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Ethanol induces the expression of α1(I) procollagen mRNA in a co-culture system containing a liver stellate cell-line and freshly isolated hepatocytes

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Abstract

To study the fibrogenic action of ethanol in vitro we used a co-culture system of freshly isolated hepatocytes and a liver stellate cell line (CFSC-2G) developed in our laboratory. Our results show that in this co-culture system ethanol induces the expression of α1(I) procollagen mRNA in a dose- and time-dependent manner. This effect of ethanol was due to its metabolism by alcohol dehydrogenase since 4-methylpyrazole prevented the ethanol-mediated increase in α1(I) procollagen mRNA. Ethanol was more efficient than acetaldehyde in inducing α1(I) procollagen mRNA expression and its effect was protein synthesis-independent. Transfection of either hepatocytes or liver stellate cells with a reporter gene, chloramphenicol acetyl transferase (CAT), driven by 3700 bp of the mouse α1(I) procollagen promoter demonstrated that only LSC expressed significant CAT activity and that this activity was enhanced by ethanol. Overall, our results suggest that this co-culture system is a useful model to study alcohol-induced fibrogenesis in vitro and that mechanisms other than acetaldehyde formation may also play an important role in alcohol-induced fibrogenesis. © 1997 Elsevier Science B.V.

Keywords: Acetaldehyde; Ethanol; Hepatocyte; Liver stellate cell; α1(I) procollagen

1. Introduction

Several factors, including genetic background, nutritional status and frequency and amount of alcohol abuse are important determinants in the development of hepatic fibrosis and cirrhosis [1]. However, the administration of isocaloric diets containing ethanol

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to baboons, suffices to induce liver fibrosis and cirrhosis in some animals [2,3]. Thus, it has been suggested that ethanol and/or its metabolite acetaldehyde are fibrogenic per se [2,3]. The exact mechanisms by which ethanol induces fibrosis are not well understood. Acetaldehyde, the first metabolite derived from ethanol, induces the production of collagen and the expression of α1(I) procollagen mRNA by fibroblasts [4,5] and primary cultures of liver stellate cells (LSC) [5–7], and its effect is accentuated when cultured LSC are incubated with cyanamide, an inhibitor of aldehyde dehydrogenase [6]. However, the acetaldehyde effect is variable and appears to depend on the degree of activation and/or confluency of the LSC [5]. Neither ethanol nor acetaldehyde increase the production of collagen by primary cultures of hepatocytes [8].

It has been previously demonstrated that acetaldehyde stimulates transcription of the α1(I) procollagen gene in LSC by a protein synthesis-dependent mechanism [4,6]. However, the mechanism(s) by which ethanol induces liver fibrogenesis remains to be elucidated. Studies performed in vivo with ethanol-fed rats and baboons demonstrated that several factors contribute to liver fibrogenesis [9,10]. In alcohol-fed rats expression of fibrogenic cytokines, such as TGF-β1 and interleukin-6, is increased in Kupffer cells and precedes liver fibrosis [11]. In addition, products derived from lipid peroxidation accumulate prior to excess collagen deposition [11,12], thus suggesting their involvement in fibrogenesis. An additional factor contributing to ethanol-induced fibrogenesis is the induction of an oxidative stress response [12]. Indeed, ethanol-induced liver fibrosis can be exacerbated with iron [13] and prevented with antioxidants [12,14,15]. Because of the complexity of the above-described mechanisms, it was important to develop an in vitro model system to study and dissect the mechanisms by which ethanol induces fibrogenesis. Thus, we took advantage of a co-culture system composed of freshly isolated hepatocytes and a LSC line developed in our laboratory [16]. This co-culture system closely mimics some of the physical and functional relationships of these two cells types in vivo and sustains the expression of liver-specific genes by hepatocytes for at least two weeks [16]. In this communication we report the effects of ethanol on α1(I) procollagen gene expression in this co-culture system.

2. Materials and methods

2.1. Co-cultures of hepatocytes and LSC

Co-cultures were prepared with freshly isolated hepatocytes and the LSC clone CFSC-2G as previously described [16]. Hepatocytes were isolated from male Sprague–Dawley rats weighing 200–250 g by a two-step perfusion method using collagenase, as described originally by Seglen [17]. Viability of hepatocytes was determined by trypan blue exclusion, and only cells with viability greater than 85% were used.

