, and liver samples taken at different time points were stained with anti-p65 (NF-κB) or anti-p53 as indicated.](image)

![Fig. 3. NF-κB and p53 are activated independently of each other after doxorubicin treatment in vivo. Liver samples obtained 4 h after doxorubicin injection were stained with anti-p65 antibodies (a–c) or p53 (d–f). a, d, wt mice; b, e, dIκBα mice; c, f, p53 KO mice.](image)
liver on doxorubicin/verapamil treatment. To assess the activation of p53 and NF-\(\kappa\)B, liver sections taken 1–24 h postinjection were immunostained for NF-\(\kappa\)B/p65 and p53. Nuclear p65 staining, indicative of NF-\(\kappa\)B activation, was observed in both hepatocytes and Kupffer cells as early as 1 h postinjection, peaking at 4 h, and becoming undetectable at 8 h (Fig. 2). In contrast, p53 staining was only evident at 8 h postinjection and was confined to hepatocyte nuclei. Because NF-\(\kappa\)B activation in the liver precedes the induction of p53, it is unlikely to be a consequence of p53 activity.

To rule out the dependence of NF-\(\kappa\)B activation on p53, we investigated NF-\(\kappa\)B activation in livers of p53 KO mice in comparison to livers of wt and dIkB\(\alpha\)/NF-\(\kappa\)B-expressing mice. Mice were injected with doxorubicin/verapamil, and liver sections were analyzed by immunohistochemistry for NF-\(\kappa\)B activation. Although no NF-\(\kappa\)B activation was detectable in hepatocytes of dIkB\(\alpha\)-expressing livers, liver sections of wt and p53 KO mice were indistinguishable, both exhibiting widespread nuclear p65 staining (Fig. 3, top panel). These results indicate that p53 is not required for NF-\(\kappa\)B induction in the liver in response to DNA damage.

As discussed earlier, numerous studies have shown that NF-\(\kappa\)B can activate the p53 gene promoter in cultured cells and that this can contribute to the accumulation of p53 in such cells in response to DNA damage. To determine whether this mechanism is also operative \textit{in vivo}, liver sections of the different treated mouse strains were immunostained for p53. As expected, there was no detectable p53 staining in liver sections of p53 KO mice (Fig. 3, bottom panel). However, p53 immunostaining of wt and dIkB\(\alpha\) mice were indistinguishable: both strains responded to doxorubicin treatment by prominent nuclear accumulation of p53 in their hepatocytes. There was no difference in the intensity of the immunostaining or the fraction of positive hepatocytes between wt and dIkB\(\alpha\) mice, nor was there a difference in the time course of the response (data not shown).

Furthermore, real-time RT-PCR analysis of p53 mRNA (data not shown) indicated that the accumulation of p53 in the doxorubicin-treated cells was not accompanied by its transcriptional up-regulation in both wt and NF-\(\kappa\)B-deficient hepatocytes. Thus, at least within the first 8 h after systemic administration of doxorubicin, NF-\(\kappa\)B does not appear to be required for the accumulation of p53. Taken together, our results do not support a significant cross-regulation of p53 and NF-\(\kappa\)B in the liver in response to DNA damage.

Finally, we evaluated the effects of p53 and NF-\(\kappa\)B on liver integrity after DNA damage. To that end, liver sections of doxorubicin/verapamil-injected mice were stained with PAS reagent and assessed for signs of tissue damage. Only minimal damage was observed in liver sections of mice with a functional NF-\(\kappa\)B pathway, at either 4 (data not shown) or 8 h postinjection (Fig. 4), and there was no observable difference between wt and p53 KO mice. By contrast, multiple foci of coagulative necrosis were noted in livers of dIkB\(\alpha\) transgenic mice. In addition, hepatocytes dropout, parenchymal inflammation (Fig. 4A), and collapse of the liver architecture evident in reticulin stain (Fig. 4B) were only present in NF-\(\kappa\)B-deficient livers. These liver damage signs were doxorubicin dependent, because non-
treated littermates of the same transgenic mice had normal liver appearance (Fig. 4, A and B). Hence, failure to mount an NF-kB response on exposure to chemotherapy is associated with enhanced liver damage similarly to the vulnerability of NF-kB-deficient tissues to other apoptotic insults (46).

Discussion

Tumor cells can evade chemotherapy-induced killing through a number of mechanisms, including effective exclusion of the drug from the cells, down-modulation of proapoptotic pathways, and constitutive activation of antiapoptotic machineries (50). It is therefore important to understand the molecular determinants that affect the response of normal and neoplastic tissues to chemotherapy in vivo. In vivo treatment with doxorubicin, a widely used chemotherapeutic agent, resulted in activation of both p53 and NF-kB in the liver in a time-dependent manner. Yet, these effects are not interdependent, because the abrogation of either has no effect on activation of the other. This is at variance with several in vitro studies, which suggested that these two effectors are cross-regulated as part of the genotoxic stress response. Our experiments could not address the role of the induced p53 in chemotherapy-inflicted damage, because neither the wt nor the p53 KO mice produced significant signs of liver damage after doxorubicin administration. On the other hand, we show that NF-kB exerts a pronounced protective effect against liver damage: abrogation of NF-kB activity, through the expression of a dominant form of IxBα (dIXBα or “IxBα super-repressor”), renders hepatocytes highly susceptible to cell death after chemotherapy (Fig. 4). These findings suggest that NF-kB activity may also endow liver-derived tumor cells with an increased survival capacity, in line with the observation that constitutive NF-kB activity is observed frequently in hepatocellular carcinomas (51). Systemic inhibition of NF-kB is therefore expected to enhance the cytotoxic effect of chemotherapy, similarly to the observations in tissue culture and xenograft mouse models (52). This mechanism provides a rational explanation for the ability of proteasome inhibitors to enhance the cytotoxic effects of various chemotherapeutic agents in xenografts and preliminary clinical trials (53). One outcome of proteasome inhibition is IxBα stabilization, yet many other mechanisms could account for the antitumor activity of proteasome inhibitors. NF-kB inhibition per se has no discernible effects in the mouse liver; deficient mice could be maintained for many months in a germ-controlled environment (46). However, our results indicate that NF-kB inhibition in the liver is a double-edged sword, because in addition to the potentiation of tumor chemotherapy, it is likely to enhance cytotoxicity against normal tissues. These in vivo results are consistent with the findings reported recently in mouse embryo fibroblasts, in which resistance to doxorubicin was restored in IKK2-reconstituted cells (44).

At present, the generality of this conclusion remains to be established. The protective effect of NF-kB against chemotherapy has been described in many, although not all, types of tumor-derived cells grown in culture (52, 54). It has been proposed that this heterogeneity is caused by differences in p53 status; although tumors that lack p53 function are protected by NF-kB, tumors retaining functional wt p53 may actually require NF-kB for optimal p53-mediated apoptosis (45). However, other studies have shown that NF-kB can also exert an antiapoptotic effect in tumor-derived cells lacking p53 (55, 56). Our in vivo data do not reveal a functional link between p53 and NF-kB in the hepatocyte response to chemotherapy, and it is likely that a similar situation may also apply to at least some types of malignant cells. Extensive attempts are presently being carried out to develop low molecular weight inhibitors of NF-kB, which target recently identified components of the pertinent signal transduction pathway (19). The future availability of such inhibitors should enable the testing of this prediction in vivo in a wide variety of tumor models.

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References


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