Differential Fibronectin Expression in Activated C6 Glial Cells Treated with Ethanol

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ABSTRACT

The central nervous system is particularly susceptible to alcohol effects and toxicity. Glial cells constitute the most common cell type in the brain and play critical roles in normal brain function and during infection and injury. Astrocytes in particular seem to be important targets for alcohol neurotoxicity during both development and in adulthood. To gain more insight into alcohol-mediated effects on astrocytes at the molecular level, gene expression in rat C6 glial cells was studied in the presence or absence of ethanol. The differential display of mRNA technique was used to screen the expressed genes in ethanol-treated rat C6 cells before and after treatment with lipopolysaccharide (LPS) combined with phorbol-12-myristate-13-acetate (PMA), conditions that mimic an infectious inflammatory state and cause immunologic activation.

The present data show that fibronectin appeared as a major gene whose expression is increased in C6 cells by LPS plus PMA stimulation and decreased by chronic ethanol exposure, both in mRNA and protein levels. Fibronectin is a dimeric glycoprotein found in the extracellular matrix of most tissues, in the blood, and on cell surfaces and is involved in many cellular processes. These results show that chronic exposure to ethanol is associated with changes in astrocyte properties during immunologic activation that reduce fibronectin expression. The discovery of astrocyte fibronectin expression as a potential regulated target for chronic alcohol abuse may be useful in understanding, preventing, and treating some brain disorders associated with alcohol abuse and alcoholism.

Beverage alcohol is clearly a toxic substance when consumed in excess, and alcoholism is a common, chronic, often progressive disorder (Blondell et al., 1996). Excessive consumption of alcohol and alcoholism are now the greatest substance abuse problems in the United States and other Western countries and are major health concerns worldwide, contributing to numerous social and medical problems (Samson and Harris, 1992; Cook, 1998). Chronic alcohol abuse results in a variety of effects, including liver and brain damage, and is associated with an increased risk of certain types of cancers. Alcohol consumption by pregnant women can result in fetal alcohol effects and the fetal alcohol syndrome. Because the brain is particularly susceptible to ethanol toxicity, understanding the pathogenesis of alcohol-related brain damage becomes very important to institute more effective treatments and preventative measures for alcohol abuse problems.

Recent advances have provided significant clues about where and how ethanol works on the brain (Samson and Harris, 1992). Interactions between the more numerous glial cells and neurons are tantamount to a fully functional brain.

Therefore, it is probable that chronic effects on glial cells contribute to the pathogenesis of alcohol-related brain damage. Astroglial cells (astrocytes) are an important target of ethanol toxicity during CNS development and are profoundly affected by prenatal ethanol exposure. Ethanol can affect DNA, RNA, and protein synthesis in primary cultures of rat cortical astrocytes and can suppress astrocyte mitogenesis (Aroor and Baker, 1997). Ethanol also reduces the capacity of astrocytes to secrete growth factors (Valles et al., 1994); induces oxidative stress in astrocytes (Montoliu et al., 1995); and alters the development, content, and distribution of several cytoskeletal proteins, including transcription of the astroglial marker glial fibrillary acidic protein (Valles et al., 1997). Thus, ethanol-induced alterations in astrocyte gene expression could be important mechanisms underlying the CNS dysfunction observed after prenatal exposure to ethanol (Guerri and Renau-Piqueras, 1997). Chronic alcohol abuse also affects adult glial cells. Human alcoholic brains show clinical and pathological evidence of significant astroglial cell loss in both gray and white matter regions (Hunt and Nixon, 1993; Korbo, 1999).

It is probable that chronic ethanol effects on astrocyte gene expression contribute to the pathogenesis of alcohol-related brain damage. Identity of these genes and their products can

ABBREVIATIONS: CNS, central nervous system; iNOS, inducible nitric-oxide synthase; LPS, lipopolysaccharide; PMA, phorbol-12-myristate-13-acetate; DDRT, differential display reverse transcription; PCR, polymerase chain reaction; RT, reverse transcription; TST, Tris/saline/Tween-20; IDV, integrated density value.

provide greater insight into the etiology of alcoholism and will supply molecular probes for future studies on animal models of alcoholism and human alcoholic specimens. Genes that play a role in CNS host defense mechanisms may be particularly important targets. We have previously identified the inducible nitric-oxide synthase (iNOS) as one such gene in astrocytes susceptible to ethanol exposure (Syapin, 1995; Syapin, 1996; Militante et al., 1997). iNOS is not normally present in healthy brain tissue but is readily expressed after brain infection and injury and in many neurological disorders. iNOS expression requires transcriptional activation of its gene. This occurs after activation of glial cells by a host of stimuli, including bacterial LPS and protein kinase C activators. A common feature of glial cell activation is the synergism observed between stimuli. For example, C6 glial cells exposed to either 400 ng/ml of PMA alone or 500 ng/ml of LPS alone respond with minimal iNOS activity. However, if exposed to the same concentrations simultaneously, an increase in iNOS activity of more than 10-fold is seen (Syapin, 1995). C6 cells activated by LPS/PMA have been extensively studied for effect of ethanol on gene expression (Syapin, 1995, 1996; Militante et al., 1997; Ren et al., 1999a; L. Q. Ren and P. J. Syapin, unpublished observations). Acute ethanol exposure during LPS/PMA activation suppresses iNOS activity in a concentration-dependent manner, an effect that does not involve the protein kinase C pathway (Syapin, 1995). Chronic ethanol exposure before iNOS induction causes a time- and dose-dependent increase in sensitivity to the ethanol inhibition. Consistent with the findings for acute ethanol exposure, chronic ethanol acts to reduce the potency of LPS, but without affecting that of the coadministered PMA (Syapin, 1995).