Frozen stocks of CFSC-2G [18] were thawed and maintained in culture with MEM (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT). Confluent dishes of CFSC-2G were trypsinized as previously described [16] and 1×10^6 cells plated in regular 100 mm culture dishes. Forty-eight hours later, when the number of LSC doubled, 10×10^6 freshly isolated hepatocytes suspended in MEM containing 5% FBS and 5 mg/l insulin (Sigma, St. Louis, MO), were plated on top of the LSC. Three hours after plating, culture medium was removed and replaced by a serum-free, hormonally defined medium [19]. Unless otherwise indicated, cells were maintained in culture for 24 h with either 200 μM acetaldehyde or 100 mM ethanol. Control dishes were identical cultures that received no treatment.

2.2. Measurements of alcohol dehydrogenase activity

Co-cultures prepared as described above were washed with PBS and layered with 2 ml of hypotonic buffer (25 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$). After incubation for 5 min at room temperature, the solution was aspirated and 400 μl of Triton lysis buffer (0.25 M Tris–HCl pH 7.8, 0.5% Triton X100) were added. Cells were scraped, transferred into 1.5 ml microcentrifuge tubes, centrifuged for 1 min at 14,000 rpm and the supernatants used for determining alcohol dehydrogenase activity. Assays were performed as described by Bonnichsen and Brink [20]. In brief, 3 ml of 0.1 M glycine–sodium hydroxide buffer pH 9.6, 0.1 ml of NAD 10 mg/ml, 100 μl of

ethanol and 100 μl of sample were mixed in a glass cuvette, and the optical density at 340 nm recorded immediately (time zero). After 3 min, the change in optical density was again recorded. Blanks consisted of samples that contained glycine buffer, NAD and sample, but without added ethanol substrate. Changes in absorbance at 340 nm were determined as described above and the values were subtracted from samples incubated with the substrate. Values correspond to quadruplicate experiments ± S.D. and are expressed as nmol of ethanol converted to acetaldehyde/minute/mg protein.

2.3. Northern blot analysis

Total RNA was extracted from harvested cells as described by Chomczynski and Sacchi [21]. Approximately 10 μg of total RNA were electrophoresed on 1% agarose gels and transferred to a GeneScreen hybridization transfer membrane (Dupont, Boston, MA), as described by the manufacturer. The following [α-^{32}P]-labeled rat cDNA probes were used for hybridization: fibronectin (0.5 Kb) [22], α1(I) procollagen (1.6 Kb) [23], albumin (0.35 Kb) [24], and fibrinogen (1.2 Kb) [25]. A human cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1.2 Kb) was obtained from ATTCC (Rockville, MD). The probes were labeled by primer extension with [α-^{32}P]dCTP, with a specific activity of 3000 Ci/mmol (Amersham, Arlington Heights, IL). Blots hybridized with rat cDNA probes were washed under stringent conditions: four washes for 15 min at 65°C with 2× standard saline citrate (SSC) containing 0.1% SDS, two washes for 15 min at 65°C with 1× SSC containing 0.1% SDS, and one wash for 15 min at 65°C with 0.1× SSC containing 0.1% SDS. Low stringency washes (four washes of 15 min with 2× SSC containing 0.1% SDS at 65°C) were used for blots hybridized with the GAPDH cDNA, as previously described [18]. All blots were exposed to Kodak X-Omat film at −70°C with intensifying screens. Quantitative data were obtained after scanning the X-ray films using the computer program Adobe Photoshop (Adobe Systems, Mountain View, CA). Blots were consecutively hybridized with the probes mentioned above. Before hybridization, the previously used probe was stripped off by washing the membrane for 45 min at 75°C with a solution containing 50% formamide and 1× SSC.

Except for the time-course experiments that were performed in duplicate, all other experiments were performed in triplicate and quadruplicate and values are expressed as means ± S.D.

2.4. Reporter genes

We used the chimeric gene COLCAT1 (kindly provided by Dr. David Brenner) that contains the nucleotide sequences corresponding to −3700 to +110 of the mouse α1(I) procollagen promoter, fused to the CAT gene [26]. To determine transfection efficiency, cells were co-transfected with SV40LUC, a plasmid that contains the luciferase gene fused to the simian virus 40 promoter (kindly provided by Dr. Francesco Ramirez).

