Induction of Early-Immediate Genes by Tumor Necrosis Factor α Contribute to Liver Repair Following Chemical-Induced Hepatotoxicity

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We and others have shown that tumor necrosis factor α (TNF-α) expression is increased in the livers of experimental animals following exposure to the chemical hepatotoxin, carbon tetrachloride (CCl_4). Because TNF-α is involved in mediating inflammatory responses, its elevated expression is presumed to be associated with potentiating hepatotoxicity and/or aiding in liver repair processes. To study the role of TNF-α in chemical-induced hepatotoxicity, mice were administered neutralizing antibodies to TNF-α before administration of low, but hepatotoxic, doses of CCl_4. Antibody treatment prevented CCl_4-mediated increases in early-immediate gene expression associated with liver regeneration, including expression of c-*jun* and c-*fos* proto-oncogenes, as well as DNA binding of the activator protein-1 (AP-1) nuclear transcription factor. Hepatocyte proliferation following CCl_4 treatment was also reduced in anti–TNF-α antibody-treated mice, as evidenced by a lack of proliferating cell nuclear antigen (PCNA) staining. Antibody treatment slightly delayed liver repair processes, as evidenced by extending the period in which plasma liver enzyme levels were increased and hepatocellular necrosis could be observed. Consistent with the above observations, injection of recombinant TNF-α into control mice induced rapid expression of c-*jun* and c-*fos* proto-oncogenes. Taken together, these results indicate that TNF-α positively modulates liver recovery following CCl_4 exposure presumably by stimulating early-immediate genes involved in hepatic mitogenesis, a phenomenon also observed following partial hepatectomy. (HEPATOLOGY 1997;25:133-141.)

The liver, unlike most organ systems, is capable of regeneration following chemical or physical injury. Carbon tetrachloride (CCl_4), a classical hepatotoxicant, causes acute, reversible liver injury characterized by centrilobular necrosis, followed by hepatic regeneration and tissue repair. Injury results initially from the metabolism of CCl_4 to the highly reactive trichloromethyl radical, which initiates lipid peroxidation, resulting in damage to hepatocellular membranes.[1,2] Secondary liver injury following CCl_4 exposure occurs from inflammatory processes originating from products of activated Kupffer cells,[3] the resident macrophages in the liver. Activated Kupffer cells release chemoattractants and activators of neutrophils. The resulting neutrophil influx promotes extensive tissue damage via the release of reactive oxygen species and, consequently, inhibitors of reactive oxygen species attenuate CCl_4-induced hepatotoxicity.[4] Following CCl_4-induced injury, the liver immediately undergoes repair processes, evidenced by the release of growth factors such as epidermal and hepatocyte growth factors, and the entry of resting hepatocytes into the proliferating cycle.[5] Hepatocyte proliferation is preceded by increased expression of growth-related proto-oncogenes, c-*jun* and c-*fos*.[6-8] These genes encode for two groups of protein, fos-related antigen (fra) and Jun proteins, which compose the activator protein-1 (AP-1) transcription factors. AP-1 binds to specific DNA sequence in the promoter of numerous genes, particularly those involved in cell growth and differentiation.[9] Increasing evidence has indicated that cytokines, particularly tumor necrosis factor α (TNF-α), are important modulators of hepatic injury and repair. For example, in liver injury TNF-α proximally mediates acute phase responses, inflammatory cell infiltration, hyperlipidemia, free oxygen radical generation, fibrogenesis, and cholestasis.[10-12] On the other hand, TNF-α stimulates manganous superoxide dismutase, which, as a scavenger of superoxide radicals, is cytoprotective.[13] TNF-α is also a hepatocyte mitogen[14] or sensitizes hepaocytes to mitogens/growth factors and is required for liver regeneration following partial hepatectomy.[15]

Elevated TNF-α levels occur in various acute and chronic liver disease, including viral and alcoholic hepatitis, fulminant liver failure, alcoholic cirrhosis, biliary obstruction, and ischemia.[16-18] Recently, elevated TNF-α levels have been observed in experimental animals treated with CCl_4,[19] as well as other chemical hepatotoxicants including cadmium chloride,[20] acetaminophen,[21] and dimethylnitrosamine.[22] While TNF-α gene expression occurs in chemical-induced hepatotoxicity, neither its role in hepatotoxicity nor molecular events responsible for its expression have been well studied. The present study employs neutralizing antibodies to TNF-α in a rodent model of acute liver injury, repair, and regeneration to address these questions.

MATERIALS AND METHODS

Experimental Design. Female B6C3F1 (C57BL/6xC3H) mice (Charles River Breeding Laboratories, Portage, MI), weighing 22 to 28g and approximately 6 to 8 weeks old, were housed in polycarbonate cages containing hardwood chip bedding at room temperature (21 ± 2°C) on a 12-hour light/dark cycle. Animals were provided chow (NIH 31, Ziegler Bros. Inc., Gardner, PA) and tap water *ad libitum*. Animals were assigned to groups randomly by weight and administered a single intraperitoneal dose of CCl_4 (Sigma Chemicals Co., St. Louis, MO) following dilution in corn oil. Control animals received

Abbreviations: TNF-α, tumor necrosis factor α; CCl_4, carbon tetrachloride; AP-1, activator protein-1; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; RT, reverse transcription; cDNA, complementary DNA; mRNA, messenger RNA.

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only corn oil. Groups of mice received 25 μg/kg of recombinant murine TNF-α (R&D Systems, Minneapolis, MN) and were sacraficed 90 minutes later. Euthanasia was performed by CO_2 asphyxia using National Institute for Occupational Safety and Health–approved guidelines for the humane treatment of laboratory rodents, and liver and serum samples were collected for study.

Polyclonal antiserum to murine TNF-α was prepared by injecting New Zealand rabbits with recombinant murine TNF-α (a gift from Genzyme, Cambridge, MA) as previously described by Kayama et al.[20] Each mouse was injected intravenously with .2 mL of prefiltered nonimmune or immune serum 1 hour before CCl_4 administration. This dose effectively inhibits increases in serum TNF-α following endotoxin administration[20] and represents 250,000 (U) of antibody (1 unit equals the amount of antibody that neutralizes 1 bioactive unit of TNF). The activity is not altered by the addition of hepatocyte or epidermal growth factors (Luster, unpublished observations, 1993). Circulating half-life of anti–TNF-α antibodies is approximately 20 days (Luster, unpublished observation, 1993).

