Expression of Variant Fibronectins in Wound Healing: Cellular Source and Biological Activity of the EIIIA Segment in Rat Hepatic Fibrogenesis

William R. Jarnagin,*§ Don C. Rockey,‡§ Victor E. Koteliansky,‖ Shao-Shean Wang,‡§ and D. Montgomery Bissell‡§

*Departments of Surgery and ‡Medicine, University of California at San Francisco, California 94143; §Liver Center Laboratory, San Francisco General Hospital, San Francisco, California 94110; and ‖Ecole Normale Supérieure, 75230 Paris, France Cedex 05

Abstract. We have examined the cell-specific expression of two fibronectin isoforms, EIIIA and EIIIB, during experimental hepatic fibrosis induced by ligation of the biliary duct. At the mRNA level, EIIIA and EIIIB were undetectable in normal liver but expressed early in injury, preceding fibrosis. The cellular sources of these changes were determined by fractionating the liver at various time points after bile duct ligation into its constituent cell populations and extracting RNA from the fresh isolates. EIIIA-containing fibronectin mRNA was undetectable in normal sinusoidal endothelial cells but increased rapidly within 12 h of injury. By contrast, the EIIIB form was restricted to hepatic lipocytes (Ito or fat-storing cells) and appeared only after a lag of 12–24 h: it was minimal in sinusoidal endothelial cells. Both forms were minimal in hepatocytes. At the protein level, EIIIA-containing fibronectin was markedly increased within two days of injury and exhibited a sinusoidal distribution. Secretion of this form by endothelial cells was confirmed in primary culture. Matrices deposited in situ by endothelial cells from injured liver accelerated the conversion ("activation") of normal lipocytes to myofibroblast-like cells, and pretreatment of matrices with monoclonal antibody to the EIIIA segment blocked this response. Finally, recombinant fibronectin peptide containing the EIIIA segment was stimulatory to lipocytes in culture. We conclude that expression of EIIIA fibronectin by sinusoidal endothelial cells is a critical early event in the liver's response to injury and that the EIIIA segment is biologically active, mediating the conversion of lipocytes to myofibroblasts.

FIBRONECTIN is a large (440 kD) glycoprotein that is widely distributed in the extracellular matrix (ECM)[1] (Hynes, 1985, 1987). Several variants exist, all of which arise by alternative splicing of three "type III" domains spaced along the carboxy-terminal half of the molecule (Schwarzbauer et al., 1983, 1987; Kornblihtt et al., 1985; Tamkun et al., 1984; Schwarzbauer, 1990). Two of the three (EIIIA and EIIIB, respectively) have been termed "extra" domains, because they are either completely included or excluded in the mature molecule. The third is termed V ("variable"), because in the human it contains several internal splice sites that result in a domain of variable length; in the rat, one internal site exists giving rise to three possible forms (completely excluded, partially excluded, or completely included) (Magnuson et al., 1991).

Although several cell- and ECM-binding regions of fibronectin have been characterized, the role of the EIIIA and EIIIB segments is poorly understood. "Plasma" fibronectin, which is produced largely by hepatocytes and circulates as a soluble protein, lacks both regions. The extra domains are present at specific stages of embryonic development and organogenesis (Norton and Hynes, 1987; ffrench-Constant and Hynes, 1989; Glukhova et al., 1990; Laitinen et al., 1991; Pagani et al., 1991). Their expression in the adult is minimal except in specific pathological circumstances such as wound healing (ffrench-Constant et al., 1989; Brown et al., 1993), epithelial fibrosis (Kuhn et al., 1989; Barnes et al., 1994), and vascular intimal proliferation (Glukhova et al., 1989). The apparently programmed expression implies an active role for the extra domains, although few clues on its nature exist.

In approaching this question, we have examined wound healing using a liver model, which facilitates analysis at the cellular level. As in cutaneous wounding, fibrogenesis in liver is heralded by the appearance of a myofibroblast-like population (Bienkowski et al., 1978; Martinez-Hernandez, 1984; Bissell et al., 1990*a*). While the myofibroblasts in cutaneous wounds are of uncertain origin, in liver injury they arise largely if not entirely from pericyte-like cells termed lipocytes (Ito or fat-storing cells) (Minato et al., 1983; Mak et al., 1984; Friedman et al., 1985; Milani et al., 1989,

Address all correspondence to William R. Jarnagin, M.D., Liver Center Laboratory, San Francisco General Hospital, Bldg. 40, Rm 4102, 1001 Potreto Ave., San Francisco, CA 94110. Tel.: (415) 206-4808. Fax: (415) 641-0517.

1. *Abbreviations used in this paper*: ECM, extracellular matrix; GGT, γ-glutamyl transpeptidase; GMNA, γ-glutamyl-4-methoxy-napthylamide; MBP, maltose-binding protein.

1990; Nakafsukasa et al., 1990; Rockey et al., 1992). In normal liver, lipocytes are synthetically quiescent, notable mainly for their abundant stores of retinoid esters. A central event in the initiation of fibrosing injury is the conversion of these cells from the resting to an "activated" state in which cytokine receptors and ECM production are sharply upregulated and features of smooth-muscle cells appear (Maher and McGuire, 1990; Friedman, 1993). Overall, the cells acquire the phenotype of myofibroblasts.

Analysis of lipocyte activation suggests that it is regulated both by soluble products of inflammation (cytokines) and by the ECM itself (Friedman et al., 1989). Among the earliest detectable changes in the ECM of the injured liver is an increase in total fibronectin (Martinez-Hernandez, 1984). Given the likelihood that this comprises splice variants as in other forms of epithelial fibrosis, we undertook a detailed examination of fibronectin expression during the early injury response. Also, having developed culture models of lipocyte activation with cells freshly isolated from normal animals (Friedman et al., 1989), we were in a position to test whether variant fibronectins—specifically the EIIIA-containing variant—directly mediates activation.

Materials and Methods

Materials

Radiolabeled cytidine-5'-triphosphate ([α-^{32}P]CTP, >800 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). Pronase, collagenase B, and DNase I were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN); DME, Ham's F-12 and Medium 199, calf and horse sera from Flow Laboratories (McLean, VA); Eagle's MEM without calcium was prepared in the laboratory using amino acids from Sigma Chemical Co. (St. Louis, MO). Larex® (arabinogalactan) was obtained from Consulting Associates (Tacoma, WA). Guanidine thiocyanate was purchased from Fluka Chemical Corp. (Ronkonkoma, NY); acrylamide and agarose from Bio-Rad Laboratories (Richmond, CA); ultra-pure urea, restriction enzymes, T4 DNA ligase, and RNase T2 from GIBCO BRL (Gaithersburg, MD); SP6 and T7 RNA polymerases, Taq DNA polymerase, and low-melting agarose from Promega (Madison, WI); Bluescript SK+ (pBSSK) cloning vector, MMLV reverse transcriptase and oligo-dT primers from Stratagene (La Jolla, CA); and a DNA sequencing kit (Sequenase) from United States Biochemical (Cleveland, OH). Avidin-biotin complex (Vectastain) was purchased from Vector Laboratories (Burlingame, CA); carboxylated paramagnetic spheres (1-2-μm-diam) and γ-glutamyl-4-methoxy-naphthylamide (GMNA) from Polysciences (Warrington, PA); deoxycholic acid and Fast Blue BB salt from Sigma. The pMAL-c2 expression vector and amylose resin were purchased from New England BioLabs (Beverly, MA). Purified plasma and "cellular" fibronectin (both human) were purchased from Fibrogenex, Inc. (Chicago, IL).

Antibodies

Biotinylated sheep anti-mouse IgG was purchased from Amersham. Two monoclonal antibodies specific to the EIIIA domain of fibronectin were used: IST-9 (Borsi et al., 1987) and ED-A (52DH1) (Vartio et al., 1987). A third monoclonal, C6F10, specific for the invariant tenth type III repeat (Darribère et al., 1992) and a polyclonal rabbit anti-type IV collagen (Irving et al., 1984) also were used. Individual monoclonals were titered by an ELISA procedure (Koteliansky et al., 1982). The titer of C_6F_{10}, per μg purified IgG, was fivefold that of IST-9 or IST-6, which were of similar titer (data not shown). In the studies to be described, the indicated dilutions do not take into account these differences.

