

# Accumulation and Cellular Localization of Fibrinogen/Fibrin During Short-term and Long-term Rat Liver Injury

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**Background/Aims:** During liver fibrosis, there is a putative pacemaker role of fibronectin. Fibrinogen is closely linked to fibronectin during clotting processes. The aim of this study was to show fibrinogen gene expression during liver damage. **Methods:** Fibrinogen/fibrin deposition in damaged livers was studied by immunohistology. Fibrinogen gene expression was analyzed *in vivo* in a model of CCl<sub>4</sub>-induced rat liver damage and *in vitro* in isolated liver cells by means of Northern blot analysis and *in situ* hybridization. **Results:** Immunohistology showed striking amounts of fibrinogen and fibrin deposits in pericentral necrotic areas (short-term damage) and within fibrotic septa (long-term damage). Total RNA extracted from short-term-damaged livers contained an increased fibrinogen messenger RNA level. By *in situ* hybridization, fibrinogen transcripts were localized in cells of the nonnecrotic areas (short-term damage) and outside fibrotic septa (long-term damage). *In vitro* studies showed fibrinogen de novo synthesis restricted to hepatocytes. **Conclusions:** The results show fibrinogen/fibrin deposition during short-term liver injury and liver fibrogenesis, which may suggest the involvement of a “clotting-like process” in short-term liver damage and liver fibrosis. The data might indicate that fibrin/fibronectin constitute a “provisional matrix,” which affects the attraction and proliferation of inflammatory and matrix-producing cells.

Liver fibrogenesis represents the uniform response of the liver to toxic, infectious, or metabolic agents and is characterized by an increased synthesis and altered deposition of newly formed extracellular matrix components. Fibrogenesis is mostly initiated by hepatocyte damage leading to recruitment of inflammatory blood cells and activation of liver Kupffer's cells (KCs) with subsequent release of cytokines and growth factors.<sup>1</sup> Ito cells seem to be the primary target cells for inflammatory stimuli.<sup>2</sup> Ito cells proliferate, transform into myofibroblast-like cells (“activated” Ito cells), and synthesize large amounts of connective tissue components such as collagen type I, III, and IV<sup>3,4</sup>; cellular fibronectin<sup>5</sup>; laminin<sup>6</sup>; entactin<sup>7</sup>; tenascin<sup>8</sup>; undulin<sup>9</sup>; and proteoglycans.<sup>10</sup> Endothelial cells (ECs) contribute to the synthesis of

cellular fibronectin,<sup>11</sup> laminin,<sup>12</sup> collagen type IV,<sup>13</sup> and entactin.<sup>7</sup>

In liver fibrosis, connective tissue components are changed quantitatively but not qualitatively; in other words, novel matrix components, except for a variant of type I collagen, are not detected. The composition in expanded portal areas, septa, and cirrhotic nodules is similar to that of the normal portal tracts and consists of collagen I, III, IV, V, and VI, various members of the structural glycoprotein family, and the major subclasses of glycosaminoglycans. Deposition of collagen type IV, perlecan, laminin, and entactin in the perisinusoidal space results in the formation of a complete basement membrane, a process called “collagenization” or “capillarization” of the sinusoids. Capillarization of the sinusoids is an early event in liver fibrogenesis and seems to be of crucial importance for liver function because the exchange of macromolecules between the sinusoidal blood and hepatocytes is fundamentally affected.<sup>14–17</sup> Current research seeks to understand the mechanism underlying early fibrosing injury. A pacemaker function of fibronectin during liver fibrogenesis was suggested because fibronectin accumulation precedes the increase of other connective tissue proteins.<sup>18</sup>

Liver fibrogenesis is suggested to be a paradigm for wound healing processes, which include the cross-linkage between fibrin and fibrinogen and fibronectin during clot formation.<sup>19,20</sup> Fibrinogen is a plasma glycoprotein consisting of three disulfide bond-linked pairs of α, β, and γ chains coded by separate genes. It is involved in the final step of blood coagulation. Thrombin-induced cleavage of α and β chains results in a conversion of the soluble protein into fibrin monomers. These fibrin monomers polymerize to form a fibrin clot.

The aim of this study was to analyze whether fibrinogen/fibrin is a component of the neomatrix of acutely and

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**Abbreviations used in this paper:** DIL-AC, dioctadecyltetramethylindocarbocyanine percholate; DMEM, Dulbecco's modified Eagle medium; EC, endothelial cells; FCS, fetal calf serum; FSC, fat-storing cells; KC, Kupffer's cells; SDS, sodium dodecyl sulfate.

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0016-5085/95/\$3.00

chronically damaged liver. We studied the localization of fibrinogen and fibrin in normal and short-term- and long-term-damaged liver by immunofluorescence detection. The temporal relationship between fibrinogen and fibrin and fibronectin deposition during experimental short-term and long-term liver damage was also investigated.

Furthermore, we were interested in the source of fibrinogen under conditions of liver damage. As it is known so far, the liver is the primary site of fibrinogen synthesis. In the liver, hepatocytes seem to be the main source of fibrinogen. Fibrinogen is stored in the  $\alpha$  granules of platelets and megakaryocytes.<sup>21,22</sup> Although recent studies could not prove fibrinogen synthesis in cells different from hepatocytes,<sup>23–25</sup> several reports indicate that purified preparations of megakaryocytes from guinea pigs, rats, and humans synthesize fibrinogen.<sup>26–28</sup> To study whether fibrinogen synthesis can take place in cells besides the hepatocyte, we analyzed fibrinogen de novo synthesis in isolated parenchymal and nonparenchymal cells and by means of *in situ* hybridization on sections of normal and damaged rat liver tissue.

## Materials and Methods

### Reagents

Normal and methionine-free Dulbecco's modified Eagle medium (DMEM) and fetal calf serum (FCS) were purchased from Flow (Bonn, Germany); L-glutamine and penicillin/streptomycin from Seromed (Berlin, Germany); collagenase of *Clostridium histolyticum* from Boehringer (Mannheim, Germany); pronase E from Merck (Darmstadt, Germany); Nyco-denz from Nyegaard & Co. (Oslo, Norway); ethylendiaminetetraacetic acid, sodium deoxycholic acid, ethidium bromide, 2-mercaptoethanol, Triton X-100, collagenase type I (125–250 U/mg), and elastase type III (20 U/mg) from Sigma (Munich, Germany); and sodium dodecyl sulfate (SDS) and acrylamide from Bio-Rad Laboratories (Munich, Germany). Formalin-fixed *Staphylococcus aureus* protein A (Pansorbin) was obtained from Calbiochem GMBH (Frankfurt, Germany); guanidinium isothiocyanate from Fluka (Bern, Switzerland); CsCl<sub>2</sub>, agarose, and the nick translation kit from BRL (Heidelberg, Germany); and [<sup>35</sup>S]methionine, [<sup>32</sup>P]deoxycytidine triphosphate, [<sup>14</sup>C]methylated protein standards, and Amplify from Amersham Buchler GMBH (Frankfurt, Germany). Acetylated low-density protein labeled with diiodotetracyclotetramethylindocarbocyanine perchlorate (DIL-AC) was purchased from Paesel and Lorei (Frankfurt, Germany). The endothelial cell medium kit was purchased from Promo Cell (Heidelberg, Germany).