Reverse Transcriptase–Polymerase Chain Reaction Amplification. Approximately 100-mg samples of liver tissue were homogenized in 1.5 mL of Triazol solution (BRL, Gaithersburg, MD), and total cellular RNA was extracted according to the manufacturers' instructions. For the synthesis of complementary DNA (cDNA), 1.0 μg of total RNA from each sample was resuspended in a 20-μL final volume of the reaction buffer (25 mmol/L TRIS-HCl [pH 8.3] 37.5 mmol/L KCl, 10 mmol/L dithiothreitol, 1.5 mmol/L $MgCl_2$, 10 mmol/L of each deoxynucleotide triphosphate [Perkin Elmer, Cetus, Norwalk, CT], and 0.5 μg oligo d[T] 12-18 primer [BRL]). After the reaction mixture reached 42°C, 400 U of reverse transcriptase (BRL) was added into each tube and the samples were incubated for 30 minutes at 42°C. Reverse transcription (RT) was stopped by denaturing the enzyme at 99°C. The reaction mixture was diluted with distilled water to a final volume of 50 μL. Commercially available polymerase chain reaction (PCR) primers for c-*fos,* c-*jun,* and glyceraldehyde 3-phosphate-dehydrogenase were purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Primers for TNF-α were purchased from Stratagene (La Jolla, CA) and contained the following sequences:

TNF-α	sense (5′-ATGAGCACAGAAAGCATGATG-3′)
	antisense (5′-TACAGGCTTGTCACTCGAATT-3′).
c-*fos*	sense (5′-GAGCTGACAGATACACTCCAAGCG-3′)
	antisense (5′-CAGTCTGCTGCATAGAAGGAACCG-3′).
c-*jun*	sense (5′-GCATGAGGAACCGCATTGCCGCCTCCAAGT-3′)
	antisense (5′-CGCAAAGTCTGCCGGCCAATAGGCCGCT-3′).
G3PDH	sense (5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′)
	antisense G3PDH 5′-CATGTGGGCCATGAGGTCCACCAC-3′).

The size of amplified PCR products were 276 bp for TNF-α, 432 bp for c-*fos,* 460 bp for c-*jun,* and 983 bp for glyceraldehyde 3-phosphate-dehydrogenase.

Five-microliter aliquots of the synthesized cDNA were added to 45 μL of PCR mix containing 5 μL of 10× PCR buffer, 1 μL deoxynucleotides (1 mmol/L each), .5 μL of sense and antisense primers (0.15 mmol/L), and .25 μL of DNA polymerase (Gene Amp PCR kit, Perkin Elmer Cetus). The reaction mixture was covered with a wax tablet and amplification was initiated by 1 minute of denaturation at 94°C for 1 cycle, followed by multiple (range, 25-35) cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute using a GeneAmp PCR system 9800 DNA Thermal Cycler (Perkin Elmer Cetus). After the last cycle of amplification, the samples were incubated for 7 minutes at 72°C. For each set of primers, dilutions of cDNA were amplified for 20, 23, 25, 28, 30, 33, 35, and 38 cycles to define optimal conditions for linearity and to permit semiquantitative analysis of signal strength.[23] When necessary, the specificity of the PCR bands was confirmed by restriction-site analysis of the amplified cDNA, which generates fragments of the expected size (data not shown).

The amplified PCR products were electrophoresed at 75 V through 2% agarose gel (BRL) for 1 hour. The pBR322 *Hae* digest was used as a molecular-weight marker (Sigma Chemical Co.). The agarose gels were stained with Tris-borate-ethylenediaminetetraacetic acid buffer (ICN, Costa Mesa, CA) containing .5 mg/mL of ethidium bromide and photographed with type 55 Polaroid (Polaroid Corp., Cambridge, MA) positive/negative film. The relative amount of messenger RNA (mRNA) transcripts was determined using an Eagle Eye II Still Video System (Stratagene). Densitometric analysis of the captured image was performed on a Macintosh computer using the NIH image 1.54 analysis software. The area under the curve was normalized for glyceraldehyde 3-phosphate-dehydrogenase content.

Histology. The caudal portion of the left lobe from the liver of each mouse was removed and fixed by immersion in 10% neutral-buffered formalin. Fixed tissues were embedded in paraffin, cut into 6-mm sections, and placed on superfrost/plus microscope slides (Fisher, Pittsburgh, PA). Slides were stained with hematoxylin-eosin for light microscope examination.

Proliferating cell nuclear antigen (PCNA) staining was performed on formalin-fixed tissues as previously described,[24] using a biotin-streptavidin method (BioGenex Laboratories, San Ramon, CA).

For immunohistochemistry, frozen tissue sections (5- to 6-μm thickness) were stained with a neutralizing goat anti-mouse TNF-α antibody (R&D Systems) as a primary antibody and peroxidase-conjugated anti-goat immunoglobulin, and were color-developed using 3′3-diaminobenzidine (ABC Elite Kit, Vector, Burlingame, CA). The sections were counterstained with hematoxylin-eosin.

Serum Chemistry. Serum alanine transaminase and aspartate transaminase were quantified using an automatic chemical analyzer.[24,25] Serum sorbitol dehydrogenase was determined as described by Gerlach and Hiby.[26]

Liver Protein Extraction. Approximately 100 mg of liver tissue was homogenized in RPMI-1640 medium with 10% fetal bovine serum for 15 seconds and immediately frozen in liquid nitrogen. After 10 minutes, homogenates were thawed at 37°C and sonicated for an additional 15 seconds. Samples were then centrifuged at 13,000*g* for 10 minutes and supernatants were stored at −70°C until assayed.

TNF-α Analysis. TNF-α activity in liver preparation was measured by quantitating cytolytic activity against the L929 target cell line in the presence of 6 μg/mL of actinomycin D.[27] Cytolysis was calculated from the reduction in mean absorbance at 570 nm measured with a UV max kinetic microplate reader (Molecular Devices Corp., Menlo Park, CA) relative to control wells incubated with medium only. Reference wells containing known amounts of recombinant murine TNF-α (Genzyme, Cambridge, MA) were used to generate a standard curve. TNF-α in the serum was also quantitated using a commercial enzyme-linked immunosorbent assay kit (Genzyme).

Electrophoretic Mobility Shift Assay. Nuclear proteins were prepared according to the method of Dignam et al.[28] AP-1 DNA binding activity was determined by a modification of the gel electophoresis DNA binding assay described by Pennypacker et al.[29] Briefly, the AP-1 (22-mer; 5′-CTAGTGATGAGTCAGCCGGATC-3′) oligomer (Research Genetics, Huntsville, AL) was labeled with $^{32}\gamma$–adenosine triphosphate (New England Nuclear/Dupont, Boston, MA) using 6-10U of T4 polynucleotide kinase (USB/Amersham, Cleveland, OH). Binding reactions were performed at room temperature. The reaction

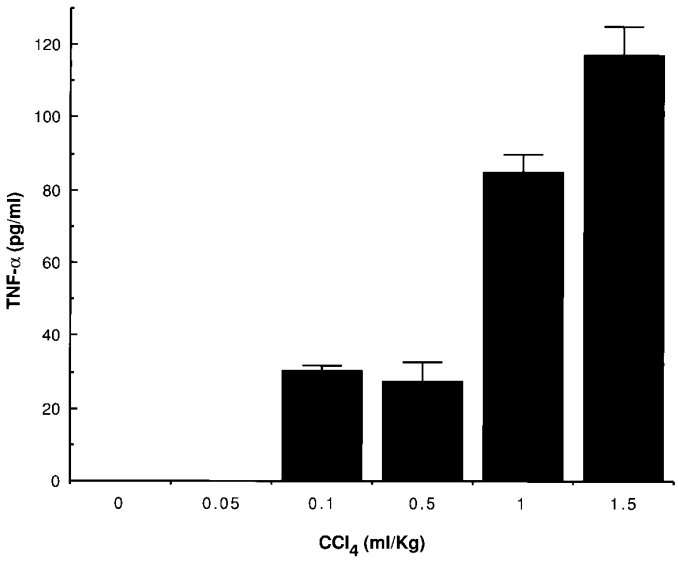

FIG. 1. TNF-α concentrations in the serum of mice following CCl_4 administration. Mice were treated with increasing concentrations of CCl_4 and serum obtained 24 hours after treatment. TNF-α was determined by enzyme-linked immunosorbent assay. Each value represents the mean ± SE of four mice per treatment group.

