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

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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) (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; Kornblith 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., 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., 1990a). 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).
other forms of epithelial fibrosis, we undertook a detailed
variant fibronectins—specifically the EIIIA-containing vari-
activation with cells freshly isolated from normal animals
response. Also, having developed culture models of lipocyte
crease in total fibronectin (Martinez-Hernandez, 1984).

the phenotype of myofibroblasts.

(Friedman et al., 1989), we were in a position to test whether

Materials and Methods

Materials

Radiolabeled cytidine-5'-triphosphate ([γ-32P]CTP, >800 Ci/mmoll was
purchased from Amersham Corp. (Arlington Heights, IL). Protein
per collateral B, and DNAse I were purchased from Boehringer Mannheim Bio-
chemicals (Indianapolis, IN); DME, Ham's F-2 and Medium 199, calf
and horse sera from Flow Laboratories (McLean, VA); Eagle's MEM with-
out calcium was prepared in the laboratory using amino acids from Sigma
Chemical Co. (St. Louis, MO). Larex® (arabino gulcan) was obtained from
Consulting Associates (Laconia, WA). Guanidine thiocyanate was pur-
blished from Fluka Chemical Corp. (Ronkonkoma, NY); acrylamide and
agarose from Bio-Rad Laboratories (Richmond, CA); ultra-pure urea, re-
striction enzymes, T4 DNA ligase, and RNase T2 from GIBCO BRL
(Gaithersburg, MD); SP6 and T7 RNA polymerases, Taq DNA polymer-
ase, and low-melting agarose from Promega (Madison, WI); Bluescript
complex (Vectastain) was purchased from Vector Laboratories (Burlin-
game, CA); carboxylated paramagnetic spheres (1-2-μm-diam) and γ-glu-
tamyl-4-methoxy-naphthylamide (GMNA) from Polycliences (Warrington,
PA); deoxycholic acid and Fast Blue BB salt from Sigma. The pMAL-c2
marker of biliary epithelial cells. Aliquots of various fresh isolates were
slides were incubated in GMNA solution (GMNA 2.5 mg/ml, Fast blue BB
salt 0.67 mg/ml, glycyglycine 0.67 mg/ml) for 30 rain 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
ultraviolet autofluorescence (Friedman and Roll, 1987), endothelial cells by
their uptake of DiI-conjugated acetoacetylated low density lipoprotein (Ir-
ing 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 hepat-
ocytes, >95% for lipocytes and endothelial cells, respectively; and for
Kupffer cells it was 90-95%, the principal contaminant being lipocytes.

Hepatocytes were isolated from control and experimental animals by col-
genase perfusion and elutriation, as previously described (Bissell et al.,
1990b). Lipocytes, Kupffer cells and sinusoidal endothelial cells were iso-
late by in situ perfusion with pronase and collagenase followed by centrifu-
gation 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 cellu-
lar debris. The bottom two interfaces contained mixed Kupffer and en-
dothei al 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 (In-
ving 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 hepat-
ocytes, >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 intrave-
nously in a volume of 0.5 ml, 5-10 min before liver perfusion. The particles
rapidly disappeared from the circulation and, within the liver, and were internal-
ized 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 aspi-
rated, and the isolate was released into fresh culture medium and again
separated magnetically; three such washes were carried out. The final iso-
lolate was 99% pure by light microscopy. The remainder of the non-
parenchymal cells were fractionated by gradient centrifugation, as de-
scribed 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 (GOT), 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, glycyglycine 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% GOT-
positive cells and the routinely prepared Kupffer cells, 5-10%. Kupffer cells
purified using magnetic beads, as described above, were free of con-
taminants.