### Antibodies and Complementary DNA Probes

The antiserum against human fibrinogen, the antiserum directed against von Willebrand factor antigen, and the monoclonal antibody against desmin were purchased from

Dako (Copenhagen, Denmark). Cross-reactivity of the antiserum directed against human fibrinogen with rat fibrinogen/fibrin was proved, and cross-reactivity with total fibronectin was excluded by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. The antiserum against human total fibronectin was purchased from Behring (Marburg, Germany); the monoclonal antibody directed against ED 2 epitope was a gift from C. Dijkstra.<sup>29</sup> Fluorescein- or rhodamin-labeled immunoglobulin G fractions directed against murine or rabbit immunoglobulins were obtained from Sigma (Munich, Germany). A complementary DNA (cDNA) probe of the  $\beta$  chain of human fibrinogen (clone pKK 424) was purchased from the American Type Culture Collection (Rockville, MD). A cDNA clone coding for total human fibronectin (clone pFH 154) was kindly provided by F. E. Baralle<sup>30</sup> and the cDNA for mouse albumin by D. Kioussis.<sup>31</sup> The chicken  $\alpha$ -actin cDNA was a gift from A. Schwarz<sup>32</sup> (Baylor College, Houston, TX). The cDNA specific for  $\alpha_2$ -macroglobulin was kindly provided by G. Fey.<sup>33</sup>

### Induction of Short-term and Long-term Liver Damage

Short-term liver damage was induced in 8-week-old female Wistar rats (body wt, ~200 g) by oral administration of a carbon tetrachloride and maize oil solution (50% vol/vol) as previously described<sup>34</sup> but modified by reduction of the CCl<sub>4</sub> dose to 75  $\mu$ L/100 g body wt. Control animals were treated with maize oil only. Four animals in each group were killed 3, 6, 12, 24, 48, 72, and 96 hours after a single high-dose CCl<sub>4</sub> administration. The liver was perfused with saline solution (0.9% NaCl) and snap-frozen in liquid nitrogen. For induction of liver fibrosis, animals were pretreated with drinking water containing phenobarbitone (0.03%) for 2 weeks and then exposed to oral application of CCl<sub>4</sub> once a week up to 10 weeks according to the method of Proctor and Chatamra<sup>35</sup> starting with 0.04 mL CCl<sub>4</sub>. Weekly, further 0.04-mL doses of CCl<sub>4</sub> were added. To study the time kinetics of fibrinogen and  $\alpha_2$ -macroglobulin messenger RNA levels after repeated CCl<sub>4</sub> administration, we analyzed rats 3, 6, 12, 24, 48, 72, and 96 hours after the sixth administration. The effect peaked after 48 hours. Thus, in our experiments, long-term liver injury was studied 48 hours after the actual administration of CCl<sub>4</sub>.

### Cell Isolation and Culture Conditions

**Isolation of liver cells.** Rat hepatocytes were isolated according to the method of Seglen<sup>36</sup> and were cultured on collagen-coated tissue culture plates in DMEM supplemented with 10% FCS, 0.05% insulin, and 10<sup>−7</sup> mol/L dexamethasone. Four hours after plating, medium supplemented with 0.2% bovine serum albumin, 0.05% insulin, and 10<sup>−7</sup> mol/L dexamethasone was used to replace the plating medium.

Fat-storing cells (FSCs) and KCs were isolated according to the method of De Leeuw et al.<sup>37</sup> as described previously.<sup>38</sup> ECs were obtained according to the method of Knook et al.<sup>39</sup> FSCs were cultured in DMEM containing 10% FCS. KCs were cultured in M199 supplemented with 15% FCS, and ECs were

cultured in EC medium (Promega) supplemented with epidermal growth factor, basic fibroblast growth factor, 0.15% insulin, and 2% fetal bovine serum. Medium was replaced 1 day (KCs and ECs) or 3 days (FSCs) after plating and every other day thereafter. Cells were kept in culture at 37°C in a 5% CO<sub>2</sub>/95% O<sub>2</sub> atmosphere and 100% humidity. Purity of the cell isolations was determined by phase-contrast microscopy, indirect immunofluorescence staining, vitamin A autofluorescence and desmin staining of the FSCs, DIL-AC-LDL incorporation of endothelial cells, and ED 2 staining of KCs. KC cultures were more than 99% pure. Purity of FSCs was more than 85%. ECs were contaminated with 2% FSCs and with 8%–10% KCs.

Freshly isolated hepatocytes, FSCs, ECs, and KCs were centrifuged onto slides in a cytopin centrifuge (Shandon, London, England) at 300 rpm (hepatocytes) and 1500 rpm (FSCs, KCs, and ECs). Cells were fixed in methanol (5 minutes) and acetone (10 seconds) at –20°C, washed with phosphate-buffered saline, and preincubated with FCS for 20 minutes at 37°C. After three washes in phosphate-buffered saline, cells were incubated with antigen-specific antibodies for 1 hour at 37°C, then rinsed again three times in phosphate-buffered saline and incubated with fluorescein-labeled immunoglobulin Gs directed against murine or rabbit immunoglobulins.

### Immunohistology

Cryostat sections (5 μm) were air-dried, fixed in methanol for 10 minutes and in acetone for 10 minutes at –20°C, and used for immunofluorescence detection as described above. Double-staining was performed as previously described.<sup>8</sup>

### Biosynthetic Labeling, Immunoprecipitation, and SDS– Polyacrylamide Gel Electrophoresis Analysis

Cells were biosynthetically labeled by exposure to methionine-free DMEM containing [<sup>35</sup>S]methionine for 4 hours and then processed for immunoprecipitation and SDS–polyacrylamide gel electrophoresis under reducing conditions according to the method of Laemmli<sup>40</sup> as described previously.<sup>41</sup>

### Northern Blot Analysis of Total RNA

Total RNA was isolated from freshly isolated and cultured hepatocytes, FSCs, KCs, ECs, and liver tissue by the guanidinium isothiocyanate and CsCl<sub>2</sub> ultracentrifugation method of Chirgwin et al.<sup>42</sup> Total RNA was separated by agarose gel electrophoresis, transferred onto nylon membrane, and finally hybridized with specific [<sup>32</sup>P]deoxycytidine triphosphate-labeled cDNA probes. Probes were labeled by nick translation. Hybridization and posthybridization washes were performed under low stringency conditions. Hybridization was performed overnight at 42°C in 2× SSC with 50% formamide. Hybridization washes were performed at 42°C in 2× SSC, 0.1% SDS for 5, 15, 30, and 45 minutes. In the case of hybridization with a cDNA for mouse albumin, stringency of washes was increased to 56°C and 0.2% SSC. Filters were exposed to Kodak X-omat film at –70°C. Filters were suc-

sively hybridized with cDNAs specific for fibrinogen, total fibronectin, albumin, and α-actin. Results were normalized to an oligonucleotide probe specific for 28S ribosomal RNA.<sup>43</sup> Total messenger RNA of each animal was analyzed separately. In the time kinetics, slight differences between the individual rats were observed. Altogether, the main changes were detectable 24–48 hours after a single intoxication. Data presented in this report are representatives of four parallel studies.

### In Situ Hybridization

An EcoRI/Pst I fragment of 1900 bases obtained from the fibrinogen cDNA clone pKK 424 was subcloned into Bluescript SK plasmid DNA. The linearized DNA template was transcribed into <sup>35</sup>S-labeled sense or antisense RNA probes using the T3 or T7 polymerase promotor of the Bluescript vector.