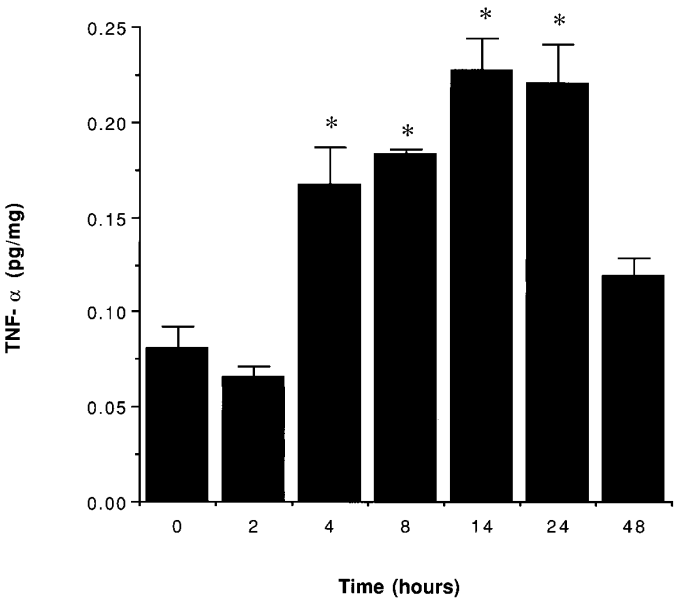

FIG. 2. Time course for TNF-α production in the liver. Mice were treated with .1 mL/kg of body weight of CCl_4 livers collected and processed as described in Materials and Methods. Each value represents the mean ± SE of four determinations. *Significantly different from control at $P < .05$.

mixture contained 40 μg of nuclear protein in 20 mmol/L HEPES (pH 7.9) 4% Ficoll 400, 50 mmol/L KCl, 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L dithiothreitol, .25 mg/mL bovine serum albumin, .1 mg/mL of sonicated salmon sperm DNA, and approximately .1 ng (2×10^5 cpm) of specific probe. Protein-DNA complexes were separated on a 4% nondenaturing polyacrylamide gel. The gels were electrophoresed at 125 V in 50 mmol/L TRIS-borate-ethylenediaminetetraacetic acid buffer, dried, and autoradiographed for approximately 3 hours. For characterization of DNA binding activity, the nuclear protein extracts were preincubated for 10 minutes before the addition of labeled probe with a 100-fold excess of unlabeled oligomer.

Statistical Analysis. All experiments were replicated and representative findings are shown. Statistical significance was determined by one-way ANOVA. When the F value was significant, the means were compared using Fisher post-hoc analysis. In all statistical comparisons, a P value $< .05$ was used to indicate a significant difference.

RESULTS

Preliminary studies were conducted to determine the effect of CCl_4 administration on TNF-α gene expression and secretion. As shown in Fig. 1, there was a dose-related increase in serum TNF-α with easily discernible levels (35 pg/mL) observed 24 hours after a dose of .1 mL/kg body weight of CCl_4. This dose was not lethal and allowed for full liver recovery, as evidenced by a return to control levels of liver-associated plasma enzymes by day 5 (data not shown). Subsequently, this dose was used in all further experiments. Because serum TNF-α concentrations are not always representative of changes that occur in specific organs or tissues, the livers were removed at various times following CCl_4 administration, homogenized, centrifuged, and concentrations of TNF-α were determined in the soluble fraction following normalization for total protein (Fig. 2). Livers from untreated mice contained low basal levels of TNF-α, while increased levels were observed within 4 hours following CCl_4 exposure and were maximum (threefold) between 14 and 24 hours, before returning to control levels by 48 hours. TNF-α mRNA transcripts isolated from livers of treated and control mice were examined by RT-PCR analysis to determine whether the increase in TNF-α was associated with relative changes in gene expression (Fig. 3). As with secreted TNF-α, livers from vehicle-treated mice expressed low constitutive levels of TNF-α mRNA, which were rapidly increased following exposure to CCl_4. Low basal levels of TNF-α mRNA have been reported previously in mouse liver,[30] and are assumed to be associated with normal physiological processes. The time-course for changes in TNF-α mRNA expression indicated a slight increase within 2 hours following CCl_4 treatment, which was statistically significant at the 8-hour time point. This was followed by a maximum increase at 14 hours and a slow return to control levels within 1 week.

To determine the localization of TNF-α protein in the liver following CCl_4 treatment, immunostaining was conducted. Immunoreactive TNF-α was not detected in control livers (Fig. 4A). In livers from mice treated with CCl_4 14 hours before killing, TNF-α was localized to centrilobular areas (Fig. 4B), which corresponded to the zones of hepatocellular necrosis seen on hematoxylin-eosin–stained sections (not shown). The granular cytoplasmic staining pattern in the centrilobular areas appeared to be localized to Kupffer cells and hepatocytes undergoing coagulative necrosis. There was no detectable staining in noncentrilobular areas.

Neutralizing antibodies to TNF-α were administered before CCl_4 to help establish its role in hepatotoxicity. As compared with vehicle-treated mice (Fig. 5A), CCl_4 administration led to the typical histological changes associated with acute CCl_4-induced hepatotoxicity, characterized by centrilobular hepatic necrosis and mild inflammation that peaked at 24 hours (Fig. 5B). Within 48 hours following CCl_4 treatment, the liver architecture had returned essentially to normal (Fig. 5C). Histological examination of liver sections from CCl_4-exposed mice admin-