cDNA Probes

Rat cDNA probes containing the alternatively spliced regions EIIA (A+),
and EIIIB (B+), and an invariant, 270 base-pair fragment (pSS270) 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 invar-
iant 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 (Paganl et al., 1991). The amplified fragment was sub-
cloned into pBSK. 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
(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 \([\alpha-32P]CTP\). Total cellular RNA (5-50 \(\mu\)g) was hybridized in solution with excess \(^{32P}\)-labeled cRNA (0.5-1.0 \(\times\) 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 1.2-2.0-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 freshly isolated cells 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), penicillin (100 U/ml), and 5% calf 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, 0.15 M 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.
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 (157-9, ED-A, or C5710) added in serum-free medium for 6 h and then washed with PBS before plating of lipocytes (0.5-1.0 x 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 quantify 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'-CTCCGATTCACATGGACCGCCTACTAAGAGCTG-3'
Reverse 5'-CTCCGACCTGACCACTGACGACTCAAGAGGAGG-3'
2. Forward 5'-CTCCGATCAGGACCATGAGGATGCGAGGAGG-3'
Reverse 5'-CTCCGACCTCAGAGAACGAGTCCAGAGGAGGAGG-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, 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 band was identical to EIIIA flanked on either side by the 11th and 12th type III repeats (Fig. 1). Recognition sites for restriction enzymes were added (5'-EcoRI, 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 (Fig. 1). The PCR-amplified fragments were cloned into the EcoRI and HindIII sites of pMAL-c2, downstream of the maltE 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 ~2 x 10^9 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 3 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 ultratiltration (Centricon-30; Amicon, Beverly, MA) and analyzed by Western blot using monoclonal antibodies EDA (52DHI) and IST9.

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
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 after liver injury are 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, a and b), but the amount deposited by cells from the ligated liver clearly exceeded that deposited by cells from normal liver (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.
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 1EIIIAl2 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.

Discussion

The appearance of the EIIIA and EIIIB variable domains during development (french-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
preformed matrices deposited in situ by endothelial cells from ei-

Western blot is shown at the upper right, with the indicated quantity
by scanning densitometry

muscle c~-actin band at 42 kD. Immoblot results were quantitated
wound healing, taking advantage of cell-isolation techniques

variable segments has remained unclear. In the present

results were proportional to the amount of smooth muscle c~-actin. The

coefficient, is shown at the lower right.

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
populations can be obtained. The results indicate that
expression were virtually identical (Maher and McGuire,
1990). With respect to A+ fibronectin also, carbon tetra-
chloride 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 fibronec-
tin EIIIA and EIIIB regions, respectively, proceeds independ-
ently (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 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
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
immoblot is shown at the upper left, with the expected smooth
muscle α-actin band at 42 kD. Immoblot 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.

Figure 8. The effect of "cellular" (cFn) or "plasma" (pFn) fibronectin
on activation of normal lipocytes. The human proteins were pur-
chased 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 adja-
cent to EIIIB, present in all forms of fibronectin (Carnemolla et al.,
1992). Fresh normal lipocytes were plated and monitored as de-
scribed in Fig. 7. The effect of the cellular form was significantly
greater than that of the plasma form and was neutralized by pretreat-
ment of the substratum with IST-9 but not by IST-6. Although the
mean value for pFn was greater than that for the collagen 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.

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-.
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 EIII B 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 EIII A 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 analogous 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 EIII A 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 EIII A 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 EIII A segment is a key element in a milieu that directs lipocyte activation. EIII A could act, in theory, by affecting the conformation. The principal cell-binding region (RGD site) is only one type III repeat removed from EIII A, and lipocytes express its receptor, the α6β1 integrin (S. S. Wang and D. M. Bissell, unpublished data). Two points, however, argue against a significant interaction of the EIII A and RGD regions. The first is that recombinant peptide containing EIII A but lacking the cell-binding site was stimulatory for lipocytes. The second is that intact "cellular" fibronectin, containing EIII A and/or EII IB, while more active than "plasma" fibronectin (without either region), was no more stimulatory to lipocytes than the EIII A peptide alone. This does not rule out other interactions, those between EIII A 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 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

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 increases 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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References

Barone, M. V., C. Henchcliffe, F. E. Baralle, and G. Paolella. 1988. Cell type specific trans-acting factors are involved in alternative splicing of human fibronectin pre-mRNA. *EMBO* (*Eur. Mol. Biol. Organ.*). J. 7:1089-1095.

Biechele, B. J., M. J. Cowan, J. A. MacDonald, and R. G. Crystal. 1978. Degradation of newly synthesized collagen. *J. Biol. Chem.* 253:4356-4363.

Bissell, D. M., and F. J. Roll. 1990. Connective tissue metabolism and hepatic fibrosis. In *Hepatology*: A Textbook of Liver Disease. D. Zakim, and T. D. National Institutes of Health. 424-444.