We have now used the DDRT-PCR technique to screen the expressed genes in ethanol-treated rat C6 glial cells after being activated with LPS/PMA. This technique detected the mRNA of fibronectin as one of those most strongly increased by activation with LPS/PMA and significantly affected by chronic exposure to ethanol. Subsequent experiments examined the temporal effects of ethanol on fibronectin mRNA and protein expression. The discovery of fibronectin as an alcohol-responsive gene during glial cell activation suggests new signaling pathways whose alterations may contribute to damage caused by chronic alcohol abuse.

Materials and Methods

Cell Culture. Rat C6 glial cells (CCL107) were obtained from the American Type Culture Collection (Manassas, VA) at passage 38 and were propagated, maintained, and used according to methods described previously (Syapin, 1995; Militante et al., 1997; Syapin et al., 1999). Stock cultures were grown in high (4.5 g/l) glucose-containing Dulbecco's modified Eagle's medium (Mediatech, Washington, DC) with 5% fetal bovine serum (Hyclone Laboratories, Logan, UT). Sister cultures for experimentation were seeded into 6-well culture plates (Falcon, Oxnard, CA) at densities of either $4 \times 10^4/cm^2$ or $4 \times 10^3/cm^2$, for use after 5 or 9 days, respectively, and grown in medium with 2.5% serum. Cultures were maintained at 37°C inside a humidified incubator with 5% CO_2/95% air and replenished with fresh media 2 or 3 days after seeding and every other day thereafter.

Ethanol Treatment. Cells were treated with ethanol as for previous studies (Militante et al., 1997; Syapin et al., 1999). The cultures were placed in holding trays in sealed 2-gallon ZipLoc bags (DowBrands, Indianapolis, IN) containing a 400-ml reservoir of aqueous ethanol at the same concentration as in the medium. The

bags, located inside a water-jacketed, 37°C, CO_2 incubator, were gassed with compressed 5% CO_2/95% air.

Cell Stimulation. LPS (*Escherichia coli*; Sigma Chemical Co., St. Louis, MO) stocks (1 mg/ml) were prepared in cartridge-purified 18 MΩ water, filter-sterilized, and stored frozen at −20°C. LPS working solutions (50 μg/ml) were prepared from stock in fresh assay medium (serum-free Dulbecco's modified Eagle's medium) and used soon thereafter. Stock PMA (Sigma) was dissolved in acetone (Fisher Scientific, Houston, TX) at 0.5 mg/ml and stored at −20°C. Working solutions (40 μg/ml) were prepared fresh on ice into assay medium under subdued lighting and used immediately thereafter. The low level of acetone added to the cells as vehicle was tested and found not to affect the response. C6 glial cells were activated by simultaneous treatment with 500 ng/ml LPS and 400 ng/ml PMA, as described previously (Syapin, 1995; Militante et al., 1997). Exposure to the stimulating agents was for 24 h in this study. Stimulation was initiated by removing the growth medium, rinsing the culture dish once with assay medium, adding fresh assay medium, and then exposing cells to LPS and PMA. For cells exposed to ethanol, the assay medium contained the appropriate concentration during the rinsing step and the subsequent 24-h incubation. Controls consisted of unstimulated cells exposed and not exposed to ethanol.

RNA Isolation and Treatment with DNase I. After the 24-h incubation, the medium was removed; cells were lysed in guanidinium isothiocyanate/mercaptoethanol solution, and total cellular RNA was extracted according to Chomczynski and Sacchi (1987). Total cellular RNAs were treated with RNase-free DNase I (Amersham Pharmacia Biotech, Piscataway, NJ) according to the following protocol. A 50-μl reaction solution, containing about 50 μg of total cellular RNA from the above step, 50 mM Tris-Cl, pH 7.8, 0.1 mM EDTA, pH 8.0, and 4 U of RNase-free DNase I, was incubated at 37°C for 1 h and extracted with 200 μl of phenol/chloroform (1:1 v/v) (Sigma). The RNA was collected by precipitation with LiCl and alcohol.

mRNA Differential Display. Differential display experiments were carried out using the HIEROGLYPH mRNA Profile Kit, the FluoroDD TMR-fluorescent anchored primer adapter kit for the HIEROGLYPH mRNA Profile Kit System, a genomyx LR Programmable DNA sequencer and a genomyx SC fluorescence scanner (all from Beckman Instruments Inc., Fullerton, CA) following the manufacturer's instructions with some modification as described below.

Reverse transcriptions of total RNAs were done with one of the twelve T7(dT$_{12}$)AP anchored primers, at 70°C for 10 min, 42°C for 5 min, 50°C for 50 min, and 70°C for 15 min. PCR amplifications were carried out with one TMR-anchored primer and one arbitrary primer on the RapidCycler PCR machine (Idaho Technology, Idaho Falls, ID) at 93°C for 3 min, then 4 cycles at 93°C for 30 s, 42°C for 45 s, 72°C for 45 s, and 35 cycles at 93°C for 30 s, 58°C for 45 s, 72°C for 45 s, and finally 10 min at 72°C.

