Chronic ethanol intake promotes double gluthatione S-transferase/transforming growth factor-α-positive hepatocellular lesions in male Wistar rats

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(Received May 9, 2007/Revised August 21, 2007/Accepted October 9, 2007/Online publication February 4, 2008)

The chronic ethanol intake influence on the gluthatione S-transferase (GST-P) and transforming growth factor α (TGF-α) expression in remodeling/persistent preneoplastic lesions (PNLs) was evaluated in the resistant hepatocyte model. Male Wistar rats were allocated into five groups: G1, non-treated, fed water and chow *ad libitum*; G2, non-treated and pair-fed chow (restricted to match that of G3 group) and a maltodextrin (MD) solution in tap water (matched ethanol-derived calories); G3, fed 5% ethanol in drinking water and chow *ad libitum*; G4, diethylnitrosamine (DEN, 200 mg/kg, body weight) plus 200 parts per million of 2-acetylaminofluorene (2-AAF) for 3 weeks and pair-fed chow (restricted to match that of G5 group) and an MD solution in tap water (matched ethanol-derived calories); G5, DEN/2-AAF treatment, fed ethanol 5% and chow *ad libitum*. All animals were subjected to 70% partial hepatectomy at week 3 and sacrificed at weeks 12 or 22, respectively. Liver samples were collected for histological analysis or immunohistochemical expression of GST-P, TGF-α and proliferating cell nuclear antigen or zymography for matrix metalloproteinases-2 and -9. At the end of ethanol treatment, there was a significant increase in the percentage of liver area occupied by persistent GST-P-positive PNLs, the number of TGF-α-positive PNLs and the development of liver tumors in ethanol-fed and DEN/2-AAF-treated groups (G5 *versus* G4, $P < 0.001$). In addition, ethanol feeding led to a significant increase in cell proliferation mainly in remodeling and persistent PNLs with immunoreactivity for TGF-α at week 22 ($P < 0.001$). Gelatinase activities were not altered by ethanol treatment. The results demonstrated that ethanol enhances the selective growth of PNL with double expression of TGF-α and GST-P markers. (*Cancer Sci* 2008; 99: 221–228)

Ethanol consumption is a widespread habit, especially in the western world, with imperative familial and socioeconomic effects. Various studies have shown that chronic ethanol abuse increases the risk of developing cancer of the breast, liver, pancreas, colon and the upper aerial-digestive tract.[1–4] Alcohol is a known risk factor for the development of hepatocellular carcinoma (HCC), mainly in patients infected with the hepatitis C virus (HCV) who drink heavily.[1,3] However, a direct correlation between alcohol consumption and the development of HCC in humans remains inconclusive.

The hepatocyte represents the major site of ethanol metabolism using three main pathways, including alcohol dehydrogenase (ADH), inducible CYP2E1 and catalase enzymes, leading to the generation of acetaldehyde and reactive oxygen and nitrogen species.[5,6] These free radicals can bind rapidly to cell constituents, including DNA, lipids and proteins, raising mainly oxidative DNA damage, lipid peroxidation, protein impairment and depletion of many antioxidant systems, including reductions in glutathione, vitamin E and phosphatidylcholine.[6,7] In addition, acetaldehyde is highly toxic, mutagenic and carcinogenic[8,9] and the induction of CYP2E1 has been demonstrated to enhance the activation of many xenobiotics, among them diethylnitrosamine (DEN), a known carcinogen, genotoxic to the liver, present in many alcoholic beverages, tobacco smoke, and dieting compounds.[10]

Recently, some studies have demonstrated that ethanol and its metabolites, including acetaldehyde, can induce the production of matrix metalloproteinases (MMPs) or inhibit the tissue inhibitors of metalloproteinases (TIMPs) in the liver.[11–13] Ethanol and acetaldehyde directly stimulate the production of transforming growth factor beta-1 (TGFβ-1) and several extracellular matrix (ECM) constituents and also alter TIMPs/MMPs regulation by direct activation of hepatic stellate cells (HSCs), inducing liver fibrosis.[11,12] In fact, acetaldehyde is a fibrogenic agent to the liver inducing the expression of type-1 and type-3 collagen genes in HSCs.[12] Also, in fibrotic liver there is an increased expression of TIMPs, which play an important role in the regulation of MMPs activities, including the gelatinases 2 and 9.[11–13]

There are some controversies in rodent liver carcinogenesis assay concerning whether ethanol is a promoting agent[14–16] or exerts cocarcinogenic effects[17,18] or neither.[19–21] The exact mechanism(s) of ethanol-associated carcinogenesis remains obscure, since this abuse drug *per se* is not considered a carcinogen in experimental hepatocarcinogenesis studies.[1,22] The disagreements between these data are probably due to differences in experimental design: like different initiators used; moment of ethanol administration (before, during or after carcinogen exposure); preneoplasia and/or neoplasia end-points, and the rodent strain.[1]

The resistant hepatocyte (RH) model is a classic for the study of the different steps of rat hepatocarcinogenesis. In this model, DEN-initiating agent induces single cells and small foci of putative initiated hepatocytes that are promoted with 2-acetylaminofluorene (2-AAF) associated with a 70% partial hepatectomy (PH).[23] 2-AAF is a mito-inhibitor to normal hepatocytes but does not inhibit a subpopulation of glutathione S-transferase (GST-P)-positive hepatocytes. Partial hepatectomy acts as a mitotic stimulus resulting in rapid growth of this subpopulation of initiated cells into larger GST-positive lesions, while the surrounding liver parenchyma remains quiescent. Some lesions eventually progress into grossly visible nodules and, over months, tumors arise.[24,25]

Two phenomena occur following the promotional stimulus of the RH protocol: remodeling (or phenotypic reversion) of lesions and the proliferation and migration of oval cells. Hepatocytes within remodeling lesions lose GST-P expression and other enzymes common to preneoplastic liver lesions (PNL) and tend to revert to a normal liver phenotype.[24–26] Remodeling occurs in

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doi: 10.1111/j.1349-7006.2007.00677.x

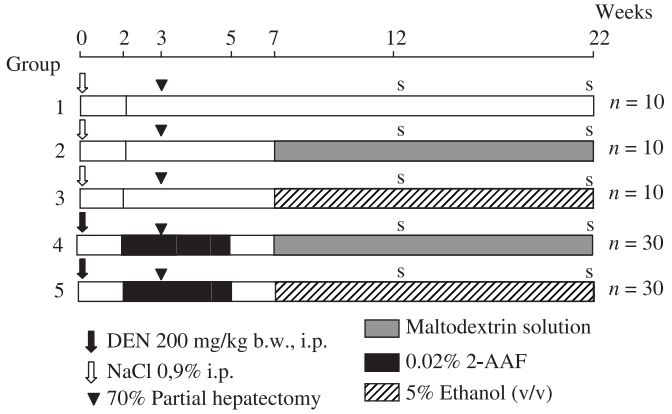

Fig. 1. Experimental protocol. b.w., body weight; *n*, number of rats; S, sacrifice.

most lesions following promotion and persistent PNL may progress to HCC after several months.[24,25] The potential effect of ethanol on the selective growth of putative preneoplastic hepatocellular lesions is not well established. Therefore, the present study was developed to investigate the differential promoting effects of ethanol on remodeling and persistent PNL expressing GST-P and transforming growth factor alpha (TGF-α) phenotypes. In addition, the selective growth of PNL was assessed by proliferating cell nuclear antigen (PCNA) and apoptosis rates. The activities of gelatinases (MMP-2 and MMP-9) were investigated by zymography.