Bissell, D. M., J. T. Boyles, and R. Schmid. 1972. Liver sinusoidal cells. Identification of a subpopulation for erythrocyte catabolism. *J. Cell Biol.* 54:107-119.

Bissell, D. M., M. A. Arenson, J. J. Maher, and F. J. Roll. 1987. Support of cultured hepatocytes by a laminin-rich gel. Evidence for a functionally significant subendothelial matrix in normal rat liver. *J. Clin. Invest.* 79: 801-812.

Bissell, D. M., S. L. Friedman, J. J. Maher, and F. J. Roll. 1990a. Connective tissue biology and hepatic fibrosis: report of a conference. *Hepatology*. 11:488-498.

Bissell, D. M., J. M. Caron, L. E. Babiss, and J. M. Friedman. 1990b. Transcriptional regulation of the albumin gene in cultured rat hepatocytes. Role of basement-membrane matrix. *Mol. Biol. Med.* 7:187-197.

Boyer, R. S., M. J. Cowan, J. A. MacDonald, and R. G. Crystal. 1978. Degradation of newly synthesized collagen. *J. Biol. Chem.* 253:4356-4363.

Bissell, J. A., M. S. Akiyama, K. M. Yamada, J. F. Theory, and J. C. Bouc. 1992. Distinct regions of human fibronectin are essential for fibril assembly in an in vivo developing system. *Dev. Dyn.* 194:63-70.

Courtoy, P. J., and J. Boyles. 1983. Fibronectin in the microvasculature: localization in the pericyte-endothelial interstitium. *J. Ultrastruct. Res.* 83: 258-273.

Carnemolla, B., A. Leprini, G. Allemanni, M. Saginati, and L. Zardi. 1992. The inclusion of the type III repeat ED-B in the fibronectin molecular generates conformational modifications that unmask a cryptic sequence. *J. Biol. Chem.* 267:24689-24692.

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-CHC13 extraction. *Anul. Biochem.* 162:156-159.

Courtoy, P. J., and J. Boyles. 1983. Fibronectin in the microvasculature: localization in the pericyte-endothelial interstitium. *J. Ultrastruct. Res.* 83: 258-273.

Darribre, T., V. E. Koteliansky, M. A. Chernousov, S. K. Akiyama, K. M. Yamada, J. F. Theory, and J. C. Bouc. 1992. Distinct regions of human fibronectin are essential for fibril assembly in an in vivo developing system. *Dev. Dyn.* 194:63-70.

Gressner, A. M. 1991. Liver fibrosis: perspectives in pathobiochemical research and clinical outlook. *Hepatology*. 15: 67-90.

