Research Article

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p53 promotes inflammation-associated hepatocarcinogenesis by inducing HMGB1 release

He-Xin Yan[1,2,*], Hong-Ping Wu[1,2], Hui-Lu Zhang[1], Charles Ashton[2], Chang Tong[2], Han Wu[1], Qi-Jun Qian[1], Hong-Yang Wang[1], Qi-Long Ying[2,*]

[1]*Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200433, China;* [2]*Eli and Edythe Broad Center for Regenerative Medicine and Stem Cell Research at USC, Department of Cell and Neurobiology, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA*

Background & Aims: Hepatocellular carcinoma (HCC) develops in response to chronic hepatic injury. Although induced cell death is regarded as the major component of p53 tumor-suppressive activity, we recently found that sustained p53 activation subsequent to DNA damage promotes inflammation-associated hepatocarcinogenesis. Here we aim at exploring the mechanism linking p53 activation and hepatic inflammation during hepatocarcinogenesis.

Methods: $p53^{-/-}$ hepatocytes expressing inducible p53 and primary wild type hepatocytes were treated to induce p53 expression. The supernatants were collected and analyzed for the presence of released inflammatory cytokines. Ethyl pyruvate was used in a rat model of carcinogen-induced hepatocarcinogenesis to examine its effect on p53-dependent chronic hepatic injury, inflammation, and tumorigenesis.

Results: Here we show that cytoplasmic translocation and circulating levels of potent inflammatory molecule high-mobility group protein 1 (HMGB1) were greater in wild type rats than in $p53^{+/-}$ rats following carcinogen administration. Restoration of p53 expression in p53-null hepatocytes or induction of endogenous p53 in wild type hepatocytes gives rise to the release of HMGB1. Administration of the HMGB1 release inhibitor ethyl pyruvate, which does not affect p53-mediated hepatic apoptosis, substantially prevented carcinogen-induced cirrhosis and tumorigenesis in rat livers.

Conclusions: These results suggest that although p53 is usually regarded as a tumor suppressor, its constant activation can promote pro-tumorigenic inflammation, at least in part, via inducing HMGB1 release. Application of HMGB1 inhibitors when restoring p53 in cancer therapy might protect against pro-tumorigenic effects while leaving p53-mediated clearance of malignant cells intact.

Keywords: HCC; Inflammation; p53; HMGB1.

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* Corresponding authors. Tel.: +86 21 81875361; fax: +86 21 65566851 (H.-X. Yan), tel.: +1 323 442 3308; fax: +1 323 442 4040 (Q.-L. Ying).

E-mail addresses: hexiny@yahoo.com (H.-X. Yan), qying@usc.edu (Q.-L. Ying).

Abbreviations: HCC, hepatocellular carcinoma; HMGB1, high-mobility group protein 1; DEN, diethylnitrosamine.

Introduction

A strong association between chronic tissue injury and tumorigenesis has been established by many clinical data [1–3]. The mammalian liver is vulnerable to damage induced by toxic chemicals and hepatitis viruses, all of which increase the risk of hepatocellular carcinoma (HCC). Chronic hepatic inflammation, as a consequence of chronic injury, frequently precedes the development of HCC [4]. However, the molecular mechanisms responsible for chronic hepatic injury and inflammation remain elusive.

The tumor suppressor p53 is activated by a wide variety of stress signals that a cell might encounter during malignant progression—genotoxic damage, oncogene activation, and hypoxia. Induction of apoptosis or senescence is the key mechanism by which p53 eliminates cancer cells. p53 can also prevent cancer development through a number of other mechanisms. p53 has been shown to promote autophagy through negative regulation of mTOR signaling and act as an antioxidant to prevent DNA damage and genome instability [5,6]. When the liver is poisoned by genotoxic chemicals, p53 induces hepatocytes to commit suicide or go into growth arrest. It is therefore conceivable that p53 contributes to chronic tissue injury especially in livers exposed to agents that inflict mutagenic DNA damage. Consistent with this notion, we recently found that in a rat model of carcinogen-induced hepatocarcinogenesis, heterozygous deficiency of *p53* results in attenuated hepatic injury, inflammatory responses, and tumorigenesis [7]. However, it is still unknown how p53 links hepatocyte death to the activation of tissue inflammation.