Materials and Methods

Animals and treatments. Four-week-old male Wistar rats were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB, UNICAMP Campinas, SP, Brazil) and housed in polypropylene cages (five animals/cage) covered with metallic grids in a room maintained at 22 ± 2°C, 55 ± 10% humidity and with a 12-h light-dark cycle. They were fed commercial Purina chow (LABINA, Paul'nia, SP, Brazil) and water *ad libitum* for a 2-week acclimation period. Then, the animals were randomly divided into five experimental groups (Fig. 1). G1: non-treated, fed water and chow *ad libitum*; G2: non-treated and pair-fed chow (restricted to match that of the G3 group) and a maltodextrin (MD, Neo-Nutri, Poços de Caldas, MG, Brazil) solution in tap water (matched ethanol-derived calories); G3: fed 5% ethanol (v/v) (Dinâmica Qu'mica, São Paulo, SP, Brazil) in tap water and chow *ad libitum*; G4: diethylnitrosamine (DEN, Sigma-Aldrich, St. Louis MO, USA) (200 mg/kg body weight) plus 200 parts per million of 2-acetylaminofluorene (2AAF, Sigma-Aldrich) for 3 weeks[23] and pair-fed chow (restricted to match that of the G5 group) and an MD solution in tap water (matched ethanol-derived calories); G5: DEN/2-AAF treatments plus 5% ethanol in tap water and chow *ad libitum*. All animals were subjected to 70% partial hepatectomy at week 3 and killed at either week 12 or 22 (5 and 15 weeks after the beginning of ethanol administration, respectively). Food and liquid intakes were measured daily to calculate the volume of solid food and of isocaloric MD solution offered to the pair-fed groups. Body weight was measured twice a week throughout the experiment. The protocols used were consistent with the Ethical Principles for Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA).

Tissue processing, histology and immunohistochemical procedures. At necropsy, the liver was removed, weighed, and fixed in 10% phosphate-buffered formalin for 24 h. After fixation, representative samples of liver were processed for paraffin embedding. The paraffin blocks were cut into 5-μm-thick sections and stained with hematoxylin-eosin (HE) for histological analysis, apoptosis rates and for immunohistochemical analysis of GST-P, PCNA or TGF-α antigens in serially sectioned liver slides.

Hepatic preneoplastic lesions (PNL, Fig. 1d) were classified as altered foci of hepatocytes (AFH) or as hyperplastic nodules (HN) with clear/eosinophilic, amphophilic, basophilic, tigroid cell foci and mixed cell types, according to previously published criteria.[27] AFH were considered PNL smaller than one hepatic lobule, whereas HN comprised spherical PNL greater than or equal to one hepatic lobule, without visible compression of the surrounding liver. Liver tumors were classified as hepatocellular adenoma (HA) or hepatocellular carcinoma (HCC), according to the previously published criteria.[27]

Proliferating cell nuclear antigen, GST-P and TGF-α expression were immunohistochemically detected as previously described.[28] Briefly, deparaffinized 5-μm-thick serial liver sections on poly L-lysine coated slides were treated with 3% H_2O_2 in phosphate-buffered saline for 15 min, non-fat milk for 60 min, anti-PCNA (clone PC10, 1:200 dilution), anti-GST-P (Medical and Biological Laboratories, Tokyo, Japan, clone 311, 1:1000 dilution) clone or anti-TGF-α (Oncogene Science Inc., New York, NY, USA, clone Ab-2, 1:200 dilution) antibodies, biotinylated horse antimouse IgG or antigoat IgG antibodies (Dako Corporation, Carpinterie, CA, USA, 1:200 dilution) for 60 min, and streptavidin-biotin-peroxidase solution (TissuGnost ABC Kit, Merck, Darmstadt, Germany, 1:1:50 dilution). A pretreatment with 0.25% trypsin (Sigma-Aldrich) for 15 min and 0.05% saponin (Calbiochem, La Jolla, CA, USA) solution for 30 min was carried out for sections submitted to TGF-α immunostaining.[28] Chromogen color development was accomplished with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich) as the substrate to demonstrate the sites of peroxidase binding. The slides were counterstained with Harris's hematoxylin (GST-P and TGF-α analysis) or with HE (PCNA analysis).

Analysis of PNL expressing GST-P and TGF-a phenotypes. Preneoplastic lesions expressing uniform (distinct borders) and non-uniform (indistinct borders) staining for GST-P were classified as the persistent (Fig. 2a) and remodeling (Fig. 2b) phenotypes, respectively.[29] GST-P-positive PNLs larger than 0.03 mm^2 (~0.20 mm in diameter) were measured using a Nikon photomicroscope (Microphot-FXA, Tokyo, Japan) connected to a KS-300 apparatus (Kontron Elektronic, Munich, Germany). Data were expressed as number (PNL/cm^2), mean size (mm^2) and area (mm^2/cm^2) of persistent and remodeling or total (persistent + remodeling) GST-P-positive PNLs.

The number of clearly discernible TGF-α-positive PNLs (Fig. 2c) per unit area of liver (cm^2) was analyzed with the aid of a KS-300 system, as indicated above. The differential expression of TGF-α in persistent and remodeling PNL was analyzed. The TGF-α-positive PNLs were expressed as a percentage of persistent and remodeling or total (persistent + remodeling) GST-P-positive PNLs.