2.5. Transfection of LSC

Transient transfection of LSC cells was carried out as previously described [27]. Fifteen micrograms of

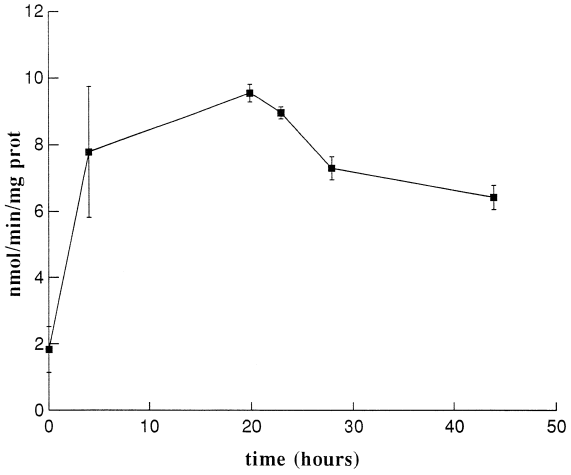

Fig. 1. Alcohol dehydrogenase activity in co-cultures of freshly isolated hepatocytes (5×10^6) and the liver stellate cell line CFSC-2G (1×10^6). The activity was determined in freshly isolated hepatocytes (time = 0), 4 h after seeding hepatocytes on the CFSC-2G culture, at the time that the plating medium was replaced by a serum-free, hormonally-defined medium (time = 4 h) and at various time-points thereafter for up to 46 h. Values are from quadruplicate experiments and are expressed as nmols of alcohol converted/min/mg protein ± S.D.