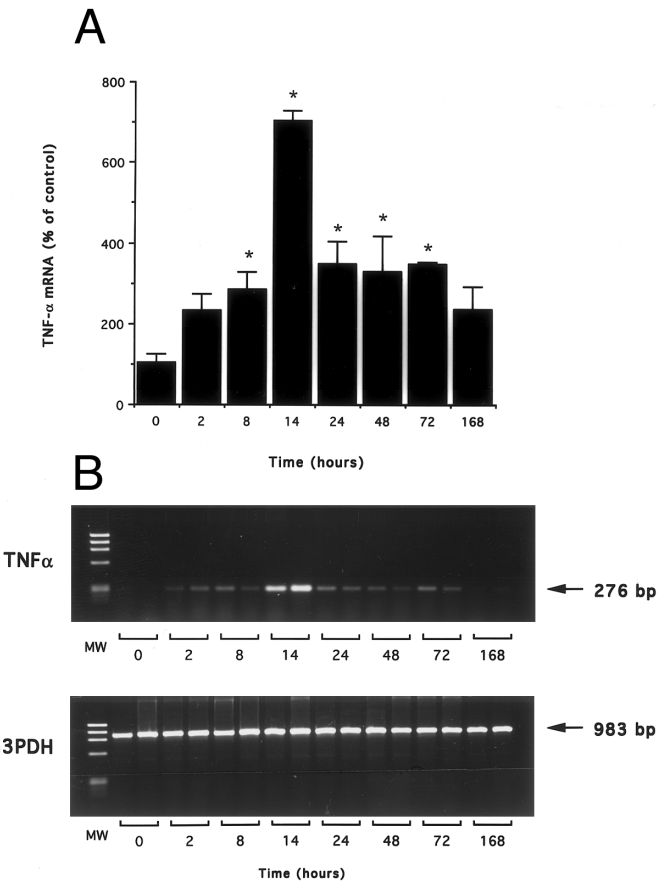

FIG. 3. Amplification of hepatic TNF-α mRNA by RT-PCR from livers of CCl_4-treated mice. Mice were treated with .1 mL/kg of CCl_4, and liver samples were obtained at times shown. Using cDNA equivalents of 10-ng RNA, samples were amplified for 35 cycles using specific TNF-α primers. Gels were scanned with a computerized laser densitometer, and the area under the curve was expressed as a percent of the control. The density of each amplified cDNA band for TNF-α was normalized relative to the density of each corresponding band for G3PDH. MW, molecular-weight marker (pBr322/*Hcc* III digest).

istered anti–TNF-α antibodies revealed centrilobular necrosis similar to CCl_4-treated mice at the 24-hour time point. However, antibody treatment delayed the recovery, as necrosis was still evident at 72 hours (Fig. 5D). After 7 days, liver architecture from all treatment groups appeared normal (Fig. 5E). Table 1 shows the change in serum concentration of liver-associated enzymes, aspartate transaminase, alanine transaminase, and sorbitol dehydrogenase in mice following the administration of CCl_4 and neutralizing anti–TNF-α antibodies. Similar to histological changes, serum liver enzymes markedly increased up to 24 hours following CCl_4 exposure before rapidly returning to control levels. Liver-associated plasma enzyme profiles in CCl_4-treated mice administered anti–TNF-α antibodies revealed a profile similar to those treated with CCl_4, except that the return to control levels was delayed, as slightly elevated levels were still observed at the 72-hour time point. There were no changes in liver histology or serum enzyme concentration in animals treated with preimmune serum or anti–TNF-α antiserum alone at any of the time points examined (data not shown).

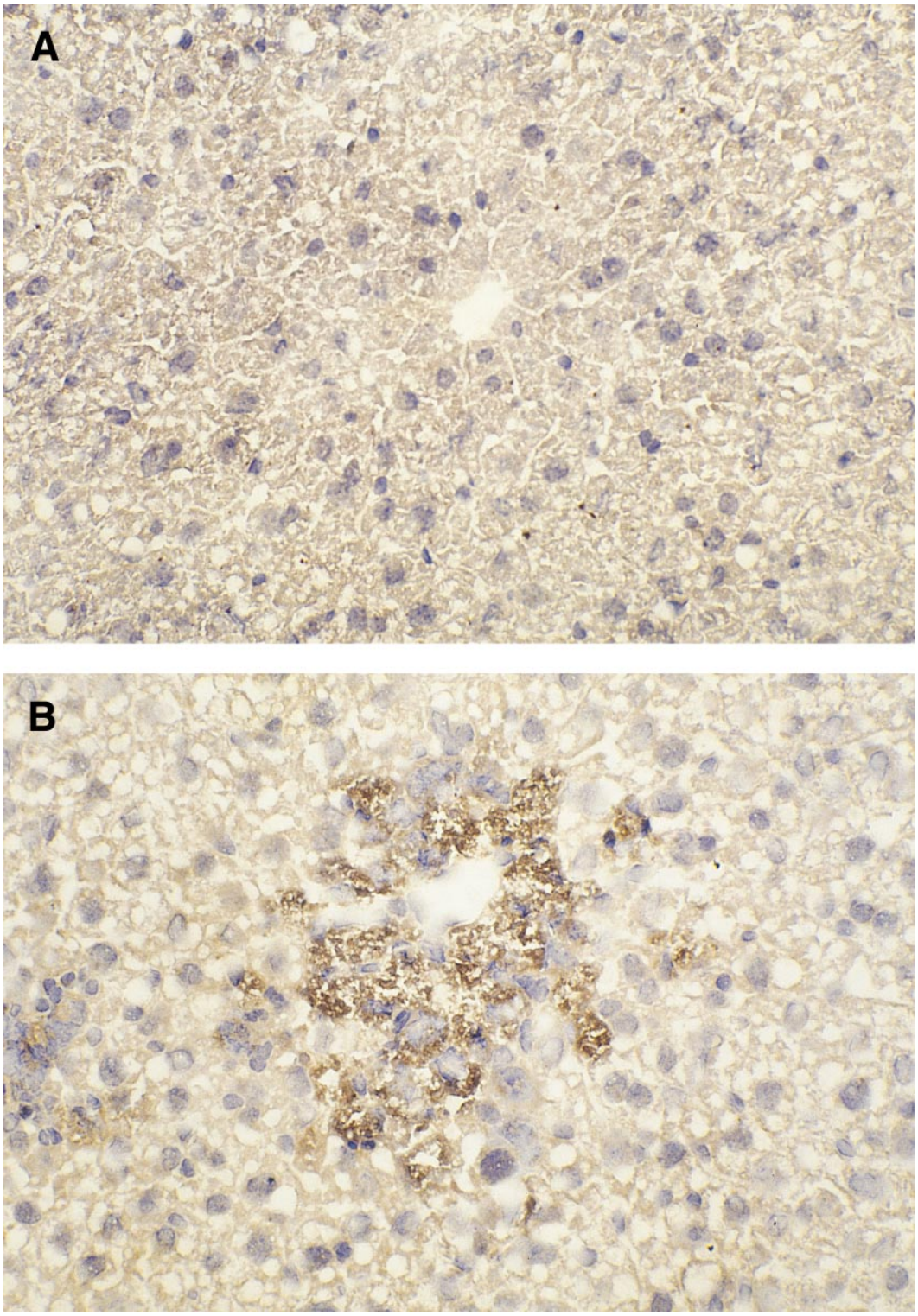

FIG. 4. Immunostaining for TNF-α. Frozen liver sections from mice treated 14 hours earlier with (A) corn oil or (B) CCl_4 were incubated with anti–TNF-α antibody and prepared as indicated in Materials and Methods. Note the intense brown staining for TNF-α localized in the centrilobular region of the livers from CCl_4-treated mice. Little staining was evident in the livers of control mice. The nuclei were counterstained blue with hematoxylin-eosin.

