PUMA–Mediated Apoptosis Drives Chemical Hepatocarcinogenesis in Mice

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Hepatocyte death and proliferation contribute to hepatocellular carcinoma development after carcinogen exposure or chronic liver inflammation. However, the role and the molecular targets of hepatocyte death in relation to compensatory proliferation have not been fully characterized. In this study, we investigated the role of p53 up-regulated modulator of apoptosis (PUMA), a BH3-only protein important for both p53-dependent and -independent apoptosis, in a diethylnitrosamine (DEN)-induced liver carcinogenesis model. PUMA deficiency significantly decreased the multiplicity and size of liver tumors. DEN treatment induced p53-independent PUMA expression, PUMA-dependent hepatocyte death, and compensatory proliferation. Furthermore, inhibition or deletion of c-jun N-terminal kinase 1 (JNK1) abrogated PUMA induction, hepatocyte death, and compensatory proliferation. Conclusion: These results provide direct evidence that JNK1/PUMA-dependent apoptosis promotes chemical hepatocarcinogenesis through compensatory proliferation, and suggest apoptotic inducers as potential therapeutic targets in liver injury and cancer. (Hepatology 2011;54:1249-1258)

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Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide.1 Viral hepatitis, alcoholic liver disease, and carcinogen exposures are the major risk factors for HCC and can cause chronic liver injury and inflammation.2 HCC development is commonly associated with hepatocyte death and compensatory proliferation, though the molecular mechanisms underlying hepatocyte death are not well understood.3 Several classes of chemicals promote HCC in rodents,2 including diethylnitrosamine (DEN), which has been studied extensively and is known to induce rapid killing of hepatocytes.1 Several signaling pathways have been implicated in DEN-induced liver carcinogenesis. Deficiency of c-jun N-terminal kinase 1 (JNK1) decreased cell death, compensatory proliferation, and liver carcinogenesis induced by DEN in mice.4 Inactivation of nuclear factor κB (NF-κB), a prosurvival factor often overexpressed in cancer, was found to enhance the development of DEN-induced HCC.5 However, lack of positive correlation between apoptosis and susceptibility to DEN-induced hepatocarcinogenesis in mice has been reported.5 Therefore, the role and specific mediators of hepatocyte death in HCC development remain to be defined.

The Bcl-2 family of proteins plays a central role in apoptosis and cancer development.6 The BH3-only members in this family initiate apoptosis in response to a wide range of stimuli and cell types.7 We and others identified p53 up-regulated modulator of apoptosis (PUMA) as a BH3-only protein that plays an essential role in p53-dependent and -independent apoptosis via the mitochondrial pathway.8-15 Reduced PUMA expression has been reported in leukemia and
melanoma, whereas increased PUMA expression has led to extensive apoptosis of a variety of human cancer cells in vitro and in xenograft models. Several lines of evidence have demonstrated a prominent role of PUMA in tumor suppression in mice. Inhibition of PUMA by short hairpin RNAs suppressed p53-dependent apoptosis, promoted oncogenic transformation of primary murine fibroblasts by E1A/ras, and dramatically accelerated Eμ-myc-induced lymphomagenesis. Loss of PUMA enhanced intestinal tumorigenesis induced by carcinogens or the loss of the APC tumor suppressor. Unexpectedly, two independent studies demonstrated that PUMA-dependent apoptosis is required for radiation-induced lymphoma in mice, suggesting a complex role of apoptosis in cancer.

Given the strong association of apoptosis and proliferation in HCC development, and the specific death-promoting function of PUMA, we investigated the role of PUMA in hepatic carcinogenesis. We found that PUMA deficiency significantly decreased DEN-induced liver cancer by blocking the acute apoptotic responses and the subsequent compensatory proliferation. The JNK1/c-Jun pathway, but not p53, appears to be responsible for PUMA induction after DEN treatment. These data provide a novel molecular link between acute tissue injury and cancer development, and offer new avenues for HCC intervention.

Materials and Methods

Mice and Treatment. The procedures for all animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. The PUMA+/- and PUMA-/- littersmates on C57BL/6 background (F10) were generated from heterozygote intercrosses. The p53-/- mice (The Jackson Laboratory) were generated by way of heterozygote intercrosses. Genotyping was performed as described. The JNK1/-/- mice on C57BL/6 background (F6) were generated by way of heterozygote intercrosses and were genotyped as described. The mice were housed and measured with a caliper. For short-term studies of DEN-induced liver injury, 8- to 12-week-old mice were injected intraperitoneally with DEN (100 mg/kg body weight) and sacrificed after 1, 3, and 10 days. Mice receiving the JNK1 inhibitor, SP600125, were injected with 20 mg/kg intraperitoneally once daily starting 1 day before DEN treatment and continued until 3 days after DEN treatment. All mice were injected intraperitoneally with 100 mg/kg of bromodeoxyuridine (Brdu) 2 hours prior to sacrifice to label cells in S-phase.