Electrophoresis was done on the genomyx LR programmable DNA sequencer at 55°C, 3000 V, and 100 W for 5 h in a 5.6% denaturing polyacrylamide gel (HR-1000 5.6% denaturing high resolution differential display gel; genomyx Co., Beckman Instruments). After scanning the gel on the genomyx SC, duplicate cDNA bands were excised from the gels, and gel pieces were immersed in 50 μl Tris/EDTA (10 mM Tris-Cl, 1 mM EDTA). They were then incubated at 37°C for 1 h and stored at 4°C for further use.

Gel band reamplification was done according to the following protocol in a 40-μl PCR reaction volume: 1 μl of gel supernatant from above, 4 μl of 2 μM M13 reverse (−48) 24-mer primer (5′-AGC GGA TAA CAA TTT CAC ACA GGA-3′) and 4 μl of 2 μM T7 promoter 22-mer primer (5′-GTA ATA CGA CTC ACT ATA GGG C-3′) (genomyx Co., Beckman Instruments) with dNTPs, Amplitaq DNA polymerase and buffer (Perkin-Elmer, Branchburg, NJ) on the RapidCycler PCR machine at 93°C for 3 min, then 5 cycles at 93°C for 30 s, 50°C for 45 s, 72°C for 45 s, and 40 cycles at 93°C for 30 s, 60°C for 45 s, 72°C for 45 s, and finally 10 min at 72°C. This PCR product was

used as the template for direct DNA sequencing on a Perkin-Elmer ABI Prism Genetic Analyzer.

Semiquantitative RT-PCR and Confirmation of Differential mRNA Expression. Total RNA (0.2 μg) from LPS/PMA-stimulated C6 cells treated or untreated with ethanol was reverse transcribed with random primers (Promega, Madison, WI) and 1 μl of the cDNA mixture was subjected to PCR using specific oligonucleotide primers (5′-CCC TCC ATT TCT GAC TGG TC-3′ and 5′-GAC AGT GAG TCC TGT GGG GT-3′) for rat fibronectin mRNA (Schwarzbauer et al., 1987; Patel et al., 1987). The same reverse transcript mixture was also subjected to PCR in separate tubes using a pair of primers (5′-ACG TCA ACA CTG CTC TAC A-3′ and 5′-CTT TGC CAT AGT CCT TAA C-3′) specific for rat ribosomal S12 RNA (Ayane et al., 1989). The expected size of the amplification product for fibronectin was 396 base pairs and the product was sequenced again to confirm the correct fibronectin sequence. The S12 cDNA, with a product size of 311 base pairs, served as internal standards to make sure equal quantities of cDNAs were amplified. It is assumed that this mRNA does not change significantly upon addition of LPS/PMA or ethanol to the cultured cells and that it allows for an estimation of the integrity of the RNA (Biber et al., 1997; Ren et al., 1998, 1999b). A number of PCR cycles and denaturation temperatures were examined by scanning densitometry to ascertain a linear working range for both PCR products. After electrophoretic separation, ethidium bromide-stained gels were photographed and scanned with an Alpha Imager 2000 (Alpha Innotech Corp., San Leandro, CA).

Western Blotting. Cell protein extracts were prepared in Cell Culture Lysis Reagent (Promega Corp.) and samples of equal protein quantity were electrophoretically separated through 5% polyacrylamide gels (30% acrylamide/bis solution, 29:1 (3.3% cross-linking); Bio-Rad Laboratories, Hercules, CA) containing 0.1% SDS [SDS Solution 10% (w/v), Bio-Rad] in running buffer (25 mM Tris base, 190 mM L-glycine, 0.1% SDS) with the Mini-PROTEAN 3 CELL (Bio-Rad) at 200 V. Proteins were electro-transferred to polyvinylidene difluoride membranes (Bio-Rad) in transfer buffer (25 mM Tris base, 150 mM L-glycine, 20% methanol) with the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) for 2 h at 90 V. Membranes were blocked for 1 h at room temperature in TST buffer [10 mM Tris base, 100 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5] containing 3% bovine serum albumin (Sigma Chemical Co.) and 0.5% Carnation natural nonfat dry milk (Nestlé, Glendale, CA) and then incubated overnight at 4°C with rabbit anti-rat fibronectin antibody (1:80,000 dilution; Calbiochem-Novabiochem Corp., La Jolla, CA) in the same blocking buffer. Membranes were then washed with TST buffer six times for 5 min each, incubated for 4 h at room temperature with horseradish peroxidase-conjugated goat polyclonal anti-rabbit IgG (1:45,000 dilution; Transduction Laboratories, Lexington, KY) and then washed again with TST buffer six times for 5 min each. Bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and autoradiography (Fuji Medical X-ray Film, Tokyo, Japan).

Band densities were quantified with the Alpha Imager 2000 (Alpha Innotech Corp., San Leandro, CA) using the automatic background mode. The bands of interest were designated manually and the software then computed the integrated density value (IDV = Σ [each pixel value − background]) within the designated area. The β-tubulin content in the samples used for fibronectin protein determination was also determined to demonstrate the specificity of the effect on fibronectin (see also Syapin et al., 1999). Western blotting conditions for β-tubulin were the same as for fibronectin with the following exceptions. The blocking buffer was TST plus 4% nonfat dry milk. Hybridization of the E-7 β-tubulin monoclonal antibody (Webster, 1997) was for 2 h at room temperature at a 1:100 dilution (E-7 was kindly provided by Dr. Daniel Webster). Hybridization of the anti-mouse IgG peroxidase conjugated secondary antibody (Sigma) was for 1 h at room temperature at a 1:3000 dilution.