Animal Model of Fibrogenesis

Hepatic injury was induced in male Sprague-Dawley rats (~400 g body weight) by ligation of the biliary duct, which reproducibly initiates a fibrogenic response (Bienkowski et al., 1978; Maher and McGuire, 1990). Sham-operated animals underwent laparotomy and bile duct manipulation without ligation. Animals were maintained postoperatively on food and water ad lib.

Liver Cell Isolation and Purification

Hepatocytes were isolated from control and experimental animals by collagenase perfusion and elutriation, as previously described (Bissell et al., 1990*b*). Lipocytes, Kupffer cells and sinusoidal endothelial cells were isolated by in situ perfusion with pronase and collagenase followed by centrifugation on a discontinuous gradient of Larex (6, 8, 12 and 15% wt/vol) (Friedman and Roll, 1987). The top two interfaces contained lipocytes, which were collected and washed twice in culture medium to remove cellular debris. The bottom two interfaces contained mixed Kupffer and endothelial cells, which were separated by centrifugal elutriation at 2,500 rpm and flow rates of 18 ml/min (endothelial cells) and 36 ml/min (Kupffer cells) (Irving et al., 1984). Lipocytes were identified by their characteristic ultraviolet autofluorescence (Friedman and Roll, 1987), endothelial cells by their uptake of DiI-conjugated acetoacetylated low density lipoprotein (Irving et al., 1984), and Kupffer cells by their ability to phagocytose fluorescein-conjugated *S. aureus* (Friedman and Roll, 1987). In isolates from normal liver, the purity of the individual fractions was 99% for hepatocytes, >95% for lipocytes and endothelial cells, respectively; and for Kupffer cells it was 90–95%, the principal contaminant being lipocytes. Preparations from injured or fibrotic liver were similarly pure except for the Kupffer cell fraction, in which 10–20% of the cells were lipocytes and 5–10% biliary epithelial cells. The Kupffer cell isolate was further purified by an adaptation of a method initially described by Rous (Rous and Beard, 1934): paramagnetic spheres (1-2-μm-diam) were administered intravenously in a volume of 0.5 ml, 5–10 min before liver perfusion. The particles rapidly disappeared from the circulation and, within the liver, were internalized exclusively by Kupffer cells (Bissell et al., 1972). After perfusion of the liver with pronase and collagenase, the unfractionated cell suspension was placed in a plastic centrifuge tube, and the iron-laden Kupffer cells were drawn to the wall of the tube with a magnet. The free suspension was aspirated, and the isolate was released into fresh culture medium and again separated magnetically; three such washes were carried out. The final isolate was 99% pure by light microscopy. The remainder of the nonparenchymal cells were fractionated by gradient centrifugation, as described above.

Histochemical Detection of γ-Glutamyl Transpeptidase

Non-parenchymal cells isolated from normal and injured liver were stained for the presence of γ-glutamyl transpeptidase (GGT), which is a specific marker of biliary epithelial cells. Aliquots of various fresh isolates were plated on glass chamber slides coated with type I collagen and, 24 h later, fixed with acetone at 4°C. After washing in 0.1 M Tris-HCl, pH 7.4, the slides were incubated in GMNA solution (GMNA 2.5 mg/ml, Fast blue BB salt 0.67 mg/ml, glycylglycine 0.67 mg/ml) for 30 min at 25°C, as previously described (Rutenburg et al., 1969). Frozen sections of normal kidney served as a positive control. Cells exhibiting the bright red reaction product were absent from isolates of normal liver. From animals subjected to bile duct ligation, the lipocyte and endothelial fractions contained 1–5% GGT-positive cells and the routinely prepared Kupffer cells, 5–10%. Kupffer cells purified using magnetic beads, as described above, were free of contaminants.

cDNA Probes

Rat cDNA probes containing the alternatively spliced regions EIIIA (A+) and EIIIB (B+) and an invariant, 270 base-pair fragment (pSR270) were provided in pGEM vectors by Dr. Richard Hynes (M.I.T., Cambridge, MA). The A+ probe spans 170 bp of EIIIA and 100bp of the adjacent invariant segment (III-12). The B+ probe spans 250 bp of EIIIB and 100 bp of the adjacent type III repeat (Schwarzbauer et al., 1987). A cDNA spanning the entire V region with 5' and 3' extensions of 67 and 102 bp, respectively, was prepared in the laboratory according to an established protocol using PCR amplification (Pagani, et al., 1991). The amplified fragment was subcloned into pBSSK. By DNA sequencing, it was identical to the published one (Schwarzbauer et al., 1983). The probe constructs are summarized in Fig. 1.

RNase Protection Assay

Total RNA was extracted from the individual cell isolates or whole liver tissue as described (Chomczynski and Sacchi, 1987) or using TRI Reagent

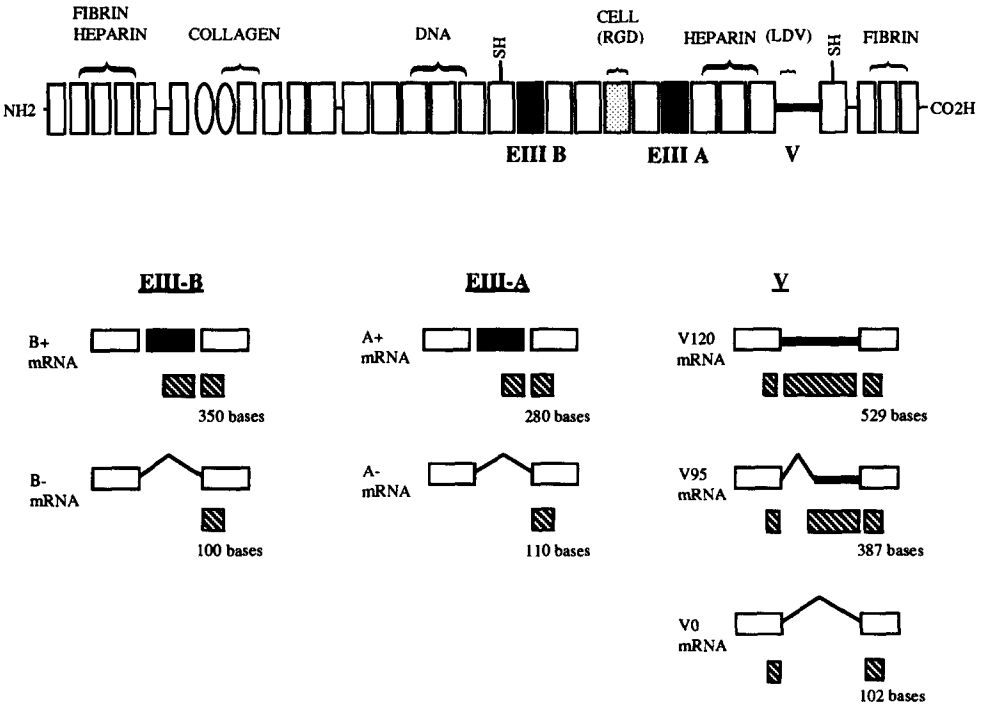

Figure 1. Summary of cDNA probes used in RNase protection assays. In the diagram, the fibronectin molecule is shown with type I, II, and III repeats as small rectangles, ovals and large rectangles, respectively. Various binding regions are indicated. Filled rectangles indicate the type-III segments, EIIIA and EIIIB, that undergo alternative splicing. A third variable region, V, also is indicated. The protected probe fragments are shown as stippled boxes. A+ and B+ indicate inclusion, and A− and B− exclusion, of EIIIA and EIIIB, respectively. V120 indicates inclusion of the entire V region; V95 denotes exclusion of a 25-amino acid fragment at the 5′ end of the V region. (V0 mRNA, indicating exclusion of the entire V region, was not detectable.) In addition to the probes shown, one termed pSR270 was also used; it represents a 270-bp invariant sequence at the carboxy-terminus and hybridizes with all forms of fibronectin.

(Molecular Research Center, Inc., Cincinnati, OH). The concentration of RNA was determined spectrophotometrically; the integrity of each sample was verified by agarose/formaldehyde gel electrophoresis. Radiolabeled probes were made by transcription of the appropriate plasmid with SP6 or T7 RNA polymerase in the presence of [α-^{32}P]CTP. Total cellular RNA (5–50 μg) was hybridized in solution with excess ^{32}P-labeled cRNA (0.5–1.0 × 10^6 cpm) for 12–16 h at 50–55°C. Unhybridized RNA was digested with ribonuclease T2 (Maher and McGuire, 1990). Intact hybrids were precipitated, denatured by boiling for 3 min in electrophoresis buffer containing 80% formamide, and separated by electrophoresis in a 5% polyacrylamide/urea gel. After drying, gels were applied to x-ray film (Kodak X-OMat AR-5) for 12–24 h. Scanning densitometry (Hoefer Scientific Instruments, San Francisco, CA) was used to quantitate the autoradiographic signals. Apparent band intensities were corrected for size differences among the various fragments.