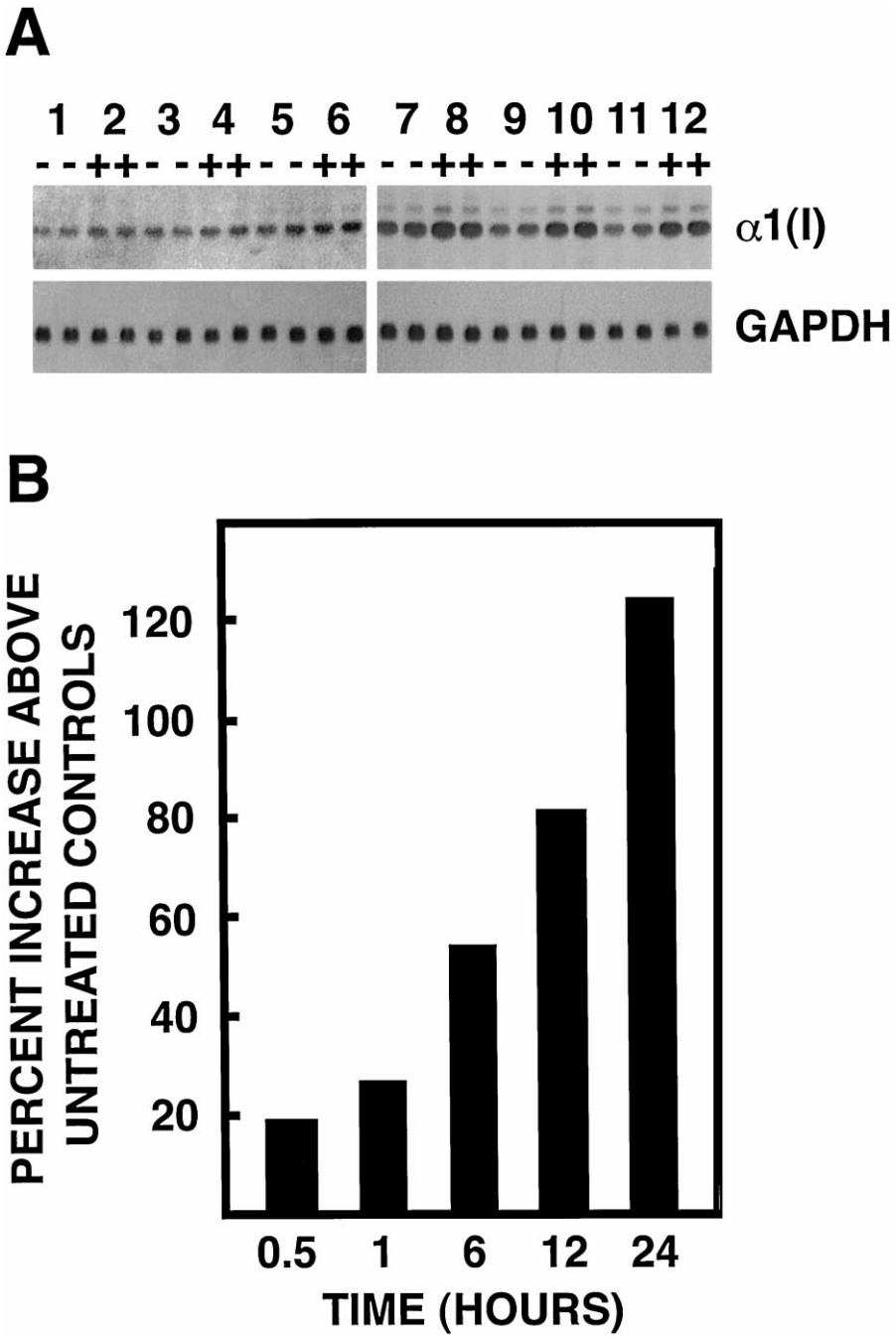

Fig. 2. Time-course of induction of α1(I) procollagen mRNA by ethanol in co-cultures of freshly isolated hepatocytes and the LSC clone CFSC-2G. The co-cultures were prepared as described in Section 2. Ethanol (100 mM) was added to the co-cultures 18–20 h after plating the hepatocytes and total RNA was extracted after 0.5, 1.0, 6.0, 12.0 and 24.0 h. Steady-state levels of α1(I) procollagen mRNA were determined by Northern blot analysis and a representative blot is shown in Panel A. All experiments were performed in duplicate. Because α1(I) procollagen mRNA expression increases with time in control co-cultures, for each set of cultures treated with ethanol (+), an identical set of controls without ethanol (−) was analyzed. The intensity of the bands was measured by laser densitometry, and values were corrected for loading using GAPDH mRNA levels as a control and were expressed as percent change above controls. Variation between duplicate experiments was less than 10%.

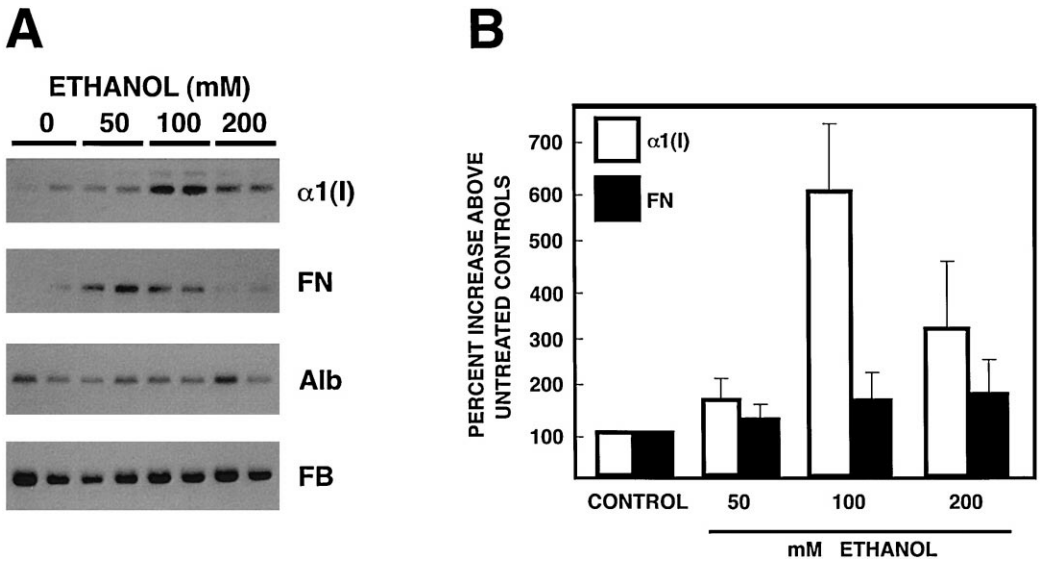

Fig. 3. Effect of 50, 100 and 200 mM ethanol on the expression of α1(I) procollagen and fibronectin mRNAs by co-cultures of hepatocytes and CFSC-2G prepared as described in Section 2. Co-cultures were harvested 24 h after addition of ethanol and mRNA analyzed as indicated in Fig. 2. The same blot was sequentially probed with cDNAs for α1(I) procollagen and fibronectin. A representative Northern blot is shown in panel A. Values are means of quadruplicate experiments ± S.D. after correction for loading using albumin mRNA levels as a control (Panel B).

COLCAT1 and 3 μg of SV40LUC in the form of a calcium phosphate precipitate, were added to dishes containing 1×10^6 LSC cells in MEM supplemented with 10% FBS. Five hours after transfection, the medium was removed and the cells were incubated with 3 ml of 15% glycerol in MEM for 2 min, washed

