

Transforming Growth Factor- β Initiates Wound Repair in Rat Liver through Induction of the EIIIA-Fibronectin Splice Isoform

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A prominent feature of the hepatic response to injury is production of a fetal isoform of fibronectin, a splice variant containing the EIIIA region, which appears very early after injury and derives from sinusoidal endothelial cells. Previous studies have shown that it is instrumental in initiating the cellular response to injury, specifically the conversion of resting stellate cells to myofibroblast-like cells. The present work describes the regulation of this change in fibronectin expression. Using sinusoidal endothelial cells from normal or injured liver in primary culture, we show that exogenous transforming growth factor β (TGF β) stimulates [EIIIA]Fn expression. To assess the role of TGF β *in vivo*, we used a chimeric IgG containing the extracellular portion of the TGF β type II receptor as a competitive inhibitor of the cytokine. Administered to animals at the time of injury, the inhibitor reduced expression of [EIIIA]Fn mRNA by 50% as compared to controls ($P < 0.01$). There was a corresponding decrease in [EIIIA]Fn protein production as judged by immunohistochemistry. Cell fractionation experiments indicated that the changes observed in whole-liver extracts were localized to sinusoidal endothelial cells. We conclude that TGF β initiates wound repair in part by stimulating endothelial expression of [EIIIA]Fn. Results with the soluble inhibitor of the TGF β type II receptor suggest a novel strategy for modulating wound repair *in vivo*. (*Am J Pathol* 2000, 156:115–124)

Transforming growth factor β (TGF β), a 25-kd homodimeric polypeptide, has been implicated in regulating repair and regeneration after tissue injury.^{1,2} Prominent among its effects in an injury milieu are the stimulation of production of other cytokines and inflammatory mediators,^{3,4} induction of extracellular matrix (ECM) synthesis,^{5–7} and inhibition of matrix degrada-

tion.^{8,9} The cellular source of TGF β in liver varies with the type of injury and may involve resident parenchymal cells, mesenchymal cells, or recruited inflammatory cells; its effects appear to be largely autocrine or paracrine.^{10,11}

An important aspect of the injury response is the elaboration of ECM, including collagens I, III, IV, VI as well as fibronectin and other glycoproteins. ECM is elaborated presumably to contain the agent of injury, close the wound, and complete healing. When ECM deposition is excessive, however, it may disrupt the organization of the tissue and lead to impaired function. From numerous recent studies, it is clear that ECM in injury derives principally from stellate cells.^{12–19} A pivotal event in the response of the liver to injury is the activation of these cells, which is characterized by the *de novo* appearance of cytokine receptors, ECM production, proliferation, and contraction.^{18,20,21} Because of its importance to fibrosis and chronic liver injury, the regulation of stellate activation has been a subject of intensive study. Both soluble mediators and the ECM itself play a role.¹⁸

Fibronectin is one of the earliest of the ECM components to be expressed after injury. Its primary transcript has three regions, termed EIIIA, EIIB, and V, that are variably spliced.^{22–24} The EIIIA and EIIB domains are either completely included or excluded within the mature molecule. Splice variants containing EIIIA are expressed during development but are minimally present in normal adult tissue. They reappear, however, in the setting of epithelial repair; this has been studied extensively in cutaneous repair.²⁵ It has been demonstrated that the EIIIA isoform is also expressed in various forms of liver injury.^{26,27} We have shown that this derives from hepatic sinusoidal endothelial cells (SEC) and that it critically modifies the local ECM so as to activate stellate cells.²⁶ Moreover, increased expression of this fibronectin variant is detectable within 12 hours of an injury, preceding any

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increase in expression of collagen and other structural ECM proteins.²⁶

The present study concerns the regulation of [EIIIA]Fn production, focusing on cytokines that are part of the injury milieu. Although TGF β is particularly prominent in the early repair process, its effects *in vivo* are not well understood with respect to cellular targets and quantitative changes in Fn isoform expression. We show that it modulates production of [EIIIA]Fn in primary cultures of hepatic sinusoidal endothelial cells and *in vivo*. The *in vivo* experiments take advantage of a novel recombinant TGF β -soluble receptor to block the action of TGF β during wound repair. The findings point to the central role of endothelial cells in wound repair and suggest that manipulation of [EIIIA]Fn expression in injury may be beneficial in reducing neomatrix formation.

Materials and Methods

Materials

Pronase and DNase were purchased from Boehringer Mannheim (Indianapolis, IN), collagenase from Serva (Heidelberg, Germany), and Ham's F-12, Medium 199, DME, and fetal calf and donor horse sera from Flow Laboratories (McLean, VA). Eagle's MEM without calcium was prepared using amino acids purchased from Sigma Chemical Co. (St Louis, Mo). Accudenz was obtained from Accurate Chemicals (Westbury, NY). Collagen I from rat tail tendon was prepared in the laboratory.

TRI reagent was from Molecular Research Center, Inc., (Cincinnati, OH). Acrylamide, bis-acrylamide and agarose were from Bio-Rad (Richmond, CA). Ultrapure urea, Trypsin-EDTA, and RNaseT2 were from GIBCO BRL (Gaithersburg, MD). T7 RNA polymerase, RQ1 DNase, and RNasin were from Promega (Madison, WI). Radiolabeled cytidine-5'-triphosphate ([α -³²P]CTP, >800 Ci/mmol) and [³H]-thymidine (>50 Ci/mmol) were from Amersham Corp. (Arlington Heights, IL); Trans³⁵S-label was from ICN (Irvine, CA). Protein A sepharose CL-4B was purchased from Pharmacia (Kalamazoo, MI). Mink lung epithelial cells (CCL64) were obtained from the University of California San Francisco Cell Culture Facility (San Francisco, CA).