**Pretreatment.** Cryostat sections (5 μm) on silan-coated slides and slides with freshly isolated or cultured cells were fixed for 20 minutes with 4% paraformaldehyde at room temperature, washed in 10 mmol/L MgCl<sub>2</sub> in phosphate-buffered saline, and stored at –70°C until further use. After predigestion with proteinase K (10 μg/mL in 5 mmol/L ethylenediaminetetraacetic acid and 50 mmol/L Tris-HCl; pH 7.4) for 10 minutes at room temperature, slides were rinsed in phosphate-buffered saline containing 0.1 mmol/L glycine and phosphate-buffered saline for 5 minutes each and postfixed in 4% paraformaldehyde for 20 minutes. Liver tissue sections were then acetylated in 0.25% acetic anhydride in 0.1 mol/L triethanolamine buffer (pH 8.0) for 10 minutes, washed again, and dehydrated in ethanol. <sup>35</sup>S-labeled RNA probes were diluted in hybridization buffer to a final concentration of 2 × 10<sup>4</sup> cpm/μL. After hybridization performed overnight at 42°C, the slides were subsequently washed in 2× SSC and treated with ribonuclease (10 μg/mL in SSC [2×]) for 30 minutes at 37°C. Successive washes were followed by dehydration in a gradual alcohol series. The slides were coated with Kodak NTB-2 emulsion, exposed for 7–12 days, developed in Kodak D-19, and fixed in Kodak rapid fixer. After counterstaining with H&E, the slides were analyzed by light microscopy.

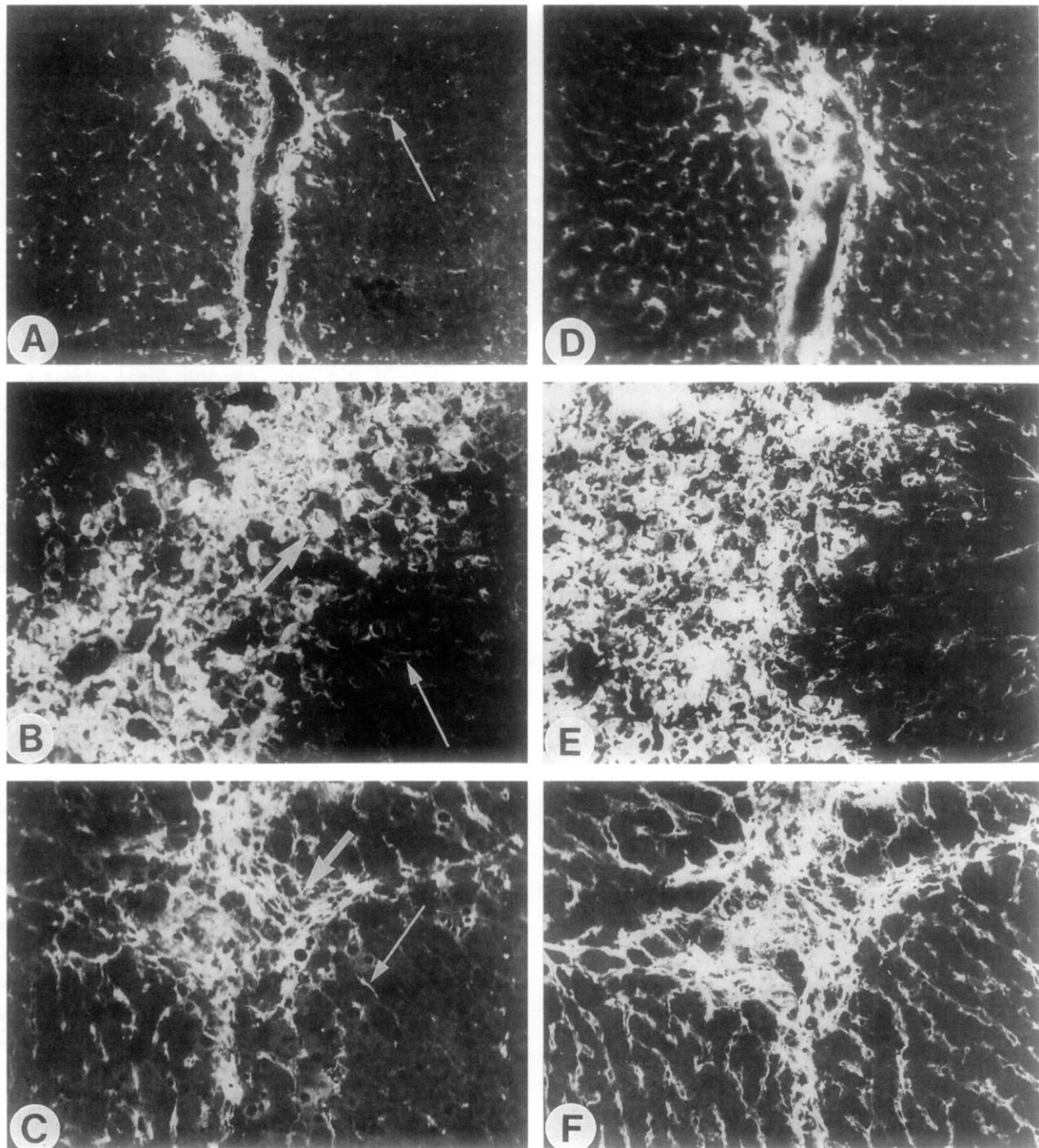
### Results

#### Histopathology of CCl<sub>4</sub>-Induced Liver Damage

Histological signs of liver necrosis were already observed 12–24 hours after a single dose of CCl<sub>4</sub> and peaked at 48 hours after intoxication. Necrotic areas were most conspicuous in zone 3 of Rappaport and extended up to the peripheral zone 1. Repeated injection resulted in rearrangement and accumulation of extracellular matrix leading to fibrosis with septa connecting portal tracts and central veins as previously shown.<sup>7,8</sup>

#### Immunohistological Detection of Fibrinogen/Fibrin in Normal and Damaged Rat Liver

Immunohistological detection of fibrinogen/fibrin in the normal rat liver (Figure 1A) showed positivity



**Figure 1.** Indirect immunofluorescence detection of fibrinogen/fibrin and total fibronectin. Sections of normal (*A* and *B*) and short-term- (*C* and *D*) and long-term- (*E* and *F*)  $\text{CCl}_4$ -damaged rat liver were stained with antisera directed against human fibrinogen (*A*, *C*, and *E*) and human total fibronectin (*B*, *D*, and *F*) followed by the incubation with a fluorescein-linked secondary antibody. Arrows indicate positivity along the sinusoids. **Bold arrows** indicate fibrinogen/fibrin-specific fluorescence within necrotic areas and fibrous septa (original magnification 100 $\times$ ).

along vessel walls of the portal tract as well as walls of the central veins and along the sinusoids. Furthermore, a diffuse intracellular fluorescence in hepatocytes indicating cytoplasmic reactions of the antiserum with parenchymal cells was observable. Fibrinogen staining showed a pattern with close similarity to total fibronectin (Figure

