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Abrogation of hepatocyte apoptosis and early appearance of liver dysplasia in ethanol-fed p53-deficient mice☆,☆☆

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Abstract

Ethanol consumption represents a major risk factor for cancer development, and a significant fraction of hepatocarcinomas arises in alcoholic liver cirrhosis. Increasing evidence indicates that ethanol acts as a tumor promoter on genetically initiated cells, by increasing the intracellular concentration of reactive oxygen species and promoting tissue necrosis/regeneration and cell proliferation. The tumor suppressor p53 restrains the expansion of carcinogen-initiated cells by inducing cell cycle arrest and apoptosis; accordingly, p53-deficient mice develop spontaneous and chemically induced neoplasms at a much higher frequency than normal mice. In normal mice exposed to a subacute (3 weeks) ethanol intoxication, a significant increase in the number of apoptotic hepatocytes was observed in concomitance with the up-regulation of the mitochondrial superoxide scavenger MnSOD, a reliable indicator of oxidative stress. Cell death occurred in the absence of liver inflammation and necrosis. Ethanol-induced hepatocyte apoptosis was completely abrogated in the p53 null background, suggesting that the tumor suppressor is necessary for hepatocyte death by ethanol. Accordingly, p53 −/− MEF were, unlike wild type cells, completely insensitive up to 0.5 M ethanol in the culture medium. Strikingly, marked and widespread signs of dysplasia, with nuclear pleomorphisms and initial loss of normal architecture, heralding malignant transformation, were scored in all the mutant mice exposed to ethanol, but not in the control-fed littermates nor in ethanol-fed normal mice. These observations suggest that p53-dependent apoptosis restrains the tumorigenic effect of ethanol on liver cells, in agreement with the frequent loss of p53 function in HCC, and reveal an unexpected carcinogenic potential of alcohol which appears to be independent from the induction of cirrhosis and hepatocyte regeneration.

Keywords: Ethanol; Apoptosis; Reactive oxygen species; MnSOD; p53; Hepatocarcinoma

Hepatocarcinoma accounts for about one-fourth of human malignancies, with significant differences in incidence among different areas of the world. Most hepatocarcinomas arise in cirrhotic/regenerating liver, and, accordingly, several hepatotoxic agents including hepatotropic viruses (HBV [1], HCV), micotoxins, and iron overload represent major risk factors for liver cancer [1].

Ethanol consumption is causally linked to several human neoplasms, including primitive liver cancer [2]. Alcohol metabolism in liver cells leads to the formation

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☆☆ *Abbreviations:* AP, alkaline phosphatase; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; HBV, Hepatitis B virus; HCV, Hepatitis C virus; MEF, mouse embryonic fibroblasts; OD, optical density; PI, propidium iodide; ROS, reactive oxygen species; TUNEL, Tdt-mediated d-UTP-x nick end labelling.
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of reactive oxygen species (ROS), which are responsible for hepatocyte damage, cellular apoptosis, and inflammation associated to excess alcohol consumption [3,4]. ROS are also involved in the tumor promoting effect of ethanol, in part through the induction of liver cirrhosis/regeneration, in part through a direct effect of oxygen species on cell proliferation and on DNA integrity [2].

The tumor suppressor protein p53 mediates the cytotoxic effect of many prooxidant agents on mammalian cells [5,6], and escape of oxidant-damaged cells from p53-dependent apoptosis can easily lead to cell initiation and eventually to cancer. A large fraction of human hepatocarcinomas (about 30%) harbors missense mutation in the gene encoding for the p53; moreover, two important co-factors for liver carcinogenesis, such as HBV and aflatoxins, reportedly target the gene or the function of p53 [1].

In spite of this indirect evidence linking ethanol to hepatocyte oxidative damage and to carcinogenesis, the role of p53 in alcohol-related liver damage and the potential synergism between ethanol consumption and loss of p53 for liver carcinogenesis have not been directly investigated so far.

Materials and methods

Mice and cell lines. p53-deficient C57Bl/6 mice (C57BL/6J-*Trp53*^{tm1Tyj}) and their wild type controls were obtained from the Jackson Laboratories (Bar Arbor, Maine). Mice were routinely maintained in the Animal facility of the Catholic University Medical School, Rome.

The E1A/Ras-transformed cell lines with or without homozygous deletion of the p53 gene were obtained from Dr. Scott Lowe (Cold Spring Harbor Laboratories) and have been described elsewhere [6].