The monoclonal antibody IST-9, which is specific for the EIIIA domain of fibronectin,²⁸ was kindly provided by L. Zardi (Genoa, Italy). Biotinylated sheep anti-mouse IgG and streptavidin-linked Texas red were purchased from Amersham. Avidin-biotin complex (Vectastatin) was from Vector (Burlingame, CA). Purified TGF β and TGF β antibodies were purchased from R&D Systems (Minneapolis, MN). Other chemicals were from Sigma.

Soluble TGF β Type II Receptor (sTGF β R)

The TGF β receptor fusion protein was synthesized using the extracellular domain of the rabbit TGF β type II receptor, which was amplified by PCR from plasmid 3F11.²⁹ The forward primer consisted of nucleotides 1–21 of the rabbit TGF β type II receptor, and the reverse primer was

the complement of nucleotides 466–486. To prepare a plasmid encoding the desired fusion protein, DNA fragments encoding amino acids 1–160 of the rabbit TGF β type II receptor were ligated to a DNA fragment encoding the Fc domain of human IgG1 in the Biogen transient expression vector SAB132. DNA sequencing revealed a glycine-to-arginine mutation in the fourth amino acid of the signal peptide. The rabbit TGF β type II receptor-IgG fusion DNA fragment was cloned into the Biogen stable expression plasmid pMDR901 to generate plasmid pMSN001, which was used to stably transfect Chinese hamster ovary cells. Fusion protein secreted by the cells was purified on a protein A sepharose affinity column as previously described.³⁰ The extracellular domain of the rabbit TGF β type II receptor is 83% homologous to that of the rat. The inhibitory activity of the protein was verified and titered in a standard bioassay for TGF β ³¹ as described below.

Animal Model of Liver Injury

Injury was induced in male Sprague-Dawley rats (~400 g body weight) by laparotomy and high ligation of the bile duct.^{21,32} Controls underwent laparotomy and manipulation of the bile duct but no ligation. The effects of this injury are well documented.²⁰ After 3 days, the liver exhibits cholestasis with periductular inflammation, and collagen I mRNA is markedly increased in stellate cells; after 5 to 7 days, periportal fibrosis is seen histologically. At the time of laparotomy, some animals received sTGF β R (5 mg/kg) or normal human IgG by slow infusion into the inferior vena cava. In preliminary experiments, this amount was found to be sufficient for maximal inhibition of TGF β . Unless otherwise stated, animals were sacrificed 24 hours later for cell isolation. In preliminary studies with varying doses of sTGF β R, 5 mg/kg was found to maximally reduce [EIIIA]Fn expression; in the experiments presented here, this dose was used.

Endothelial Cell Isolation and Culture

SECs were isolated by *in situ* perfusion with pronase and collagenase and fractionation by centrifugation on a discontinuous gradient of Accudenz (15.6 and 8.2%),³³ then further purified by centrifugal elutriation.³⁴ Purity was monitored by labeling the cells *in vivo* with fluorescent acetoacetylated low density lipoprotein, administered before cell isolation,³⁴ and was >90%. SECs were plated at confluent density in 60 mm culture dishes precoated with a thin layer of type I collagen (~40 μ g/60 mm dish) to facilitate cell attachment. The culture medium was a modified Medium 199³⁵ with 20% serum (10% calf, 10% horse), insulin (4 mU/ml), and gentamicin (40 μ g/ml). For comparison of treated and control cultures, cells from a single animal were used, initiated in culture under identical conditions.

Quantitation of mRNA

Total RNA was isolated from whole liver tissue or from purified SECs using TRI reagent. Its integrity was as-

sessed routinely by visualization of 18S and 28S ribosomal bands on agarose/formaldehyde gel electrophoresis. Radiolabeled probe for [EIIIA]Fn was prepared as described²⁶ and mRNA quantified by RNase protection. Fibronectin mRNA containing the EIIIA domain protected a 280-bp fragment of the labeled cRNA probe, and that lacking EIIIA protected 109 bp. Samples were assayed also for S-14 mRNA, which encodes a ribosomal protein.³⁶ S-14 exhibits minimal variation after bile duct ligation (BDL)²⁶ and was used to control for mRNA loading.

Probes were hybridized with either 20 μ g (EIIIA) or 5 μ g (S14) of total RNA at 55°C for 16 to 18 hours. Unhybridized RNA was digested with ribonuclease T2.²⁰ The RNA-cRNA hybrids were extracted in phenol-chloroform, precipitated with ammonium acetate and ethanol, and then dissociated by boiling for 3 minutes in electrophoresis buffer containing 80% formamide. Samples were run on a 6% (EIIIA) or 5% (S-14) polyacrylamide/urea gel, dried, and visualized by autoradiography (Kodak X-OMat AR-5). Specific bands were quantitated by scanning densitometry (Hoefer Scientific Instruments, San Francisco, CA) with correction of the raw data for the size difference of the [EIIIA]-positive and [EIIIA]-negative fragments.

Nuclear Run-on Assay for Fibronectin Gene Transcription

Fibronectin transcription was measured by a modification of the nuclear run-on assay described by Kavanaugh et al.³⁷ SECs were isolated and cultured for 24 hours in 20% serum growth medium. After a further overnight incubation in serum-free medium, the cells were incubated for 2 hours with either TGF β (7.5 ng/ml) or vehicle, then lysed in ice cold 10 mmol/L Tris-HCl, pH 7.4, 10 mmol/L NaCl, 3 mmol/L MgCl₂, and 0.5% NP-40. The lysate was centrifuged to pellet nuclei, which were washed and resuspended in storage buffer (20 mmol/L Tris-HCl, pH 8.0, 50% glycerol, 60 mmol/L NaCl, 100 μ mol/L EDTA, 1 mmol/L DTT) to a concentration of 1 to 2 \times 10⁷ nuclei/100 μ l. An aliquot was inspected by light microscopy and the remainder snap-frozen in liquid nitrogen until use.