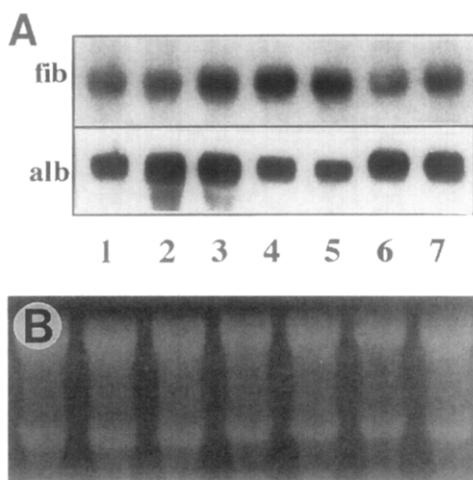
1*B*) staining. In short-term-damaged livers, a significant accumulation of amorph fibrinogen and fibrin-related fluorescence comparable with the total fibronectin detection could already be observed 12 hours after intoxication in the areas of necrosis (data not shown). Maximum levels were reached 48 hours after intoxication (Figure 1*C* and

D) in parallel to the largest extension of necrotic areas. In sections of short-term-damaged liver, indirect immunofluorescence staining did not allow the identification of possible cellular sources of fibrinogen. After the second administration of  $\text{CCl}_4$ , a fiberlike positivity could be observed by immunofluorescence detection of fibrinogen and fibrin. Immunodetection in fibrotic livers showed a fibrinogen/fibrin positivity within the fibrous septa similar to the pattern of total fibronectin immunodetection (Figure 1E and F).

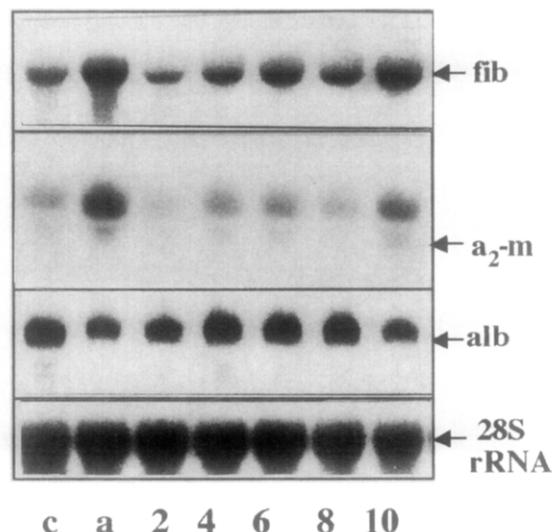
Double immunodetection with antibodies directed against fibrinogen in parallel to either desmin (FSC), ED1 (KCs), or von Willebrand factor (ECs) did not allow us to assign fibrinogen-specific fluorescence to nonparenchymal cells. Because von Willebrand factor does not seem to be a suitable marker for recognition of rat sinusoidal ECs, it remained unclear if the fibrinogen pool reflects localization inside sinusoidal cells or assembly into the extracellular matrix.

#### Fibrinogen Transcript Level in Total RNA Extracted From Normal and Damaged Rat Livers

The immunohistological study showed striking amounts of fibrinogen/fibrin in the damaged livers. Changes in fibrinogen transcript content after single or repeated liver damage were studied by Northern blot analysis of total RNA extracted from rat liver tissue (Figure 2A). Total RNA extracted from normal (Figure



**Figure 2.** (A) Northern blot analysis of total RNA extracted from normal rat liver tissue (lane 1) and short-term-damaged liver 6 (lane 2), 12 (lane 3), 24 (lane 4), 48 (lane 5), 72 (lane 6), and 96 hours (lane 7) after a single administration of  $\text{CCl}_4$ . RNA was electrophoresed (10  $\mu\text{g}$  of total RNA per lane), blotted, and subsequently hybridized with [ $^{32}\text{P}$ ]deoxycytidine triphosphate-labeled cDNA probes specific for the  $\beta$  chain of fibrinogen (fib) and albumin (alb). (B) Ethidium bromide-stained agarose gel indicating that equal amounts of total RNA were loaded.



**Figure 3.** Northern blot analysis of total RNA extracted from normal rat liver tissue (lane c), short-term-damaged liver 48 hours after a single intoxication (lane a), and long-term-damaged livers 48 hours after 2 $\times$  (lane 2), 4 $\times$  (lane 4), 6 $\times$  (lane 6), 8 $\times$  (lane 8), and 10 $\times$  (lane 10) administrations of  $\text{CCl}_4$ . RNA was electrophoresed (10  $\mu\text{g}$  of total RNA per lane), blotted, and subsequently hybridized with [ $^{32}\text{P}$ ]deoxycytidine triphosphate-labeled cDNA probes specific for the  $\beta$  chain of fibrinogen (fib),  $\alpha_2$ -macroglobulin ( $\alpha_2\text{-m}$ ), albumin (alb), and an oligonucleotide recognizing 28 S ribosomal RNA (28S rRNA).

2A, lane 1) and short-term-damaged rat liver tissue (lane 2), 12 (lane 3), 24 (lane 4), 48 (lane 5), 72 (lane 6), and 96 hours (lane 7) after a single intoxication was hybridized with a cDNA specific for the  $\beta$  chain of human fibrinogen and for mouse albumin. After a single  $\text{CCl}_4$  administration, amounts of fibrinogen-specific transcripts already increased 6 hours after intoxication with a maximum level between 24–48 hours persisting until the beginning of tissue recovery at 72 hours. Total RNA extracted from normal (Figure 3, lane c), short-term-, (48 hours after a single intoxication) (lane a), and long-term-damaged rat liver tissue 48 hours after 2 (lane 2), 4 (lane 4), 6 (lane 6), 8 (lane 8), and 10 (lane 10) intoxications was hybridized with a cDNA specific for the  $\beta$  chain of human fibrinogen, for mouse albumin, and for  $\alpha_2$ -macroglobulin. The amount of  $\alpha_2$ -macroglobulin-specific transcripts increased after a single-shot intoxication, indicating a short-term-phase reaction and, concordantly, fibrinogen was reduced after the second administration. Albumin-specific transcripts decreased in the short-term-damaged livers as shown earlier.<sup>44</sup> For induction of long-term liver damage, the weekly dose of  $\text{CCl}_4$  was continuously increased. Repeated administration of low amounts of  $\text{CCl}_4$  did not induce an enhancement of fibrinogen-specific transcripts. When the amount of administered  $\text{CCl}_4$  was gradually enhanced by 0.04 mL  $\text{CCl}_4$  each week, starting at the fourth intoxication with

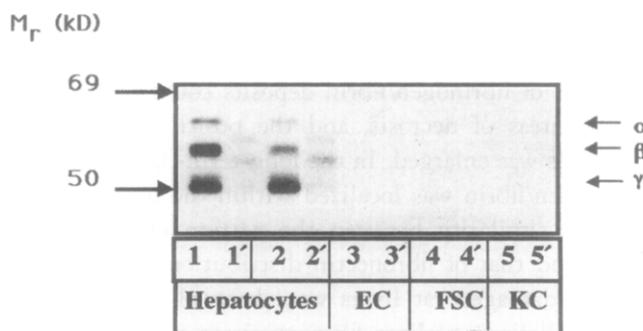
0.16 mL of CCl<sub>4</sub>, a moderate increase of fibrinogen- and  $\alpha_2$ -macroglobulin messenger RNA steady-state levels was found.