Results

Differential Expression of Fibronectin mRNA in LPS/PMA-Stimulated C6 Glial Cells. The mRNA differential display technique (Liang and Pardee, 1992) was used to isolate genes that are up- or down-regulated in ethanol-treated rat C6 glial cells after stimulated with LPS/PMA. One of the mRNAs we found regulated under these conditions was a cDNA referred to as Band15c38–1. This band was detected using the TMR-Anchored primer 5′- ACG ACT CAC TAT AGG GCT TTT TTT TTT TTG G-3′ and the arbitrary primer 5′-ACA ATT TCA CAC AGG AGC TAG CAG AC-3′ in the DDRT-PCR (Fig. 1). After reamplification of this band, sequence analysis of the PCR product revealed 97% nucleotide sequence identity in a 467-base-pair fragment that overlapped the rat fibronectin mRNA sequence (Patel et al., 1987; Schwarzbauer et al., 1987). This result clearly indicated that this differentially expressed cDNA is rat fibronectin. From Fig. 1, we can see expression of this mRNA was markedly increased by 24 h LPS/PMA stimulation of C6 glial cells. Figure 1 also indicates that chronic 50 mM ethanol treatment (9 days ethanol treatment before LPS/PMA stimulation plus 24 h ethanol/LPS/PMA treatment) seemed to reduce fibronectin mRNA expression in C6 glial cells after 24 h LPS/PMA stimulation.

Chronic Ethanol Treatment Reduces the Expression of Fibronectin mRNA in C6 Glial Cells after LPS/PMA Stimulation. To confirm differential expression of fibronectin mRNA in LPS/PMA-stimulated C6 glial cells under conditions with or without chronic ethanol treatment, we designed a new pair of primers (see under *Materials and Methods*) specific for rat fibronectin cDNA (Patel et al., 1987;
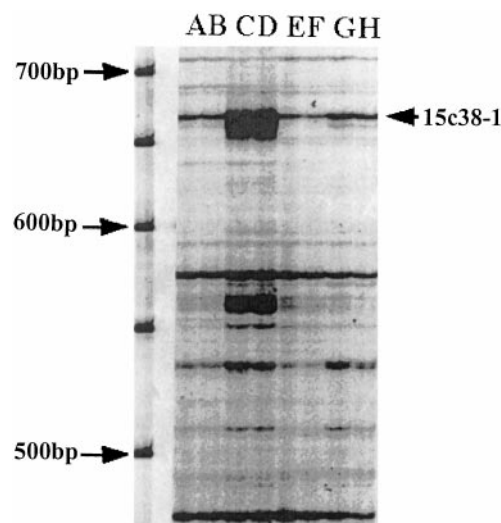

Fig. 1. Identification of gene expression changes in 24 h LPS/PMA-stimulated rat C6 glial cells treated chronically with 50 mM ethanol using DDRT-PCR. mRNA differential display was carried out using an anchored primer (5′- ACG ACT CAC TAT AGG GCT TTT TTT TTT TTG G-3′) and an arbitrary primer (5′- ACA ATT TCA CAC AGG AGC TAG CAG AC-3′). PCR products were resolved in a 5.6% denaturing polyacrylamide gel in the following order: lanes A and B, control of untreated and unstimulated C6 glial cells; lanes C and D, LPS/PMA-stimulated C6 glial cells; lanes E and F, ethanol-treated C6 glial cells; lanes G and H, LPS/PMA-stimulated C6 glial cells treated with ethanol. The band indicated by an arrowhead (designated as Band15c38–1) showed a marked change in expression in response to LPS/PMA stimulation and ethanol treatment. The online version of this article contains the complete gel. The online version is available at http://molpharm.aspetjournals.org

Schwarzbauer et al., 1987). We then used a semiquantitative RT-PCR technique with S12 cDNA as internal standard to measure fibronectin mRNA levels in total RNA from control and LPS/PMA-stimulated C6 glial cells with or without chronic 50 mM ethanol exposure. As seen in Fig. 2, basal fibronectin mRNA expression was found in control C6 glial cells, but LPS/PMA stimulation greatly enhanced the mRNA expression ($P < .01$) and chronic ethanol treatment clearly reduced the mRNA expression in LPS/PMA stimulated C6 glial cells ($P < .01$).