Protocol for ethanol intoxication. Wild type (p53 +/+) and p53 null (p53 −/−) C57Bl6 mice (18–26 g initial body weight) were fed ad labium for 21 days a semi-synthetic basal diet used in a previous experiment [10] and were allowed to drink ad libitum a mixture containing increasing concentrations of ethanol (10%: 2 days; 15%: 3 days and thereafter 20%) in 20% sucrose as the only source for drinking fluid during the entire period of treatment. Control groups (wild type and p53 null) (19–27 g initial body weight) were offered water and their solid diets were based on the final regimens (basal diet plus ethanol–sucrose solution) consumed by the alcohol groups, but Et-OH was replaced isocalorically by a mixture of 50% sucrose–50% corn starch. These control animals were pair fed isocalorically with those corresponding alcohol groups. Animals in the alcohol groups were kept without Et-OH for 18 h before death, but were allowed free access to drinking water. Animals from all groups were starved for food overnight.

Liver pathology. After animal sacrifice livers were either fixed in paraformaldehyde or flash-frozen in liquid nitrogen and stored at −80 °C. Fixed specimens were included in paraffin, sectioned, and stained with hematoxylin/eosin for pathology.

In situ apoptosis assay. The percentage of apoptotic cells in liver sections was determined by Tdt-mediated d-UTP-x nick end labelling (TUNEL) (in situ cell death detection kit, AP from Boehringer–Mannheim), according to the manufacturer's recommendations. Data were analyzed by Student's *t* test (Microsoft Excel).

Assay for ethanol toxicity in vitro. p53 +/+ and p53 −/− oncogene-transformed mouse fibroblasts were exposed to 500 mM ethanol in standard growth medium (DMEM–10% FBS) for 48 h. Cell viability was determined by flow cytometry after sample staining with propidium iodide (PI).

Western blot analysis for MnSOD. Two micrograms of total protein lysates was subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto nitrocellulose membrane. After blocking in 3% non-fat milk membranes were immunoblotted with an anti-MnSOD rabbit antiserum (Upstate Biotechnology) and immunocomplexes were detected by enhanced chemiluminescence (ECL).

Results and discussion

Exposure of rodents to subacute or chronic ethanol intoxication has been reported to induce characteristic pathological changes in the liver, including steatosis, inflammation, and cellular apoptosis [7,8]. These changes are largely dependent on the generation of ROS and are prevented by antioxidants.

In order to investigate the role of p53 in ethanol-induced liver damage and carcinogenesis, we have exposed wild type and p53 null mice to 3 weeks (subacute) ethanol intoxication, and compared them with respect to liver pathology and hepatocyte apoptotic index. Both mouse strains displayed occasional steatosis with no correlation with the mouse phenotype, and absence of necrosis and regeneration, likely due to the short duration of the treatment (Fig. 1). However, hematoxylin/eosin liver sections of four out of four p53 null mice exposed to ethanol revealed marked and widespread hallmarks of dysplasia, with nuclear pleomorphism, increased nuclear and cellular size, abundant nucleoli, and initial disorganization of the trabecular architecture of the parenchyma (Figs. 1D and E). None of these changes was observed in wild type mice irrespective of the treatment, while only one out of five p53 null mice which had not been fed ethanol was scored positive for liver dysplasia. These observations suggest that, in spite of the absence of other alcohol-related pathological hallmarks, subacute ethanol intoxication induces/accelerates the appearance of histological changes reminiscent of initial stages of malignant transformation in the liver of tumor-prone mice lacking p53.

Since p53 prevents tumor formation mainly by inducing apoptosis of mutated or oncogene-deregulated cells [9], early appearance of alcohol-induced pre-neoplastic changes in p53 null mice may reflect a defect in p53-dependent apoptosis. TUNEL analysis of liver sections from wild type (p53 +/+) mice revealed a significant ($p < 0.05$) increase in the percentage of apoptotic hepatocytes following exposure to ethanol, although with some variability among the different animals (Fig. 2A). In contrast, no apoptosis was induced by ethanol in p53 null mice, in which also the basal level of cell positivity to TUNEL was much lower than in control mice (Fig. 2A, a).

