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Mechanism of the Pathogenesis of Hepatocellular Carcinoma during Chronic Administration of Ethanol in Mice

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Abstract

Background & Aims: Epidemiological evidence indicates that chronic intake of alcohol increases the risk of gastrointestinal and hepatic carcinogenesis. However, the incidence and the mechanism of pathogenesis of hepatocellular carcinoma (HCC) are not clear.

Methods: We administered ethanol through drinking water to male ICR mice for 70 weeks at concentrations of 5% (first week), 10% (next 8 weeks), and 15% thereafter. The control animals received equal amount of water. Half of the control and treated mice were sacrificed at 60 weeks and the remaining at 70 weeks. Histopathology and immuno-histochemistry were performed on liver sections.

Results: At 60th week, 40% of mice in the ethanol group had visible white nodules (5-10 mm) in the liver, while nodules were completely absent in control mice. At 70th week, several larger nodules (5-22 mm) were present in the livers of 50% mice in ethanol group. In control group, one mouse (10%) developed a single nodule. All nodules were histologically trabecular HCC composed of eosinophilic and vacuolated cells. In the livers of both control and ethanol group, several foci were present with cellular alteration. The incidence and size of the foci in ethanol group was significantly higher ($P < 0.01$) than those in the control group. Immunohistochemical staining for cytochrome P4502E1 (CYP2E1), 4-hydroxy-nonenal and c-Myc depicted dramatic upregulation of all these molecules in the foci of cellular alteration.

Conclusions: Our data demonstrate that chronic consumption of ethanol induces CYP2E1 and generates highly reactive oxygen species that produce mutations in c-Myc gene leading its persistent expression and pathogenesis of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and is always associated with a dismal prognosis. Even though there are several risk factors for the pathogenesis of HCC, chronic alcohol consumption is considered as one of the major risk factors for hepatocarcinogenesis in human. Despite the identification of major etiological agents, the molecular mechanisms leading to the development of HCC remains poorly understood and appear to be extremely complex. Ethanol is not considered as a carcinogen and the mechanism of ethanol-associated carcinogenesis is still obscure. It is well known that alcohol induces cytochrome P4502E1 (CYP2E1), an enzyme responsible for metabolism of large number of toxins, chemicals and pro-carcinogens. Conversion of pro-carcinogens into carcinogens occurs through the CYP2E1 mediated drug-metabolizing system present in the microsomes. This induction of CYP2E1 by ethanol could be partly responsible for the high incidence of gastrointestinal and hepatic cancer in chronic alcoholic individuals.

Although there are several studies based on the assumption of increased conversion of pro-carcinogens into carcinogens through microsomal CYP2E1, there is no experimental evidence to confirm the pathogenetic correlation between alcohol abuse and development of cancer. An insight of the underlying molecular mechanisms by which chronic alcohol consumption promotes carcinogenesis is important for development of appropriate strategies for prevention and/or treatment of alcohol-associated cancers. The aim of the present investigation was to study the role of ageing and consistent administration of ethanol in the incidence and pathogenesis of hepatocellular carcinoma in mice.

Experimental protocol

About 5 weeks old ICR mice (Institute for Cancer Research) were administered water containing 5% ethanol during the first week, 10% ethanol during the next 8 weeks, and 15% ethanol thereafter *ad libitum* for 70 weeks. ICR mice are known for susceptibility to induced colon cancer. A group of animals were sacrificed at weeks 60 and the remaining at weeks 70 after the start of the study. The livers were carefully removed, examined under a stereoscopic microscope and photographed. The liver tissue was cut into slices of 5 mm and examined under a stereoscope for the development of nodules and photographed. A portion of the liver was fixed in 10% phosphate-buffered formalin and processed for histopathology. The liver sections were stained with Hematoxylin and Eosin (H&E) and examined under a microscope. The foci of cellular alteration (FCA) was identified and photographed. Immunohistochemical staining was carried out for CYP2E1, 4-hydroxy-nonenal (4-HNE) and c-Myc on paraffin sections and the staining intensity was quantified using WinRoof image analyzing software.

Results

Table 1

Table 1. The incidence and size of hepatic tumors at 60 and 70 weeks during the study.