Jarnagin et al. *Fibronectin Variants in Hepatic Fibrosis*
ing hepatic fibrosis in vivo. J. Clin. Invest. 86:1641–1648.
Magnuson, V. L., M. Young, D. G. Schattenberg, M. A. Mancini, D. Chen, 
B. Steffensen, and R. J. Klebe. 1991. The alternative splicing of fibronectin 
pre-mRNA is altered during aging and in response to growth factors. J. Biol. 
Chem. 266:14654–14662.
Mak, K. M., A. M. Leo, and C. S. Lieber. 1984. Alcoholic liver injury in ba-
boons: transformation of lipocytes to transitional cells. Gastroenterology. 
87:188–200.
Martinez-Hernandez, A. 1984. The hepatic extracellular matrix. II. Electron 
immunohistochemical studies in rats with CCl4-induced cirrhosis. Lab. In-
vest. 53:166–186.
McGuire, R. F., D. M. Bissell, J. Boyles, and F. J. Roll. 1992. Role of ex-
tracellular matrix in regulating fenestrations of sinusoidal endothelial cells 
isolated from normal rat liver. Hepatology. 15:989–997.
Miliuli, S., H. Herbst, D. Sehuppan, E. (3. Hahn, and H. Stein. 1989. In situ 
hybridization for procollagen types I, III and IV in RNA in normal and 
fibrotic rat liver: evidence for predominant expression in non-parenchymal 
cells. Hepatology. 10:84–92.
Milani, $., D. Sehuppan, H. Herbst, E. O. Reicken, E. G. Hahn, C. Surrenti, 
and H. Stein. 1990. Procollagen expression by nonparenchymal rat liver 
cells in experimental hiliary cirrhosis. Gastroenterology. 98:175–184.
Minato, Y., Y. Hasamura, and J. Takeuchi. 1983. The role of fat-storing cells 
in Disse space fibrogenesis in alcoholic liver disease. Hepatology. 3:559– 
566.
Nakasuaka, H., P. Nagy, R. P. Evarts, C.-C. Hsia, E. Marsden, and S. S. 
Thorngierson. 1990. Cellular distribution of transforming growth factor-B1 
and procollagen types I, III, and IV transcripts in carbon tetrachloride-
induced rat liver fibrosis. J. Clin. Invest. 85:1833–1843.
Norton, P. A., and R. O. Hynes. 1987. Alternative splicing of chicken fibrone-
citin in embryos and in normal and transformed cells. Mol. Cell. Biol. 
7:4297–4307.
Odenthal, M., K. Neubauer, F. E. Baralle, H. Peters, K. H. Meyer zum 
Buschenfelde, and G. Ramadori. 1992. Rat hepatocytes in primary culture 
synthesize and secrete cellular fibronectin. Exp. Cell Res. 203:289–296.
Odenthal, M., K. Neubauer, K. H. M. Meyer zum Buschenfelde, and G. 
Ramadori. 1993. Localization and messenger RNA steady-state level of cel-
lular fibronectin in rat liver undergoing a CCl4-induced acute damage or 
fibrosis. Biochim. Biophys. Acta. 1181:266–272.
Odermatt, E., J. W. Tamkun, and R. O. Hynes. 1985. Repeating modular struc-
ture of the fibronectin gene: relationship to protein structure and subunit vari-
ation. Proc. Natl. Acad. Sci. USA. 82:6571–6575.
Page, F. L., Zagato, C. Vergani, G. Casari, A. Siodi, and F. E. Baralle. 
1991. Tissue-specific splicing pattern of fibronectin messenger RNA proc-
ers during development and aging in rat. J. Cell Biol. 113:1223–1229.
Rhoods, D. D., A. Dixit, and D. J. Roufa. 1986. Primary structure of human 
ribosomal protein S14 and the gene that encodes it. Mol. Cell. Biol. 
6:2774–2783.
Rockey, D. C., J. K. Boyles, G. Gabbiani, and S. L. Friedman. 1992. Rat he-
patic lipocytes express smooth muscle actin upon activation in vivo and in 
culture. J. Submicr. Cytol. Pathol. 24:193–203.
Roux, P., and J. W. Beard. 1934. Selection with magnet and cultivation of 
reticulo-endothelial cells (Kupffer cells). J. Exp. Med. 59:577–591.
Rutenburg, A. M., H. Kim, J. W. Fischbein, J. S. Hanker, H. L. Wasserkrug, 
and A. H. Sligman. 1969. Histochemical and ultrastructural demonstration 
of c-glutamyl transpeptidase activity. J. Histochem. Cytochem. 17:517–526.
Schwarzbauer, J. E. 1990. The fibronectin gene. In Extracellular Matrix Genes. 
L. J. Sandell, and C. D. Boyd, editors. Academic Press, San Diego.
195–219.
Schwarzbauer, J. E., J. W. Tamkun, I. R. Lemischka, and R. O. Hynes. 1983. 
Three different fibronectin mRNAs arise by alternative splicing within the 
coding region. Cell. 35:421–431.
Schwarzbauer, J. E., R. S. Patel, D. Fonda, and R. O. Hynes. 1987. Multiple 
sites of alternative splicing of the rat fibronectin gene transcript. EMBO (Eur. 
Mol. Biol. Organ.) J. 6:2573–2580.
Tamkun, J. W., J. E. Schwarzbauer, and R. O. Hynes. 1984. A single rat 
fibronectin gene generates three different mRNAs by alternative splicing of 
a complex exon. Proc. Natl. Acad. Sci. USA. 81:5140–5144.
Varti, T., L. Laitinen, O. Naranvanen, M. Cuto, L.-E. Thornell, L. Zardi, 
and I. Virtanen. 1987. Differential expression of the ED sequence-containing 
form of cellular fibronectin in embryonic and adult tissue. J. Cell Sci. 
88:419–430.