Cell proliferation and apoptosis analysis. Liver PCNA labeling (PCNA LI%) and apoptosis (AI%) indices for each animal from groups G1 to G3 were calculated by counting the number of immunohistochemically-labeled hepatocyte nuclei (S-Phase) per total number of counted hepatocytes (~1000) × 100. The PCNA LI% and AI% indices within persistent or remodeling PNL, expressing or not TGF-α phenotype, and in the surrounding (non-focal) liver were determined (~200–500 hepatocytes per PNL and ~2000 hepatocytes in non-focal liver, respectively). Criteria for identification and quantification of hepatocytes in apoptosis were taken from the published reports.[30]

Gelatin zymography for MMP-2 and MMP-9. Frozen samples of liver (~200 mg) were mechanically homogenized in 50 mM Tris buffer pH 7.5 plus 0.25% triton-X 100 with a Polytron for 30 s at 4°C, then centrifuged, and protein extracted on supernatant was quantified by the Bradford method.[31] Aliquots (30 μg protein) from liver extracts (nonaltered or with PNL) were subjected to

doi: 10.1111/j.1349-7006.2007.00677.x

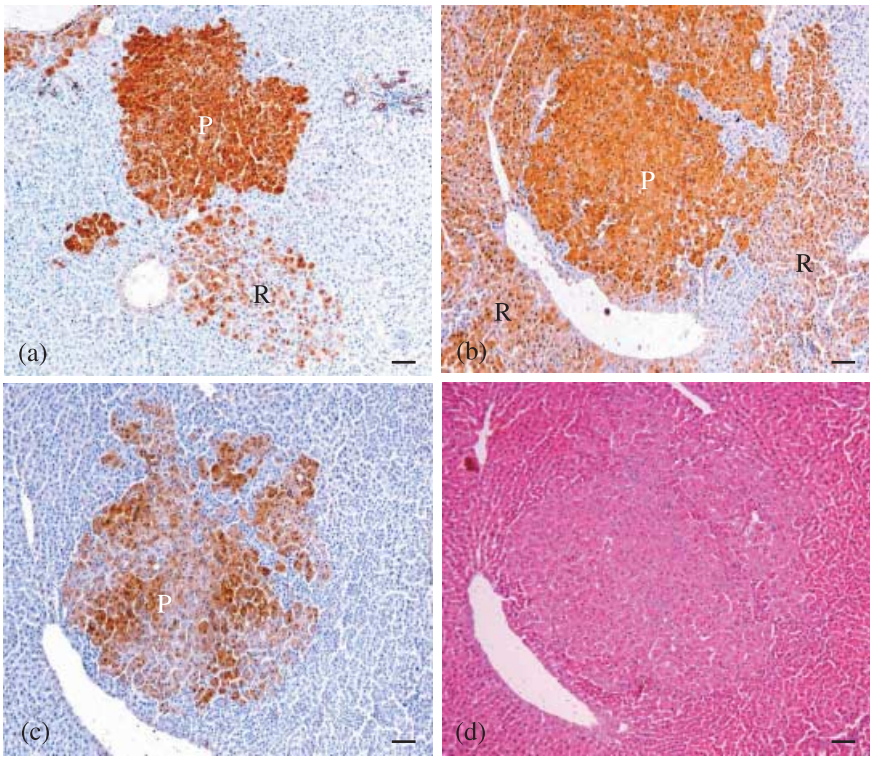

Fig. 2. (a) Remodeling (R) and persistent (P) gluthatione S-transferase (GST-P)-positive preneoplastic lesions (PNLs) (20× objective); (b–e) Serial liver section showing a persistent PNL with immunoexpression for GST-P (b) or transforming growth factor-α (TGF-α) (c) and hematoxylin-eosin (HE) stained (d). PCNA, proliferating cell nuclear antigen. Bar, 100 μm.

electrophoresis in gelatin containing polyacrylamide (8% acrylamide) gels in the presence of sodium dodecyl sulfate under non-reducing conditions. The gelatin substrate was present at 0.1% final concentration in the gel. The gels (0.75-mm thick) were electrophoresed for 2 h at 100 V, 4°C, in a Bio-Rad MiniProtean II system (Bio-Rad Laboratories, Richmond, CA, USA). Following electrophoresis, the gels were washed by gentle shaking at room temperature with 2.5% Triton X-100 (two changes) for 1 h. The gels were incubated overnight (18–20 h) in 50 mM Tris-HCl (pH 8.4) containing 5 mM CaCl2 and 1 μM $ZnCl_2$ at 37°C. Following incubation, the gels were stained by Coomassie Blue. Areas of proteolysis appeared as clear zones against a blue background. Molecular mass determinations were made with reference to prestained protein standards (Bio-Rad Laboratories) co-electrophoresed into the gels. Image gels were captured and the corresponding bands for each enzyme form were quantified by densitometry as Integrated Optical Density (IOD) in an Image Master VDS version 3.0 (Pharmacia Biotechnology, Piscataway, NJ, USA). The values were analyzed statistically and plotted in histograms.

Statistical analysis. Statistical analysis was made using the Jandel Sigma Stat Software (Jandel Corporation, San Rafael, CA, USA). Body weight, body-weight gain, absolute and relative liver weights, PCNA-labeling and apoptosis indices and lesion multiplicity were analyzed by Student's *t*-test or Mann–Whitney test. The incidence of different types of PNL and neoplastic lesions was examined using χ^2 or the Fischer exact tests. Data were considered statistically significant when $P < 0.05$.

Results

General findings and biochemical analysis of ALT and AST. A total of eight rats from DEN/2AAF-treated groups, six rats in DEN/2-AAF-treated group (G4) and two rats in ethanol-fed and DEN/2-AAF-treated group (G5), died during treatment with 2-AAF. After 70% partial hepatectomy, some severe clinical signs of toxicity such as reduced food intake and body-weight gain and rough hair coat were observed in the majority of 2-AAF-treated animals. A complete necropsy was not carried out in four rats due to advanced postmortem changes. The presumed cause of death was severe hepato-toxicity induced by 2-AAF.[32]

At the end of weeks 12 and 22, no differences in the final body weight and body-weight gain were observed between the experimental groups. The absolute and relative liver weights from the DEN/2-AAF-treated groups (G4 and G5) were slightly higher than the non-initiated/promoted groups (G1 to G3) but not significantly (data not shown). The average daily intake of ethanol was ~2.23–2.70 g/kg per day and did not differ between the ethanol-treated groups (G3 and G5). Values for alanine aminotransferase and aspartate aminotransferase were higher in the ethanol fed and DEN/2-AAF-treated group (G5) than in the other groups but without significance at the end of week 22 (data not shown).

Histological findings. Liver histological analysis did not show PNL or neoplastic lesions in the non-initiated/promoted groups (G1 to G3) at weeks 12 and 22 (data not shown). Similarly, lesions indicative of hepatic injury such as steatosis, fibrosis and inflammation were not observed in ethanol-fed animals (G3) (data not shown).

Table 1 summarizes data for the incidence of PNL and liver tumors such as hepatocellular adenomas and carcinomas in DEN/2-AAF-treated groups (G4 and G5). Treatment with 5% ethanol did not alter the incidence of the different types of PNL or neoplastic lesions at the end of weeks 12 and 22. In contrast, the mean number/group and multiplicity of liver tumors were significantly higher in the ethanol-fed and DEN/2-AAF-treated group (G5) when compared with the DEN/2-AAF-treated group (G4) at week 22 ($P = 0.001$, $P = 0.002$, respectively) (Table 1).