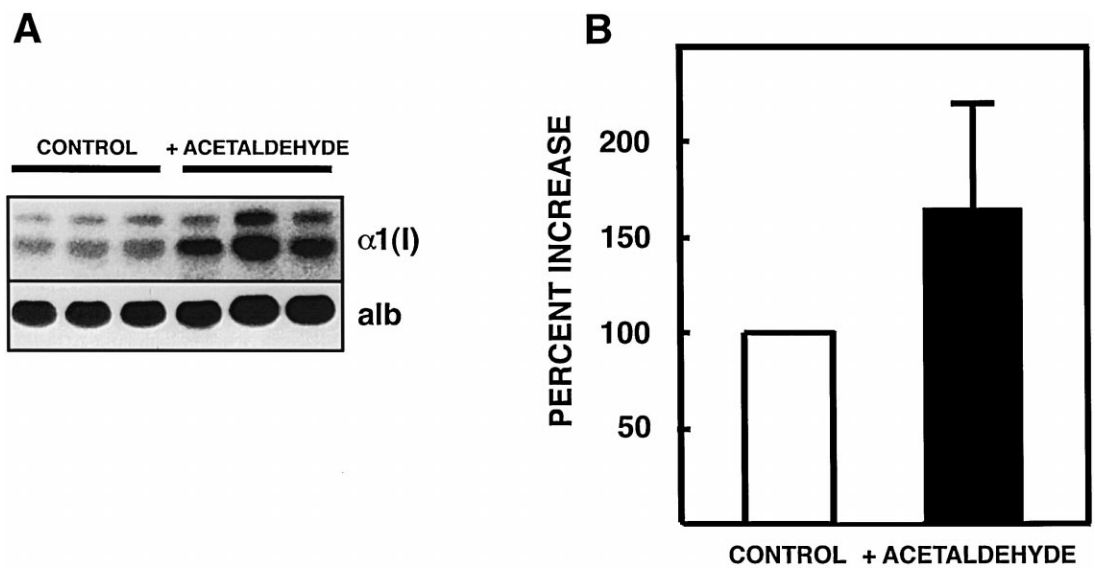

Fig. 4. Effect of acetaldehyde on the expression of α1(I) procollagen mRNA by co-cultures of freshly isolated hepatocytes and CFSC-2G. The experiments were performed as described in Fig. 3, except that the cells were incubated with 200 mM acetaldehyde. A representative Northern blot is shown in Panel A. Experiments were performed in triplicate and values are means ± S.D. after correction for loading using albumin mRNA levels as a control (Panel B).

3 times with PBS, and covered with fresh serum-supplemented medium. After 12 additional hours of incubation, the medium was aspirated and 5×10^6 hepatocytes were plated in MEM with 5% FBS and 5 mg insulin/l. Hepatocytes were allowed to attach overnight at 37°C in a humidified 5% CO_2 atmosphere. Cells were then washed twice with PBS and the medium replaced by a serum-free, hormonally defined culture medium [19]. The remainder of the experiment was performed as described above (see Section 2.1). Cells were harvested 48 h after transfection, washed twice with PBS (pH 7.4), scraped with a rubber policeman and pelleted by centrifugation. Protein extracts from transfected cells were prepared after three cycles of freeze-thawing in 250 mmol/l Tris–HCl pH 7.5. Protein concentration was determined according to the method of Lowry et al. [28]. Luciferase activity was measured with a luminometer (Moonlight 2010, Analytical Luminescence Laboratory, San Diego, CA) using a kit from Promega (Madison, WI). CAT activity was determined as previously described [29] and expressed as percent of acetylation after correcting for transfection efficiency. These experiments were performed at least in quadruplicate, and values were corrected for the efficiency of transfection using luciferase activity as a control. Values are means ± S.D.

2.6. Transfection of hepatocytes

Hepatocytes were transfected in suspension by electroporation [30]. Briefly, cells were transfected at room temperature with a single low-voltage pulse (160 V, 960 μF), using an extended capacitance gene pulser system (Bio–Rad Laboratories, Bethesda, MD). Immediately after isolation, hepatocytes were resuspended at a concentration of 25×10^6 cells/ml in sterile PBS containing 5% FBS. Eight-hundred microliters of suspension were mixed with 30 μg of COLCAT1, 6 μg of SV40LUC and 400 μg of sonicated salmon sperm DNA, and transferred into an electroporation cuvette. Cells were left in contact with the DNA for 10 min before applying the pulse. Hepatocytes were then incubated at room temperature for 10 min, and plated at a density of 5×10^6 cells onto 100 mm diameter culture plastic dishes containing 1×10^6 CFSC-2G cells. The remainder of the experiments was performed as described above.

