Regulation of fibronectin splicing in sinusoidal endothelial cells from normal or injured liver

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Communicated by Rudi Schmid, University of California, San Francisco, CA, November 15, 2004 (received for review September 15, 2004)

Fn containing an extra type III domain (EIIIA in the rat, ED1 or EDA in humans) is commonly termed "fetal" fibronectin, but it is prominent during the injury response of adult tissues and mediates important early events in the response. This form is particularly apparent in acute liver injury, where it has been shown that sinusoidal endothelial cells produce EIIIA-fibronectin. This fibronectin isoform arises by alternative splicing of the primary transcript. In the present experiments, we have studied the regulation of fibronectin splicing in primary sinusoidal endothelial cells by transfecting a minigene containing the EIIIA exon and its flanking introns, driven by various promoters. The results indicate that fibronectin splicing in endothelial cells from normal liver is in part promoter-dependent. However, in cells from injured liver in which expression of both total and EIIIA-fibronectin is strikingly increased, promoter effects disappear. Because fibronectin splicing is known to be regulated in part by TGFβ, we also examined the effect of a soluble inhibitor of the TGFβ type 2 receptor. This agent had no effect on splicing by normal endothelial cells. By contrast, for endothelial cells from the injured liver, the splicing pattern reverted to that of normal cells, i.e., it became promoter-dependent. We conclude that, in the setting of injury *in vivo*, TGFβ overrides the promoter dependence of fibronectin splicing in normal cells. The data suggest that TGFβ modifies the spliceosome, if not through its known signaling intermediates, then through the products of genes regulated by this cytokine.

extracellular matrix | fibrosis | spliceosome | TGFβ

The large extracellular matrix protein fibronectin (Fn) is encoded by a single gene that expresses several variants. The latter arise by alternative splicing of the primary transcript: two "type III" domains in the carboxyl terminal half of the molecule (EIIIA and EIIIB) are either included or excluded, and a third region (V) is variably excluded (1). In the embryo, their expression coincides with cell migration events (2). In the adult, the variant forms have very limited expression except in the setting of wound repair, when EIIIA-Fn is strikingly increased (3). Jarnagin *et al*. (4) examined this response in liver injury, showing that increased expression of EIIIA-Fn is a very early event in the injury response, arising within 12 h of the insult, and that its locus is sinusoidal endothelial cells (SECs). Moreover, EIIIA-Fn was shown to stimulate the conversion of resting stellate cells to myofibroblast-like cells, consistent with a role in initiating fibrogenesis in wound repair (4).

As SECs react to liver injury, their total Fn production increases severalfold. Concomitantly, the fraction of total Fn mRNA containing the EIIIA domain changes from ≈15% to ≈70% (4). These responses are regulated in part by TGFβ (5, 6). Cramer *et al*. (7) examined the role of promoter structure in the regulation of Fn splicing by using a minigene construct consisting of the EIIIA (human ED1 or EDA) domain and its flanking introns with the upstream and downstream splice junctions. They placed the native Fn promoter, a mutated Fn promoter, or a heterologous promoter, such as α-globin, in front of the splice reporter. Liver-derived cells (HepG3) were transfected with various promoter–minigene constructs, and the ratio EIIIA$^+$/EIIIA$^-$ minigene mRNA was examined by PCR. The ratio was found to depend on the structure of the 5′ promoter but was independent of promoter strength (7).

The extent to which these data from cell lines model Fn splicing by native cells is unknown. To our knowledge, splicing activity using a direct sampling method has never been examined in native cells representing contrasting physiological states. In the present study, we have transfected EIIIA-Fn minigene constructs into primary SECs isolated from normal or acute injured liver. To ensure that the primary cells were representative of the *in vivo* state, we minimized their time in culture. We found that minigene splicing with inclusion of the EIIIA domain increases dramatically in cells isolated from the injured liver. However, in contrast to the results from cell lines, the change occurs regardless of the type of promoter. When the TGFβ receptor was blocked, the pattern reverted to that of normal SECs, indicating a central role of TGFβ in spliceosome function.

Methods

Cell Lines and Plasmid Constructs. The following cell lines were used: PY4-1, mouse endothelial (8); PAC-1, rat smooth muscle cells from pulmonary artery (9); Huh-7, a human hepatoma cell line (10); and HeLa, a human cervical carcinoma cells (11). The plasmids used in these studies have been described (7). The reporter is the EIIIA (EDA) exon with its flanking introns and portions of the upstream and downstream constant exons (III-11 and III-12, respectively) (Fig. 1). This cassette is placed within the third α-globin exon to make pSVED-A Tot (12). The promoters driving this construct consisted of the native Fn proximal promoter (220/+44, FN-pSVEDA); a mutated Fn promoter with substitutions in the CRE and CCAAT elements (mutFN-pSVEDA); and the human α-globin promoter (691-bp NruI/*Hin*dIII fragment, aG-pSVEDA). Plasmids were grown in competent bacteria (One Shot, Invitrogen) and purified by using a kit from Qiagen (Valencia, CA).