RNA samples were probed also with a cDNA encoding 585 bp of ribosomal protein S-14 (Rhoads et al., 1986) both to verify the integrity of the mRNA in a sample and also to control internally for the amount of mRNA present in an individual assay. Preliminary experiments were conducted to evaluate the constancy of this mRNA among liver cell types and under different experimental conditions. The level of S-14 mRNA increased less than 1.4-fold in whole liver after bile duct ligation (Fig. 2) and did not change significantly in endothelial cells or lipocytes (Fig. 3).

Immunohistochemical Detection of A+ Fibronectin in Normal and Injured Liver

The liver, with or without bile duct ligation, was perfused under low pressure with PBS until free of blood, via a catheter inserted into the portal vein. The tissue was then removed, cut into small (0.5 cm^2) pieces and immersed in liquid nitrogen. Cryostat sections of frozen liver were placed on positively charged glass plates and stored at 4°C overnight. Specimens were fixed in acetone at −20°C for 10 min, then incubated at 25°C in a blocking solution consisting of PBS with 0.1% nonfat milk, 0.15 M ammonium acetate and 2% sheep serum.

Monoclonal antibodies specific for A+ fibronectin (IST-9 and ED-A), directed at epitopes within the EIIIA domain of the molecule, were diluted 1:200 in PBS containing 0.1% nonfat milk, 15 mM ammonium acetate, and 2% sheep serum. Sections were incubated with antibody overnight at 4°C, then washed three times with PBS and incubated with biotinylated sheep anti-mouse IgG for 2 h at 25°C. After three washes with PBS, the specimens were exposed to streptavidin-linked Texas red for 30 min, washed with PBS, and mounted. Negative controls consisted of specimens incubated with nonimmune mouse IgG and processed in parallel. Sections were viewed with a Nikon Microphot-FX fluorescence microscope and photographed with Ilford HP5-plus (ASA 400) film.

Preparation of Endothelial Cell-derived Extracellular Matrices

Native cell matrices with differing levels of A+ fibronectin were prepared as follows: sinusoidal endothelial cells were isolated from either normal liver or from liver that had been subjected to bile duct ligation (BDL) 12 h earlier. The fresh isolates were plated at confluent density in 35-mm cell culture dishes or on chamber-well slides (Nunc, Inc., Naperville, IL) in Medium 199 with 20% serum (10% calf, 10% horse), insulin (4 mU/ml) and penicillin (100 U/ml). All culture dishes were precoated with a thin layer of type I collagen (~10 μg/35-mm dish) to facilitate cell attachment.

Three days after plating, the cells were removed from the underlying extracellular matrix with 0.5% deoxycholic acid in Dulbecco's PBS for 1 h at room temperature followed by extensive washing with the same buffer. Complete removal of the cells was confirmed by microscopy. The presence of A+ fibronectin in the endothelial cell-derived substrata was established immunohistochemically. Cell layers or stripped preparations on chamber-well slides were fixed in methanol at 4°C for 10 min, followed by incubation at 25°C with PBS containing 0.1% nonfat milk, 0.15 M ammonium acetate and 5% sheep serum, to block nonspecific binding of antibody. Anti-fibronectin monoclonal antibodies or anti-type IV collagen (all as purified IgG) were added (IST-9 diluted 1:100, ED-A 1:200, C6F10 1:100, IST-6 1:100 and anti-type IV collagen 1:100 in PBS with 0.1% dry milk, 15 mM ammonium acetate, and 2% sheep serum). In some studies, IST-9 hybridoma culture medium was used at a 1:20 dilution with results identical to those for purified IgG. After overnight incubation at 4°C, the slides were washed several times with PBS and incubated with biotinylated sheep

anti-mouse IgG for 2 h at 25°C. After multiple washes with PBS, streptavidin-linked Texas red was added for 30 min. After washes, the specimens were viewed and photographed, as described above.

Lipocyte Activation in Culture

Culture dishes (35 mm) were prepared with a thin layer of type I collagen or with matrices formed in situ by endothelial cells from normal or injured liver (see above). Where indicated, some matrices were pretreated with antifibronectin antibodies (IST-9, ED-A, or C6F10) added in serum-free medium for 6 h and then washed with PBS before plating of lipocytes ($0.5–1.0 \times 10^6$ cells/dish) in serum-free medium. By immunofluorescence, there was no loss of bound IgG from the matrices during 3 d of incubation, with or without lipocytes (data not shown). Cells in each group were harvested at 3 d after plating in a buffer containing 62.5 mM Tris–HCl, 1% SDS, 10% glycerol, 20 mM dithiothreitol, and 2% β-mercaptoethanol. The samples were boiled and the protein concentration determined (Bio Rad Labs., Richmond, CA). Equivalent amounts of protein were analyzed by SDS-PAGE (8% polyacrylamide). Immunoblotting and detection of smooth muscle-specific α-actin, a marker of lipocyte activation, was carried out as described previously (Rockey et al., 1992). Scanning densitometry (reflectance mode) was used to quantitate the results. The data are expressed relative to smooth muscle α-actin levels in cells plated on type I collagen, arbitrarily set at one. The quantitative nature of the assay was established by parallel assay of graded amounts of protein extract from rat aorta.

Studies with Fibronectin Fusion Proteins

cDNA clones encoding the desired fusion proteins were prepared utilizing an RNA extract of whole liver from an animal that had undergone bile duct ligation and contained the mRNA for both A+ and A− fibronectin (see Fig. 2). Reverse transcription was performed with an oligo dT primer, according to the manufacturer's protocol (GIBCO BRL, Gaithersburg, MD). For amplification of specific fibronectin sequences with or without the A+ segment, two sets of oligonucleotide primers complementary to the published sequence of rat fibronectin were prepared. (Schwarzbauer et al., 1983; Odermatt et al., 1985)

1. *Forward* 5′-CTCCGAATTCAACATTGACCGCCCTAAAGGACTG-3′
   Reverse 5′-CTCCAAGCTTTGTGGACTGGACTCCAATCAGGGG-3′
2. *Forward* 5′-CTCCGAATTCGAAATTGACAAGCCATCCCAGATG-CAG-3′
   Reverse 5′-CTCCAAGCTTCTCCAGAGTCGTGACGACTCCCTG-AGC-3′

The first primer pair spanned just the 270-bp EIIIA segment. The second primer pair was designed to extend from the amino terminus of the eleventh type III repeat to the carboxy terminus of the twelfth type III repeat (see Fig. 1). Recognition sites for restriction enzymes were added (5′-EcoRI, 3′-HindIII). Amplification of the first strand cDNA was accomplished by 30 cycles of polymerase chain reaction (1 min at 94°C, 30 s at 45°C, 1 min at 72°C). The first primer pair yielded a single band at ~280 bp which on sequencing was identical to the Fn EIIIA fragment (EIIIA). The second primer pair yielded two bands, one at 550 and one at 840 bp. The latter fragment was identical to EIIIA flanked on either side by the 11th and 12th type III repeats (11EIIIA12), while the former comprised the 11th and 12th repeats without the EIIIA segment (1112A−).

The PCR-amplified fragments were cloned into the EcoRI and HindIII sites of pMAL-c2, downstream of the malE gene, which encodes the maltose-binding protein (MBP) and results in expression of an MBP fusion protein. Transformed *Escherichia coli* were grown to a density of $\sim 2 \times 10^8$ cells/ml. After addition of isopropyl-β-D-thiogalactoside to a final concentration of 0.9 mM, the bacteria were agitated at 37°C for 2 h, centrifuged at 4,000 *g* for 20 min, resuspended in buffer (20 mM Tris, 200 mM NaCl, 1 mM NaEDTA, pH, 7.4), and disrupted by freezing/thawing and sonication. The bacterial lysate was centrifuged at 9,000 *g* for 30 min and the supernatant was applied to an amylose resin column. After extensive washing with column buffer, the fusion protein was eluted in 10 mM maltose, dialyzed against 10 mM Tris–Cl, 100 mM NaCl, pH, 8.0, concentrated by ultrafiltration (Centricon-30; Amicon, Beverly, MA) and analyzed by Western blot using monoclonal antibodies EDA (52DH1) and IST9.