Analysis of GST-P and TGF-α-positive PNL. Data from GST-P analysis of the groups submitted to the resistant hepatocyte protocol is summarized in Table 2. The non-initiated/promoted groups (G1 to G3) did not develop any GST-P-positive PNLs above the established cut off value (0.20 mm in diameter) at the end of 12 and 22 weeks (data not shown). In week 12, the number and

Table 1. Incidences of liver preneoplastic lesions (PNLs) and neoplastic lesions in DEN/2-AAF treated groups

Treatment (Group)	Types of PNL[§]			Neoplastic lesions			
	CCF	ECF	BCF	HA	HCC	Number/group	Multiplicity[†]
12 weeks							
DEN + 2-AAF + MD (G4, *n* = 7)[‡]	6 (86%)	7 (100%)	6 (86%)	5 (71%)	0	1.56 ± 2.24	2.29 ± 2.21 (22)[‡]
DEN + 2-AAF + 5% EtOH (G5, *n* = 8)	6 (75%)	8 (100%)	5 (63%)	6 (75%)	0	3.14 ± 3.28	4.40 ± 2.73 (16)
22 weeks							
DEN + 2-AAF + MD (G4, *n* = 15)	7 (46%)	15 (100%)	15 (100%)	13 (87%)	0	1.87 ± 1.13	2.15 ± 0.90 (29)
DEN + 2-AAF + 5% EtOH (G5, *n* = 18)	8 (44%)	18 (100%)	18 (100%)	17 (94%)	1 (6%)	5.00 ± 3.24*	5.29 ± 3.08 (90)[§]
P-value						= 0.001	= 0.002

2-AAF, 200 parts per million of 2-acethylaminofluorene; 5% EtOH, 5% ethanol (v/v); BCF, basophilic cell PNL; CCF, clear cell PNL; DEN, 200 mg/kg of diethylnitrosamine; ECF, eosinophilic cell PNL; HA, hepatocellular adenoma; HCC, hepatocellular carcinoma; MD, maltodextrin solution; *n*, effective number of animals. [†]Number of tumors/tumor-bearing rats; [‡]Total number of tumors/group; [§]Significantly different from G4 group.

Table 2. Analysis of gluthatione S-transferase (GST-P)-positive preneoplastic lesions (PNLs) with remodeling and/or persistent phenotypes in DEN/2-AAF-treated groups[†]

Treatment (Group)	Number of GST-P-positive PNLs per unit area of liver (cm²)			Mean size of GST-P-positive PNLs (mm²)			Area occupied by GST-P-positive PNLs (%)		
	Total[‡]	*P*	R	Total	*P*	R	Total	*P*	R
12 weeks									
DEN + 2-AAF + MD (G4)	73.69 ± 15.48	26.40 ± 4.07	47.29 ± 13.23	0.62 ± 0.41	0.92 ± 0.48	0.57 ± 0.26	45.01 ± 26.01	22.12 ± 14.91	22.89 ± 12.64
DEN + 2-AAF + 5% EtOH (G5)	60.15 ± 5.74	22.06 ± 5.58	38.09 ± 2.14	0.79 ± 0.39	1.17 ± 0.16	0.57 ± 0.08	48.20 ± 8.04	26.47 ± 10.34	21.72 ± 3.11
22 weeks									
DEN + 2-AAF + MD (G4)	66.59 ± 14.67	25.55 ± 7.72	41.04 ± 8.77	0.72 ± 0.59	0.92 ± 0.38	0.62 ± 0.34	47.04 ± 14.35	21.45 ± 5.27	25.58 ± 9.94
DEN + 2-AAF + 5% EtOH (G5)	55.42 ± 18.91	28.52 ± 5.30	26.89 ± 5.31[§]	1.1 ± 0.81*	1.53 ± 0.39[§]	0.57 ± 0.36	62.59 ± 16.57[§]	41.11 ± 11.44[§]	21.49 ± 9.46
P-value			< 0.001	= 0.002	< 0.002		= 0.010	< 0.001	

[†]Values are means ± standard deviation. [§]Significantly different from the G4 group. [‡]Total = persistent + remodeling PNL. 2-AAF, 200 parts per million of 2-acethylaminofluorene; 5% EtOH, 5% ethanol (v/v); DEN, 200 mg/kg of diethylnitrosamine; MD, maltodextrin solution; *P*, persistent PNL; R, remodeling PNL.

Table 3. Analysis of development of preneoplastic lesions (PNLs) with double expression for transforming growth factor-α (TGF-α)/gluthatione S-transferase (GST-P) in DEN/2-AAF treated groups[†]

Treatment (Group)	Number of TGF-α-positive PNL per unit area of liver (cm²)			Relation between GST-P and TGF-α-positive staining (%)[§]		
	Total[‡]	*P*	R	GST-P + /TGF-α[+§]	*P*[¶]	R[¶]
12 weeks						
DEN + 2-AAF + MD (G4)	5.21 ± 2.26	4.94 ± 2.09	0.27 ± 0.32	9.21 ± 5.23	95.59 ± 5.34	4.41 ± 5.34
DEN + 2-AAF + 5% EtOH (G5)	4.69 ± 3.75	4.41 ± 3.53	0.27 ± 0.26	10.97 ± 8.71	94.16 ± 4.44	5.84 ± 4.44
22 weeks						
DEN + 2-AAF + MD (G4)	3.91 ± 1.41	3.66 ± 1.29	0.25 ± 0.21	9.23 ± 4.80	94.31 ± 4.82	5.69 ± 4.82
DEN +2-AAF + 5% EtOH (G5)	10.77 ± 6.78[††]	10.12 ± 5.91[††]	0.65 ± 0.90[‡‡]	19.97 ± 10.23[††]	94.86 ± 3.77	5.14 ± 3.77
P-value	< 0.001	< 0.001	= 0.095	= 0.008		

[†]Values are means ± SD. [‡]Total = persistent + remodeling PNL.[§]Percentage positive for both GST-P and TGF-α immunostaining. [¶]Percentage of remodeling or persistent PNL positive for TGF-α. [††]Significantly different from the G4 group. [‡‡]Trend toward difference from the G4 group. 2-AAF, 200 parts per million of 2-acethylaminofluorene; 5% EtOH, 5% ethanol (v/v); DEN, 200 mg/kg of diethylnitrosamine; MD, maltodextrin solution; *P*, persistent PNL; R, remodeling PNL.

percentage of liver area occupied by total (persistent + remodeling), persistent or remodeling GST-P-positive PNLs were not influenced by treatment with 5% ethanol. The number of remodeling PNLs was higher than the persistent PNLs but the mean size of persistent PNLs was approximately 1.6–2.0 times larger than those of remodeling PNLs, resulting in similar values of the percentage liver area occupied by these PNLs for both G4 and G5 groups. However at week 22, the number of remodeling PNLs per unit area of liver was significantly lower in the ethanol-fed and DEN/2-AAF-treated group (G5) when compared with the DEN/2-AAF-treated group (G4) ($P < 0.001$). The mean size and percentage of liver area occupied by total (persistent + remodeling) and persistent GST-P-positive PNLs were significantly higher in the ethanol-fed and DEN/2-AAF-treated group (G5) when compared with the DEN/2-AAF-treated group (G4) ($P = 0.002$, $P < 0.002$ and $P < 0.001$, respectively). The mean size of persistent PNLs was approximately 2.7 times larger than remodeling PNLs, resulting in significantly higher values of percentage liver area occupied by this latter PNL when compared with the G4 group. Also, in this group persistent PNLs were approximately 1.5 larger than those of remodeling PNLs.