These data clearly involve p53 in cellular apoptosis induced by hepatocyte exposure to alcohol. The role

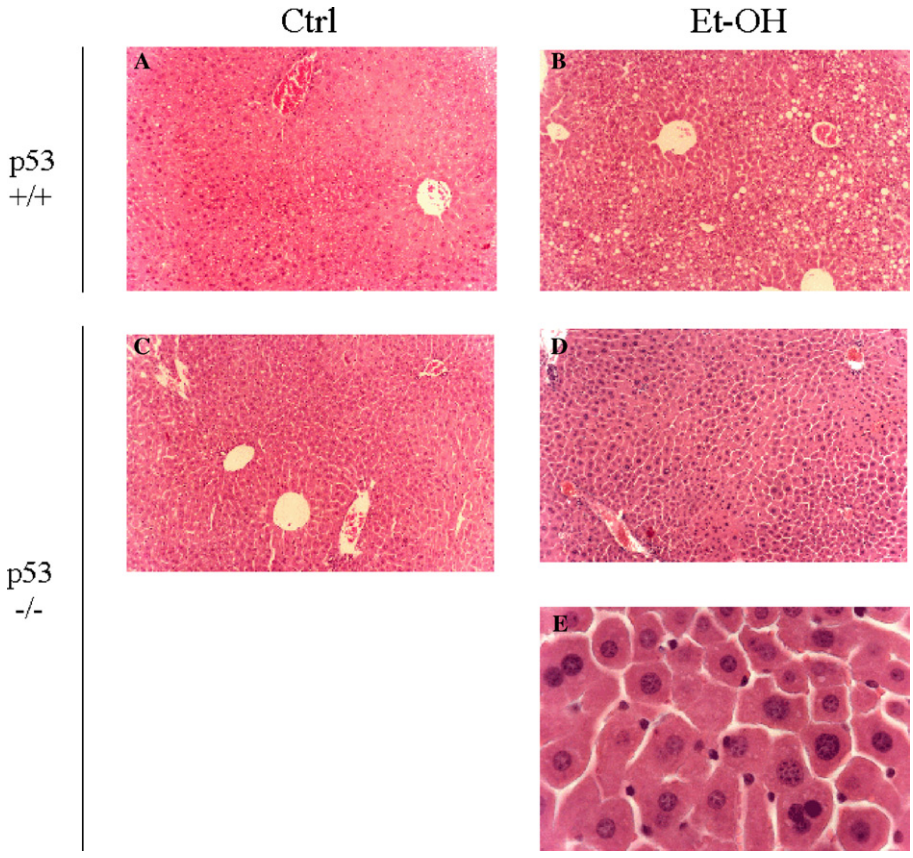

Fig. 1. Representative liver sections from control and ethanol-fed mice with and without p53. (A) Wild type control: the liver shows small droplets of fat in periportal areas. Nuclei are normal. Hematoxylin–eosin. 100×. (B) Wild type + ethanol: moderate fatty changes in periportal areas. Hematoxylin–eosin. 100×. (C) Normal aspect of the liver from an animal of p53 −/− without ethanol group. Hematoxylin–eosin. 100×. (D) Marked liver cell dysplasia from a p53 −/− ethanol-fed mouse. Hematoxylin–eosin. 100×. (E) High power view of nuclear dysplasia in a liver section from an ethanol-fed p53 −/− mouse. 600×.

of p53 in mediating the cellular toxicity of ethanol was also confirmed in experiments in vitro, in which mouse embryonic fibroblasts (MEF) derived from either wild type or p53-deficient mice were given ethanol (500 mM, equal to 2.5% v/v) in the culture medium. As shown in Fig. 2A, b, a significant percentage of dead (PI permeant) cells was scored after 48 h exposure to ethanol in the p53 proficient population, but not in fibroblast cultures from p53-deficient mice. This suggests that the involvement of p53 in cellular damage by ethanol is a general phenomenon and is not restricted to liver cells.

Cell up-regulation of the mitochondrial antioxidant enzyme MnSOD is a reliable indicator of oxidative stress [10]. In the liver of wild type mice exposed to ethanol, the amount of immunoreactive MnSOD was significantly increased, as revealed by Western blot analysis, in comparison to untreated animals. MnSOD induction conceivably represents a protective response against ethanol-induced ROS [11]. As previously reported [6], MnSOD levels are constitutively high in p53 null livers (Fig. 2B), which likely accounts for the resistance of mutant mice to apoptosis by alcohol. To this end, it should be noted that MnSOD is confined in mitochondria, where it prevents the oxidative triggering of apoptosis, but is unlikely to interfere with the mutagenic and growth-promoting effect of ROS generated in the cytosol [12].