Groups	Incidence	Size and incidence of the tumor (mm)			
		< 5	> 5–10 <	> 10–15 <	> 15
Control (60 W)	0/10 (0%)	-	-	-	-
Ethanol (60 W)	4/10 (40%)**	0	4	0	0
Control (70 W)	1/10 (10%)	-	1	-	-
Ethanol (70 W)	5/10 (50%)**	0	3	1	1

** P < 0.01 compared to mean control values

Table 2

Table 2. Size and incidence of focus of cellular alteration in HCC at 60 and 70 weeks.

Groups	Incidence / hepatic lobule (n=10)	Diameter (μm)	Size (μm²)
Control (60 W)	1.5 ± 0.6	69.6 ± 20.1	3,800 ± 1,400
Ethanol (60 W)	2.3 ± 1.2*	83.8 ± 22.5*	5,500 ± 1,900*
Control (70 W)	1.8 ± 0.8	70.4 ± 23.3	3,900 ± 1,700
Ethanol (70 W)	2.7 ± 1.1**	88.8 ± 25.2**	6,200 ± 2,500**

* P < 0.05 and ** P < 0.01 compared to mean control values

Figure 1

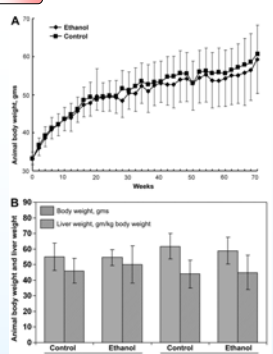

Figure 1. (A) Alteration of body weight in control and ethanol treated mice during the entire period of study. (B) Body weight and liver weight at 60 and 70 weeks of the study. Values are Mean ± S.D. (n=10).

Figure 2

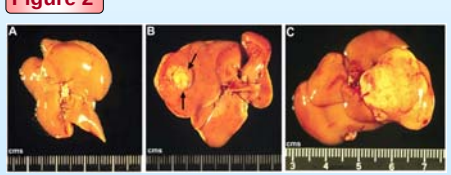

Figure 2. Stereoscopic images of the liver tissue of ethanol administered mice at weeks 60 and 70. (A) Control group (60 W) Hepatic tumor was absent. (B) Ethanol group (60 W) Hepatic tumor was observed. (C) Ethanol group (70 W) Liver tissue with a single large hepatic tumor measuring 22 mm.

Figure 3

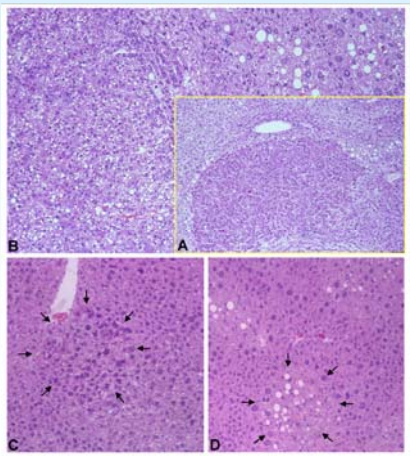

Figure 3. Hematoxylin and Eosin staining of mouse liver tissue after administration of ethanol for 70 weeks. (A) Conspicuous hepatic tumor depicting invasion results in compression of the surrounding tissue (x100). The hepatic nodules were histologically trabecular hepatocellular carcinoma. (B) Higher magnification of ethanol induced hepatic tumor (x400). The tissue was composed of eosinophilic and vacuolated cells. (C) Eosinophilic focus of cellular alternation (x400). (D) Clear cell focus of cellular alternation (x 400).

Figure 5

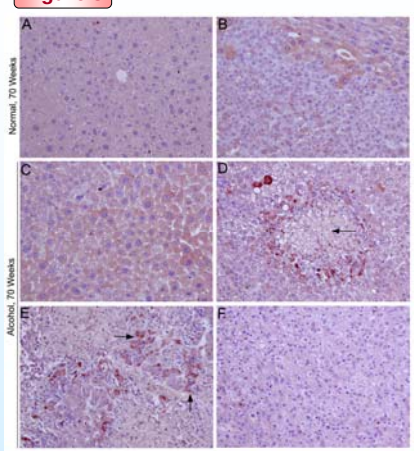

Figure 5. Immunohistochemical staining for 4-hydroxy-nonenal (4-HNE). (A) Control mice (x100). Absence of 4-HNE staining. (B) Control mice liver depicting well developed tumor and normal area (x100). Absence of 4-HNE staining in tumor area and moderate staining in normal area. (C) Alcohol treated mice showing normal liver (x100). Moderate staining of 4-HNE in pericentral hepatocytes. (D) Alcohol treated mice liver showing normal and precancer area (x100). Strong staining of 4-HNE in cells surrounding precancer zone indicating increased oxidative stress. (E) Alcohol treated mice liver with normal and tumor area depicting progressive carcinogenesis. Marked staining of 4-HNE in borderline cells indicating enhanced oxidative stress (x100). (F) Alcohol treated mice liver showing well developed hepatocellular carcinoma (x100). Complete absence of 4-HNE staining.