Table 3 summarizes data from double-positive foci for TGF-α and GST-P markers in the groups submitted to the resistant hepatocyte protocol. The immunohistochemical study of hepatocellular

doi: 10.1111/j.1349-7006.2007.00677.x

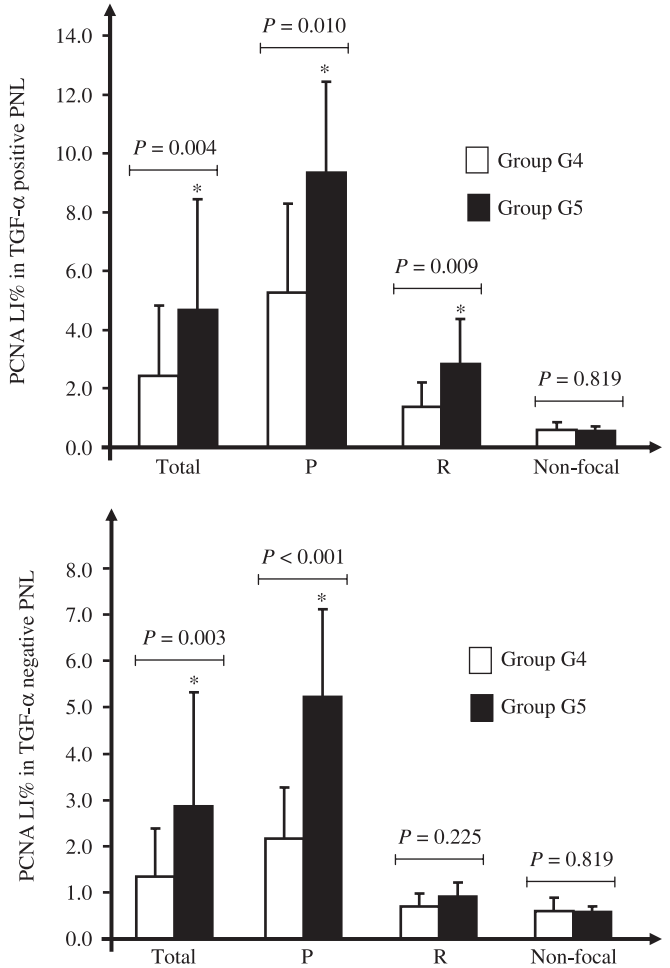

Fig. 3. Effects of ethanol at 5% on proliferating cell nuclear antigen (PCNA) LI% in total, persistent and remodeling preneoplastic lesions (PNLs) expressing or not expressing transforming growth factor-α (TGF-α) and in the surrounding liver at 22 week. Total (persistent plus remodeling PNL). *P*, remodeling PNL; R, remodeling PNL. *Significantly different from the G4 group.

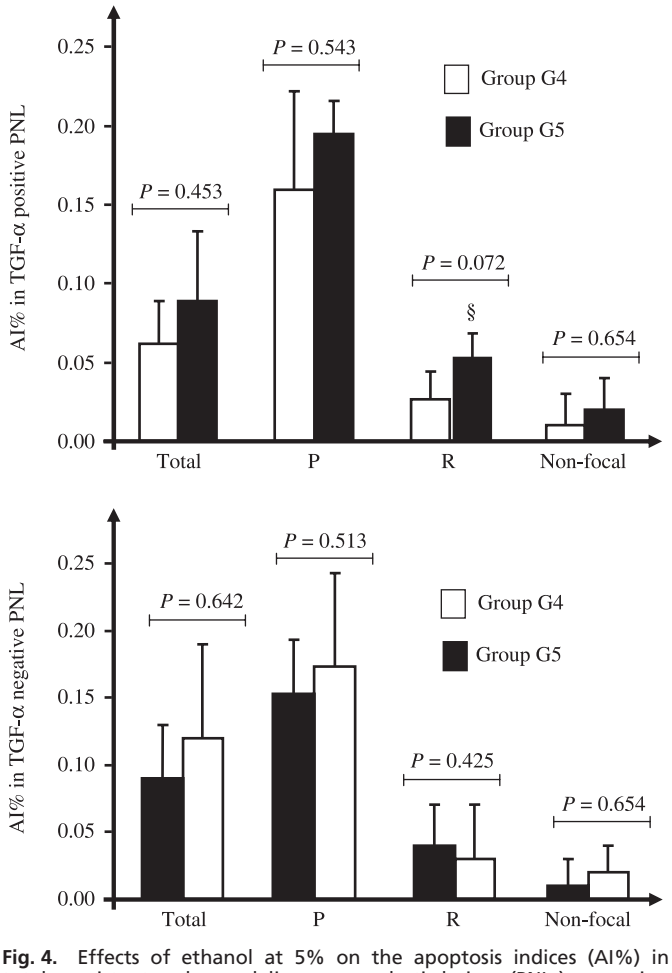

Fig. 4. Effects of ethanol at 5% on the apoptosis indices (AI%) in total, persistent and remodeling preneoplastic lesions (PNLs) expressing or not expressing transforming growth factor-α (TGF-α) and in the surrounding liver at 22 week. Total (persistent plus remodeling PNL). *P*, remodeling PNL; R, remodeling PNL. §Trend toward difference from the G4 group.

lesions revealed that the immunoreactivity for TGF-α was homogenous and intense in HA and HCC and often heterogeneous in most PNLs and was more frequently observed in persistent rather than in remodeling GST-P-positive PNLs (Fig. 1b,c) in both DEN/2-AAF-treated groups (G4 and G5). At week 22, a significant increase in the number of PNLs (per unit area of liver [cm^2]) with expression of TGF-α, and in the percentage of PNL with double expression for TGF-α/GST-P were observed in the ethanol-fed and DEN/2-AAF-treated group (G5) when compared with the DEN/2-AAF-treated group (G4) ($P < 0.001$ and $P < 0.008$, respectively). Besides the clear promoting effects of ethanol feeding on total (persistent and remodeling) and in persistent GST-P/TGF-α phenotypes, a trend toward an increase in the number of remodeling PNL/TGF-α-positive was also observed in the ethanol-fed and DEN/2-AAF-treated group (G5) although not at a statistically significant level.