#### Fibrinogen Synthesis in Purified Liver Cells

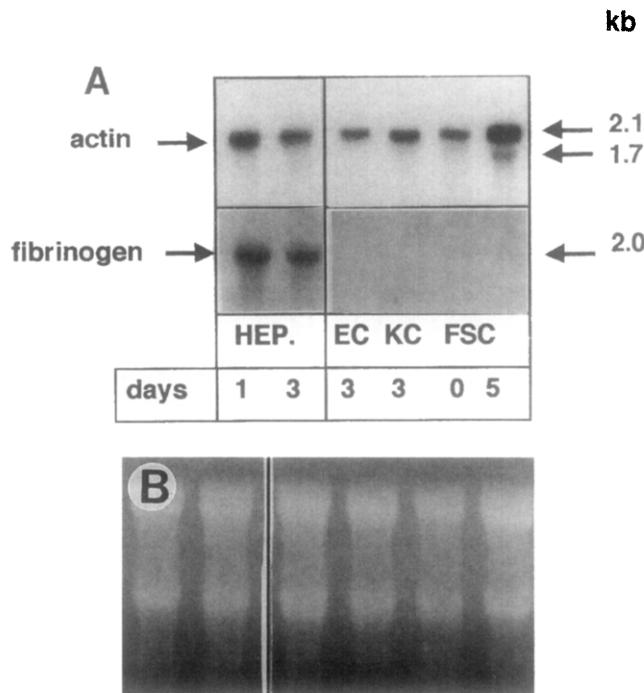
**SDS-polyacrylamide gel electrophoresis analysis of biosynthetically labeled proteins.** Fibrinogen de novo synthesis was analyzed by means of SDS-polyacrylamide gel electrophoresis of biosynthetically labeled proteins of cultured hepatocytes, ECs, FSCs, and KCs (Figure 4). Immunoprecipitation showed the  $\alpha$ ,  $\beta$ , and  $\gamma$  chains of fibrinogen in hepatocyte-derived samples but not in cell lysates or pulse media of nonparenchymal cells. Newly synthesized fibrinogen was detectable in cell lysates of primary hepatocyte cultures. Secreted fibrinogen was converted into fibrin. SDS-polyacrylamide gel electrophoresis of immunoprecipitates derived from the pulse media resulted in two light bands consistent with  $\alpha$  and  $\beta$  fibrin bands. Fibrinogen gene expression decreased, like albumin synthesis, during prolonged hepatocyte culture.

#### Northern Blot Analysis of Total RNA Extracted From Purified Liver Cells

Total RNA (10  $\mu$ g) was size-selected per lane. Hybridization and following washes were performed at low-stringency conditions. Northern blots were exposed 7 days for fibrinogen and 4 days for actin hybridization. Fibrinogen-specific transcripts for the  $\beta$  chain could only be identified in hepatocytes (Figure 5A). In accordance with the results of protein synthesis and secretion, de-



**Figure 4.** Fibrinogen de novo synthesis in liver cells. Immunoprecipitation with an antiserum directed against human fibrinogen was followed by SDS-polyacrylamide gel electrophoresis. Lanes 1–5 represent  $\alpha$ ,  $\beta$ , and  $\gamma$  chain of fibrinogen precipitated from lysates of a primary rat hepatocyte culture at day 1 (lane 1), day 3 (lane 2) after isolation, rat EC (lane 3) at day 3, rat FSC (lane 4) at day 3, and KCs (lane 5) at day 3 after isolation. Lanes 1'–5' represent fibrin converted into fibrinogen that was precipitated from the pulse media obtained from cell cultures pulsed for 4 hours with [ $^{35}$ S]methionine-containing medium.

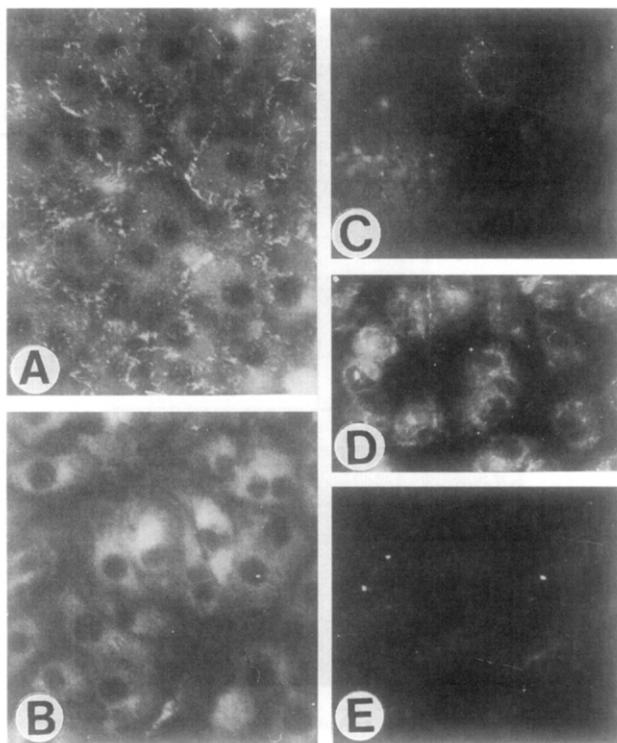


**Figure 5.** (A) Northern blot analysis of total RNA extracted from primary cultures of hepatocytes at day 1 and 3 after isolation, from FSCs at day 0 and 5 after isolation, from KCs at day 3 after isolation, and from ECs at day 3 after isolation. Total RNA was separated on agarose gel, blotted, and hybridized with [ $^{32}$ P]deoxycytidine triphosphate-labeled cDNA probes specific for the  $\beta$  chain of fibrinogen and chicken  $\alpha$ -actin. (B) Ethidium bromide-stained agarose gel indicating that equal amounts of total RNA were loaded.

creasing amounts of fibrinogen-specific transcripts were found during prolonged time of culture.

#### ImmunocytoLOGY

Hepatocytes were cultivated in the absence of FCS. Nonparenchymal cells were washed in phosphate-buffered saline for 15 minutes to remove fibrinogen and fibrin containing FCS before fixation. Three-day cultured hepatocytes (Figure 6A and B) EC (Figure 6E), KC (Figure 6D), and FSC (Figure 6C) were immunostained with the antibody directed against fibrinogen. Immunofluorescence detection showed fibrinogen-specific fluorescence exclusively in the hepatocyte cultures. The positivity was located in the extracellular matrix of hepatocyte cultures in the form of fiberlike structures as shown earlier.<sup>45</sup> As the liver cells were cultured in the absence of heparin, secreted fibrinogen was converted into fibrin. The highest amounts of fibrinogen/fibrin deposits in the extracellular matrix of hepatocyte cultures were observable at day 1 after isolation decreasing with prolonged culture. A diffuse fluorescence was detectable inside the hepatocytes. In cultured KCs, there was a diffuse intracellular positivity observable, which may represent exogenous fibrin after phagocytosis by the KC.



**Figure 6.** Immunodetection of fibrinogen/fibrin in (A) cultured hepatocytes, (E) ECs, (D) KCs, and (C) FSCs with an antiserum directed against human fibrinogen. (B) Control hepatocytes were incubated with rabbit serum before application of fluorescein-labeled anti-rabbit immunoglobulins (original magnification 200 $\times$ ).