High-mobility group box 1 (HMGB1) is a nuclear constituent loosely bound to chromatin that signals tissue damage when released into the extracellular medium, and thus acts as a damage-associated molecular pattern (DAMP) [8]. Extracellular HMGB1 is responsible for the inflammatory response to hepatic injury, as shown in models of acute liver toxicity and liver ischemia-reperfusion [9]. Here we show that sustained p53 activation causes chronic liver injury and gives rise to the release of

HMGB1, which may contribute to inflammation-associated hepatocarcinogenesis.

Materials and methods

Animals

Generation of rats lacking one or both alleles of *p53* by gene targeting of DAc8 rat ES cells has been described previously [10]. Rats that were *p53* heterozygous were intercrossed to produce rats with no, one, or two *p53*-null alleles. These rats were designated wild type, heterozygotes ($p53^{+/-}$) or homozygotes ($p53^{-/-}$), respectively, and were monitored for DEN-induced hepatocarcinogenesis.

HCC induction by DEN

HCCs in rats were chemically induced by weekly intraperitoneal (i.p.) administration of DEN (70 mg/kg body weight; Sigma-Aldrich) for 10 weeks as described previously [11,12]. At the indicated times, rats were sacrificed and the livers immediately removed, weighed, and placed in ice-cold phosphate-buffered saline (PBS). The externally visible tumors ($\geqslant$2 mm) were counted and measured by stereomicroscopy. Tumor size was measured using a vernier caliper. Parts of the livers were fixed in 4% paraformaldehyde and paraffin-embedded for histological evaluation. $p53^{-/-}$ rats were excluded from the study due to early spontaneous tumor development. $p53^{+/-}$ rats that developed hemangiosarcoma after DEN treatment were also excluded from analysis.

Animal treatment

For ethyl pyruvate (EP) treatment, pathogen-free male Sprague-Dawley (SD) rats (weighing 160–180 g) were grouped randomly and treated with saline or 40 mg/kg ethyl pyruvate (Sigma-Aldrich) i.p. every other day. The EP regimen started 1 week prior to DEN injection and continued until six weeks after the 10-week DEN treatment had ended. For *in vivo* adenoviral delivery of HMGB1, 200 µl of saline or 3×10^9 pfu of Ad5-CMV-GFP or Ad5-CMV-HMGB1 were administered via tail vein injection into male SD rats (5 animals per group). All procedures were performed according to investigator's protocols approved by the USC Institutional Animal Care and Use Committee (IACUC) or the Second Military Medical University Animal Ethics Committee.

Cell culture

Hepatic Kupffer cells and stellate cells were isolated according to the methods of Mello *et al.* [13]. Primary hepatocytes were isolated from 250–300 g WT, $p53^{+/-}$, and $p53^{-/-}$ (*p53*-null) rats by a two-step perfusion method. The animals were anesthetized by injecting sodium pentobarbital solution (0.1 ml/100 g of body wt) intraperitoneally and their livers were removed intact. The liver was first perfused *in vitro* via the portal vein with warmed (37 °C) Ca^{2+} and Mg^{2+} free Hanks balanced salt solution at a flow rate of 5–8 ml/min for 15 min, and then perfused with 0.05% collagenase (Sigma, Type IV) in the same solution supplemented with $CaCl_2$ to a final concentration of 5 mM and HEPES to a final concentration of 50 mM. The reperfusion with collagenase solution lasted 25 min at a rate of 5 ml/min at 37 °C. After 10 min of incubation (37 °C) with gentle shaking, the suspension was filtered and hepatocytes were sedimented at 70*g* for 1 min. All harvests yielded hepatocytes with viability exceeding 90% as indicated by trypan-blue dye exclusion. Isolated hepatocytes were seeded on rat tail collagen I-coated tissue culture dishes and cultured in DMEM/F12 with ITS (Sigma) plus growth factors EGF and HGF (20 ng/ml of each, Peprotech) as the standard medium. Propagation of the *p53*-null hepatocyte line was performed by passaging cells at a ratio of 1:3 twice per week in the standard medium.