Culture substrata were prepared by mixing purified fusion protein (100 μg) with 50 μg type I collagen in a volume of 200 μl which was spread evenly over 35-mm culture dishes and allowed to dry at 25°C overnight. The presence of the fibronectin fusion proteins after rehydration was confirmed by immunoperoxidase staining. Lipocytes from normal liver were plated on these substata and on collagen alone ($0.5–1 \times 10^6$ cells/dish); at 3 d, the cells were analyzed for smooth muscle α-actin by immunoblot as described above.

Recombinant peptides were prepared also using the 6xHis system (Qiagen, Chatsworth, CA), which eliminates MBP from the fusion product. The 6xHis-tagged peptides were purified on Ni-NTA resin and used as described above.

Statistical Analysis

All numerical data represent the mean ± SEM of at least three independent experiments, with cells prepared from different animals. Results with $P < 0.05$ by Mann-Whitney U-test were considered significant.

Results

Quantitation of Fibronectin mRNAs

Whole Liver. Total fibronectin mRNA, detected by the pSR270 probe, increased sevenfold after liver injury (Fig. 2 *A*); the signals for A− and B− fibronectin increased in parallel, indicating that much of the change is attributable to fibronectin that is A+ only, B+ only or A−/B− (Fig. 2, *B* and *C*). The mRNAs for A+ and B+ fibronectin were essentially undetectable in normal whole-liver extracts but clearly present seven days after bile duct ligation. V95 and V120 mRNAs similarly increased. S-14 mRNA increased 1.3-fold after bile duct ligation in relation to total liver RNA (Figure 2, *E* and *F*).

Hepatocytes. A+ (EIIIA containing) and B+ (EIIIB containing) fibronectins were not detectable in hepatocytes from normal liver or from injured liver up to 14 d after bile duct ligation. Moreover, total fibronectin mRNA in hepatocytes remained constant throughout the injury response as indicated by the intensity of the A− and B− bands (data not shown). The data for V-region mRNA in hepatocytes were similar to those for whole-liver extracts. The relative amounts of V120 and V95 mRNA in hepatocytes did not change during injury (data not shown).

Sinusoidal Endothelial Cells. As in whole liver, normal non-parenchymal cells expressed very little fibronectin mRNA. By contrast, in liver injury, A+ mRNA in sinusoidal endothelial cells increased rapidly to a peak at 12–24 h and constituted most (>80%) of the total fibronectin mRNA (Figs. 3 and 4, *A* and *C*). Under the same conditions, B+ fibronectin was minimally increased (Figs. 3 and 4 *B*). Small increases in endothelial cell A+ mRNA occurred also in the 12- and 24-h sham-operated animals, representing less than 10% of the values for bile duct-ligated animals and disappearing by 48 h (Fig. 4 *A*, inset). V120 mRNA levels paralleled those for A^+, peaking at 12 h then declining over several days (data not shown).

Lipocytes. A small increase in A+ mRNA was noted in lipocytes at 24 h after liver injury (Figs. 3 and 4 *A*), but this was present also in sham-operated animals. Beyond 24 h, increased A+ expression persisted in the injury model but not in the sham-operated animals. In contrast to endothelial cells from the injured liver, lipocytes displayed an increase also in B+ mRNA, which reached statistical significance at 24 h and progressively increased (Figs. 3 and 4 *B*). The A+ form, representing 7% of total fibronectin mRNA in normal cells, increased to 42% at 7 d after liver injury; B+ was <1% in normal cells and increased to 9% at 7 d (Fig. 4 *C*); V120 mRNA was fourfold above normal at 7 d (data not shown).

Kupffer Cells. Initial studies on a conventionally prepared Kupffer cell fraction from injured liver suggested relatively

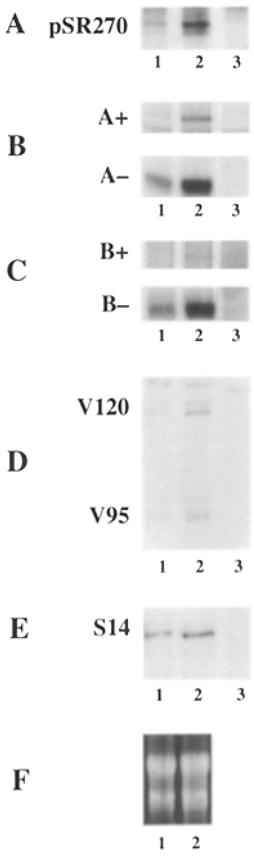

Figure 2. Expression of total fibronectin and variants after liver injury in whole-liver extracts. A representative RNase protection assay with 10 μg total cellular RNA is shown; 5 μg RNA was used for the assay of S-14. Lane *1*, normal liver; lane *2*, 7 d bile duct-ligated liver; lane *3*, negative control (yeast tRNA). (*A*) Total fibronectin (pSR270) mRNA. (*B*) A+ fibronectin mRNA. A+ indicates inclusion and A− exclusion, respectively, of the EIIIA region of fibronectin. (*C*) B+ fibronectin mRNA expression. B+ indicates inclusion and B− exclusion, respectively, of the EIIIB region of fibronectin. (*D*) V-region mRNA. V120 and V95 indicate inclusion of the respective fibronectin V-region mRNA fragments. (*E*) S-14 mRNA, an internal control for the mRNA content of each extract. (*F*) Denaturing gel electrophoresis of typical extracts, showing the 18S and 28S ribosomal bands stained with ethidium bromide; normal liver (lane *1*), and injured liver 7 d after bile duct ligation (lane 2). Sham operation yielded data essentially identical to that from unoperated normal controls.

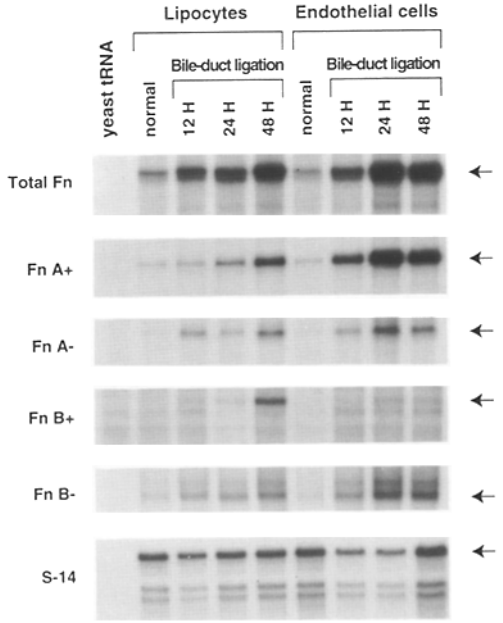

Figure 3. Fibronectin and S-14 mRNA expression in normal and injured liver. Representative RNAse protection assays are shown in which the same RNA extract was used for all probes at a given time point. Lipocytes and endothelial cells for each time point were harvested from a single animal. 5 μg total RNA was used with S-14; 20 μg RNA was used with the remaining probes. Arrows indicate the position of the specific bands. Yeast tRNA served as the negative control. Total fibronectin mRNA was measured using pSR270. A+ indicates inclusion and A− exclusion, respectively, of the EIIIA region. B+ indicates inclusion and B− exclusion, respectively, of the EIIIB region.

high levels of mRNA for both A+ and B+ fibronectin, but the isolates contained significant numbers of lipocytes and, to a lesser extent, biliary epithelial cells. Magnetically purified Kupffer cells (see Methods) expressed a low level of A+ mRNA and no B+ mRNA (data not shown).

In summary, the earliest change in fibronectin mRNA expression after liver injury is a transient, but striking, increase in the A+ form in sinusoidal endothelial cells. This is followed by a gradual increase in B+ expression by lipocytes.