Time Course of Fibronectin mRNA Expression in Chronic Ethanol-Treated C6 Glial Cells after LPS/PMA Stimulation. To get further information about how ethanol affects fibronectin mRNA expression in C6 glial cells, we selected three different time periods (24 h, 5 days, and 9 days) for treatment of C6 glial cells with 50 mM ethanol. As above, we used semiquantitative RT-PCR techniques to detect the fibronectin mRNA in these cells. As seen in Fig. 3, 5
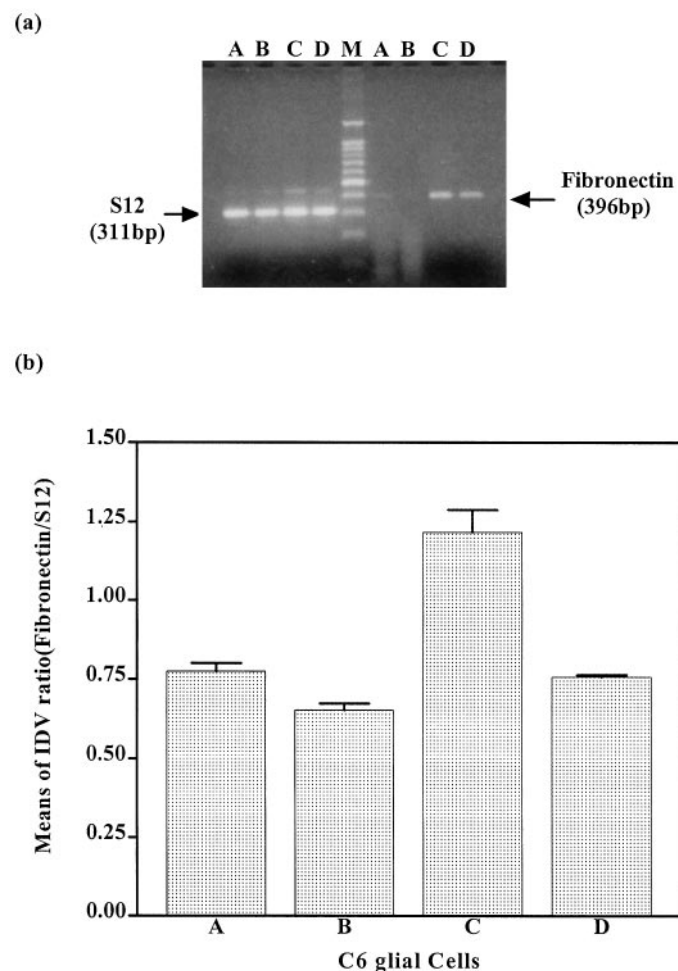
days' chronic 50 mM ethanol treatment is sufficient to decrease fibronectin mRNA expression in C6 glial cells after 24 h of LPS/PMA stimulation ($P < .01$). However, 24 h of ethanol treatment did not reduce fibronectin mRNA expression in C6 glial cells after LPS/PMA stimulation. Although fibronectin mRNA expression appears increased in unstimulated cells treated for 24 h with ethanol (Fig. 3), the difference was not statistically significant ($P > .05$).

Acute Effects of Ethanol Treatment on Fibronectin mRNA Expression in C6 Glial Cells after LPS/PMA Stimulation. As can be seen from the above results (Fig. 3), 24-h ethanol exposure did not reduce fibronectin mRNA expression in C6 glial cells and perhaps even enhanced its
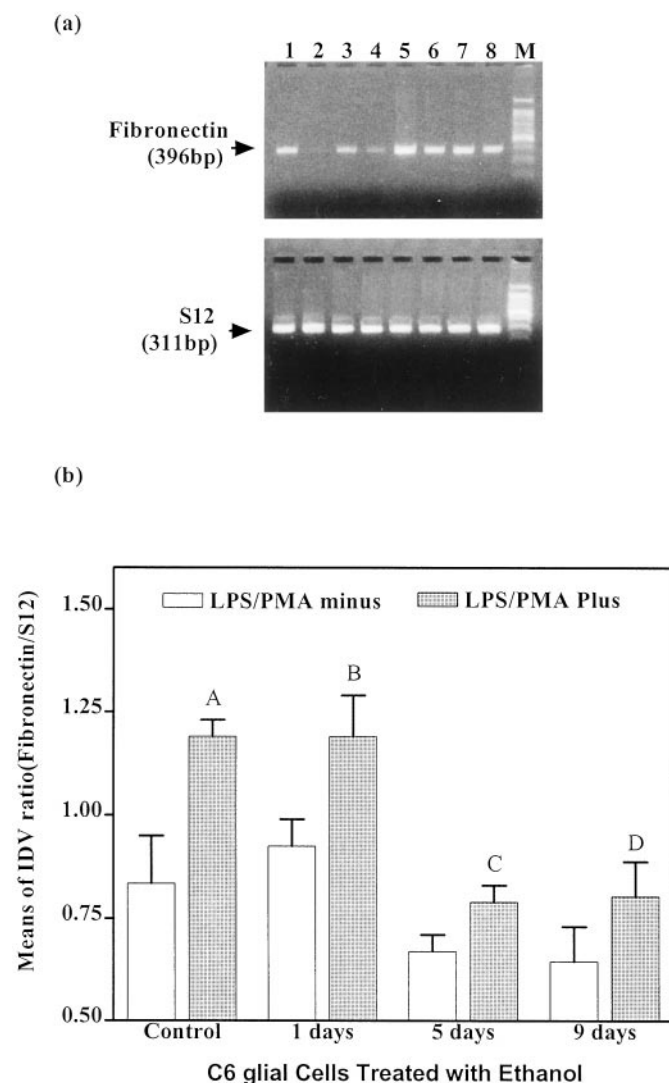

Fig. 2. Confirmation of the differential expression of fibronectin mRNA in 24 h LPS/PMA-stimulated rat C6 glial cells treated chronically with 50 mM ethanol using semiquantitative PCR. PCR was carried out using the primer pairs for fibronectin and S12 listed under *Materials and Methods*. a, PCR productions were resolved in a 1.2% agarose gel in the following order: lane A, LPS/PMA-stimulated C6 glial cells treated with ethanol; lane B, ethanol-treated C6 glial cells; lane C, LPS/PMA-stimulated C6 glial cells; lane D, control of untreated and unstimulated C6 glial cells; lane M, DNA molecular size markers. b, normalization of fibronectin PCR product to S12 product as IDV ratios (means with S.E.M., $n = 6$ per group). Group designations same as a. P values (Student's t test): D versus C, < 0.01; C versus A, < 0.01.