Figure 4

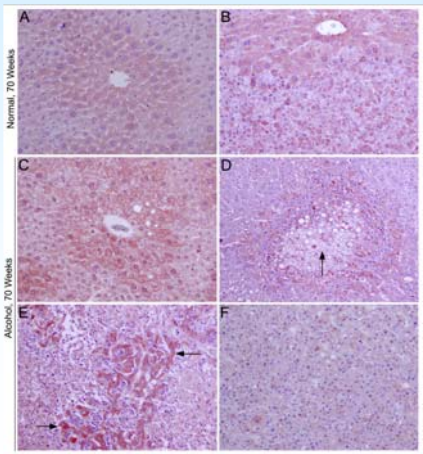

Figure 4. Immunohistochemical staining for cytochrome P4502E1 (CYP2E1). (A) Control mice showing normal liver (x100). Moderate staining of CYP2E1 in pericentral area. (B) Control mice liver depicting normal area and well developed hepatic tumor (x100). Moderate staining of CYP2E1 in normal pericentral area and feeble focal staining in tumor area. (C) Alcohol treated mice showing normal liver (x100). Strong staining of CYP2E1 in pericentral area. (D) Alcohol treated mice liver showing normal and precancer area (x100). Marked staining of CYP2E1 in cells surrounding precancer zone indicating dramatic upregulation. (E) Alcohol treated mice liver with normal and tumor area depicting progressive carcinogenesis. Conspicuous staining of CYP2E1 in borderline cells demonstrating marked upregulation (x100). (F) Alcohol treated mice liver showing well developed hepatocellular carcinoma (x100). Feeble staining of CYP2E1.

Figure 6

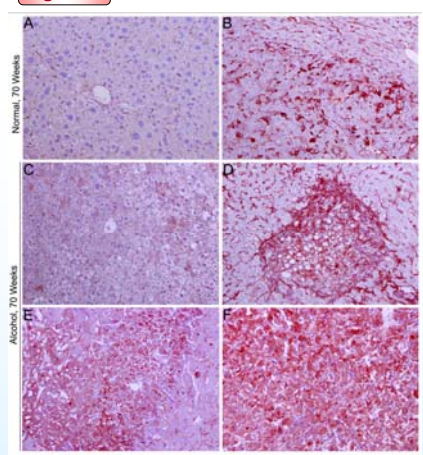

Figure 6. Immunohistochemical staining for c-Myc (A) Control mice showing normal liver (x200). Absence of c-Myc staining. (B) Control mice liver depicting normal area and well developed tumor (x100). Moderate staining of c-Myc in the normal area and strong staining in the tumor area. (C) Alcohol treated mice showing normal liver (x100). Focalized feeble staining for c-Myc. (D) Alcohol treated mice liver showing normal and precancer area (x100). Marked staining of c-Myc in the precancer zone indicating dramatic upregulation. (E) Alcohol treated mice liver depicting normal and tumor area demonstrating progressive carcinogenesis with intense staining of c-Myc in the borderline areas (x100). (F) Alcohol treated mice liver showing well developed hepatocellular carcinoma (x200). Conspicuous staining of c-Myc in the entire tumor area indicating remarkable upregulation.

Conclusions

- **Chronic administrations of ethanol produced visible white tumors in 50% of the mice at 70 weeks.**
- **One mouse (1/10) developed a single nodule among control mice received pure water without ethanol.**
- **Several foci cellular alteration were present in the livers of both control and ethanol group.**
- **Staining for CYP2E1, 4-hydroxy-nonenal and c-Myc depicted dramatic upregulation of all the molecules in the foci of cellular alteration.**
- **Chronic consumption of ethanol induces CYP2E1 and generates free radicals and highly reactive oxygen species that drives persistent expression of c-Myc gene.**
- **Dramatic upregulation of c-Myc drives unregulated expression of several oncogenes leading pathogenesis of HCC.**

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