Immunohistochemical Detection of A+ Fibronectin in Injured Liver. The observed changes in A+ fibronectin expression were present also at the protein level as seen immunohistologically in whole-liver sections. Staining of normal or sham-operated liver with monoclonal antibody IST-9 yielded faint, but specific, reaction within portal triads, which appeared to be localized to arterioles. Staining within the lobule was undetectable (Fig. 5 *A*). By contrast, 2 d after bile duct ligation, perisinusoidal staining was extensive (Fig. 5 *B*); at 5 and 9 d, it was virtually continuous (Fig. 5, *C* and *D*). The monoclonal antibody, ED-A, derived independently but having the same specificity as IST-9, gave identical results (not shown). When either monoclonal antibody was replaced by nonimmune IgG, no specific staining was observed (not shown).

Lipocyte Activation by Endothelial Cell-Derived A+ Fibronectin

The next set of studies examined production of A+ fibronectin by endothelial cells and the effect of endothelial cell-derived matrices on lipocyte activation. In preliminary work, it was noted that sinusoidal endothelial cells isolated from normal liver and cultured on a plastic substratum undergo spontaneous changes that include increased expression of A+ fibronectin. The latter was evident at 72 h of culture and increased progressively. A similar phenomenon occurs in cultured large-vessel endothelium (Burke and Danner, 1991). For this reason, the period of culture was limited to three days, which sufficed for deposition of a stable ECM. Cultures were examined for the presence of A+ fibronectin by fluorescence immunohistochemistry with monoclonal antibody to the EIIIA segment (Fig. 6). Endothelial cells from both normal liver and bile duct-ligated liver produced detectable A+ fibronectin (Fig. 6, *g* and *h*), but the amount deposited by cells from the ligated liver clearly exceeded that deposited by cells from normal liver (Fig. 6, *a* and *b*). In keeping with the increase in total fibronectin mRNA after bile duct ligation (Fig. 3), staining by anti-fibronectin antibody to an invariant segment (III-9) was increased in endothelial cell cultures from bile duct-ligated liver (Fig. 6, *c* and *d*). In contrast, deposition of type IV collagen was similar in the two types of culture (Fig. 6, *e* and *f*). Type IV collagen is known to be produced by normal sinusoidal endothelial cells (Irving et al., 1984; Maher and McGuire, 1990).

Normal lipocytes were plated on the various substrata, and their activation was monitored using smooth-muscle α-actin as a marker (Fig. 7). Fresh isolates contained no detectable

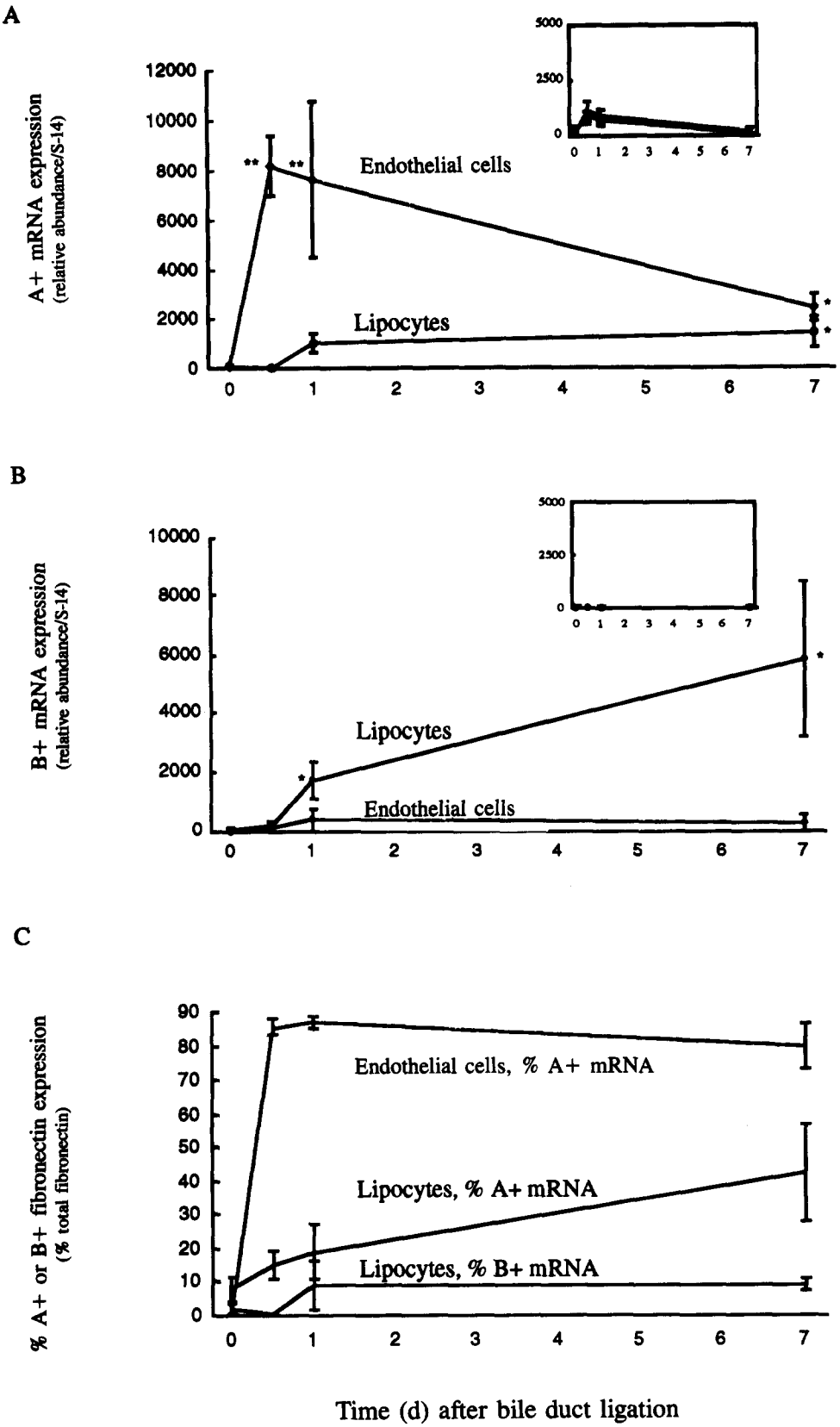

Figure 4. Cell-specific changes in fibronectin splicing with liver injury. Each point represents the mean of at least three separate experiments ± SEM. The signal for A+ or B+ mRNA has been corrected for the level of S-14 mRNA in the same samples. Values obtained following sham operation (*inset*) have been subtracted. Note different scale of inset graphs. (*A*) A+ mRNA relative abundance. The difference between endothelial cells and lipocytes is significant ($p < 0.05$) at all time points after injury except at 7 d. $^{**}P < 0.05$ versus lipocytes at the same time points and control endothelial cells. $^{*}P < 0.05$ versus normal controls. (*B*) B+ mRNA relative abundance. $^{*}P < 0.05$ versus control (0− time) lipocytes. (*C*) Fibronectin variant mRNA as a percentage of total fibronectin expressed for each cell type. The percentage of A+ and B+ mRNA, respectively, was calculated after correction of the autoradiographic signals for the size of the protected fragment.