Alanine Aminotransferase Measurement. Venous blood of mice was taken from the tail vein at 0, 24, and 72 hours of DEN treatment. Blood was kept at 4°C for overnight and centrifuged at 200 g for 20 minutes to isolate serum. Alanine aminotransferase (ALT) levels were measured using the Infinity ALT kit (Thermo Scientific, Middletown, VA) and are reported as the mean ± SD. Briefly, 10 μL serum was added into 100 μL ALT reagent and measured at 37°C at 340 nm. Each sample was measured in triplicate, and three mice were used in each group.

Western Blot Analysis. Total protein was prepared from freshly isolated liver tissue. Approximately 300 mg liver tissue was minced and homogenized in 1 mL homogenization buffer (0.25 M sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 1 mM ethylene glycol tetraacetic acid). Extracts were centrifuged at 1,000 × g for 10 minutes, and the supernatant was collected and analyzed by way of NuPage gel (Invitrogen) electrophoresis as described. Detailed information on antibodies can be found in the Supporting Information.

Total RNA Extraction and Real-Time Reverse-Transcription Polymerase Chain Reaction. Approximately 100 mg of fresh tissue was minced and put into 600 μL lysis buffer (Promega). Total RNA was isolated, and complementary DNA was then generated for real-time polymerase chain reaction (PCR) analysis as described. Real-time PCR was performed on a Mini Opticon Real-Time PCR system (Bio-Rad) with SYBR Green (Invitrogen) and primers specific for PUMA and β-actin. Melting curve and agarose gel electrophoresis of the PCR products were used to verify the specificity of PCR amplification.

Histological Analysis, Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate Nick-End Labeling, and BrdU Staining. Liver tissue was fixed in 10% formalin for 24 hours followed by processing. Sections (5 μm) from paraffin-embedded liver tissue were subjected to hematoxylin and eosin staining for histological analysis and various staining. Protocols for terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) and BrdU staining have been described. The apoptotic or BrdU index was scored in 900 hepatocytes/mouse and are reported as the mean ± SD. Three or more mice were used in each group.
Immunohistochemical and Immunofluorescent Staining. Slides were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide. Antigen retrieval was performed by boiling the sections for 10 minutes in 0.1 M Citrate Buffer Antigen Retrieval Solution (pH 6.0). Nonspecific antibody binding was blocked using 15% goat serum for 30 minutes. Detailed information and staining conditions can be found in the Supporting Information.24,25 Cells with positive staining were scored in at least 100 hepatocytes and are reported as the mean $\pm$ SD. Three or more mice were used in each group.

Statistical Analysis. Statistical analysis was performed using GraphPad Prism V software. Data are presented as the mean $\pm$ SD. Statistical significance was calculated using the Student t test. $P < 0.05$ was considered significant.

Results

PUMA Deficiency Attenuated DEN-Induced Liver Cancer. A single injection of DEN to 15-day-old male mice results in efficient HCC induction.26 To determine a potential role of PUMA in chemical hepatocarcinogenesis, we compared the tumor incidence and size in wild-type (WT) and PUMA knockout (KO) littermates 9 months after DEN treatment. All the mice developed tumors by 9 months (Fig. 1A). Surprisingly, the tumor incidence in PUMA KO mice decreased by approximately two-fold compared with WT mice (12.1 $\pm$ 5.4 versus 32.2 $\pm$ 7.8) (Fig. 1A,B). The overall tumor load was also significantly reduced in PUMA KO mice. For example, the relative liver weight versus body weight in PUMA KO mice was reduced by 1.5-fold compared with WT mice (2.1 $\pm$ 1.1 versus 5.0 $\pm$ 1.9%) (Fig. 1C). The maximal or average size of tumors significantly decreased in PUMA KO mice compared with those in WT mice (Fig. 1C and Supporting Fig. 1A). DEN induces formation of microfoci in the livers of WT mice within 4.5 months of treatment,23 which decreased significantly in PUMA KO mice (4.3 $\pm$ 2.1 versus 1.9 $\pm$ 1.6) (Fig. 1D and Supporting Fig. 1B). These results suggest that PUMA is required for efficient HCC induction in response to DEN.