PCNA labeling and apoptosis analysis in PNL. The effects of ethanol on cell proliferation and apoptosis rates in remodeling and persistent PNLs are shown in Figs 3 and 4, respectively. In general, PCNA LI% and AI% indices were high in persistent PNLs and a very low percentage of apoptosis was observed in remodeling and surrounding liver. In week 12, ethanol feeding (G5) did not change the PCNA LI% within either the persistent and remodeling PNLs or in the surrounding non-focal liver when compared with the DEN/2-AAF-treated group (G4) (data not shown). On the other hand, at week 22, a significant increase in PCNA LI% was observed mainly in persistent and remodeling PNLs expressing TGF-α phenotype in the ethanol-fed and DEN/2-AAF-treated group (G5) when compared with the DEN/2-AAF-treated group (G4) ($P = 0.010$ and $P = 0.009$, respectively). Ethanol at 5% did not change the apoptosis rates within the remodeling and persistent PNLs in both TGF-α-positive and TGF-α-negative PNLs at week 12 (data not shown). A slight not significant increase in AI% was observed in remodeling PNLs expressing TGF-α phenotype in the ethanol-fed and DEN/2-AAF-treated group (G5) at week 22.

In the non-initiated/promoted groups (G1 to G3), neither ethanol nor maltodextrin intake changed cell proliferation or apoptosis rates in the liver (data not shown).

MMP-2 and -9 activities. The activity of MMP-2 and -9 was measured by gelatin zymography at weeks 12 (not shown) and 22 (Fig. 5a). The zymography of all experimental groups showed clear bands of gelatinolytic activity with 92, 72, 64 and 59 kDa, which corresponded to active-MMP-9, pro-MMP-2, intermediate-MMP-2 and active-MMP-2, respectively. Analysis of the integrated optic density (IOD) of the bands revealed that liver gelatinase activities were not significantly altered either by ethanol treatment or by exposure to the chemical carcinogens (DEN/2-AAF) in

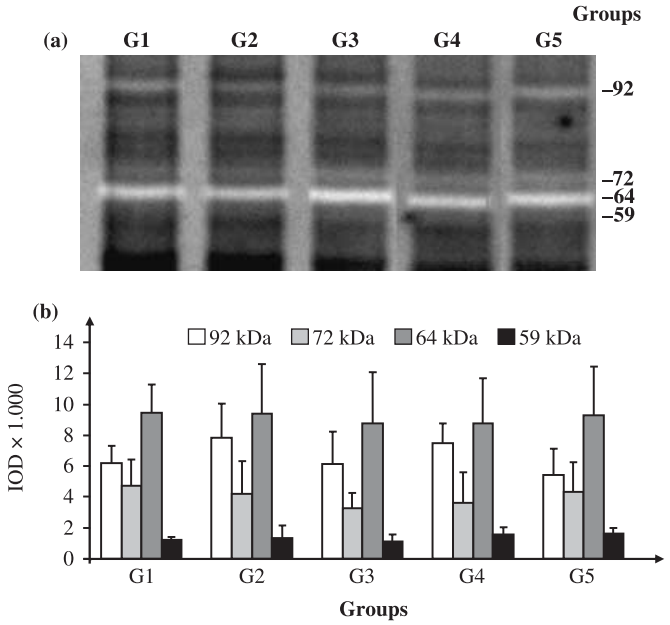

Fig. 5. Gelatin zymography analysis of matrix metalloproteinase-2 (MMP-2) and MMP-9 activities in the liver at week 22. (a) Clear bands with 92, 72, 64 and 59 kDa correspond to pro-MMP-9, pro-MMP-2, intermediate-MMP-2 and active-MMP2, respectively. (b) Densitometric analysis of gelatinolytic bands. Data are expressed as means ± standard deviation (SD).

both non-initiated/promoted and DEN/2-AAF-treated groups (Fig. 5b).

Discussion

The present study showed that chronic 5% ethanol administration promotes chemically-induced rat hepatocarcinogenesis in the resistant hepatocyte model. The tumor-promoting potential of ethanol involved mainly the expansion of double GST-P/TGF-α-positive PNL with enhancement of cell proliferation, without alterations in the apoptosis rates or gelatinase activities. Also, ethanol increased tumor burden by enhancing the number and multiplicity of neoplastic lesions in the liver of the DEN/2-AAF-treated animals. Kushida *et al*. (2005) have determined a threshold level for the promoting effect of ethanol on rat hepatocarcinogenesis.[16] These authors observed that ethanol in concentrations lower than 3% has a weak or even nonexistent effect on the promotion of preneoplastic GST-P-positive liver foci. In the present study, a 5% ethanol concentration was used, and our findings are in agreement with the previously proposed threshold for ethanol on the promotion of rat hepatocarcinogenesis.[16]

The remodeling, or phenotypic reversion is a process where the initiated hepatocyte gradually loses the expression of some preneoplastic markers, such as GST-P and γ-GGT, and returns to a normal phenotype.[24–26,33] This process occurs naturally in the early stages of the multistep process of chemically induced hepatocarcinogenesis and is under genetic control.[34] GST-P is a suitable marker for putative preneoplastic foci of altered hepatocytes and hepatic nodules (PNLs) in chemically-induced rat hepatocarcinogenesis.[24–26] A typical feature of PNLs is their capability of expressing one of two options: spontaneously remodeling to a normal appearing liver by the majority (95–98%) or persistence with cell proliferation and evolution to cancer by a small minority (2–5%).[25,26] Persistence of PNLs could indicate a block in remodeling by loss of differentiation or apoptosis and appears to be linked to enhanced evolution of hepatocellular carcinoma.[25,26] The present study demonstrated that chronic intake of 5% ethanol increased the mean size and the percentage of liver area occupied by total (persistent and remodeling) and persistent GST-P-positive PNLs, with reduction in the number of remodeling GST-P-positive PNLs. Taken together, these data show that ethanol has a selective effect on the expansion of persistent PNLs. This is similar to the other tumor promoters of hepatocarcinogenesis like phenobarbital and nafenopin,[30] which act by inhibiting or delaying the normal remodeling process and by enhancing cell growth and size of the persistent lesions, which are thought to be precursors of HCC.

Various alterations in the expression of proto-oncogenes occur during the process of hepatocarcinogenesis, including an increase in the expression of TGF-α.[35–38] This growth factor activates the tyrosine kinase activity of the epidermal growth factor receptor, and its overexpression has been linked to tumorigenesis in transgenic mice, which were found to present a high incidence of spontaneous malignant liver tumors.[39,40] Immunohistochemical expression of TGF-α has been demonstrated in rodent hepatic preneoplastic and neoplastic lesions[35–38] although a few GST-P-positive PNLs (~10%) exhibit immunoreactivity for TGF-α.[38] It has been shown that PNLs with double positivity for GST-P and TGF-α show higher levels of cell proliferation than TGF-α-negative PNLs and this could represent a potential lesion for progression to liver tumors.[37,38] The selective increase of TGF-α-positive PNLs induced by ethanol could indicate a promoting effect, since this growth factor may act as a potent hepatocyte mitogen in the progression of liver carcinogenesis.[37,38] Also, high concentrations of TGF-α were found in an *in vitro* assay using HepG2 cells exposed to ethanol, suggesting that this growth factor may play an important role in the liver response to this abuse drug.[41] In the present study we have found that chronic ethanol intake enhanced the number of TGF-α-positive PNLs per unit area of liver, and this enhancement mainly occurred in the persistent PNLs, suggesting that these lesions may have a growth advantage over the remodeling PNLs. Interestingly, almost 95% of the total TGF-α-positive PNLs exhibited a persistent phenotype, which corroborates the hypothesis that this growth factor may be a key peptide for tumor progression in experimental hepatocarcinogenesis.[34,37,38]