Taken together, the above findings indicate that p53-deficient mice are less sensitive to the hepatotoxic and apoptogenic effect of a subacute ethanol intoxication, but significantly more prone to the tumor promoting effect of the same treatment. This notion is in line with the current view on the role of p53 in limiting the clonal expansion of DNA-damage and/or oncogene-transformed cells through the induction of programmed cell death [13].

It is possible that oxidants generated in response to ethanol, and here indirectly revealed by the cell up-regulation of MnSOD, induce DNA damage and cell initiation, thereby activating p53 and triggering p53-dependent cell death. In the absence of p53 these mutated cells would be allowed to expand, leading to the initial neoplastic changes observed in p53 −/− animals exposed to ethanol. Alternatively, oxidants generated by ethanol may release abnormal mitogenic signals by

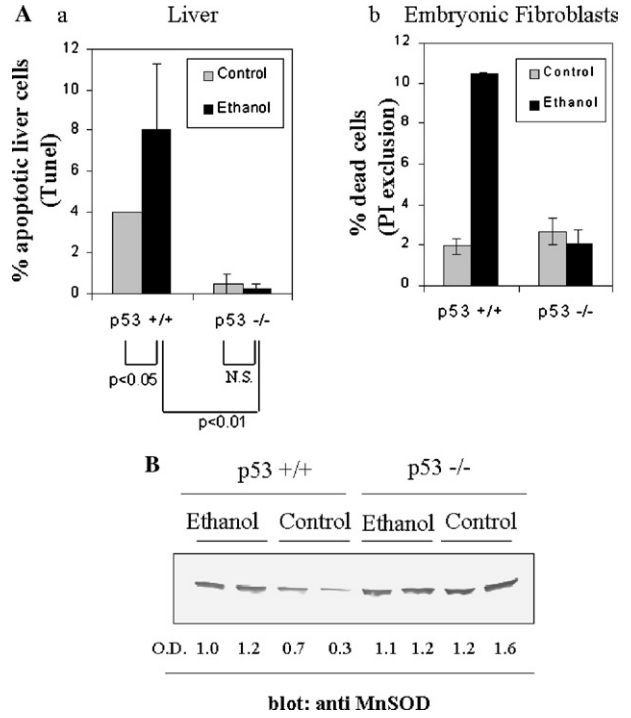

Fig. 2. (A) Essential role of p53 in ethanol-induced cell death. (a) TUNEL analysis of liver sections from control and ethanol-fed mice. Ethanol-induced apoptosis, observed in wild type mice, is abrogated in the absence of p53. Wild type control: $n = 4$ wild type ethanol: $n = 5$; p53 −/− control: $n = 2$; p53 −/− ethanol: $n = 4$. Data are means ± SD; p was calculated by a two-tailed Student's t test. (b) Ethanol toxicity on oncogene-transformed mouse fibroblasts in vitro. p53-deficient cells are resistant to the toxic effect of alcohol (500 mM, 2.5% v/v). Numbers are percentages of dead (i.e., PI-positive)cells. Values are means ± SD of duplicate samples. (B) Liver expression of MnSOD is induced by ethanol in wild type mice and is constitutive in p53-deficient animals. Equal amounts of liver homogenate proteins from two mice for each genotype were loaded on an SDS–PAGE gel. MnSOD content was determined by anti-MnSOD immunoblotting. Band densitometry was performed on digital scans with the Quantity 1 Biorad Software. Densitometry values (OD) are indicated. Equal protein loading throughout the gel was confirmed by reversible Ponceau S staining.

interfering with intracellular signal transduction cascades [14], in a fashion which normally activates p53-dependent apoptosis. Finally, since p53 mediates ethanol toxicity, alcohol abuse may also indirectly promote liver carcinogenesis by positively selecting pre-existing p53 mutated clones [15]. Notably, all these postulated pro-carcinogenic effects of ethanol are independent of the classical cirrhosis/regeneration paradigm, as it is consistent with our finding of early dysplasia in an otherwise normal tissue.

In conclusion, although further work will be required to establish whether and to which extent pre-malignant liver lesions observed in ethanol-fed p53 −/− mice will progress to full malignancy in time, our finding of defective apoptosis and early dysplasia in ethanol exposed p53 null livers establishes a novel and intriguing link between ethanol consumption, p53 and liver carcinogenesis, and may provide further biological explanation to the frequent finding of p53 mutations in liver cancer.

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