Run-on analysis was performed in a total volume of 250 μ l comprising 125 μ l of nuclei brought to a final concentration of 50 mmol/L Tris-HCl, pH 8.0, 5 mmol/L MgCl₂, 0.5 mmol/L MnCl₂, 100 mmol/L KCl, 0.25 mg/ml BSA, 250 U/ml RNasin, 50 μ mol/L EDTA, 1 mmol/L DTT, 0.5 mmol/L each of rCTP, rGTP, and rATP, and 125 μ Ci of α -³²P-labeled rUTP (3000 mCi/mol, Amersham). Following incubation on a rotary shaker for 30 minutes at room temperature, the reaction was terminated with the addition of 12.5 units of DNase I for 20 minutes. Proteinase K (200 μ g/ml), SDS (0.2%), and EDTA (5 mmol/L) were added, and the mixture was incubated at 37°C for 30 minutes. Nuclei were extracted using TRI reagent, and the final RNA pellet was resuspended in RNase-free water. An aliquot was counted, and an equal amount of radioactivity in each sample was used for hybridization to denatured plasmid DNA (5 μ g) slot blotted on nitrocellulose filters. Hybridization was carried out at 65°C for 40 hours in a buffer consisting of 50 mmol/L PIPES, pH 6.8, 10

mmol/L EDTA, 0.2% SDS, 2.5 \times Denhardt's, 100 μ g/ml herring DNA, 100 μ g/ml poly(A), and 600 mmol/L NaCl. The filters were then washed serially in 2 \times SSC, 2 \times SSC containing RNase A (10 μ g/ml, 37°C), and finally in 2 \times SSC. After air drying, they were examined by autoradiography.

Immunohistochemistry of [EIIIA]Fn

After low pressure perfusion with buffered saline via the portal vein, the liver was cut into 0.5-cm² pieces, which were snap-frozen in liquid nitrogen and stored at -70°C until use. Cryostat sections were stained for [EIIIA]Fn with IST-9 mouse anti-EIIIA antibody; the secondary antibody was sheep anti-mouse.²⁶ Negative controls consisted of sections incubated with nonimmune mouse IgG and processed in parallel.

Metabolic Labeling and Immunoprecipitation of [EIIIA]Fn

SECs in culture (~80% confluent) were placed in serum free medium containing 0.5% bovine serum albumin for 16 hours before metabolic labeling. Cells were labeled for 6 hours with Trans³⁵S-label (200 μ Ci/ml) in serum free medium without methionine or cysteine. Newly synthesized [EIIIA]Fn protein in conditioned medium and in cell lysates was immunoprecipitated as described,³⁸ with modifications. Cells were lysed on ice for 30 minutes in buffer (0.1 mol/L Tris-HCl, pH 8.3, 0.1% SDS, 1 mmol/L N-ethylmaleimide, 1 mmol/L iodoacetic acid, 2 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L EDTA, 0.5% NP-40, 0.5% deoxycholate). The lysate DNA was then sheared by repeated passage through a 23 G needle, and the mixture was spun at 10,000 \times g at 4°C for 10 minutes. The extent of radiolabel incorporation into cellular proteins was determined by trichloroacetic acid precipitation.

Before immunoprecipitation with specific antibody the supernatant was precleared by shaking for 45 minutes at 4°C with protein A sepharose beads previously equilibrated in lysis buffer containing 5 mg/ml ovalbumin. The beads were pelleted (14,000 rpm for 10 seconds), and the supernatant was incubated overnight at 4°C with the primary antibody (IST-9) or nonimmune mouse IgG. One hour before the end of the incubation, polyclonal rabbit anti-mouse IgG was added, and the mixture was incubated for 45 minutes at 4°C in a rotary shaker with 50 μ l of protein A sepharose. The beads were washed four times in buffer, resuspended in an equal volume of 2 \times buffer (6% SDS, 125 mmol/L Tris-HCl, pH 6.8, 20% glycerol) containing 6% β -mercaptoethanol, and boiled for 5 minutes to dissociate antigen-antibody complexes. After centrifugation at 14,000 rpm for 1 minute to sediment sepharose beads, the proteins in the supernatant were resolved on a 5% SDS-PAGE gel.³⁹ The gel was dried and exposed to X-ray film for 2 to 7 days.

Quantitation of TGF β Secretion

Bioactive TGF β in conditioned medium was measured by its ability to inhibit the proliferation of mink lung epithelial cells.³¹ Mink lung cells (CCL64) were maintained in DMEM/10% FCS in 75-cm² flasks. For the bioassay, subconfluent cultures between passages 3 and 10 were used. Cells were washed and trypsinized, and 1×10^4 cells were seeded onto 96-well microtiter plates in 190 μ l of DMEM/0.2% FCS/10 mmol/L HEPES (pH 7.4). SECs from normal or BDL rats were isolated and plated directly in serum-free Medium 199. The conditioned medium was harvested 24 hours later and assayed directly for active TGF β . For determining total TGF β , samples were acidified for 10 minutes (0.05 N HCl final concentration), followed by neutralization with 0.05 N NaOH and HEPES, pH 7.4 (0.025 mol/L final concentration); this treatment converts latent TGF β to the active form.³¹ Ten microliters of sample were then added to each well and incubated at 37°C for 24 hours. The cells were subsequently incubated with ³H-thymidine (0.1 μ Ci) for 4 hours, lysed at -70°C, and the incorporated radioactivity trapped onto glass fiber filters (Cambridge Technology Inc., Watertown, MA) using a cell harvester (Cambridge Technology). The radioactivity was measured in a liquid scintillation counter. Each assay was carried out in triplicate; the concentration of TGF β was determined by comparison with a standard curve generated using purified TGF β 1.

Statistical Analysis

Data are presented as means \pm SE of at least three independent experiments. Differences between groups were analyzed using the unpaired Student's *t*-test (2 groups) or the one-factor analysis of variance (multiple comparisons). The significance level for all tests was *P* < 0.05.