The increase in cell proliferation and apoptosis rates in preneoplastic and neoplastic lesions during the multisteps of liver carcinogenesis have been described.[42,43,45] One of the mechanisms proposed for the promoting effect of ethanol on rat hepatocarcinogenesis is by enhancement of cell proliferation observed during or after interruption of long-term alcohol exposure.[14,15,44] In the present study, the mean rates of cell proliferation were significantly higher in TGF-α-positive than in TGF-α-negative PNLs. Ethanol feeding at 5% resulted in clear promoting activity as observed by the increased percentage of the liver occupied by PNLs and mean PNL size. The cell proliferation increase in the remodeling PNLs with immunoexpression for TGF-α could be related to a trend toward the persistent phenotype, thus reducing the number of this type of hepatocellular lesion.

Ethanol feeding did not change the apoptosis rates within persistent or remodeling PNLs in both TGF-α-positive and -negative phenotypes at 12 and 22 weeks. Thus, apoptosis may not play an important role in the promoting effects of ethanol in the preneoplasia stage of rat hepatocarcinogenesis. In fact, previous studies have shown that PNL growth during the initial stages of rat hepatocarcinogenesis is not consistent with the concept of apoptosis failure.[42,43,45]

Matrix metalloproteinases are a family of proteases involved in many physiological and pathological processes[45] such as angiogenesis, tumor invasion and metastasis. MMP-2 and -9 are produced in the liver by activated HSCs[12,13] in response to extracellular matrix changes induced by many agents, including ethanol.[13] Ethanol itself is a stimulating agent for the release of activated gelatinases in cultured fibroblasts and breast cancer

doi: 10.1111/j.1349-7006.2007.00677.x

cells.[47,48] In the present study, ethanol feeding did not change the activity of these proteases in either the non-initiated/promoted or DEN/2-AAF groups. This is in agreement with the histological findings of the absence of liver inflammation, necrosis and fibrosis in the groups exposed to ethanol.

Some studies demonstrated that ethanol exerts a promoting activity on rat hepatocarcinogenesis.[14–16] However, few investigations have examined this effect at low ethanol doses and little attention has been given to the differential effects of ethanol on PNL phenotypic diversities. The results of the present study show that chronic feeding of ethanol at a low dose promotes a selective growth of remodeling and persistent PNLs with double GST-P/TGF-α markers. Thus, the expression of TGF-α may be a pathway for the promoting activity of ethanol toward rat hepatocarcinogenesis. Further studies on the role of ethanol in the mechanisms of hepatocarcinogenesis should continue to be carried out.

Acknowledgments

This study was supported by CAPES, FAPESP and TOXICAM. P.W. Pires was a recipient of a fellowship from CAPES.