### In Situ Hybridization

In freshly isolated and cultured hepatocytes, hybridization with a fibrinogen-specific antisense riboprobe showed positive signals distributed over parenchyma and nuclei (Figure 7A). Hybridization with the sense probe showed unspecific signals homogeneously distributed over cells and intercellular spaces (Figure 7B). In situ hybridization on hepatocytes shown in Figure 7A and B was exposed for 8 days. In freshly isolated and cultured FSCs, KCs, and ECs, unspecific signals were in and around the cells. To make sure that there is no difference between sense and antisense hybridization, ECs were exposed for 11 days (Figure 7C) and KCs (Figure 7E) and FSCs (Figure 7D) for 14 days. No difference between the hybridization with sense and antisense probe could be observed; the grains were homogeneously distributed over cells and intercellular space.

### Localization of Fibrinogen-Specific Transcripts on Sections of Normal and Damaged Rat Liver

Cellular localization of fibrinogen transcripts in the normal and damaged rat liver was studied by in situ hybridization with fibrinogen-specific antisense ribo-

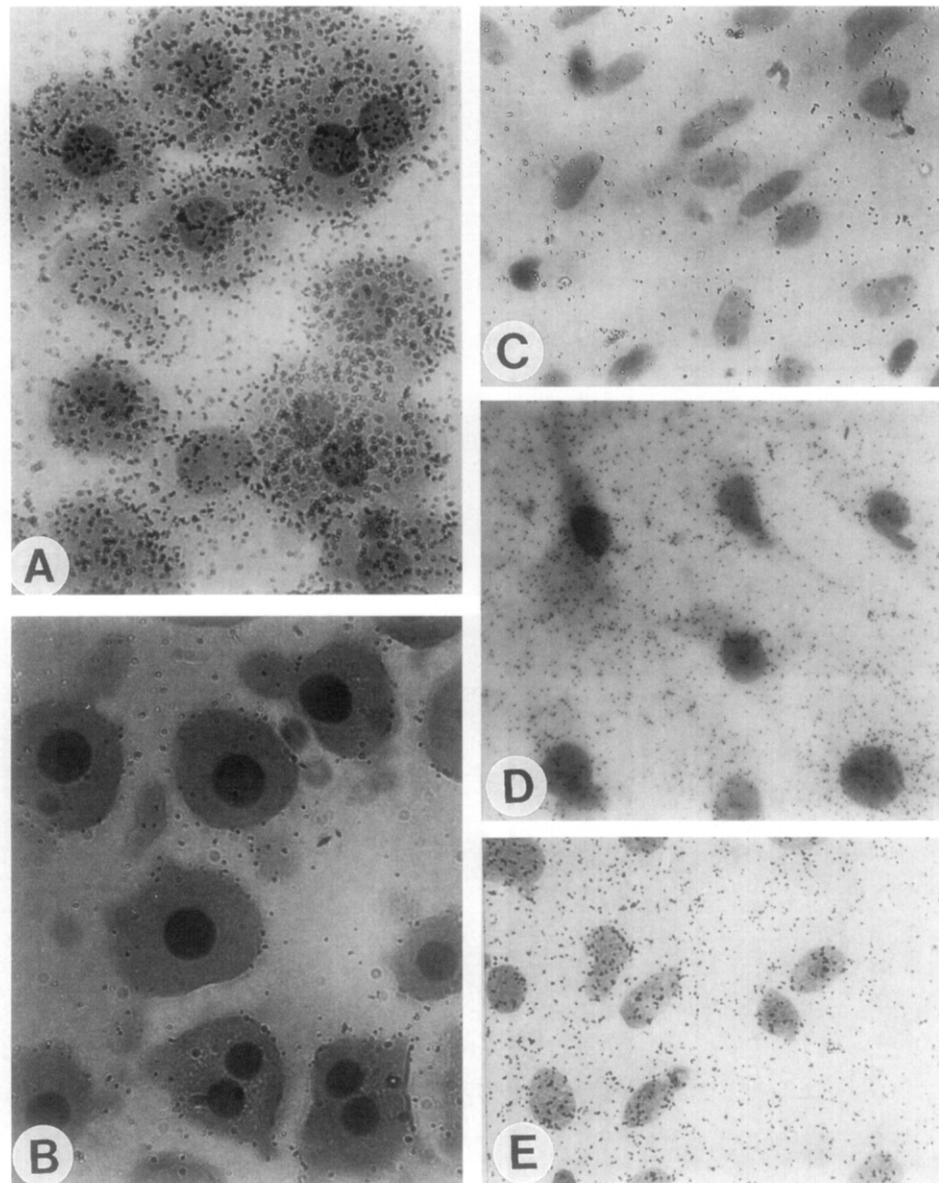
probes (Figure 8). In control livers obtained from healthy rats, a moderate amount of signals was distributed over the parenchymal cells (Figure 8A). Mesenchymal cells could be distinguished from hepatocytes by their smaller and more dense nuclei. No specific signals could be identified in mesenchymal cells of the vessel walls or along the sinusoids. In short-term-damaged livers, abundant fibrinogen-specific transcripts were localized in the non-damaged areas, whereas fibrinogen-specific transcripts were almost undetectable in the necrotic areas (Figure 8C and D). No fibrinogen-expressing cells in the vessel walls or in the sinusoids of the short-term-damaged livers could be detected. After repeated liver damage, fibrinogen-specific transcripts were localized only in hepatocytes. No signals could be detected in cells located within fibrotic septa (Figure 9A, C, and D).

### Discussion

Liver fibrogenesis is characterized by the accumulation and redistribution of extracellular matrix following repeated liver injury. The mechanisms leading to the imbalance between matrix deposition and matrix degradation still need to be clarified. The early changes in matrix formation after liver damage might be of great importance. Current concepts point out that liver fibrogenesis may represent a paradigm of wound healing. During wound healing, early steps of reparation include the clot formation. Locally produced cellular fibronectin and fibrin form a provisional matrix, which is important for the proliferation of fibroblasts and their capacity to secrete collagens.<sup>46</sup> It was shown that fibronectin shows specific domains allowing the binding to fibrin and, on the other hand, the binding to cell surfaces.

In this study, we show that fibrinogen/fibrin is immunologically detectable along vessels and sinusoids of the normal rat liver. In the short-term-damaged liver, large amounts of fibrinogen/fibrin deposits could be detected in the areas of necrosis, and the positivity along the sinusoids was enlarged. In the long-term-damaged liver, fibrinogen/fibrin was localized within the fibrous septa. A close similarity between the pattern of fibrinogen/fibrin and that of fibronectin distribution in normal as well as damaged rat livers was observed. In respect to the parallelisms of liver fibrogenesis to wound healing, the accumulation of fibrinogen/fibrin and fibronectin deposits in the sinusoidal space, the necrotic areas, and the fibrous septa account for the assumption that a "clotting-like process" may be involved after short-term liver injury and in fibrosing liver damage.