Results

[EIIIA]Fn mRNA Quantitation in Endothelial Cells after BDL

One day after BDL, [EIIIA]Fn mRNA in fresh isolates of SECs was increased eightfold compared to sham-operated controls (Figure 1; *P* < 0.0001). Although expression of [EIIIA]-negative Fn also was increased (Figure 1), [EIIIA]-positive Fn mRNA as a percent of total Fn mRNA rose from 32% in controls to 42% in SECs from BDL rats; this difference was significant (*P* < 0.001). The stability of the observed changes in primary culture was examined by placing freshly isolated SECs from normal or BDL rats in culture for 24 hours under serum-free conditions. Over this period of culture, [EIIIA]Fn mRNA remained elevated about sixfold (Figure 2, A and B) in SECs from BDL animals compared to controls (*P* < 0.001). However, the previously noted spontaneous change in splicing²⁶ was confirmed: after 24 hours in culture the proportion of [EIIIA]Fn was similar in all cultures, whether derived from

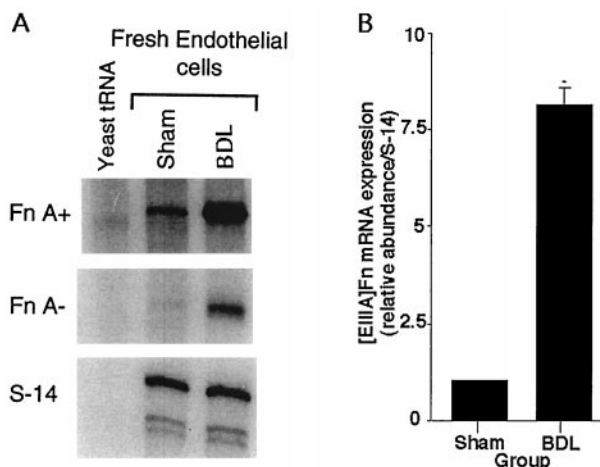


Figure 1. Expression of [EIIIA]Fn and S-14 mRNA in fresh isolates of sinusoidal endothelial cells from sham-operated or 24-hour BDL rat liver. **A:** A representative RNase protection assay using 20 μ g of total RNA for the fibronectin probe and 5 μ g for S-14. Yeast tRNA is a negative control. **B:** Relative abundance of [EIIIA]Fn mRNA in endothelial cells derived from sham-operated (Con) and 24-hour BDL rat liver. Values represent the mean \pm SE of at least four separate experiments. The signal for [EIIIA]Fn has been corrected for the level of S-14 mRNA in the same samples. * *P* < 0.0001.

control or BDL rats (57% [EIIIA]-positive in cells from controls, 61% in cells from BDL animals).

Exogenous TGF β Up-Regulates [EIIIA]Fn Expression; Blocking Effect of sTGF β R

We isolated SECs and plated them for 24 hours in medium with 20% serum to allow for recovery from the isolation procedures. After two washes the medium was replaced with one without serum, containing TGF β 1 (from a stock in 4 mmol/L HCl and 1 mg/ml BSA) or vehicle. TGF β 1 produced a dose-dependent increase in [EIIIA]Fn mRNA up to threefold (Figures 3, A and B; *P* < 0.05); expression of [EIIIB]Fn in the same samples was minimal (data not shown). Concentrations greater than 7.5 ng/ml

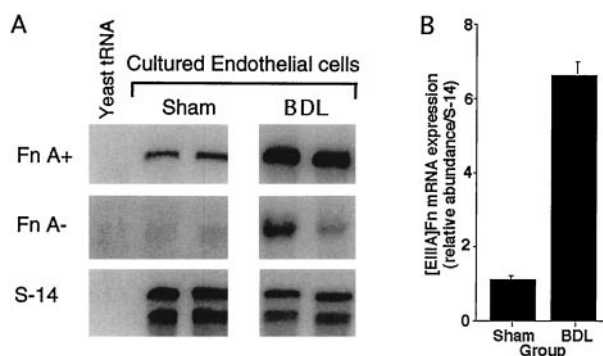


Figure 2. Stability of [EIIIA]Fn expression in cultured sinusoidal endothelial cells isolated from sham-operated and 24-hour BDL rat liver. The cells from 4 different animals (2 sham-operated and 2 subjected to BDL) were placed in culture for 24 hours under serum-free conditions. Total RNA was isolated and the level of [EIIIA]Fn mRNA determined using an RNase protection assay. **A:** A representative result for two livers is shown, with RNA loading as for Figure 1. **B:** Relative abundance of [EIIIA]Fn mRNA in endothelial cells in culture. Values represent the mean \pm SE of at least four separate experiments. The signal for [EIIIA]Fn has been corrected for the level of S-14 mRNA in the same samples. * *P* < 0.001.

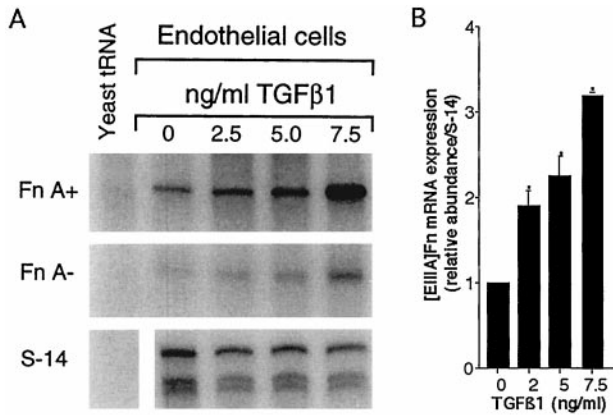


Figure 3. Effect of TGF β 1 on SEC expression of [EIIIA]Fn. Endothelial cells were placed in 20% serum medium for 24 hours, then washed and incubated in serum-free medium for 24 hours with the indicated concentrations of TGF β 1. **A:** A representative RNase protection assay, with RNA loading as for Figure 1. **B:** Relative abundance of [EIIIA]Fn mRNA in endothelial cells treated with varying doses of TGF β 1. Values represent the mean \pm SE of at least three separate experiments. The signal for [EIIIA]Fn has been corrected for the level of S-14 mRNA in the same samples. * $P < 0.05$ versus cultures without TGF β 1.