Because CCl_4 exposure is associated with liver damage as well as repair, characterized by hepatocyte proliferation, and TNF-α is associated with hepatic proliferation,[31] the effect of anti–TNF-α antibody treatment on PCNA staining was examined. Forty-eight hours following CCl_4 exposure, a marked increase in the percent of hepatocyte in S-phase could be detected by nuclear staining (Fig. 6B) compared with control liver. Nuclear PCNA staining was nearly reduced to control levels in mice pretreated with anti–TNF-α antiserum (Fig. 6C and D). However, hepatocytes from the antibody-treated group revealed a faint dispersed nuclear staining pattern consistent with cells in G-1 phase. Neither administration of preimmune sera nor anti–TNF-α antiserum alone affected PCNA staining (data not shown).

Hepatocyte proliferation is associated with binding of the AP-1 nuclear transcription factor to cis-acting elements in the promoter region of various genes. To examine the changes in AP-1 DNA binding activity, nuclear proteins isolated from livers of CCl_4-treated mice were examined using an electrophoretic mobility shift assay. AP-1 DNA binding activity was detectable at low levels in the liver from control mice, but was significantly increased within 2 hours following CCl_4 exposure (Fig. 7). Administration of anti–TNF-α antibodies, before CCl_4 exposure almost completely prevented the increase in DNA binding activity, indicating that TNF-α mediates activation of AP-1 DNA binding.

AP-1 binding positively regulates the transcription of c-*jun* and c-*fos* proto-oncogenes, which encode for proteins that

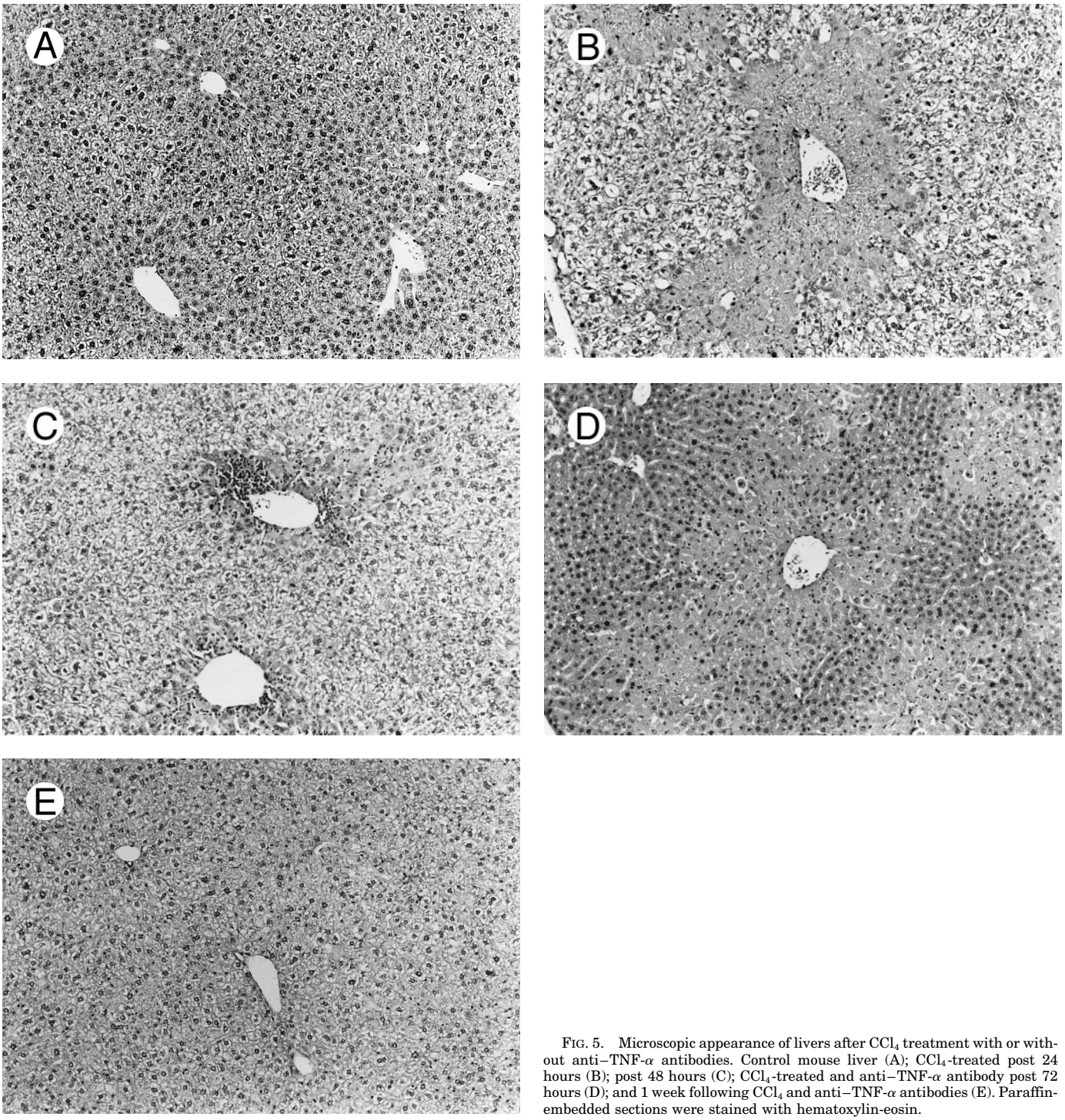

FIG. 5. Microscopic appearance of livers after CCl_4 treatment with or without anti–TNF-α antibodies. Control mouse liver (A); CCl_4-treated post 24 hours (B); post 48 hours (C); CCl_4-treated and anti–TNF-α antibody post 72 hours (D); and 1 week following CCl_4 and anti–TNF-α antibodies (E). Paraffin-embedded sections were stained with hematoxylin-eosin.

TABLE 1. Effect of Anti–TNF-α Antibodies on Liver-Associated Plasma Enzyme Levels in CCl_4-Treated Mice

		Hours After CCl_4						
Enzyme (IU/L)	Anti-TNF-α	0	2	14	24	48	72	168 days
AST	—	48 ± 15	51 ± 19	1,645 ± 1,141	3,710 ± 1,435	129 ± 27	43 ± 17	33 ± 3
	+	61 ± 16	43 ± 6	1,970 ± 662	3,650 ± 1,525	770 ± 90*	233 ± 12*	43 ± 8
ALT	—	36 ± 23	36 ± 10	2,150 ± 1,702	7,040 ± 2,354	295 ± 75	78 ± 14	28 ± 3
	+	34 ± 7	34 ± 2	2,265 ± 600	7,125 ± 2,250	1,740 ± 857*	114 ± 11*	32 ± 5
SDH	—	27 ± 13	18 ± 2	2,005 ± 1,546	4,810 ± 1,307	350 ± 75	92 ± 19	25 ± 1
	+	25 ± 12	26 ± 1	1,940 ± 593	4,893 ± 1,397	2,613 ± 881*	140 ± 6*	26 ± 4

NOTE. Animals received .1 mL of CCl_4/kg. Anti–TNF-α antibodies (.2 mL per mouse) were administered 1 hour before CCl_4 administration. Values represent means ± SD; n = 4.