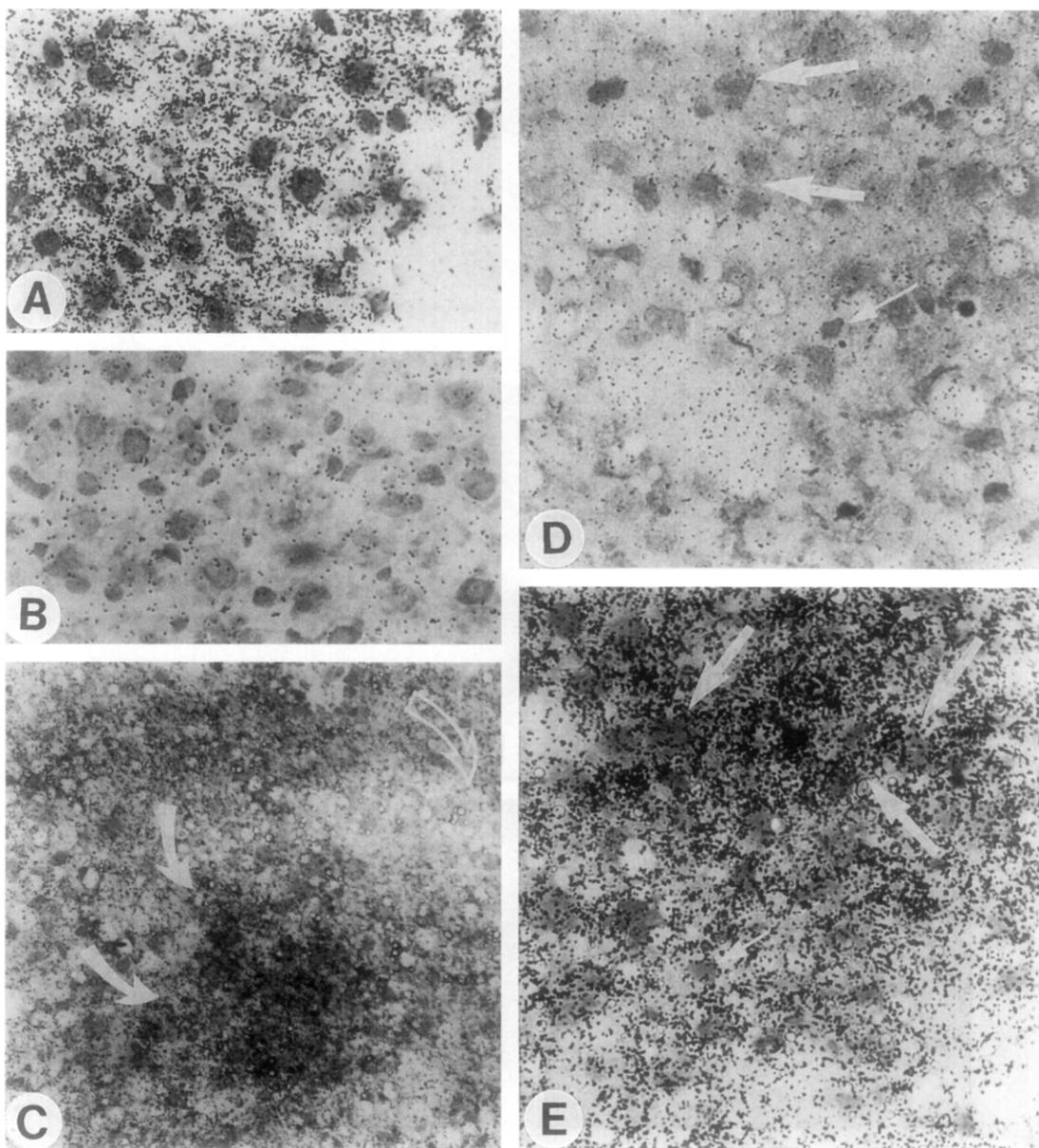
However, the immunohistological studies did not allow us to clarify if the fibrinogen staining represents intracellular fibrinogen expression in small vessels and



**Figure 7.** Detection of fibrinogen-specific transcripts by *in situ* hybridization in freshly isolated rat hepatocytes. (A) Hepatocytes, (C) ECs, (D) FSCs, and (E) KCs were hybridized with  $^{35}\text{S}$ -labeled fibrinogen-specific (A, C, D, and E) antisense and (B) sense RNA probes (original magnification 400 $\times$ ). Cells shown in A and B were exposed for 8 days. In freshly isolated and cultured FSCs, KCs, and ECs, unspecific signals were in and around the cells. To be sure that there is no difference between sense and antisense hybridization, ECs were exposed for (C) 11 days and (E) KCs and (D) FSCs for 14 days.

sinusoids or the assembly of fibrinogen/fibrin into the extracellular matrix of the normal and damaged liver. Stamatoglou et al. showed that serum-free cultured hepatocytes elaborate a fibrin and fibronectin-containing extracellular matrix.<sup>45</sup> We recently showed the coassembly of total fibronectin and cellular fibronectin in the extracellular matrix of primary cultures of hepatocytes.<sup>47</sup> Our studies of cultured rat hepatocytes show a fiber-shaped fibrinogen/fibrin-specific fluorescence in primary hepatocyte cultures. The immunohistological detection showed striking fibrinogen/fibrin-specific fluorescence in sections of normal and damaged rat liver tissue, predominantly

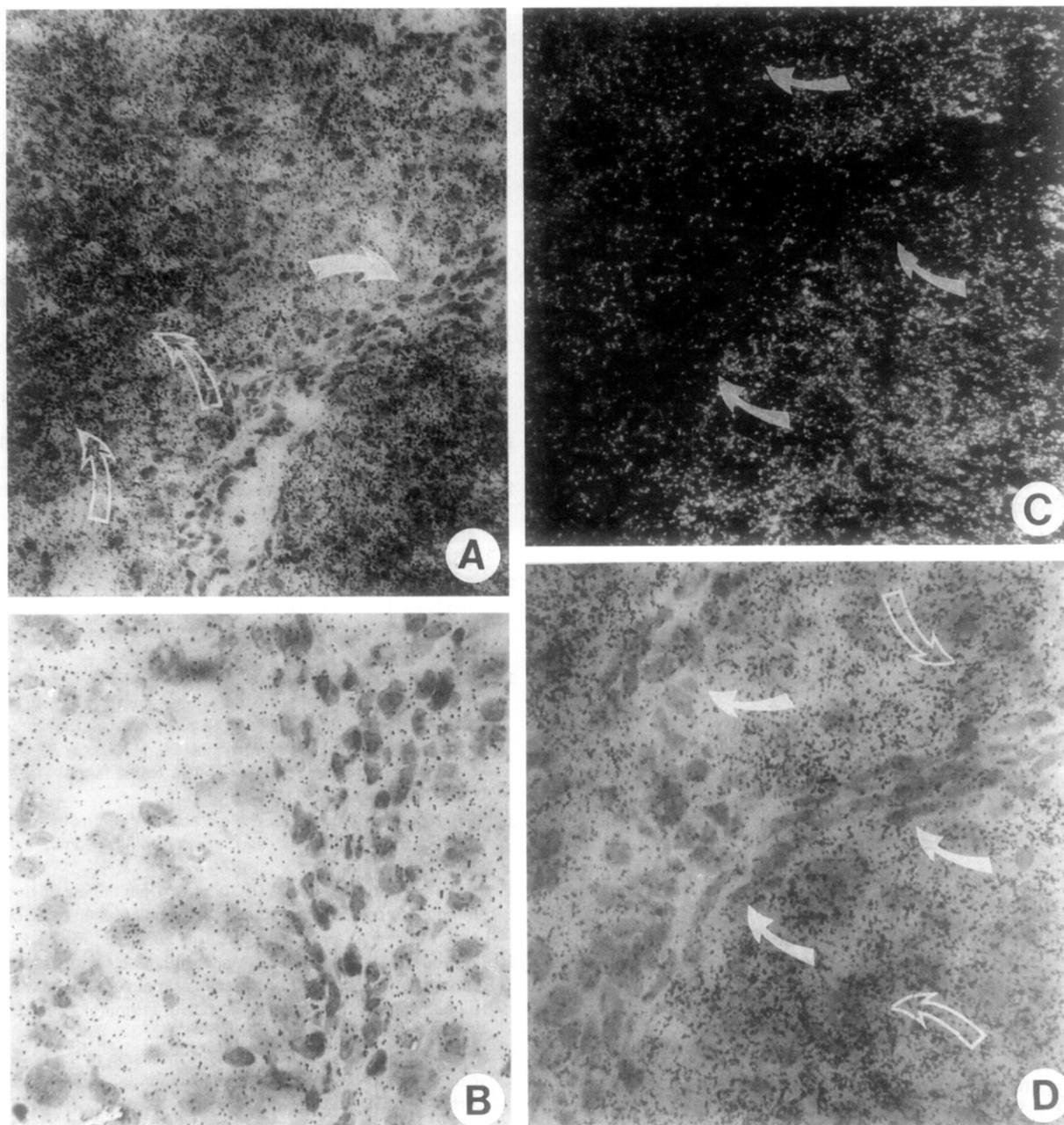
in the vicinity of nonparenchymal cells. The capacity of fibrinogen to bind to human fibroblasts was shown by Dejana et al.<sup>48</sup> The requirement of plasma fibronectin for fibrin binding to fibroblasts was pointed out by Grinnell et al.<sup>46</sup> Also, the interaction between fibrinogen and ECs was observed.<sup>49</sup> KCs are responsible for clearance and degradation of circulating fibrin and contribute to the resolution of extravasated fibrin deposits. High-affinity fibrin-binding sites on murine macrophages have been documented by Shainhoff et al.<sup>50</sup> Several investigators reported the dependence of fibrin binding by macrophages on fibronectin.<sup>51,52</sup>