DEN Induced PUMA Expression in the Liver. PUMA expression is low or undetectable in unstressed tissues, and is induced by DNA damage or nongenotoxic stimuli by p53-dependent or independent pathways.12 DEN is known to induce DNA damage.27 We therefore analyzed PUMA expression in the livers of WT mice after DEN treatment. Using quantitative reverse-transcription PCR, we found that PUMA messenger...
RNA was induced by 2.5-fold at 24 hours compared with untreated mice (Fig. 2A). PUMA protein expression was also significantly elevated at day 3 compared with untreated mice (Fig. 2B). Immunostaining indicated that PUMA protein was selectively induced in the hepatocytes around the centrilobular regions 24 hours after DEN treatment, whereas the basal level was undetectable by way of immunofluorescence (Fig. 2C). A few other BH3-only proteins, such as Bim, but not Bad, Noxa, or Bid, were also induced by DEN, whereas multi BH-domain containing Bcl-2 family members did not show a consistent change (Supporting Fig. 2).

**PUMA Deficiency Abrogated DEN-Induced Hepatocyte Apoptosis.** DEN induces extensive hepatocyte apoptosis in WT mice within 3 days (Fig. 3A,B),3,4 notably, in the centrilobular regions where PUMA is induced (Figs. 2C and 3B). The apoptosis was suppressed by over 40% in PUMA KO mice (Fig. 3A,B and Supporting Fig. 3). Apoptosis decreased at 10 days in WT mice, but was still higher than that seen in PUMA KO mice (Figs. 3A,B and Supporting Fig. 3). Active caspase-3 staining confirmed these results (Fig. 3C). DEN treatment increased the serum levels of the liver enzyme ALT at day 3, which were 40% less in PUMA KO mice compared with those in WT mice (Fig. 3D). Because DEN can induce oxidative DNA damage in the liver,3 we evaluated DNA double-strand breaks and oxidative DNA damage in the liver with p-H2AX and 8-oxoHdG staining,28 respectively. No difference in DNA damage was found between WT and PUMA KO mice 3 or 10 days after DEN treatment (Supporting Fig. 4A-C). Double strand breaks markedly decreased in both WT and PUMA KO livers at 10 days (Supporting Fig. 4A,B).

We then analyzed DNA double-strand breaks and apoptosis in HCCs. Very little staining of p-H2AX or apoptosis was found in the tumors or in adjacent
normal tissues (Supporting Fig. 4E,F). The levels of apoptosis and p-H2AX did not differ between WT and PUMA KO mice (Supporting Figs. 4E,F and 5). These results indicate that PUMA is a critical mediator of DEN-induced hepatocyte apoptosis and tissue damage, but does not affect basal apoptosis in hepatocytes or established HCCs.

**PUMA Deficiency Attenuated DEN-Induced Compensatory Proliferation.** DEN-induced hepatocyte death is associated with compensatory proliferation and HCC development.\(^3,4\) As expected, elevated proliferation was found in the livers of DEN-treated WT mice at 10 days by way of proliferating cell nuclear antigen (PCNA) and BrdU staining (Fig. 4A and Supporting Fig. 6A,B). The proliferation centered around the centrilobular regions where apoptotic cells were detected (Supporting Fig. 6C). The proliferation was significantly reduced in PUMA KO mice compared with WT mice, particularly on day 10 (Supporting Figs. 4A and 6). Staining with a mitosis marker phosphorylated histone 3 also

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**Fig. 3.** PUMA mediated DEN-induced apoptosis in the liver. Liver tissue or blood was harvested from WT and PUMA KO mice 0, 1, 3, or 10 days after a single 100-mg/kg injection of DEN. (A) Index of TUNEL-positive cells in the liver was measured by counting 900 cells/mouse. Values are means ± SD, n = 3 mice per group. (B) Examples of TUNEL immunohistochemical staining (brown) in the liver are indicated by red arrows (magnification ×400). (C) Index of active caspase-3-positive cells in the liver was measured by counting 900 cells/mouse (magnification ×400). Values represent the mean ± SD (n = 3 mice per group). The lower panels show examples of active caspase-3 immunofluorescent staining (red) in the liver with nuclei counterstained with DAPI (magnification ×400). (D) Serum ALT levels were determined at the indicated time points after DEN treatment. Values represent the mean ± SD (n = 3 mice per group).
showed reduced mitotic cells in DEN-treated PUMA KO mice compared with WT mice (Fig. 4B).