Abbreviations: AST, aspartate transaminase; ALT, alanine transaminase; SDH, sorbitol dehydrogenase.

* Significantly higher than the CCl_4-treated animals at indicated time point at $P < .05$.

compose AP-1. The relative expression of c-*jun* and c-*fos* mRNA transcripts determined by RT-PCR were increased within 2 hours following CCl_4 treatment (Fig. 8). Prior administration of anti–TNF-α antiserum completely prevented the increase in c-*fos* mRNA expression and significantly reduced c-*jun* mRNA transcripts. The expression of G3PDH, a constitutive expressed gene, was analyzed to allow determination of the relative expression of c-*jun* and c-*fos*.

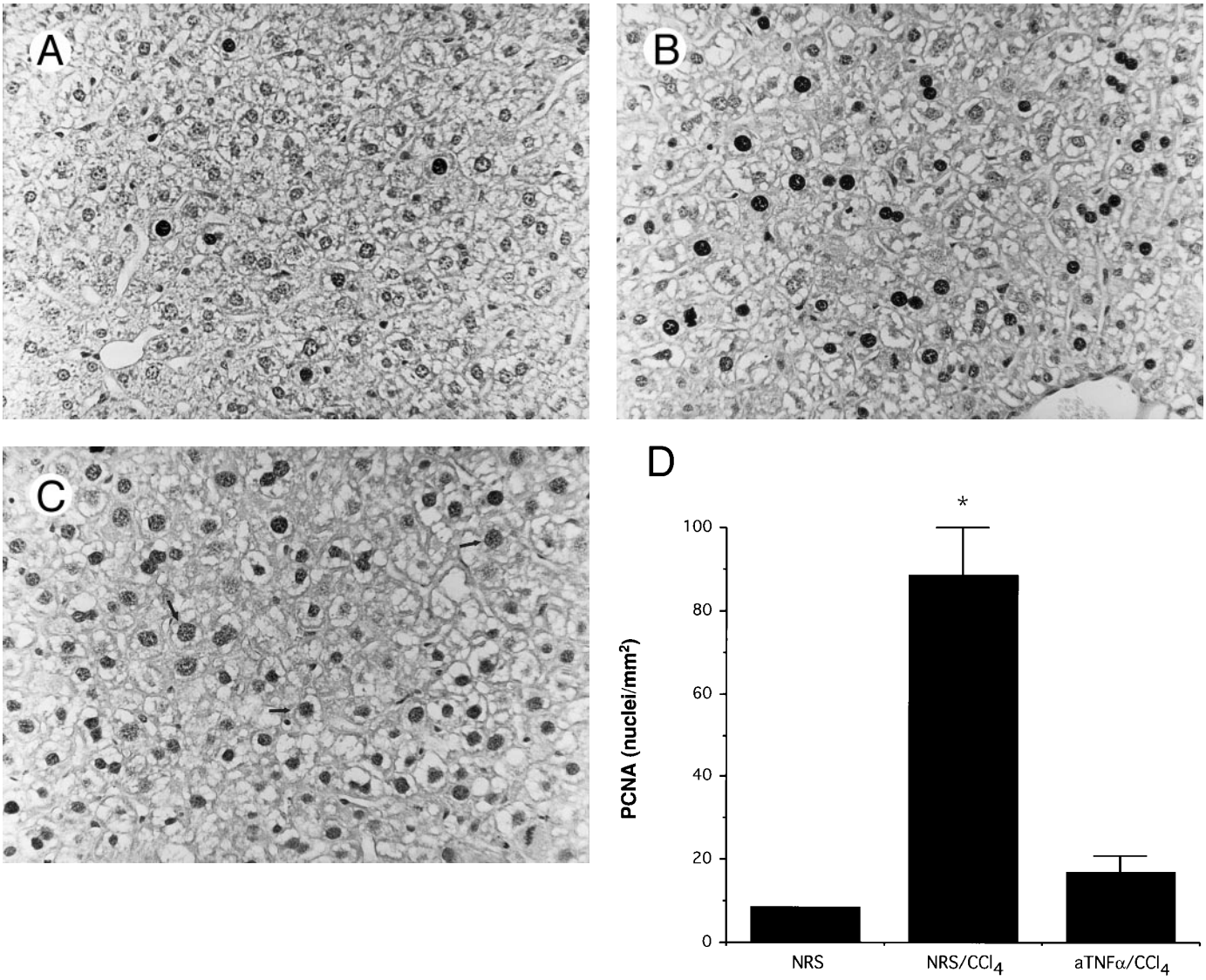

FIG. 6. PCNA immunostaining. Mice were administered either (A) normal rabbit serum, (B) CCl_4, or (C) CCl_4 plus anti–TNF-α antibodies as indicated in Materials and Methods. S-phase nuclei are indicated by the arrow. (D) Quantification of nuclei in the S-phase of the cell cycle (per square millimeter) from tissue stained with PCNA antibody as described; *significantly different from control at $P < .05$. Tissues were collected 48 hours after administration of .1 mL/kg of CCl_4 with or without anti–TNF-α antibody pretreatment.

Because neutralizing antibodies to TNF-α prevent CCl_4-induced c-*jun* and c-*fos* activity, we determined whether TNF-α could directly activate these proto-oncogenes. A single dose of recombinant murine TNF-α was administered to mice and livers collected for RT-PCR analysis. As shown in Fig. 9, TNF-α administration induced an increase in both proto-oncogenes within 90 minutes following injection.

DISCUSSION

In addition to CCl_4,[19] exposure to a number of hepatotoxicants, including acetaminophen,[21] dimethylnitrosamine,[22] cadmium chloride,[20] and ethanol,[17] increase hepatic TNF-α expression. Because TNF-α is involved in inflammatory responses, it has been assumed that its expression is associated with the induction of the hepatic acute phase response. In the present studies, the role of TNF-α in CCl_4-induced hepatotoxicity was studied in an animal model using neutralizing doses of anti–TNF-α antibodies. The results were consistent with the notion that TNF-α also serves as a positive regulator for regenerative processes. This was evidenced by a delayed, albeit modest, recovery in liver architecture and in serum enzymes in CCl_4-exposed mice pretreated with neutralizing antibody. More significantly, neutralizing anti–TNF-α antibodies prevented cell proliferation, which occurs following CCl_4-induced hepatotoxicity. Consistent with the latter observation, increases in early-immediate gene expression, usually associated with cell proliferation and differentiation (i.e., AP-1 activation and c-*jun* and c-*fos* expression), was prevented or reduced by administration of neutralizing TNF-α antibodies.