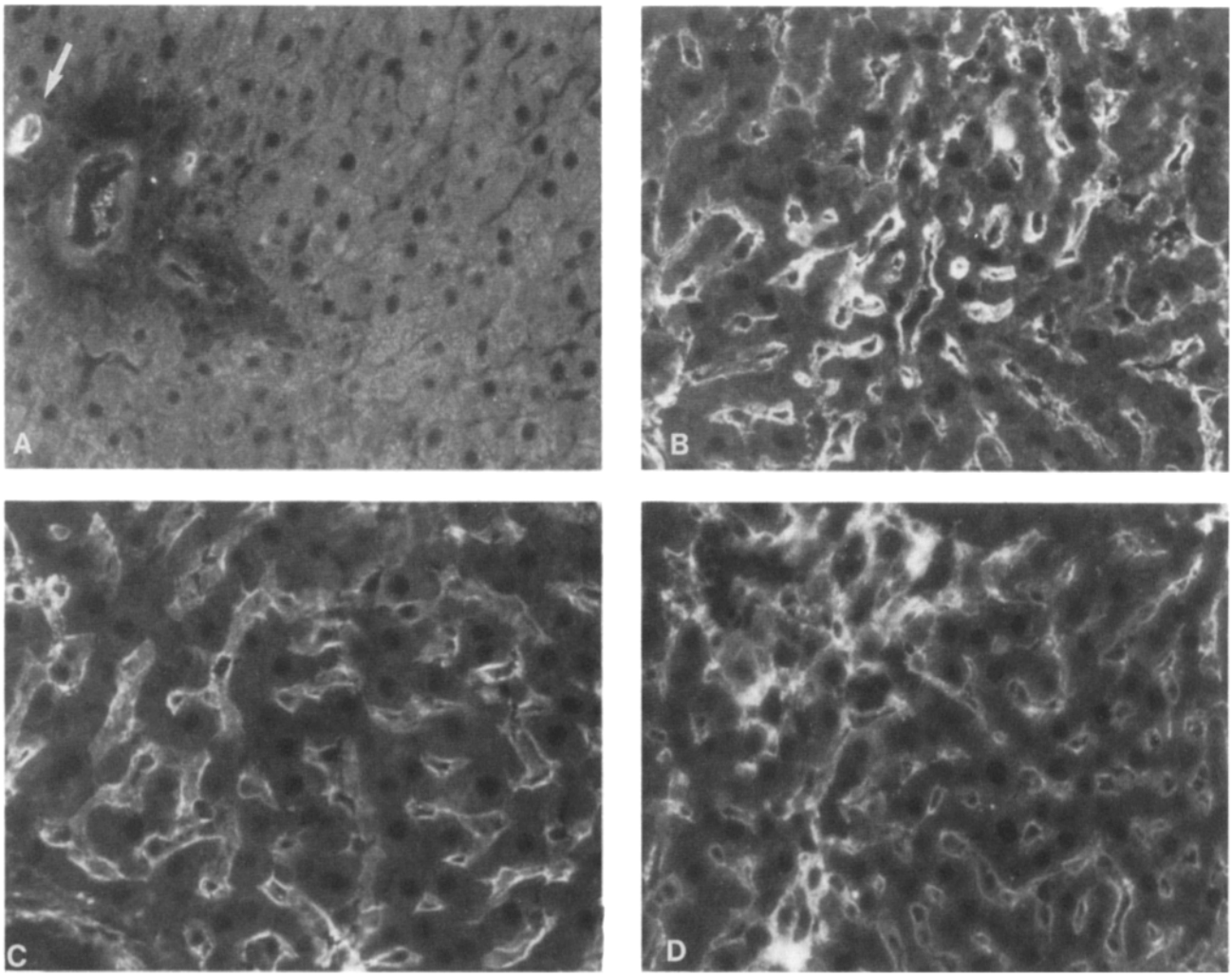

Figure 5. Immunohistochemical detection of A+ fibronectin deposition in the rat liver after bile duct ligation. Photomicrographs of whole liver sections stained with IST-9, a monoclonal antibody specific for A+ fibronectin, are shown. (*A*) Normal whole liver. Reaction is present around a portal arteriole, indicated by the arrow. There is no specific A+ staining along sinusoids. Staining was identical in sham-operated controls (not shown). (*B*) Liver 2 d after bile duct ligation. Specific staining is present along many sinusoids. (*C*) Liver 5 d after bile duct ligation. A+ fibronectin is prsent along essentially all sinusoids. (*D*) Liver 9 d after bile duct ligation. The pattern is similar to that shown in *C*.

smooth-muscle α-actin but in culture on type I collagen underwent spontaneous activation that was detectable after 2–3 d and increased progressively (Rockey et al., 1992). Lipocytes incubated for the same period of time on a matrix containing A+ fibronectin (deposited by endothelial cells from the injured liver) expressed substantially higher levels of smooth muscle α-actin, while lipocytes plated on a matrix with a low level of A+ fibronectin (elaborated by endothelial cells from normal liver) exhibited a small but nonsignificant increase in smooth-muscle α-actin. The activating effect of the endothelial cell-derived matrices was completely blocked by IST-9 or EDA, two independently derived monoclonal antibodies to the EIIIA segment. An antibody to an adjacent invariant region (C6F10) had no significant effect (Fig. 7).

While the blocking effect of IST-9 clearly pointed to a role of the EIIIA segment, lipocyte activation conceivably was related in part to the difference in total fibronectin in the matrices deposited by endothelial cells from normal or injured liver (Fig. 6, *c* and *d*). To address this issue, we prepared culture substrata of plasma (EIIIA−, EIIIB−) or cellular fibronectin (EIIIA+ and/or EIIIB+), presented in type I collagen. Normal lipocytes were plated and monitored as before (Fig. 7). The substratum containing cellular fibronectin induced twice the level of smooth-muscle α-actin as did that containing plasma fibronectin (Fig. 8); the increase was blocked by IST-9, confirming that activation was related to the presence of the EIIIA segment.

Lipocyte Activation by Fibronectin Fusion Proteins Containing the EIIIA Domain

The fibronectin fusion proteins were characterized by SDS-PAGE and Western blot. Single bands of appropriate size for all three proteins were visualized by Coomassie blue staining, and the immunological reactivity of fusion proteins EIIIA and 11EIIIA12 was confirmed by Western blot with monoclonal antibodies IST-9 and ED-A (not shown); 1112A−, which lacks the EIIIA segment, did not react. Tis-

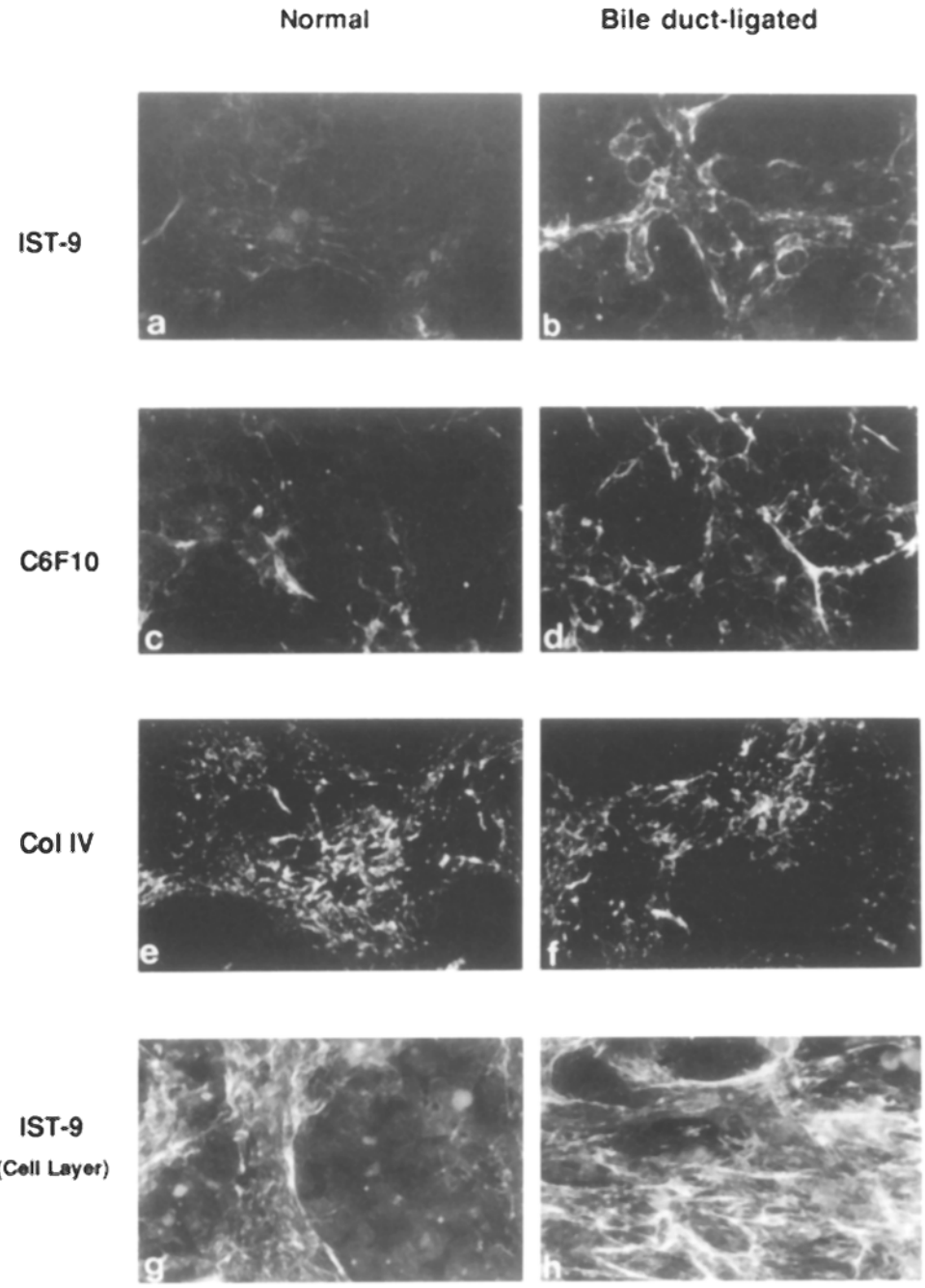

Figure 6. Fibronectin and type IV collagen immunostaining of extracellular matrix and cells from normal and injured liver, in primary culture. After 3 d of culture, cells were fixed as described in Methods and stained with the indicated antibody. IST-9 recognizes the EIIIA region; C6F10 is specific for the tenth type III repeat, common to all forms of Fn. Type IV collagen is elaborated by both normal and bile duct-ligated endothelial cells. Normal (*a*, *c*, and *e*) and bile duct-ligated (*b*, *d*, and *f*) endothelial cell matrix: the cell monolayers were stripped and then stained with the indicated antibodies, as described in Methods. Intact normal (*g*) and bile duct-ligated (*h*) endothelial cells, before stripping, were fixed and stained with IST-9.