We then analyzed proliferation in the liver tumors in WT and PUMA KO mice 9 months after DEN treatment. The numbers of BrdU- and PCNA-positive cells were reduced by over 70% in PUMA-deficient tumors compared with WT tumors (Fig. 4C,D and Supporting Fig. 7A,B). However, the proliferation in normal hepatocytes adjacent to the tumors did not differ significantly between WT and PUMA KO mice (Supporting Fig. 7C,D). In addition, the proliferation in the foci was also reduced significantly after 4.5 months of DEN treatment in PUMA KO mice compared with WT mice, but not in the adjacent normal regions (Supporting Fig. 8). The numbers and sizes of foci were reduced in PUMA KO mice compared with WT mice (Fig. 1D). These results demonstrated that PUMA deficiency attenuates DEN-induced compensatory proliferation in

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Fig. 4. PUMA deficiency suppressed DEN-induced compensatory proliferation in hepatocytes and tumor proliferation. (A) Liver tissue was harvested from WT and PUMA KO mice 0, 3, and 10 days after injection of 100 mg/kg of DEN. PCNA index at the indicated time points was quantitated by counting 900 cells/mouse. Values represent the mean $\pm$ SD (n = 3 mice per group). The right panel shows representative photomicrographs of PCNA staining (red) with nuclei counterstained with DAPI (magnification $\times400$). (B) Phosphorylated histone 3 (P-H3) staining index in the liver at the indicated times after DEN treatment was quantitated by counting 900 cells/mouse. Values represent the mean $\pm$ SD (n = 3 mice per group). The right panel shows representative photomicrographs of P-H3 staining (green) with nuclei counterstained with DAPI (magnification $\times200$). (C) PCNA index in DEN-induced liver tumors 9 months after treatment was quantitated by counting 900 cells/mouse. Values represent the mean $\pm$ SD (n = 3 mice per group). The right panel shows representative photomicrographs with tumors circled (magnification $\times100$ [top] and $\times200$ [bottom]). (D) BrdU incorporation index in DEN-induced liver tumors 9 months after treatment was quantitated by counting 900 cells/mouse. Values represent the mean $\pm$ SD (n = 3 mice per group).
hepatocytes and proliferation in HCCs, but has little or no effect on basal proliferation of hepatocytes.

**Increased PUMA Expression and Apoptosis in DEN-Induced Liver Damage Is p53-Independent.** PUMA mediates p53-dependent apoptosis after DNA damage. In agreement with other reports, we found that DEN slightly induced p53 protein levels in WT mice (Supporting Fig. 2B). To determine whether p53 is required for the induction of PUMA by DEN, we compared PUMA expression and apoptosis in the livers of WT and p53 KO mice. PUMA was induced by DEN to similar levels in WT and p53-deficient mice at day 3 (Fig. 5A). TUNEL and PCNA staining revealed that p53 deficiency increases, rather than suppresses, DEN-induced hepatocyte death and proliferation (Fig. 5B,C and Supporting Fig. 9). These results indicate that DEN-induced PUMA expression and cell death in hepatocytes is p53 independent.

**JNK1/c-Jun Pathway Is Involved in DEN-Induced PUMA Induction and Apoptosis.** We further explored the mechanism of PUMA induction by DEN. JNK1 has been implicated in DEN-induced liver injury and HCC, and can regulate PUMA expression during fatty acid-induced hepatocyte apoptosis. We identified several clustered c-Jun/AP1 binding sites in the mouse and human PUMA promoters (Supporting Fig. 10). DEN induced JNK activation in the liver of WT mice as indicated by increased phosphorylated-JNK1 (p-JNK1) and phosphorylated-c-Jun (p-c-Jun) (Fig. 6A and Supporting Fig. 2). PUMA deficiency did not affect the activation of JNK1 (data not shown).

We then determined a potential role of JNK in DEN-induced PUMA expression, apoptosis, and proliferation. The JNK inhibitor SP600125 attenuated DEN-induced PUMA expression in the liver without affecting its basal levels (Fig. 6B and Supporting Fig. 11A). SP600125 also blocked DEN-induced hepatocyte apoptosis and compensatory proliferation by over 70% (Fig. 6B and Supporting Fig. 11C-F). Similarly, DEN-induced PUMA expression, hepatocyte apoptosis, and compensatory proliferation were suppressed by over 50% in JNK1 knockout mice (Fig. 6C,D and Supporting Fig. 12). Collectively, these data strongly suggest that the JNK1/PUMA axis contributes critically to DEN-induced hepatocyte apoptosis and subsequent proliferation.