appeared to be cytotoxic as judged by cellular morphology. TGF β 1 also produced a dose-dependent increase in [EIIIA]Fn mRNA splicing; at a concentration of 7.5 ng/ml TGF β 1, [EIIIA]Fn, as a proportion of total Fn, increased from 57% at baseline to an average of 70% after incubation with TGF β 1 (7.5 ng/ml). By nuclear run-on analysis, the change in fibronectin expression was at least in part transcriptional (Figure 4). Exogenous TGF β 1 also induced [EIIIA]Fn protein synthesis, as shown by metabolic labeling and immunoprecipitation (Figure 5).

This system was used to test the inhibitory effect of sTGF β R on TGF β -mediated events. SEC isolates from normal rats were initiated in culture in 20% serum, then

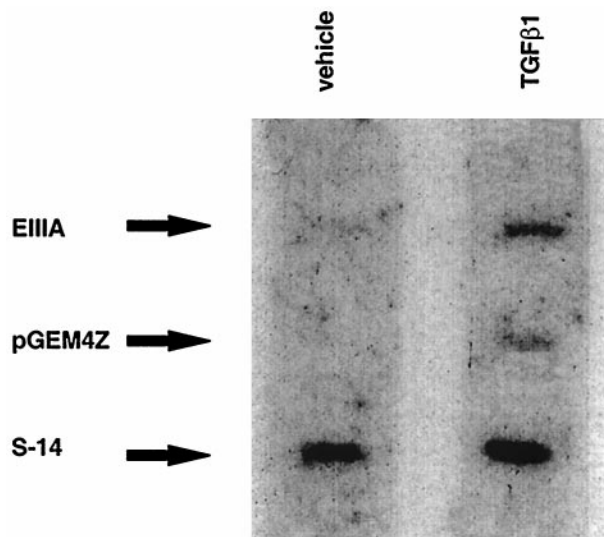


Figure 4. Transcriptional regulation by TGF β . SECs were incubated for 2 hours with either TGF β 1 (7.5 ng/ml) or vehicle, then lysed for harvesting of nuclei (see Methods). Isolated nuclei were probed for fibronectin transcription using a 280-bp rat cDNA as described in Methods. S-14 served as an internal control. The pGEM4Z plasmid served as a negative control for the specificity of hybridization conditions.

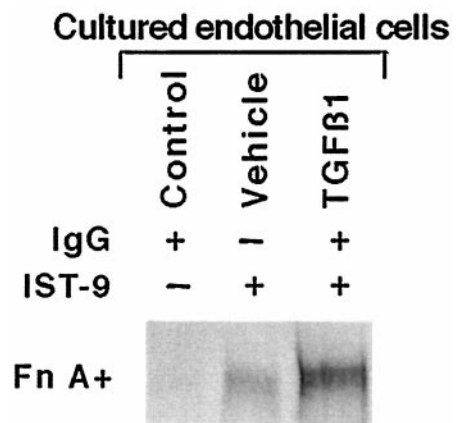


Figure 5. [EIIIA]Fn protein synthesis in endothelial cell cultures treated with TGF β 1. Cells were obtained as for Figure 4 and incubated with either vehicle (4 mmol/L HCl and 1 mg/ml BSA, lanes 1 and 2) or 7.5 ng/ml TGF β 1 (lane 3). Following metabolic labeling [EIIIA]Fn protein was immunoprecipitated using the IST-9 antibody (lanes 2 and 3). The negative control (lane 1) was handled identically with non-immune mouse IgG in place of IST-9. Cell lysates containing equal amounts of radiolabel were used for immunoprecipitation.

changed to serum-free medium with or without TGF β 1 (7.5 ng/ml). Plates treated with TGF β 1 were simultaneously incubated with either sTGF β R (50 μ g/ml) or control IgG at the same dose. As shown in Figure 6, the inhibitor reduced [EIIIA]Fn mRNA to the level in control cultures; there was relatively little effect on [EIIIA]Fn.

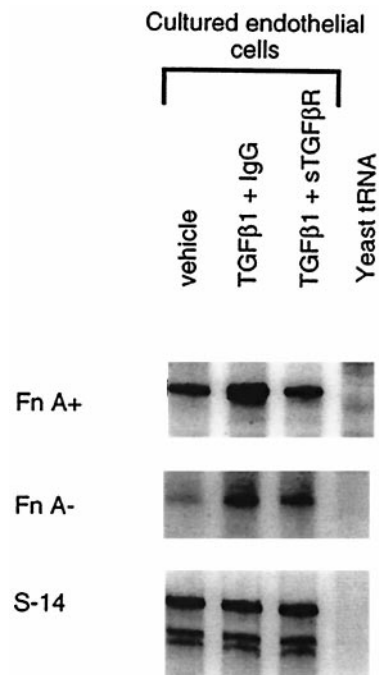


Figure 6. Effect of sTGF β R on TGF β -mediated stimulation of fibronectin mRNA. Endothelial cells in serum-free culture were incubated with either TGF β 1 or vehicle. TGF β 1-treated plates were incubated simultaneously with sTGF β R or control peptide (IgG). Yeast tRNA served as the negative control.