Fig. 3. The differential expression of fibronectin mRNA in 24-h LPS/PMA-stimulated rat C6 glial cells treated with ethanol for different times using semiquantitative PCR. PCR was carried out using the primer pairs for fibronectin and S12 listed under *Materials and Methods*. a, PCR productions were resolved in a 1.2% agarose gel in the following order: lanes 1, 3, and 5, LPS/PMA-stimulated C6 glial cells treated with 50 mM ethanol for 9 days, 5 days, and 24 h, respectively; lanes 2, 4, and 6, unstimulated C6 glial cells treated with 50 mM ethanol for 9 days, 5 days, and 24 h, respectively; lane 7, LPS/PMA-stimulated C6 glial cells; lane 8, control of untreated and unstimulated C6 glial cells; lane M, DNA molecular size markers. b, normalization of fibronectin PCR product to S12 product as IDV ratios (means with S.E.M., $n = 6$ per group). P values (Student's t test): A versus C, < 0.01; A versus D, < 0.05.

expression. Because the 24-h ethanol exposure coincided with the 24 h of LPS/PMA stimulation, this treatment was actually acute ethanol exposure. Further investigations were aimed at identifying acute effects of ethanol on fibronectin mRNA expression in C6 glial cells. C6 glial cells were stimulated with LPS/PMA in the presence of 0, 50 mM, or 200 mM concentrations of ethanol for 24 h, followed by semiquantitative RT-PCR to measure fibronectin mRNA in the cells. As shown in Fig. 4, acute treatment with 50 mM ethanol did not significantly decrease fibronectin mRNA expression, whereas inhibition was observed at 200 mM acute ethanol ($P < .05$) in these cells after LPS/PMA stimulation. Furthermore, acute ethanol exposure had no effect on fibronectin mRNA expression in unstimulated cells (Fig. 4).

Effect of Ethanol on Fibronectin Protein Expression in C6 Glial Cells after LPS/PMA Stimulation. The effect of 9 days' chronic treatment of C6 glial cells with 50 mM ethanol on fibronectin protein expression was also examined. After stimulation of control and chronic exposed cells with LPS/PMA for 24 h, total cellular protein was used for Western blotting of fibronectin protein. As seen in Fig. 5, 24 h stimulation with LPS/PMA increases fibronectin protein levels in C6 glial cells ($P < .05$), but chronic ethanol treatment

decreases fibronectin protein expression in these cells after LPS/PMA stimulation ($P < .05$). In contrast, when cells were acutely exposed to 50 mM ethanol for 24 h, there was no difference in fibronectin protein expression, either with or without LPS/PMA stimulation (Fig. 6).

Discussion

Ethanol at intoxicating blood levels is known to modify several properties and functions of astrocytes, but the mechanisms involved have not been well characterized, especially at the gene expression level. To facilitate such an analysis, we have used the technique of differential display of mRNA (Liang and Pardee, 1992) to investigate ethanol-induced changes in gene expression after activation of rat C6 glial cells with LPS/PMA.

The rat glial cell line C6 possesses extensive chemical and functional analogy to normal rat brain astrocytes, and has served as a useful astroglial cell model for decades, including studies on acute and chronic ethanol effects. This cell line
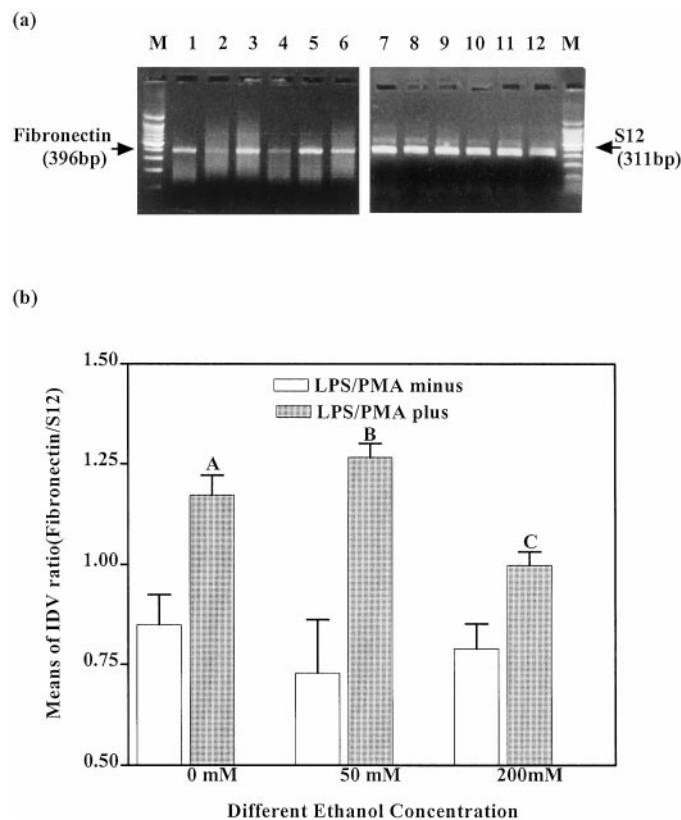