3. Results

We have recently developed a co-culture system of freshly isolated hepatocytes and a LSC line (CFSC-2G) in which the former sustain some of their cell-

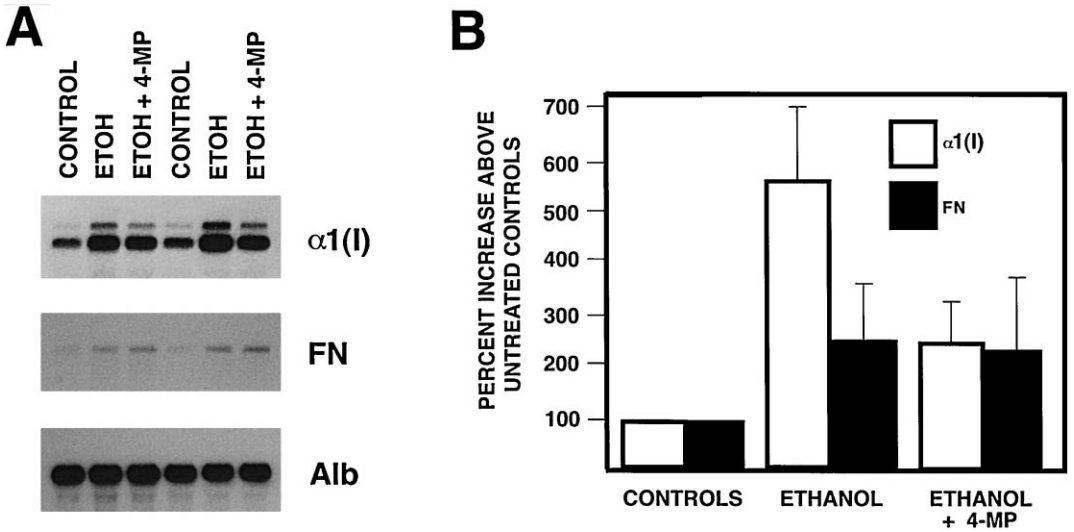

Fig. 5. Effect of 4-methylpyrazole on the induction of α1(I) procollagen and fibronectin mRNAs by ethanol in co-cultures of freshly isolated hepatocytes and CFSC-2G. The experiments were performed as described in Fig. 3. Two mM 4-MP and 100 mM ethanol were added to the co-cultures and the cells harvested 24 h later. A representative Northern blot is shown in Panel A. Values are means of quadruplicate experiments ± S.D. after correction for loading using albumin mRNA levels as a control (Panel B).

specific functions observed in vivo [16]. Therefore, we thought it was important to investigate whether alcohol dehydrogenase activity was maintained in these co-cultures, and whether the system could be implemented as an in vitro model system to study ethanol-induced liver fibrogenesis. Because our previous data showed that differentiated functions of the hepatocytes are best preserved when co-cultures contain 5 times more hepatocytes than LSC [16], all the experiments described in this communication were performed using regular culture dishes containing a 5:1 hepatocyte:CFSC-2G ratio.

We first determined whether the co-culture system was suitable to study conversion of ethanol to acetaldehyde. Thus, we measured alcohol dehydrogenase activity and compared the values with those obtained with freshly isolated hepatocytes. As shown in Fig. 1, ADH activity of freshly isolated hepatocytes (time = 0) was 1.8 ± 0.7 nmol/min/mg protein. As also shown in this figure, co-cultures contain ADH activity which is 4-fold greater than that of freshly isolated cells. This activity is sustained at least for the duration of the experiments (48 h). In contrast to these results, ADH activity in CFSC-2G was below detectable levels. However, we cannot rule out the possibility that CFSC-2G contribute to total ADH activity when placed in co-culture with hepatocytes. Co-cultures also sustain the expression of Cyt P450 2E1 mRNA for two weeks, an enzyme suggested to play a significant role in ethanol metabolism in chronic alcoholics [31] (not shown).

Under the experimental conditions described below, steady-state levels of albumin, fibrinogen and GAPDH mRNAs remained constant. Neither one of these mRNAs was modified by ethanol administration. Thus, when mRNA levels were corrected for loading differences using either albumin or GAPDH mRNA levels, the values were similar.

Fig. 2 shows that the effect of ethanol on steady-state levels of α1(I) procollagen mRNA is time-dependent. It increases to 53% above control values by 6 h, 81% by 12 h and a maximal expression of 124% by 24 h. Accordingly, all further experiments were performed with cells treated with ethanol for 24 h. The effect of ethanol is dose-dependent and a maximal increase of $609 \pm 127\%$ ($p < 0.001$ vs. untreated controls) in the expression of α1(I) procollagen mRNA was obtained with 100 mM ethanol (Fig. 3). When a 200 mM ethanol concentration was used, the expression of α1(I) procollagen mRNA was still increased. However, steady state levels of this mRNA were only $317 \pm 137\%$ above control values ($p < 0.05$). The expression of fibronectin mRNA was not significantly induced by ethanol ($p > 0.05$).