Table 1. Secretion of Total and Active TGF β Protein by Endothelial Cells Isolated from Normal and Bile Duct-Ligated (BDL) Rats

Source of cells	TGF β production (pg/mg protein/24 h)	
	Total TGF β	Active TGF β
Control rats	135 \pm 14	59 \pm 2
BDL rats	426 \pm 50*	157 \pm 29†

Endothelial cells were isolated placed in serum-free conditions in primary culture and incubated for 24 hours before assay. Total (acid-activated) and active TGF β were determined using the mink lung cell assay (see Methods).

* $P < 0.01$.
 † $P < 0.05$.

Regulation of [EIIIA]Fn Expression in SEC by Autocrine TGF β

We have postulated that effects of TGF β on hepatic stellate cells involve predominantly autocrine stimulation.¹² To investigate this, we first measured release of the cytokine by SECs from injured or normal rat liver. Freshly isolated SECs from BDL rats were placed in serum-free medium, and TGF β protein secretion was quantitated after 24 hours using the mink lung cell growth inhibition assay. Endothelial cells from bile duct ligated animals produced 2.7-fold greater active ($P < 0.05$) and 3.2-fold greater total ($P < 0.01$) TGF β protein than did SECs isolated from sham-operated controls (Table 1).

To study the role of autocrine TGF β in regulating expression of [EIIIA]Fn, we placed SEC isolated from BDL rats in serum-free medium for 24 hours with sTGF β R (50 μ g/ml) or control IgG. Incubation with the inhibitor, compared to control IgG, significantly reduced [EIIIA]Fn expression (Figure 7, A and B); the fraction of total Fn as [EIIIA]Fn also decreased (sTGF β R 32% versus IgG 39%; $P < 0.001$). The soluble receptor was similarly effective in preventing the spontaneous increase in [EIIIA]Fn expression exhibited by normal SEC placed in culture.²⁶ [EIIIA]Fn protein paralleled mRNA in these experiments,

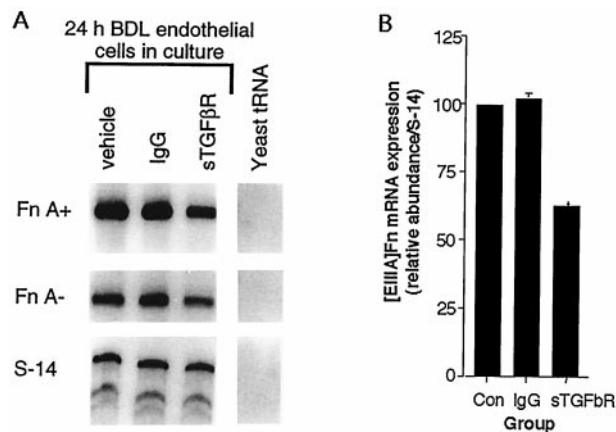


Figure 7. Inhibition of autocrine TGF β by sTGF β R. Endothelial cells from BDL rats were placed in serum-free culture for 24 hours with sTGF β R or control IgG. **A:** Representative RNase-protection assay of mRNA. **B:** Relative abundance of [EIIIA]Fn mRNA in endothelial cell cultures treated with sTGF β R. Data are mean \pm SE of four experiments.

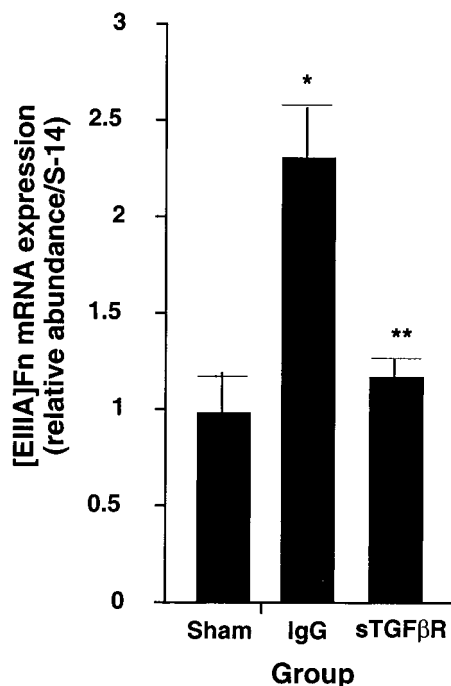


Figure 8. Effect *in vivo* of sTGF β R on [EIIIA]Fn mRNA from whole liver. Rats underwent sham operation or BDL and infusion with either sTGF β R or control IgG. The liver was harvested 24 hours later and RNA extraction performed (see Methods). Values represent the mean \pm SE of five separate experiments. The signal for [EIIIA]Fn has been corrected for the level of S-14 mRNA in the same samples. * $P < 0.02$ relative to sham-operated; ** $P < 0.01$ compared to animals infused with the control peptide.

as documented by studies in which SEC were metabolically labeled with radiomethionine and [EIIIA]Fn isolated by immunoprecipitation (data not shown).

Administration of sTGF β R in Vivo Reduces EIIIA Fibronectin Expression

Five rats in each treatment group were subjected to either sham operation, BDL with infusion of the sTGF β R, or BDL with infusion of control human IgG. Whole liver tissue was extracted 24 hours later for assay of fibronectin mRNA. [EIIIA]Fn mRNA in whole liver extracts rose 2.3 \pm 0.2-fold in bile duct-ligated rats receiving control IgG compared to sham-operated controls ($P < 0.02$). This was similar to the increase observed in untreated BDL rats. By contrast, the level in rats infused with the recombinant peptide increased only 1.2 \pm 0.1-fold. The difference between this and the value from animals infused with control IgG was significant (Figure 8; $P < 0.01$). Infusion of sTGF β R also reduced [EIIIA]Fn mRNA splicing compared to rats receiving control peptide (Table 2; 6.2 \pm 1.3% versus 10.4 \pm 1.5%; $P < 0.01$). The percent of [EIIIA]Fn in rats receiving IgG was not different from that in sham-operated animals. We next examined [EIIIA]Fn mRNA expression and splicing in SECs isolated from from BDL rats that had been treated with sTGF β R or IgG (Figure 9A). In the animals receiving sTGF β R, [EIIIA]Fn mRNA expression was less than one-half that in controls (Figure 9B; $P <$

Table 2. Effect of sTGF β R *In Vivo* on Fibronectin Splicing

Procedure	[EIIIA]Fn mRNA (percent of total Fn)
Sham operation	5.3 \pm 0.9
Infusion with control IgG	10.4 \pm 1.5*
Infusion with sTGF β R	6.2 \pm 1.3†

Animals were treated as described for Fig 8. The value for total fibronectin was calculated by adding the densitometry values for [EIIIA⁺]Fn and [EIIIA⁻]Fn mRNAs after correcting for the difference in probe length. In total liver RNA [EIIIA]Fn comprises only ~5% of fibronectin RNA reflecting the large amount of [EIIIA⁻]Fn from hepatocytes. Mean \pm SE; n = 5.