**Discussion**

Our results indicate that JNK1-dependent PUMA induction mediates DEN-induced hepatocyte apoptosis, proliferation, and carcinogenesis. The acute, PUMA-mediated apoptotic response in hepatocytes is a direct cause of compensatory proliferation and ensuing carcinogenesis, underscoring the importance of proliferation as a strong tumor promoter in the liver. Overexpression of Bcl-2 or loss of BH3-only protein Bid suppressed DEN-induced liver cancer and hepatocyte proliferation in mice. Mice deficient in NF-κB...
signaling or the antiapoptotic Bcl-2 family member Mcl-1 developed spontaneous HCC secondary to hepatocyte death. These studies strongly argue that apoptosis drives a compensatory proliferation to facilitate carcinogenesis through expansion of mutant cells, consistent with early observations that carcinogens alone resulted in minimal tumor yield in the absence of hepatocyte proliferation. Such a conclusion is supported by mathematical modeling, in which a high rate of cell death correlated with a great number of cell divisions allows for clonal expansion of cells with more mutations and/or genomic instability. PUMA deficiency had little effect on DEN-induced acute DNA damage or repair. It would therefore be interesting to determine whether PUMA status modulates chromosomal aberrations in HCC.

A dual role of PUMA and apoptosis in cancer is somewhat surprising, but it is not without precedent. PUMA deficiency was recently reported to protect hematopoietic stem/progenitor cells against irradiation-induced cell death, and suppresses compensatory proliferation and lymphomagenesis, which is abrogated by dexamethasone-induced T cell killing. Several genes can either promote or suppress tumorigenesis depending on the context. For instance, inactivation of NF-κB suppressed colitis-induced colon cancer but enhanced the development of DEN-induced HCC. Overexpression of Bcl-2 suppressed liver cancer induced by DEN, transforming growth factor α, c-myc, or SV40-T-Ag, in contrast to its well-established oncogenic role in other systems. The model discussed above might be particularly relevant in understanding carcinogenesis in tissues with high regenerative capacity, such as the liver and hematopoietic system, where strong proliferative responses after acute injuries might favor the selection of cells with deleterious mutations.

Previous studies have established that either p53-dependent or -independent induction of PUMA mediates the apoptotic response to a wide range of...
stresses. JNK1 is important for hepatocyte proliferation under homeostasis and is required for DEN-induced HCC. Our studies uncover JNK1-dependent but not p53-dependent PUMA induction and apoptosis in DEN-induced HCC, perhaps independent of JNK1’s ability to regulate cell proliferation (this study). Additional JNK1- or apoptosis-dependent mechanisms might contribute to DEN-induced HCC. JNK1 promoted production of cytokines such as tumor necrosis factor α (TNF-α), interleukin-6, and hepatocyte growth factor and activation of Bim. Bim was induced by DEN treatment in the liver (Supporting Fig. 2), and regulated Fas-mediated liver damage and hepatitis induced by other agents. Mutations in p53, β-catenin, or H-ras have been reported in human or rodent HCCs but have not been found in DEN-induced HCCs in C57BL/6J WT or PUMA KO mice (Supporting Table 1). Future work is needed to understand and model how cell death and compensatory proliferation can cooperate to initiate and promote the progression of DEN-induced HCC by using specific signaling pathways and their significance in human HCC.

HCC is highly correlated with liver inflammation in human and various experimental animal models. DEN or obesity induces TNF-α production to promote liver tumorigenesis. Suppression of Myd88-dependent interleukin-6 production by estrogen explains the resistance of female mice to DEN-induced HCC. Mice deficient in NF-κB signaling were highly susceptible to HCC development and displayed sustained JNK activation and production of inflammatory cytokines, even in the absence of carcinogen. PUMA and apoptosis were induced by TNF-α or during inflammation through NF-κB. Interestingly, PUMA-deficient HCCs or liver foci showed suppressed proliferation long after the initial DEN insult, but showed little change in apoptosis. Therefore, it is possible that JNK1-mediated production of inflammatory cytokines promotes PUMA induction, apoptosis, and HCC development beyond the initial acute injury phase, providing additional mechanisms between chronic liver injury, inflammation, and cancer. This is consistent with higher levels of PUMA in liver tumors compared with adjacent normal tissues in HCC patients.

In conclusion, our study has demonstrated that PUMA-mediated apoptosis contributes to carcinogen-induced liver injury, compensatory proliferation, and cancer. Inhibition of apoptosis may offer an effective way to protect against hepatic injury and HCC development by reducing the pressure for clonal expansion. Emerging evidence suggests BH3-only proteins as potential therapeutic targets in injury-associated liver disease, including cancer. Recently developed PUMA inhibitors might be used to test this hypothesis.

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