Generation of ip53-hepatocytes

To achieve post-translational regulation of p53 in these cells, rat p53 was fused to an FK506- and rapamycin-binding protein (FKBP)-based destabilizing domain and a transgene for expressing this fusion protein was stably integrated into the *p53*-null hepatocyte cell line. Expression of this p53 fusion protein can be reversibly modulated by adding or removing the synthetic stabilizing ligand Shield1 (1:2000, Clontech).

ELISArray and ELISA assay

Amounts of inflammatory cytokines in serum of rat and supernatants of macrophages or hepatocytes were measured by a commercially available EILSA kit (Peprotech). To survey p53-induced pro-inflammatory cytokines, we treated the ip53-hepatocytes or primary WT hepatocytes with Shield1 (S1) or Nutlin-3 for 6 or 24 h. The conditioned media were filtered and qualitatively assayed for various cytokines using Rat Inflammatory Cytokines Multi-Analyte ELISArray Kit (Qiagen).

Detection of HMGB1 release and immunodepletion of HMGB1 from supernatants

For detection of secreted HMGB1 by Western blotting, serum or cell culture-conditioned medium was first filtered through a Centrifugal Filter Unit with Ultracel-50 membrane (Millipore) to clear the samples of cell debris and macromolecular complexes. Samples then were concentrated 15-fold with a Centrifugal Filter Unit with Ultracel-10 membrane and subjected to Western blotting. Amounts of HMGB1 in serum of rats were also measured by a commercially available EILSA kit (IBL international). HMGB1 depletion was performed in two rounds by incubating 0.5 ml of cell supernatant with 20 µg anti-HMGB1 for 16 h at 4 °C. Immune complexes were removed by incubation in the presence of 75 µl protein G sepharose for 1 h at 4 °C under agitation.

LDH (lactate dehydrogenase) release assay

Cell viability was measured by calculating the ratio of released LDH activity to total activity as described by the manufacturer (CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit, Promega).

Histological analysis

Immunohistochemistry staining was performed on formalin-fixed 5-µm paraffin-embedded tissue sections using UltraVision Quanto Detection System AP (Alkaline Phosphatase) or HRP. Quantification was done using Image J (National Institutes of Health, Bethesda, MD, USA) and Photoshop software (Adobe Corporation, San Jose, CA, USA) using at least 50 images per rat at 20× magnification for cytoplasmic HMGB1 staining.

Antibodies

The antibodies used and the dilution factors were as follows: anti-p53 (1C12) (Cell Signaling; 1:1000 for WB), anti-cleaved caspase-3 (5A1E) (Cell Signaling; 1:1000 for WB), anti-HMGB1 (ab18256) (Abcam; 1:1000 for WB, 1:500 for IHC). IHC, immunohistochemistry; WB, Western blot.

ROS detection

The generation of hydrogen peroxide and superoxide in cell culture was detected using CellROX™ Deep Red dye and MitoSOX™ Red mitochondrial superoxide indicator (Invitrogen), respectively, following the manufacturer's instructions.

Statistical analysis

All data are expressed as means with error bars representing the standard deviation. Statistical calculations were performed using Prism (Graphpad). For comparisons of two groups, two-tailed unpaired Student's *t*-tests were performed. For multiple group comparisons with normal distribution, ANOVA with Tukey's analysis was performed. A p value <0.05 was considered statistically significant.

Results

p53 deficiency does not affect the innate inflammatory response of non-parenchymal cells in the liver

We recently found that *p53* deficiency mitigates carcinogen-induced hepatic inflammation, cirrhosis, and tumorigenesis