sue culture dishes were coated with the proteins (see Methods), incubated in medium and then stained to verify that the proteins were present and uniformly distributed. Normal lipocytes plated on substrata containing proteins EIIIA or 11EIIIA12 exhibited a threefold increase in smooth muscle α-actin expression compared with cells plated on 1112A− (Fig. 9); the latter substratum was not different from collagen controls. To exclude an effect of the maltose-binding protein within the fusion product, recombinant peptide containing only the 6xhis tag (see Methods) was studied and produced similar results. To test the effect of the ECM environment, the peptides were presented in EHS gel, in place of collagen. The EHS gel is rich in laminin and serves as a model basement membrane (Bissell et al., 1987). In this environment also, the EIIIA segment caused significantly greater lipocyte activation than did the control peptide (flanking segments without EIIIA), although the magnitude of stimulation was somewhat less than that of peptide presented within a collagen matrix (Fig. 10).

Discussion

The appearance of the EIIIA and EIIIB variable domains during development (ffrench-Constant and Hynes, 1989; Laitinen et al., 1991; Pagani et al., 1991), their near-total disappearance in the adult and reappearance in the context of wound-healing suggest a specific biological role for these variant fibronectins. However, while mapping has defined

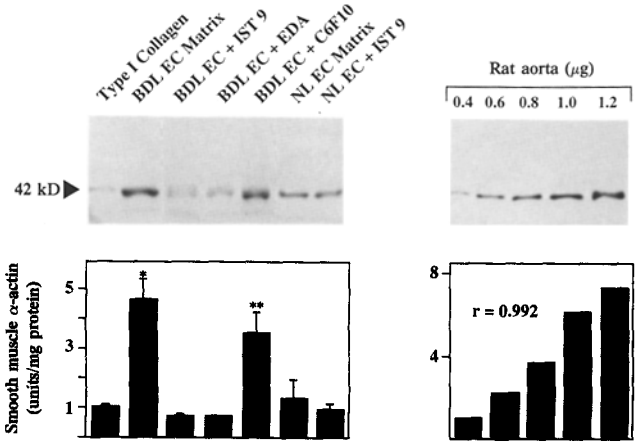

Figure 7. Expression of smooth muscle-specific α-actin by lipocytes cultured on various substrata. Lipocytes were isolated from normal liver, and aliquots from the same preparation were plated on preformed matrices deposited in situ by endothelial cells from either normal liver (*NL EC*) or liver subjected to bile duct ligation (*BDL EC*). In the latter case, the endothelial cells were isolated 12 h after the injury (see Methods). Some BDL EC matrices were pretreated with fibronectin monoclonal antibodies (4 μg IgG) prior to lipocyte plating. IST-9 and EDA recognize the EIIIA region; C6F10 is directed against the adjacent, invariant III-9 segment (see Methods). Type I collagen, which moderately induces lipocyte activation (Rockey et al., 1992), served as a positive control. A typical immnoblot is shown at the upper left, with the expected smooth muscle α-actin band at 42 kD. Immnoblot results were quantitated by scanning densitometry (*lower left*). The bars represent the mean of four separate experiments ± SEM (except for *BDL EC + EDA*, which was performed twice). The difference between BDL EC matrix and BDL EC matrix + C6F10 was not statistically significant. $*P < 0.05$ versus all conditions; $**P < 0.05$ versus all conditions except NL EC matrix. Graded amounts of protein extract from rat aorta were used in the same assay to demonstrate that the signal was proportional to the amount of smooth muscle α-actin. The Western blot is shown at the upper right, with the indicated quantity of protein; the scanning densitometry results, with the correlation coefficient, is shown at the lower right.

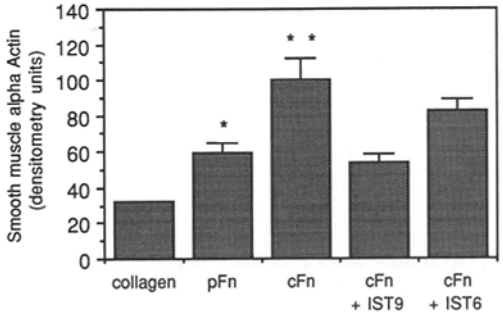

Figure 8. The effect of "cellular" (*cFN*) or "plasma" (*pFn*) fibronectin on activation of normal lipocytes. The human proteins were purchased from Fibrogenex (Chicago, IL), and 220 μg were mixed with 50 μg of type I collagen and applied to culture plates. Collagen alone served as a control substratum. Some plates containing the cellular form were pretreated with IST-9, as described for Fig. 7; the control antibody was IST-6, which reacts with a segment adjacent to EIIIB, present in all forms of fibronectin (Carnemolla et al., 1992). Fresh normal lipocytes were plated and monitored as described in Fig. 7. The effect of the cellular form was significantly greater than that of the plasma form and was neutralized by pretreatment of the substratum with IST-9 but not by IST-6. Although the mean value for pFn was greater than that for the collgen control, the difference was not significant. * pFn vs. cFn, $p < 0.01$, $n = 4$; ** cFn vs. cFn+IST9, $P < 0.01$, $n = 4$; cFn vs. cFn+IST6, $P > 0.2$, $n = 4$.

several important regions of fibronectin, the function of the variable segments has remained unclear. In the present studies, we have examined this question in a liver model of wound healing, taking advantage of cell-isolation techniques available for this tissue. Individual isolates yield sufficient RNA for immediate analysis, so that expansion of starting samples in culture is unnecessary. By this approach, an "in vivo" profile of fibronectin mRNA expression for individual cell populations can be obtained. The results indicate that A+ fibronectin increases rapidly after liver injury and that it derives from sinusoidal endothelial cells.

Bile duct ligation was selected as the injury model because it rapidly and reproducibly induces fibrosis in association with up-regulation of ECM gene expression in lipocytes (Maher and McGuire, 1990). The injury is centered on portal areas, in contrast to other models. Open to question is whether the observed endothelial cell response is unique to bile duct ligation. Injury produced by administration of carbon tetrachloride contrasts with that caused by bile duct ligation in being mechanistically distinct and involving centrilobular areas rather than portal tracts. In a direct comparison of the two models, the changes in lipocyte gene expression were virtually identical (Maher and McGuire, 1990). With respect to A+ fibronectin also, carbon tetrachloride elicits an increase resembling that produced by bile duct ligation (Odenthal et al., 1993). Taken together, the data suggest that early production of A+ fibronectin by endothelial cells in fibrosing injury is independent of the type of liver injury and likely to be broadly relevant to the repair response in wound healing.

Previous work has suggested that splicing of the fibronectin EIIIA and EIIIB regions, respectively, proceeds independently (Barone et al., 1989; ffrench-Constant and Hynes, 1989). The present data support this conclusion, showing that not only the time course of expression of the two forms differ following liver injury but that different cell types are

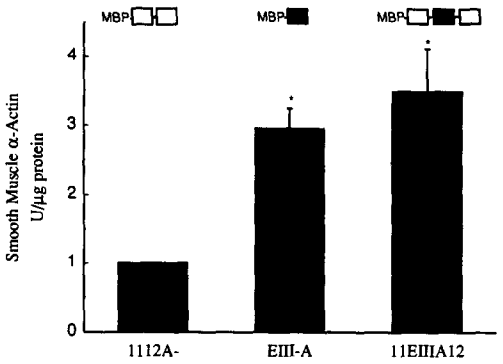

Figure 9. Smooth muscle α-actin expression in lipocytes cultured on MBP-fibronectin fusion proteins. The graph shows the combined scanning densitometry results of three separate experiments ± SEM. In each experiment, the values obtained with MBP-1112A- were similar to the collagen control and arbitrarily set at 1. $* P <$ 0.05 by comparison with MBP-1112A-.