References

1 Pöschl G, Seitz HK. Alcohol and cancer. *Alcohol Alcohol* 2004; **39**: 155–65.
2 Bofetta P, Hashibe M. Alcohol and cancer. *Lancet Oncol* 2006; **7**: 149–56.
3 Brown LM. Epidemiology of alcohol-associated cancers. *Alcohol* 2005; **35**: 161–8.
4 Voight MD. Alcohol in hepatocellular cancer. *Clin Liver Dis* 2005; **9**: 151–69.
5 Vidal F, Toda R, Gutiérrez C *et al*. Influence of chronic alcohol abuse and liver disease on hepatic aldehyde dehydrogenase activity. *Alcohol* 1998; **15**: 3–8.
6 Lieber CS, Abittan CS. Pharmacology and metabolism of alcohol, including its metabolics effects and interactions with other drugs. *Clin Dermatol* 1999; **17**: 365–79.
7 Bunout D. Nutritional and metabolic effects of alcoholism: their relationship with alcoholic liver disease. *Nutrition* 1999; **15**: 583–9.
8 Niemellä O. Distribution of ethanol-induced protein adducts in vivo: relationship to tissue injury. *Free Rad Biol Med* 2001; **31**: 1533–8.
9 Brooks TJ, Theruvathu JA. DNA adducts from acetaldehyde: implications for alcohol-related carcinogenesis. *Alcohol* 2005; **35**: 187–93.
10 Verna L, Whysner J, Williams GM. N-Nitrosodiethylamine mechanistic data and risk assessment: bioactivation, DNA-Adduct formation, mutagenicity and tumor initiation. *Pharmacol Ther* 1996; **71**: 57–81.
11 Friedman SL. Mechanisms of disease: mechanisms of hepatic fibrosis and therapeutic implication. *Nat Clin Pract Gastroenterol Hepatol* 2004; **1**: 98–105.
12 Purohit V, Brenner DA. Mechanisms of alcohol-induced hepatic fibrosis: a summary of the Ron Thurman symposium. *Hepatology* 2006; **43**: 872–8.
13 Arthur MJP. Fibrogenesis: metalloproteinases and their inhibitors in liver fibrosis. *Am J Physiol Gastrointest Liver Phisiol* 2000; **279**: 245–9.
14 Tatsuta M, Iishi H, Baba M *et al*. Enhancement by ethyl alcohol experimental hepatocarcinogenesis induced by N-nitrosomorpholine. *Int J Cancer* 1997; **71**: 1045–8.
15 Karim MR, Wanibuchi H, Wei M, Morimura K, Salim E, Fukushima S. Enhancing risk of ethanol on MeIQx-induced rat hepatocarcinogenesis is accompanied with increased levels of cellular proliferation and oxidative stress. *Cancer Lett* 2003; **192**: 37–47.
16 Kushida M, Wanibuchi H, Morimura K *et al*. Dose-dependence of promotion of 2-amino-dimethylimidazo[4,5-f]quinoxaline-induced rat hepatocarcinogenesis by ethanol: evidence for a threshold. *Cancer Sci* 2005; **96**: 747–57.
17 Stickel F, Schuppan D, Hahn EG, Seitz HK. Cocarcinogenic effects of alcohol in hepatocarcinogenesis. *Gut* 2002; **51**: 132–9.
18 Croager EJ, Smith PGJ, Yeoh GCT. Ethanol interactions with a choline-deficient, ethionine-supplemented feeding regime potentiate pre-neoplastic cellular alterations in rat liver. *Carcinogenesis* 2002; **23**: 1685–93.
19 Wanibuchi H, Wei M, Karim R *et al*. Existence of no hepatocarcinogenic effect levels of 2-amino-dimethylimidazo[4,5-f]quinoxaline with or without coadministration with ethanol. *Toxicol Pathol* 2006; **34**: 232–6.
20 Yanagi S, Yamashita M, Hiasa Y, Kamiya T. Effect of ethanol on hepatocarcinogenesis initiated in rats with 3′-methyl-4-dimethylaminoazobenzene in the absence of liver injuries. *Int J Cancer* 1989; **44**: 681–4.
21 Cho KJ, Jang JJ. Effects of carbon tetrachloride, ethanol, and acetaldehyde on diethylnitrosamine-induced hepatocarcinogenesis in rats. *Cancer Lett* 1993; **70**: 33–9.
22 Holmberg B, Ekstrom T. The effects of long-term oral administration of ethanol on Sprague-Dawley rats – a condensed report. *Toxicology* 1995; **96**: 133–45.
23 Solt D, Farber E. New principles for the analysis of chemical carcinogenesis. *Nature* 1976; **263**: 701–3.
24 Semple-Roberts E, Hayes MA, Armstrong D, Becker RA, Racz WJ, Farber E. Alternative methods of selecting hepatocellular nodules resistant to 2-acetylaminefluorene. *Int J Cancer* 1987; **40**: 643–5.
25 Tatematsu M, Nagamine Y, Farber E. Redifferentiation as a basis for remodeling of carcinogen-induced hepatocyte nodules to normal appearing liver. *Cancer Res* 1983; **43**: 5049–58.
26 Wood GA, Sarma DSR, Archer MC. Resistance to the promotion of glutathione S-transferase 7-7-positive liver lesions in Copenhagen rats. *Carcinogenesis* 1999; **20**: 1169–75.
27 Bannasch P, Zerban H. Predictive value of hepatic preneoplastic lesions as indicators of carcinogenic response. In: Vainio H, Magee PN, McGregor DB, McMichal AJ, eds. *Mechanism of Carcinogenesis in Risk Identification*. Lyon: IARC. Sci Publications, no. 116 1992; 389–427.
28 Pinheiro F, Faria RR, de Camargo JL, Spinardi-Barbisan AL, da Eira EF, Barbisan LF. Chemoprevention of preneoplastic liver foci development by dietary mushroom Agaricus blazei Murrill in the rat. *Food Chem Toxicol* 2003; **41**: 1543–50.
29 Schulte-Hermann R, Kraupp-Grasl B, Bursch W, Gerbracht U, Timmermann-Trosiener I. Effects of non-genotoxic hepatocarcinogens phenobarbital and nafenopin on phenotype and growth of different populations of altered foci in rat liver. *Toxicol Pathol* 1989; **17**: 642–9.
30 Levin S, Bucci TJ, Cohen SM *et al*. The nomenclature of cell death: recommendations of an ad hoc Committee of the Society of Toxicologic Pathologists. *Toxicol Pathol* 1999; **27**: 484–90.
31 Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–54.
32 Bitsch A, Hadjiolov N, Klöhn P-C, Bergmann O, Zwwirner-Baier I, Neumann H-G. Dose-response of early effects related to tumor promotion of 2-acetylaminofluorene. *Toxicol Sci* 2000; **55**: 44–51.
33 Imai T, Masui T, Ichinose M *et al*. Reduction of glutathione S-transferase P-form mRNA expression in remodeling nodules in rat liver revealed by *in situ* hybridization. *Carcinogenesis* 1997; **18**: 545–51.
34 De Miglio MR, Simile MM, Muroni MR *et al*. Phenotypic reversion of rat neoplastic liver nodules is under genetic control. *Int J Cancer* 2003; **105**: 70–5.
35 Kaufmann WK, Zhang Y, Kaufmann DG. Association between expression of transforming growth factor-alpha and progression of hepatocellular foci to neoplasms. *Carcinogenesis* 1992; **13**: 1481–3.
36 Tamano S, Merlino GT, Ward JM. Rapid development of hepatic tumors in transforming growth factor alpha transgenic mice associated with increased cell proliferation in precancerous hepatocellular lesions initiated by N-nitrosodiethylamine and promoted by phenobarbital. *Carcinogenesis* 1994; **15**: 1791–8.
37 Burr AW, Toole K, Mathew J, Hines JE, Chapman C, Burt AD. Transforming growth factor-α expression is altered during experimental hepatocarcinogenesis. *J Pathol* 1996; **179**: 276–82.
38 Kitano M, Wada J, Ariki Y *et al*. Possible tumour development from double positive foci for TGF-alpha and GST-P observed in early stages on rat hepatocarcinogenesis. *Cancer Sci* 2006; **97**: 478–83.
39 Sandgren EP, Luetteke NC, Qiu TH, Palmiter RD, Brinster RL, Lee DC. Transforming growth factor alpha dramatically enhances oncogene-induced carcinogenesis in transgenic mouse pancreas and liver. *Mol Cell Biol* 1993; **13**: 320–30.
40 Thorgeirsson SS, Santoni-Rugiu E. Transgenic mouse models in carcinogenesis: interaction of c-myc with transforming growth factor alpha and hepatocyte growth factor in hepatocarcinogenesis. *Br J Clin Pharmacol* 1996; **42**: 43–52.
41 Kato J, Sato Y, Inui N *et al*. Ethanol induces transforming-growth factor-α expression in hepatocytes, leading to a stimulation of collagen synthesis by hepatic stellate cells. *Alcohol Clin Exp Res* 2003; **27**: 58S–63S.
42 Zerban H, Radig S, Kopp-Schneider A, Bannasch P. Cell proliferation and cell death (apoptosis) in hepatic preneoplasia and neoplasia are closely related to phenotypic cellular diversity and instability. *Carcinogenesis* 1994; **15**: 2467–73.
43 Grasl-Kraupp B, Ruttkay-Nedecky B, Müllauer H, Taper H, Huber W, Bursch W. Schulte-Hermann R Inherent increase of apoptosis in liver tumors: implications for carcinogenesis and tumor regression. *Hepatology* 1997; **25**: 906–12.
44 Tanaka T, Hirota Y, Kuriyama M, Nishiguchi S, Otani S. Time course of change in glutathione s-transferase positive foci and ornithine decarboxylase

activity after cessation of long-term alcohol administration in rats. *Asian Pac J Cancer Prev* 2001; **2**: 131–4.
45 Mazzantini RP, de Conti A, Moreno FS. Persistent and remodeling hepatic preneoplastic lesions present differences in cell proliferation and apoptosis, as well as in p53, Bcl-2 and NF-kappaB pathways. *J Cell Biochem* 2007.
46 Foda HD, Zucker S. Matrix metalloproteinases in cancer invasion, metastasis and angiogenesis. *Drug Discov Today* 2001; **6**: 478–82.
47 Aye MM, Cuiling MA, Lin H, Bower KA, Wiggins RC, Luo J. Ethanol-induced *in vitro* invasion of breast cancer cells. the contribution of MMP-2 by fibroblasts. *Int J Cancer* 2004; **112**: 738–46.
48 Ke Z, Lin H, Fan Z *et al*. MMP-2 mediates ethanol-induced invasion of mammary epithelial cells over-expressing ErbB2. *Int J Cancer* 2006; **119**: 8–16.

doi: 10.1111/j.1349-7006.2007.00677.x