Fig. 4. The expression of fibronectin mRNA in 24 h LPS/PMA-stimulated rat C6 glial cells treated with different concentrations of ethanol using semiquantitative PCR. PCR was carried out using the primer pairs for fibronectin and S12 listed under *Materials and Methods*. a, PCR productions were resolved in a 1.2% agarose gel in the following order: lanes 1, 3, and 5: LPS/PMA-stimulated C6 glial cells treated with 200, 50, or 0 mM ethanol for 24 h; lanes 2, 4, and 6: unstimulated C6 glial cells treated with 200, 50, or 0 mM ethanol for 24 h; lanes 7 to 12 are results of S12 amplification for respective samples in lanes 1 to 6; lane M, DNA molecular size markers. b, normalization of fibronectin PCR product to S12 product as IDV ratios (means with SEM, $n = 6$ per group). *P* values (Student's *t* test): A versus C, < 0.05.
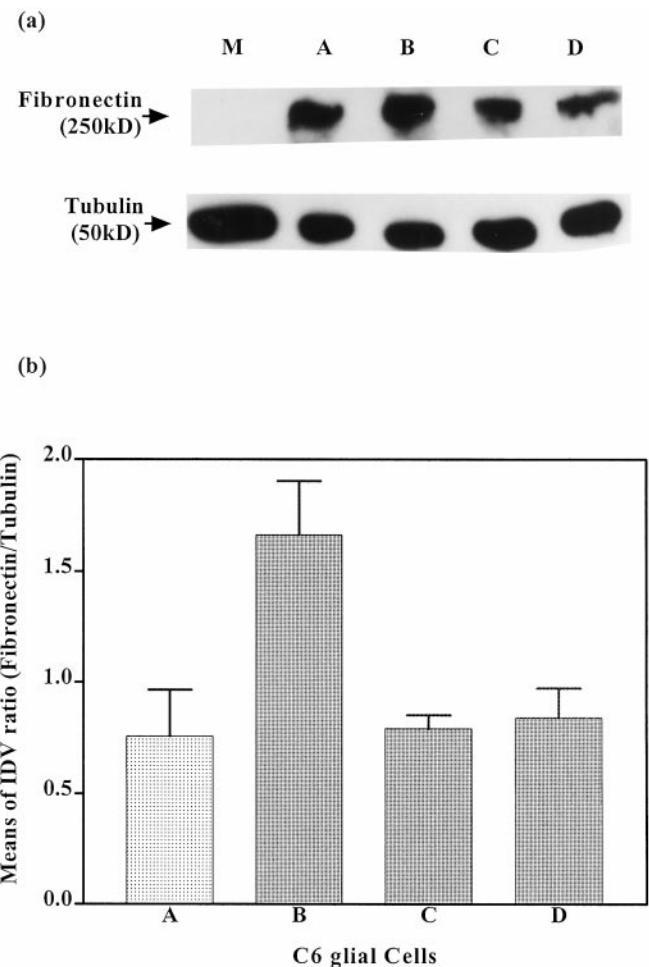

Fig. 5. Identification of the differential expression of fibronectin protein in 24 h LPS/PMA-stimulated rat C6 glial cells treated with 50 mM ethanol for 9 days using Western Blotting. Lane A, unstimulated and untreated C6 glial cells; lane B, LPS/PMA-stimulated C6 glial cells; lane C, C6 glial cells treated with ethanol; lane D, LPS/PMA-stimulated C6 glial cells treated with ethanol; lane M, β-tubulin protein standard. a, protein samples resolved in 5% SDS-polyacrylamide gel. b, normalization of fibronectin protein content to tubulin protein content as IDV ratios (means with S.E.M., $n = 6$ per group). *P* values (Student's *t* test): A versus B, < 0.05; B versus D, <0.05.

shares remarkable similarity with astrocytes in its sensitivity to ethanol. For example, ethanol dose dependently inhibits proliferation of C6 glial cells (Isenberg et al., 1992; Resnicoff et al., 1994) at the same concentrations that inhibit astrocyte proliferation. Other ethanol effects shared between astrocytes and C6 glial cells include inhibition of glucose uptake (Singh et al., 1999), free radical production (Gonthier et al., 1997), and reduction of glutamine synthase activity (Davies and Vernadakis, 1986), to name a few. Our previous work has demonstrated that iNOS induction by activated rat astrocytes and C6 glial cells is inhibited to similar degrees by acute and chronic ethanol exposure (Syapin, 1995; Syapin, 1996; Militante et al., 1997). Together, these studies demonstrate that C6 glial cells are a good model for the study of the effects of ethanol on astrocytes.

It is known that glial cell activation encompasses a much larger cellular response than iNOS induction alone (Benveniste and Benos, 1995). Therefore, to gain a more comprehensive understanding of ethanol-mediated gene regulation in activated astrocytic cells, we isolated and identified additional LPS/PMA-induced mRNAs from C6 cells whose ex-

Fig. 6. Identification of the differential expression of fibronectin protein in 24 h LPS/PMA-stimulated rat C6 glial cells treated with 50 mM ethanol for 24 h using Western blotting. Lane A, unstimulated and untreated C6 glial cells; lane B, LPS/PMA-stimulated C6 glial cells; lane C, C6 glial cells treated with ethanol; lane D, LPS/PMA-stimulated C6 glial cells treated with ethanol; a, protein samples resolved in 5% SDS-polyacrylamide gel. b, normalization of fibronectin protein content to tubulin protein content as IDV ratios (means with S.E.M., $n = 6$ per group). P values (Student's t test): A versus B, < 0.01; B versus D, >0.05.

pression seemed modulated by chronic exposure to 50 mM ethanol. From this study, fibronectin was identified as one of the major molecules whose expression is increased markedly at both mRNA and protein levels by LPS/PMA stimulation, and decreased by chronic ethanol treatment. It has repeatedly appeared in subsequent differential display analyses using the same set of experimental conditions (L. Q. Ren and P. J. Syapin, unpublished observations). Interestingly, lower concentrations of acute ethanol known to significantly inhibit iNOS expression (Syapin, 1995; Militante et al., 1997) do not seem to modify fibronectin expression, whereas chronic exposure seems to be equally effective. This suggests that down-stream mechanisms set into action by LPS/PMA have differential sensitivities to suppression by acute ethanol.