Moshague et al. [8] have shown that acetaldehyde is the ethanol metabolite responsible for inducing the expression of α1(I) procollagen mRNA in cultured LSC. Therefore, we performed experiments to determine whether ethanol was fibrogenic per se, or whether its metabolite, acetaldehyde, was responsible for the induction of collagen mRNA reported previously (see Figs. 2 and 3). In co-cultures incubated with 200 μM acetaldehyde, α1(I) procollagen mRNA is induced $164 \pm 55\%$ (see Fig. 4), a value which is similar to that previously reported for primary cultures of LSC treated with acetaldehyde [5,6]. In order to show that alcohol metabolism is required for the induction of α1(I) procollagen mRNA in co-cultured cells, some co-cultures were incubated with 4-meth-

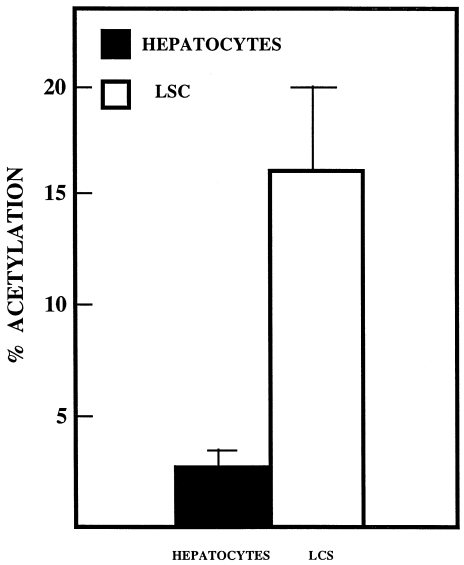

Fig. 6. CAT activity of co-cultures of hepatocytes and LSC in which either one or the other cell-type were co-transfected with a CAT reporter gene driven by 3700 bp of the mouse α1(I) collagen promoter and a luciferase control vector (See Section 2). CAT activity was determined 48 h after establishing the co-cultures, and values are means ± S.D. of at least quadruplicate experiments after correcting for transfection efficiency using luciferase activity as a control.

ylpyrazole (4-MP), a known inhibitor of ethanol metabolism. As shown in Fig. 5, ethanol-dependent induction of $\alpha 1$(I) procollagen mRNA was inhibited by $309 \pm 77\%$ with 4-MP ($p < 0.05$).

Previous work from our laboratory has shown that de novo protein synthesis is required for acetaldehyde to induce $\alpha 1$(I) procollagen mRNA in cultured LSC [6]. However, in this co-culture system, cycloheximide, at a dose that inhibits protein synthesis by 95% in both cultures of primary LSC [6] and the LSC clone used for the co-culture [27], only inhibited by 30%, the expression of $\alpha 1$(I) procollagen mRNA (not shown).

To determine the contribution of LSC and hepatocytes in co-culture to the expression of $\alpha 1$(I) procollagen mRNA, either CFSC-2G or hepatocytes were transfected with a reporter gene driven by 3700 bp of the $\alpha 1$(I) procollagen promoter and a luciferase reporter gene driven by the SV40 promoter prior to establishing the co-cultures (see Section 2). As illustrated in Fig. 6, LSC and not hepatocytes expressed CAT activity and this activity was induced by ethanol (Fig. 7), and prevented by preincubation of the co-cultures with 2 mM 4-MP 30 min prior to the addition of ethanol. As a positive control for this experiment, we show the induction of CAT activity by recombinant IL-6 [27].

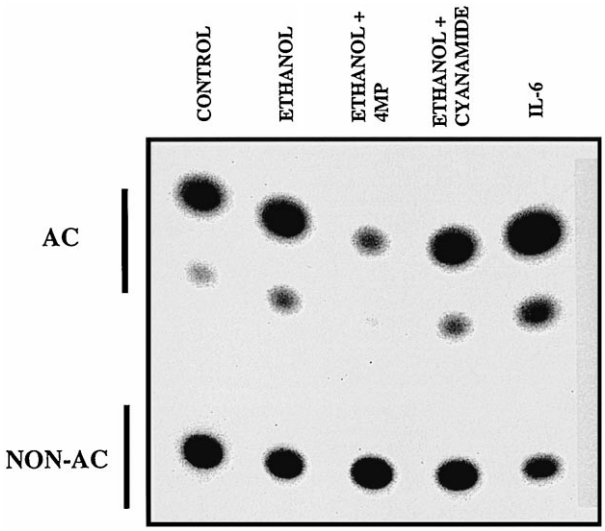

Fig. 7. Representative experiment of CAT activity of co-cultures prepared as described in Section 2 that were incubated with the following reagents: Controls (lane 1), 100 mM ethanol (lane 2), 2 mM 4-MP added 30 min prior to adding 100 mM ethanol (lane 3), 100 mM ethanol + 100 μM cyanamide (lane 4). As an internal control to determine the responsiveness of the LSC, some co-cultures were incubated with 20 ng recombinant IL-6 (kindly provided by Dr. T. Hirano) (lane 5).