Liver regeneration is a complex, tightly orchestrated process that involves proliferation of hepatocytes and nonparenchymal cells, in addition to stromal cell reconstitution and remodeling.[32,33] These events are regulated by cell-to-cell contact as well as by growth factors, such as epidermal and hepatocyte growth factors. The importance of TNF-α as a regulator of hepatic repair was recently demonstrated in rats in which administration of TNF-α antibodies before partial hepatectomy prevented hepatocyte and nonparenchymal cell proliferation and liver regeneration.[15] This is consistent with the reported ability of TNF-α to increase hepatic DNA and RNA synthesis and hepatic mitosis.[31,34] On the other hand, TNF-α has also been reported to be a proximal mediator of several forms of liver injury.[10] In this respect, Czaja et al.[35] recently reported that TNF-α contributes to hepatotoxicity associated with CCl_4. We were unable to confirm this observation and believe the differences are related to the high dose (5 mL/kg of body weight) of CCl_4 used in their studies, which would result in extensive hepatic necrosis and production of TNF-α. In contrast, the dose used in our studies (.1 mL/kg of body weight) allowed all animals to fully recover within 1 week following treatment and provided only moderate increases in TNF-α. In thymocytes, high levels of TNF-α induce expression of TNF-R1 receptors (55 kd), which are responsible for cytotoxic events, while low concentrations of TNF-α stimulate TNF-R2 receptor (75 kd) expression, which are involved in cell proliferation.[36] The occurrence of similar phenomena in the liver would be consistent with the present observations.

PCNA expression is closely correlated with DNA synthesis. The antigen first appears in a diffuse pattern in the nucleus during late G1-phase, becomes maximal during S-phase, and declines during the G2 and M phases.[37,38] PCNA staining induced by CCl_4 treatment was concentrated in centrilobular areas, where cell injury was histologically prevalent and where immunostaining of TNF-α occurred. PCNA staining, characteristic of cells in S-phase, was not apparent following pretreatment with antibodies to TNF-α in CCl_4-exposed mice. There was, however, a preponderance of cells with diffuse staining nuclei, characteristic of G1-phase, and would

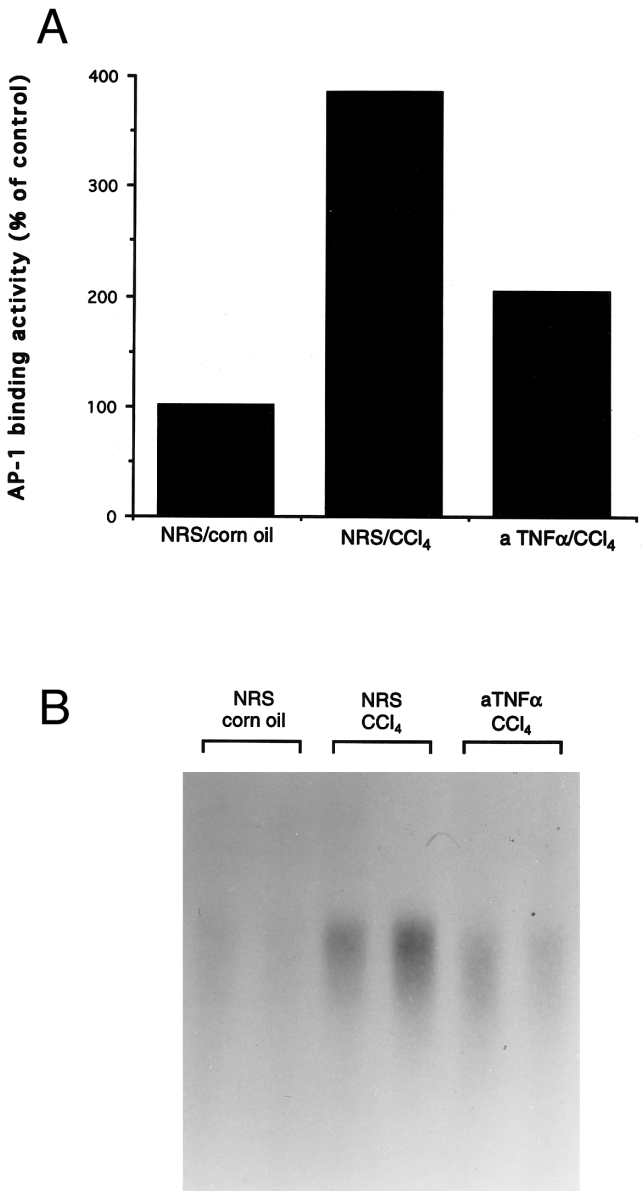

FIG. 7. Expression of AP-1 transcription factor in the mouse liver. Mice were treated with CCl_4 and anti–TNF-α antibodies, and liver samples were obtained after 2 hours as described in Materials and Methods. (A) Nuclear protein extracts (40 μg) were analyzed by a gel shift assay. (B) The film was scanned with a computerized laser densitometer, and the area under the curve was expressed as a percent of the control. NRS, normal rabbit serum.

be consistent with reports that TNF-α acts as a hepatic mitogen.[34] In contrast to PCNA staining, the delay in recovery of liver architecture and liver-associated serum enzymes caused by anti–TNF-α antibody treatment was moderate, because recovery was delayed by only 2 to 3 days. Whether this phenomenon is associated with inhibition of hepatic regeneration is uncertain.

The molecular regulation of the expression of many genes is controlled by binding of transcription factors to cis-acting elements located in the promoter region. The proto-oncogenes, c-*jun* and c-*fos,* encode for Jun and Fos proteins, respectively, which are components of the AP-1 nuclear transcription factor family. AP-1 participates in cell proliferation and differentiation by positively or negatively regulating the transcription of genes that contains AP-1 binding sites including c-*jun* and c-*fos* expression.[39,40] Recently, it has been reported that c-*jun* and c-*fos* expression are elevated in livers