Fibronectin in the brain seems to play roles similar to those that it plays in other tissues (Mosher, 1984). It is involved in cell migration during organ development, in immune cell trafficking, during injury, and it is a component of the extracellular matrix. Changes in protein expression in the 40 to 50% range observed in this study have been found to occur in vivo and are of physiological significance (Saba, 1989; Thompson et al., 1992). Thus, one can speculate that ethanol-induced changes in fibronectin expression may contribute to some of the pathophysiological effects of chronic alcohol abuse, particularly the CNS morphological defects associate with the fetal alcohol syndrome and suppression of astrocyte mediated immune response (Aschner, 1998).

The consequences stemming from reduced fibronectin expression subsequent to chronic ethanol exposure are potentially many, given the wide array of processes in which it functions (Mosher, 1984). One likely consequence in the brain is a blunting of the CNS immune response by ethanol. In response to invasion by viruses and microorganisms, resident cells in the brain, such as astrocytes, can fully mount an immune response. The secretion of numerous cytokines by astrocytes is widely accepted to indicate that this cell actively participates in an integrative communicative pathway between resident immune cells of the CNS and those of the periphery (Aschner, 1998). During times of injury or infection they act to regulate, in concert with microglial cells, the recruitment and activity of infiltrating hematogenous cells through their expression of cytokines and proteases, protease inhibitors, adhesion molecules, and extracellular matrix components such as fibronectin. Activated lymphocytes seem to use fibronectin when interacting with tissues during inflammatory processes. Furthermore, the presence at the lymphocyte surface of components of different molecular weight precipitated by anti-fibronectin antibodies (Sundqvist et al., 1994) suggests that fibronectin or its fragments bind to components of the lymphocyte surface such as integrins to initiate additional cell signaling events (Giancotti and Ruoslahti, 1999).

The mechanism underlying ethanol inhibition of fibronectin expression remains to be discovered. Pellegatta et al. (1994) reported that iNOS is involved in enhancing fibronectin production by endothelial cells, but Trachtman et al. found that stimulation of rat mesangial cell nitric oxide release with γ-interferon and LPS resulted in reduced production of fibronectin. This change was reversed by the addition of L-N^{ω}-nitro-arginine methyl esther, a nonselective nitric-oxide synthase inhibitor (Trachtman et al., 1995). Thus, a possible connection may exist between induction of nitric
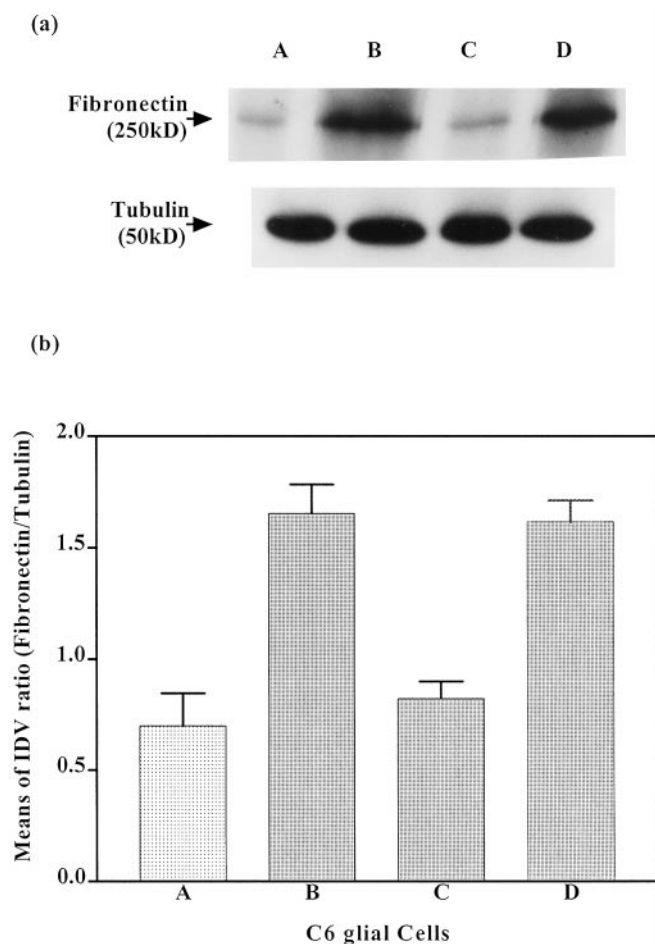
oxide synthesis and the production of fibronectin, but the exact interaction is possibly cell-type specific. Whether the reduced nitric oxide production resulting from our previously observed ethanol-induced suppression of iNOS expression is related to the present finding of reduced fibronectin expression is unknown.

In conclusion, we have discovered that fibronectin expression is up-regulated by LPS plus PMA exposure in C6 glial cells and that chronic (but not acute) treatment of C6 glial cells with 50 mM ethanol can reduce the enhanced fibronectin expression at both mRNA and protein levels. The specific consequences of these changes are unclear at this time, but could result in reduced cell signaling through components of the integrin network. The integrin signaling pathway is implicated in several cellular events, including cell adhesion, proliferation, and expression of antiapoptotic proteins (Giancotti and Ruoslahti, 1999). Thus, ethanol-induced reductions in inducible fibronectin expression may contribute to the observed effects of ethanol on these cellular events in brain astrocytes and other cell types.

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