*P < 0.004 relative to sham-operated.

†P < 0.002 compared to animals infused the control peptide.

0.0003); [EIIIA]Fn as a percent of total fibronectin was also reduced by 16% (P < 0.05).

The influence of sTGF β R on [EIIIA]Fn protein expression *in vivo* was examined by immunohistology. At 2 days after BDL, liver was harvested, fixed, and stained using the monoclonal antibody IST-9, which is specific for the EIIIA segment of fibronectin. In sections from normal or sham-operated rat liver, specific staining was limited to vessels within portal triads, as shown previously.²⁶ By contrast, 2 days after BDL, extensive perisinusoidal staining was noted (Figure 10A). In animals receiving sTGF β R after BDL, the intensity of perisinusoidal staining was similar to that seen in control liver (Figure 10B).

Discussion

The initial stages of the epithelial repair response to injury involve the coordinated action of several cell types under the influence of an inflammatory milieu that includes cytokines, other small molecules (eg, oxyradicals, NO), and a neo-ECM. Our previous work documented that the neo-ECM contains a novel fibronectin isoform, [EIIIA]Fn, and that its production precedes detectable up-regulation of

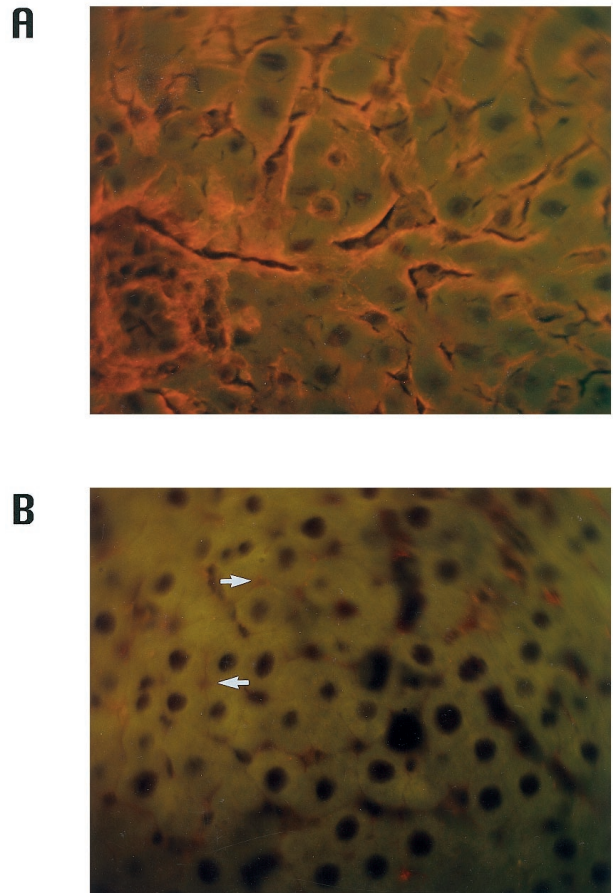


Figure 10. Immunohistochemical detection of [EIIIA]Fn in rat liver after BDL. Photomicrographs of whole liver sections stained with the IST-9 monoclonal antibody are shown. **A:** Two days after BDL. At the time of surgery rats were infused with a control peptide (human IgG). There is extensive staining for [EIIIA]Fn in a perisinusoidal distribution. **B:** Two days after BDL. At the time of surgery rats were infused with sTGF β R. Positive staining is sharply reduced (arrow), resembling that in control (sham-operated) liver, as shown previously.²⁶

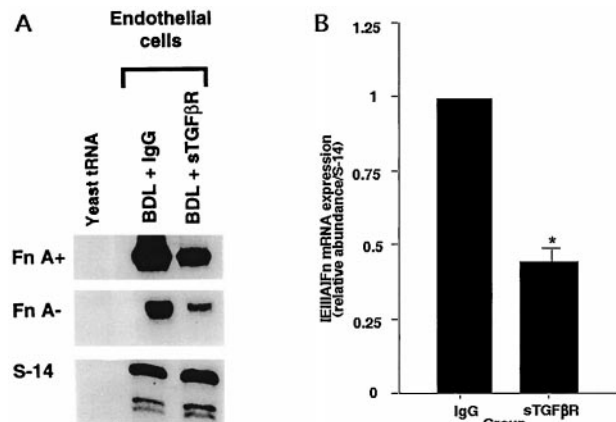


Figure 9. Effect of TGF β blockage *in vivo* on [EIIIA]Fn mRNA in isolated SEC. Rats were subjected to BDL and given either sTGF β R or control peptide. After 24 hours SEC were prepared, and the fresh isolates were extracted for RNA. **A:** Representative RNase protection assay is shown with RNA loading as for Figure 1. **B:** Relative abundance of [EIIIA]Fn mRNA in fresh endothelial cells derived from 24-hour BDL rats infused at the time of surgery with either sTGF β R or control peptide. Each group consisted of 3 animals. The signal for [EIIIA]Fn has been corrected for the corresponding level of S-14 mRNA.

collagen and other ECM proteins by stellate cells.²⁶ Moreover, [EIIIA]Fn has an important regulatory role with respect to the activation of stellate cells.²⁶ This has led us to examine the regulation of [EIIIA]Fn synthesis, with the finding that TGF β plays a central role.