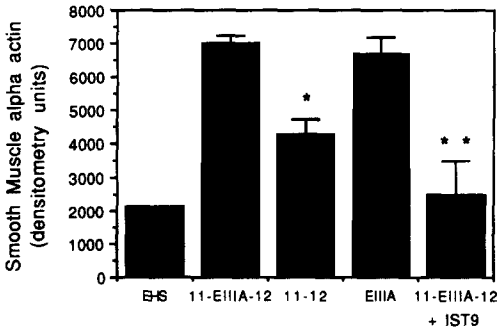

Figure 10. Activation of normal lipocytes cultured on recombinant 6xhis-tagged fibronectin peptides. Substrata were prepared by combining the indicated peptides (100 μg) with 100 μl of EHS extract prepared as described (Bissell et al., 1987) and applying the solution to culture plates; a similar amount of EHS extract alone served as control. Some matrices were pretreated with IST-9, to block the EIIIA segment. Fresh normal lipocytes were plated and monitored as described for Fig. 7. * 11-12 vs. 11-EIIIA-12 or EIIIA, $P < 0.01$, $n = 4$; ** 11-EIIIA-12 + IST9 vs. 11-EIIIA-12 or EIIIA, $P < 0.01$, $n = 4$; 11-EIIIA-12 + IST9 vs. 11-12 $P > 0.2$, $n = 4$.

involved. These findings are consistent with studies of the developing embryo (Pagani et al, 1991) and of fibrosing injury in adult tissues. In human renal allograft rejection, for example, the A+ form predominates early in the process; in chronic rejection, both A+ and B+ are expressed, and in fibrotic glomeruli only B+ is detected (Gould et al., 1992).

This study has not addressed the role of the EIIIB and V regions, except to show that B+ fibronectin expression after bile duct ligation is localized to lipocytes and that its profile is similar to that for collagen I mRNA in the same injury model (Maher and McGuire, 1990). This may be consistent with a role in matrix assembly. With regard to the V region, the V120 form increased after bile duct ligation in sinusoidal endothelial cells, paralleling the change in the A+ form. However, the mRNA for V120 is constitutively expressed by normal hepatocytes and also by lipocytes, implying that the corresponding protein is present normally in the perisinusoidal matrix. For this reason, we have chosen to focus on the increase in A+ fibronectin as being unique to the wound healing process. At no time were the A+ and B+ forms detectable in hepatocytes. The reported production of A+ fibronectin by hepatocytes maintained in culture appears to be a culture phenomenon only and is quantitatively minor (Magnuson et al., 1991; Odenthal et al., 1992).

Previous studies of lipocytes in primary culture have indicated the sensitivity of these cells to extracellular matrix: lipocytes maintained on basement membrane-like ECM retain their normal quiescent phenotype, while cells on fibrillar (type I) collagen undergo spontaneous change that closely mimics in vivo activation (Friedman et al., 1989). The perisinusoidal ECM in normal liver, which presumably surrounds lipocytes, consists of basement-membrane proteins (Bissell et al., 1987) and may play a role in maintaining lipocyte quiescence. Extrapolating from the culture data, one may speculate that injury impacts on lipocytes not only by presenting de novo the EIIIA region but also by perturbing the structure of the existing ECM. The data add to the evidence that the basement membrane ECM in epithelia is heterogeneous, with regions of local specialization. A heterogeneous distribution of fibronectin has been documented previously in morphological studies of the microcirculation. For example, capillary pericytes and endothelial cells are joined by patches of fibronectin-rich ECM (Courtoy and Boyles, 1983). While it is uncertain whether analagous patches exist around the sinusoids of normal liver, the present data suggest that they arise in liver injury.

In these studies, we used very early primary cultures of sinusoidal endothelial cells and lipocytes, respectively, for examining the direct effect of the EIIIA region on lipocyte activation. The rationale initially was to model the in vivo interaction of these cells as closely as possible. It emerged that use of early primary culture was essential to demonstrating the effect of endothelial-derived ECM on lipocytes, due to the rapidity with which liver cells in primary culture undergo phenotypic change. This has been well-documented for hepatocytes in conventional culture (Bissell and Guzelian, 1980). Also for lipocytes plated on plastic, changes that mimic activation in vivo proceed spontaneously and are marked after 3–5 d of primary culture (Friedman, 1993). With regard to sinusoidal endothelial cells, we showed previously that the number of open fenestrae, which are characteristic of these cells, decreases by 90% in conventional primary culture within 48 h of plating (McGuire et al., 1992). The present studies indicate that production of EIIIA-fibronectin accompanies this morphological change. In brief, beyond the first three days of culture of lipocytes and sinusoidal endothelial cells, spontaneous change supervenes to the extent that the cell culture models no longer reflect the differences that exist in vivo between "normal" and "injured."

The response of lipocytes to A+ fibronectin, monitored as increased expression of smooth-muscle α-actin, becomes evident only after 1–2 d in culture and may require longer than this in vivo (Rockey et al., 1992). Lipocyte activation constitutes a program of phenotypic change that proceeds through several days. We monitored smooth-muscle α-actin in the present studies not because it is the earliest marker of activation (receptors for PDGF or TGF-β appear earlier) but because it presumably represents a commitment of the cell to a myofibroblast-like phenotype.

We suggest that elaboration of A+ fibronectin by endothelial cells is a very early, if not the initial, change in the perisinusoidal ECM in liver injury and that the EIIIA segment is a key element in a milieu that directs lipocyte activation. EIIIA could act, in theory, by affecting the conformation of a second region that interacts with lipocytes. A precedent is the finding that EIIIB affects the conformation of its immediately adjacent type III repeat (III-8) (Carnemolla et al., 1992). The principal cell-binding region (RGD site) is only one type III repeat removed from EIIIA, and lipocytes express its receptor, the $\alpha_5\beta_1$ integrin (S. S. Wang and D. M. Bissell, unpublished data). Two points, however, argue against a significant interaction of the EIIIA and RGD regions. The first is that recombinant peptide containing EIIIA but lacking the cell-binding site was stimulatory for lipocytes. The second is that intact "cellular" fibronectin, containing EIIIA and/or EIIIB, while more active than "plasma" fibronectin (without either region), was no more stimulatory to lipocytes than the EIIIA peptide alone. This does not rule out other interactions, those between EIIIA and the V region, for example: the question merits further study.

Lipocyte activation is an event with important clinical ramifications, in that hepatic fibrosis often results in clinically evident liver disease. Because inflammation generally accompanies fibrosis, a number of studies have explored the role of proinflammatory cytokines in this process (Bissell and Roll, 1990; Gressner, 1991; Friedman, 1993). Most proceed from the assumption that cytokines initiate lipocyte activation. To date, however, exposure of lipocytes to such factors in culture has yielded unimpressive results, suggesting that cytokines act in concert with other elements of the injury milieu. The present findings point to the possibility that endothelial-derived A+ fibronectin forms part of that milieu. Similarly, A+ fibronectin appears to accelerate, rather than absolutely control, the activation response and thus may function cooperatively rather than in isolation; its interaction with lipocytes may render these cells sensitive to specific cytokines, perhaps by modulating receptor expression. The latter remains to be examined. Similarly, the ECM context may influence the biological effect of the EIIIA segment. Although not formally examined here, this is suggested by the general greater effect of A+ fibronectin within a "complete" endothelial-derived ECM relative to that of cellular fibronectin presented with collagen I.

A final issue concerns factors that regulate A+ fibronectin production by sinusoidal endothelial cells, and proinflammatory cytokines are obvious candidates. Indeed, in cultured human fibroblasts (Borsi et al., 1990) and in human umbilical vein endothelial cells (Kocher et al., 1990), transforming growth factor-β increased the expression of A+ fibronectin. Studies of the effect of this and other candidate factors on the ECM phenotype of hepatic sinusoidal endothelial cells are anticipated.

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