4. Discussion

The co-culture model described in this communication appears to be an appropriate model to study cell–cell interactions and the effect of hepatotoxic agents, such as ethanol on collagen expression by LSC. It is composed of hepatocytes, the only cells in the liver that contain appreciable amounts of alcohol dehydrogenase [32,33] and LSC, the main producers of type I collagen [34]. Although co-cultures of hepatocytes and LSC have been used for alcohol [7] and CCl_4 studies [35], the co-culture system used in this communication is easy to prepare, has unique properties, and reproduces in vitro some of the biological activities of hepatocytes and LSC observed in vivo [16]. In this communication, we show that alcohol dehydrogenase activity is sustained at least for the duration of the experiments (48 h). Moreover, Cyt P450 E21 mRNA expression was preserved for two weeks. Overall, these results suggest, as previously shown for other hepatocyte-specific genes [16], that the expression of two ethanol-metabolizing systems is sustained in this co-culture system.

The results obtained in this communication indicate that the induction of $\alpha 1$(I) procollagen mRNA is dependent on ethanol. This suggestion is based on the inhibition by 4-MP of the ethanol-dependent induction of $\alpha 1$(I) procollagen mRNA and expression of CAT activity by co-cultures.

Our results also showed that the expression of fibronectin mRNA was not significantly induced by ethanol. This result differs from those reported previously with LSC treated with acetaldehyde in which fibronectin mRNA was significantly increased [6].

Although in this (see Fig. 4) and other studies [6,7], acetaldehyde increased 2-fold the expression of $\alpha 1$(I) procollagen mRNA in LSC, alcohol appears to be more effective, since in multiple experiments the expression of collagen was induced up to $609 \pm 127\%$ (see Fig. 3). These data could suggest that either hepatocytes deliver acetaldehyde more efficiently to LSC, and/or that in this co-culture system, ethanol

induces collagen gene expression via formation of acetaldehyde, as well as by other mechanisms, all of them dependent on ethanol metabolism. These could include, among others, changes in redox potential [36], formation of reactive oxygen species [37] and lipoperoxidation [35]. We cannot rule out however, that the differences in α1(I) procollagen gene induction by acetaldehyde as compared to ethanol treated co-cultures are related to their differences in vapor pressure or to a direct solvent effect of ethanol.

Another interesting finding obtained with this co-culture system was that the acetaldehyde effect on collagen gene expression is protein synthesis-independent, while in LSC cultured alone is protein synthesis-dependent [6]. These findings could suggest that factors needed for the induction of α1(I) procollagen mRNA are already present in LSC maintained in co-culture. However, from these data we cannot determine whether they were induced by a paracrine stimulation of LSC by hepatocytes, or whether the needed factors were induced by the establishment of gap junctions between these two cell types [16]. This effect is not surprising since other inductive effects of hepatocytes on LSC have been reported. We [16] and others [38] have shown that hepatocytes co-cultured with LSC induce proliferation of the latter. Others have shown that hepatocytes induce the expression of metalloproteinases by sinusoidal cells maintained in co-culture [39]. Thus, these results stress the need of further studies with co-cultures when exploring changes in collagen gene expression in LSC induced by agents that perturb the metabolism of hepatocytes.

We also presented evidence showing that hepatocytes do not contribute to α1(I) procollagen gene expression in this co-culture system. We showed that hepatocytes transfected with a reporter gene linked to 3700 bp of the mouse α1(I) procollagen promoter (COLCAT1) [26] did not express significant CAT activity.

Overall, our data indicate that this co-culture system [16] offers several advantages over other systems for studies of liver fibrogenesis. It provides the target cells that respond to the injurious agent (hepatocytes) and contains the effector cells that produce collagen (LSC). This co-culture system could be used also to study the contribution of inflammatory cells to fibrosis and to test the efficacy of hepatoprotective anti-inflammatory and/or antifibrogenic substances.

Acknowledgements

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