This cytokine has been widely implicated in the wound repair response as a direct stimulus to ECM production by fibroblasts and myofibroblasts.^{7,40-44} Although it acts similarly on stellate cells in culture,^{15,19} its effects are modest relative to the change in ECM expression that occurs in liver injury *in vivo*.²⁰ This suggests either that it acts indirectly or that other factors are at play in stellate activation. The present results provide both cell-culture and *in vivo* evidence that much of the effect of TGF β is indirect, through stimulation of [EIIIA]Fn production by sinusoidal endothelial cells. Previous studies of fibrosing injury to the kidney⁴⁵ or lung⁴⁶ have suggested that [EIIIA]Fn expression is modulated by TGF β but have provided no information as to the cellular source(s) or target(s) of this fibronectin variant.

The source of TGF β in liver injury is an interesting and important question. In the current paradigm, injury elicits inflammatory cells, which release TGF β among other cytokines and may locally stimulate SEC and stellate cells. This is a model of paracrine signaling. Although platelets are considered the richest source of this cytokine,²⁶ mononuclear cells express it also.⁴⁷ When TGF β is elevated in liver (for example, in liver infected with the hepatitis C virus⁴⁸), it may be due in part to the mononuclear cell infiltrate that is characteristic of this injury. However, it is reasonable to consider also the possibility that TGF β in injury derives from resident liver cells. We have shown previously that TGF β is produced constitutively by SEC and Kupffer cells.¹¹ Moreover, these same cells express type I and type II TGF β receptors.⁴⁹ Thus, a potential pathway for autocrine signaling is present. As discussed elsewhere,¹¹ autocrine signaling may be a mechanism for locally restricting the effects of TGF β within the intact tissue. The present results are consistent with autocrine effects of this cytokine on SEC production of [EIIIA]Fn, at least in the context of wound repair.

The findings do not preclude a role for other factors in the initiation of stellate activation. The direct effect of TGF β on sinusoidal endothelial cells involves a threefold increase in [EIIIA]Fn at the mRNA level. Although this is significant, it is less than the approximately eightfold increase exhibited by SECs isolated directly from the injured liver. This suggests that TGF β is responsible for only a portion of the change in [EIIIA]Fn expression. Consistent with this, the effect of inhibitory sTGF β R on the culture-induced increase, although significant, was incomplete. Though some TGF β may be inaccessible to the soluble inhibitor, the findings more likely reflect the presence of other regulators of stellate activation. From the perspective of using this kind of inhibitor as antifibrotic therapy, incomplete inhibition is desirable: total inhibition of the repair process would raise significant safety concerns.

Information from culture is valuable in that it is direct. Its confirmation *in vivo*, however, is required, given that culture can induce rapid change in the cell phenotype. Moreover, in autocrine or paracrine pathways, the actual (physiological) concentration of an agonist at the receptor is unknowable. Thus, the relevance of culture experiments in which agonist is added at an arbitrary concentration is necessarily uncertain. Some *in vivo* models have the same shortcoming, eg, infusion or transgenic overexpression of TGF β . In a transgenic model involving constitutive overproduction and secretion of TGF β by hepatocytes under the albumin promoter, liver fibrosis was surprisingly limited, suggesting barriers to paracrine stimulation of stellate cells. Rather, the principal site of damage was the kidneys, reflecting the high circulating levels of active TGF β .⁴³

Modulation of the endogenous cytokine avoids some of the issues inherent in transgenic animals. This was accomplished by introducing a competitive inhibitor of the type II receptor, consisting of human IgG into which a peptide representing the ligand-binding extracellular domain of the receptor was inserted. The chimeric IgG

competes for endogenous TGF β , preventing its binding to receptors on endothelial (and other) cells. As an IgG, it circulates with a half-life of several days. A single injection blocked the induction of [EIIIA]Fn by about 50%, which is entirely consistent with the culture data and confirms *in vivo* the role of TGF β in the early phase of the hepatic injury response. Because the [EIIIA]Fn elaborated by SEC is capable of directly stimulating stellate cells,¹⁵ the possibility exists that blockade of TGF β at the level of the SEC would have significant downstream effects on fibrogenesis. In ongoing work, we have found that administration of sTGF β R reduces markers of stellate cell activation including collagen I expression.⁵⁰

The apparently transcriptional effect of TGF β and the fact that this cytokine causes both increased expression of [EIIIA]Fn and an altered pattern of splicing also are of interest. Although the change in splicing in response to TGF β appears small, it is statistically highly significant; moreover, it is similar to previously published data.⁷ The mechanisms for alternate splicing and its relationship to transcription and posttranscriptional processing are not clearly defined. Recent studies demonstrated that the extent of EIIIA splicing is dependent on promoter architecture.⁵¹ Our data support the postulate that regulation of transcription and splicing are intimately associated. A comparison of promoter architecture in SECs and hepatocytes, respectively, could prove informative, given the complete absence of a response to injury in hepatocytes. Such studies are anticipated.

Finally, it is worth noting that these data may be broadly applicable to epithelial fibrosis. Mechanisms of fibrogenesis in kidney appear to be very similar to those in liver, including an injury-related increase in [EIIIA]Fn expression.^{45,52} By analogy with the liver, it seems likely that the source of [EIIIA]Fn in the kidney is the microvascular endothelium, although this remains to be shown. Moreover, the demonstrated antifibrogenic effect of administered anti-TGF β in this tissue⁵³ may well reflect inhibition of [EIIIA]Fn expression. In short, the regulatory effect of TGF β on [EIIIA]Fn production likely is of general relevance to epithelial injury.

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