**Figure 8.** Detection of fibrinogen-specific messenger RNA in normal and short-term-damaged rat liver by *in situ* hybridization. Sections of normal rat liver (*A* and *B*) and short-term-damaged rat liver 48 hours after a single intoxication (*C–E*) were hybridized with  $^{35}\text{S}$ -labeled fibrinogen-specific (*A*, *C*, and *D*) antisense and (*B* and *E*) sense riboprobes. Large white arrows indicate fibrinogen-expressing parenchymal cells. Open arrows indicate necrotic areas. Small white arrows indicate the small, more dense nuclei of nonparenchymal liver cells. All sections were exposed for 10 days (original magnification: *A*, *B*, *D*, and *E*,  $400\times$ ; *C*,  $200\times$ ).

On sections of rat liver tissue, as in primary culture of liver cells, the pattern of fibrinogen detection corresponds to the pattern of fibronectin immunodetection. The binding of fibrinogen/fibrin to the extracellular matrix and to nonparenchymal cells in vitro might correspond to fibrin binding to the extracellular matrix or nonparenchymal cells along vessels, sinusoids, necrotic areas, and fibrous septa in vivo. Fibrinogen was thought to be exclusively produced by hepatocytes. However, several studies suggested that megakaryocytes represent an additional site of fibrinogen synthesis,<sup>26–28</sup> although re-

cent studies did not prove fibrinogen synthesis in megakaryocytes.<sup>23–25</sup> The nonparenchymal fibrinogen de novo synthesis was analyzed by immunoprecipitation, followed by SDS–polyacrylamide gel electrophoresis and Northern blot analysis. It is well known that during liver fibrogenesis, there is a transformation of nonparenchymal liver cells, which are now capable of synthesizing various extracellular matrix proteins. Elevated collagen, laminin, and fibronectin synthesis could be shown in FSC lines obtained from cirrhotic livers.<sup>53</sup> Recent data also show<sup>54</sup> that cellular fibronectin is locally synthesized by mesen-



**Figure 9.** Detection of fibrinogen-specific messenger RNA in long-term-damaged rat liver tissue taken 48 hours after the tenth administration of  $\text{CCl}_4$ . Sections of rat liver were hybridized with  $^{35}\text{S}$ -labeled fibrinogen-specific (*A*, *C*, and *D*) antisense and (*B*) sense riboprobes. White arrows indicate fibrotic septa containing mesenchymal cells, which do not express fibrinogen. Open arrows indicate fibrinogen-expressing parenchymal cells. *C* and *D* show the same part of the liver section; *D* represents dark-field microscopy. All sections were exposed for 10 days (original magnification: *B–D*,  $400\times$ ; *A*,  $200\times$ ).

chymal cells after liver damage. The possible contribution of activated liver cells to fibrinogen synthesis was studied by *in situ* hybridization on sections of normal and damaged rat livers. Fibrinogen-specific transcripts could only be detected in nonnecrotic areas and outside the fibrotic septa of the damaged livers by means of *in situ* hybridization. Northern blot analysis of total RNA extracted from normal and damaged livers indicated an increased steady-state level of fibrinogen transcripts with

a maximum between 24 and 48 hours after a single liver intoxication. Next to fibrinogen, which is known to be a positive short-term-phase protein,  $\alpha_2$ -macroglobulin expression also increased, whereas albumin as a negative short-term-phase protein decreased. The elevation of fibrinogen-specific transcripts is probably due to a short-term-phase reaction after a severe  $\text{CCl}_4$  intoxication. During the induction of fibrogenesis, repeated administration of a weekly enlarged amount of  $\text{CCl}_4$  did not induce

an elevation of fibrinogen- or  $\alpha_2$ -macroglobulin-specific transcripts until the amount of CCl<sub>4</sub> was increased to 0.16 mL, which was in the fourth week. Beginning with 0.16 mL CCl<sub>4</sub>, the fibrinogen- and  $\alpha_2$ -macroglobulin transcript levels were enhanced after each further weekly intoxication, which indicates the induction of a short-term-phase reaction also after repeated liver injury.

Because no mesenchymal production of fibrinogen could be proven, it should be taken into consideration that fibrinogen and fibrin deposits in the damaged liver are mostly recruited from the plasma circulating fibrinogen that is synthesized by the hepatocytes. Hepatocytes might be activated by the CCl<sub>4</sub>-induced short-term-phase reaction and by stimulation of fibrinogen and fibrin degradation products because a positive feedback of fibrinogen/fibrin degradation products on the fibrinogen synthesis has been shown in hepatocyte culture.<sup>55</sup> After repeated liver damage, there are almost no detectable transcripts specific for total fibronectin in total RNA extracted from damaged liver tissue, whereas there is a striking positivity shown by immunofluorescence staining of total fibronectin. In fact, the cross-linkage of fibrin to plasma fibronectin during clot formation might account for plasma fibronectin as a main source of fibronectin deposits in the damaged liver. Contemporary and colocated deposition of fibrinogen/fibrin and total fibronectin therefore might suggest plasmatic clotting-like processes at sites of necrosis and septum formation, which confirms the concept of basic similarities in the pathophysiology of wound healing processes and liver fibrogenesis. Martinez-Hernandez suggests that fibronectin deposition may be the crucial event in early fibrotic processes. We would like to emphasize the importance of fibrinogen because it might indicate the involvement of clotting-like processes in short-term and long-term liver damage and might represent, next to fibronectin, a component of the provisional matrix found in the areas of liver necrosis and within the fibrotic septa influencing inflammatory, angiogenic, and fibrotic processes after liver damage.

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Received June 16, 1994. Accepted December 14, 1994.

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Supported by grants of the Deutsche Forschungs-Gemeinschaft (SFB402).