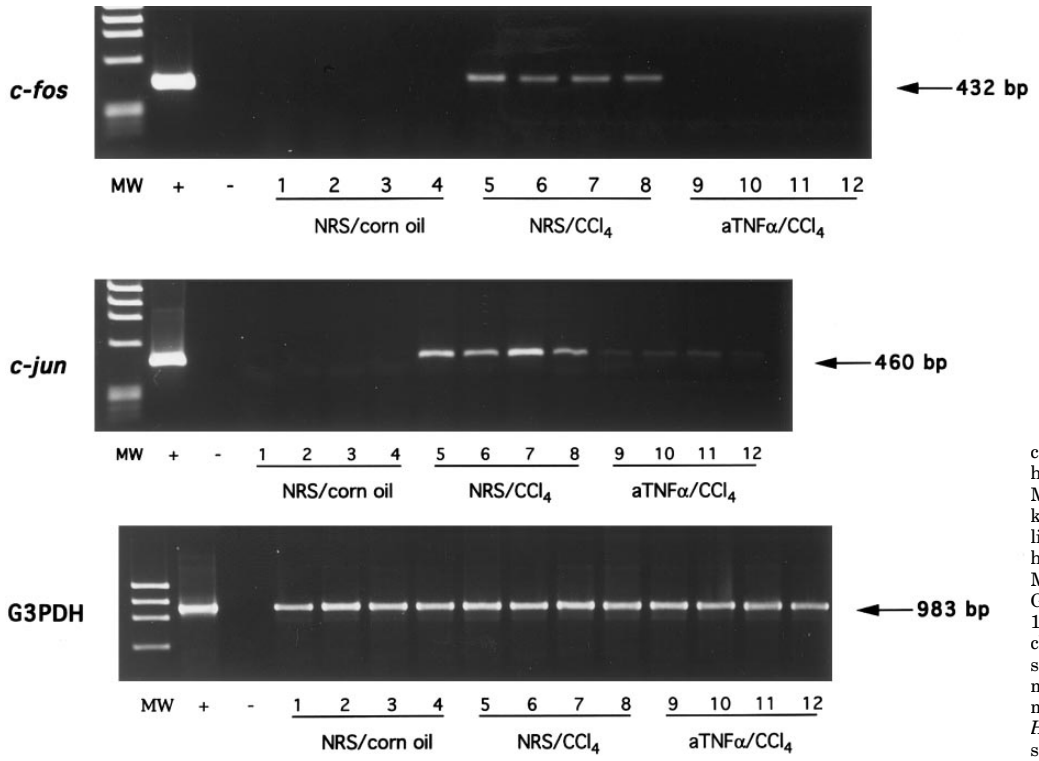

FIG. 8. Amplification of c-*jun* and c-*fos* mRNA transcripts by RT-PCR of hepatic RNA from CCl_4-treated mice. Mice were treated with CCl_4 .1 mL/kg and anti–TNF-α antibodies, and liver samples were obtained after 2 hours as described in Materials and Methods. (A) c-*fos;* (B) c-*jun;* and (C) G3PDH. Using cDNA equivalents of 10-ng mRNA, PCR was run for 35 cycles using specific primers. Liver samples were tested from four animals for each treatment group. MW, molecular-weight markers (pBr322/*Hcc* III digest); NRS, normal rabbit serum.

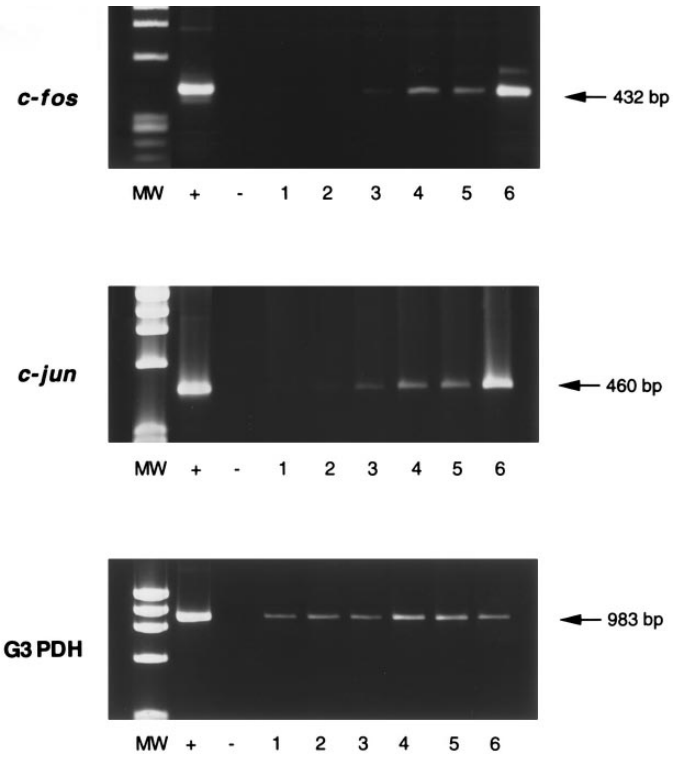

FIG. 9. Amplification of c-*jun* and c-*fos* transcripts by RT-PCR of hepatic RNA from TNF-α–treated mice. Mice were administered 25 μg/kg of recombinant murine TNF-α, and livers were collected after 90 minutes. Using cDNA equivalents of 10-ng RNA, samples were amplified for 35 cycles. Lanes 1-2, liver samples from two randomly selected mice injected with saline; and lanes 3-6, liver sample from a randomly selected mouse that received TNF-α. MW, molecular-weight standard (pB4322/*Hcc*III digest). +, commercial positive control; −, negative (water) control.

of rodents following partial hepatectomy[41] or treatment with necrogenic doses of CCl_4.[6,42] Additionally, c-*jun* was found to be expressed in the absence of c-*fos* following exposure to hepatic mitogens such as lead nitrate or ethylene dibromide.[43] Some of these treatments have been associated with liver cell proliferation, as indicated by increased mitotic indices. Our studies demonstrate that hepatic c-*jun* and c-*fos* are expressed within 30 minutes following CCl_4 administration, and this is accompanied by increased DNA binding of the AP-1 transcription. Furthermore, TNF-α mediates this response, as pretreatment with neutralizing antibodies prevents immediate-early gene expression and AP-1 DNA binding activity. Consistent with these observations, Diehl et al.[44] have recently shown that TNF-α is responsible for c-*jun* expression after partial hepatectomy in rats. Furthermore, the authors provide evidence that TNF-α stimulates c-*jun* activity by stimulating a novel c-*jun* kinase (JnK), which phosphorylates serine residues in the c-*jun* activation domain, influencing the ability to bind the AP-1 consensus motifs in cis-acting elements. While maximum TNF-α expression did not occur until 14 hours after CCl_4 treatment, slight increases, albeit not statistically significant, were observed in mRNA expression even at the earliest time point examined following CCl_4 treatment (2 hours). Thus, it is possible that low concentrations of TNF-α are provided immediately following CCl_4 treatment, which are sufficient to induce AP-1 activities.

Taken together, our studies indicate that TNF-α is produced in the liver following exposure to CCl_4 and plays a role in initiating the hepatic repair process. This may occur via the ability of TNF-α, or perhaps TNF-α–inducible mediators, to induce growth-related proto-oncogenes, such as c-*jun* and c-*fos,* which allow for AP-1 DNA binding and additional gene expression. Because neutralizing TNF-α antibodies appear to prevent cells from entering the S-phase, but not G1, it would appear that other trophic factors (e.g., epidermal and hepatocyte growth factors) are required for TNF-α to function as a complete mitogen. In this respect, it has been shown that,

in human fibroblasts, TNF-α acts to stimulate expression of epidermal growth factor receptors.[45]

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