Animals and Surgery. Male Wistar rats aged 6–8 weeks, with an average body weight of 250–300 g, were purchased from Charles River Breeding Laboratories. All animals received care as outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. Rats were supplied with food and water ad libitum and exposed to a 12-h light/dark cycle. Two groups (10 animals per group) underwent either ligation of the common bile duct (CBDL) or a sham operation. Surgery was carried out under isoflurane anesthesia.

Abbreviations: Fn, fibronectin; sTBr, soluble TGFβ receptor antagonist; SEC, sinusoidal endothelial cell; CBDL, common bile duct ligation.

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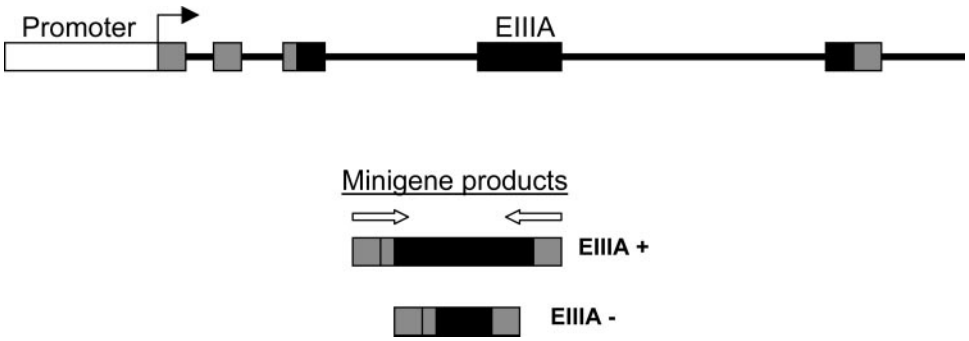

Fig. 1. The minigene construct used for transfecting primary endothelial cells. Black boxes indicate Fn exons, and gray boxes indicate α-globin exons. The heavy horizontal lines are intron sequences. The right-angle arrow is the start site of transcription. The lower part of the figure shows the minigene products with the EIIIA exon included or excluded. The block arrows show the position of the forward and reverse PCR primers (7).

CBDL was carried as follows: a 1.5-cm medium incision was made over the upper abdomen, and the duct was mobilized, triple-ligated, and transected between the second and third ties. Sham-operated animals received the same treatment up to bile duct ligation. The abdomen was closed with a silk suture, and the animals were monitored postoperatively until they resumed normal activity.

SEC Isolation and Culture. We required preparations that attach with high efficiency in primary culture and withstand DNA transfection. Moreover, it was necessary to use early primary cultures because, beyond 3 days of culture, SECs from normal liver begin spontaneously to express the injury phenotype (6). In preliminary studies, tissue dispersion using both pronase and collagenase (13) yielded large numbers of cells, but attachment efficiency, survival, and transfectability were reduced. This result led us to use collagenase only, as described by Krause *et al*. (14), with the following modifications. At 24 h after bile duct ligation, the liver was perfused *in situ* at 37°C for 3–5 min with PBS-EGTA (calcium and magnesium-free PBS, with 0.5 mM EGTA) until free of blood. The perfusate then was switched to PBS containing 0.05% collagenase (Crescent Chemical, Hauppauge, NY), which was run at a flow rate of 10 ml/min for 10–15 min or until the liver was visibly softened. The tissue was transferred to a flask with RPMI medium 1640 supplemented with 1% FBS, and the capsule was opened with forceps. The released digest was dispersed by gentle agitation in a circulatory shaker at room temperature for 10 min. Hepatocytes were separated from nonparenchymal cells by differential centrifugation: 300 rpm for 2 min, followed by 500 rpm for 3 min; the pellet (hepatocytes) was discarded. Nonparenchymal cells were pelleted from the supernatant (2,000 rpm for 7 min), then resuspended in the same medium. Elutriation (JE-5.0 rotor, Beckman J2-21M/E centrifuge) was carried out as described (14). Recovered cells were pelleted at 2,000 rpm for 7 min and resuspended in culture medium (DMEM/Ham's medium, 1:1) containing 5% FBS and 5% horse serum. The yield from two rat livers was 140–160 $\times$ 10^6 cells, with purity 85–90% and viability 90–95%.

Where indicated, recombinant rat vascular endothelial growth factor (R & D Systems) was added at a concentration of 10 ng/ml. The cells were plated onto 35-mm dishes precoated with collagen. The seeding density was 6–10 million cells per dish. After 2–4 h at 37°C under a gas phase of air and CO_2 (95:5), the culture medium was changed. When the TGFβ receptor antagonist (sTbR, or "soluble receptor") was added, its concentration was 5 μg/ml. This concentration was found to be optimal in previous work (15).

Transfection. In preliminary studies, we tested most of the commercially available systems for DNA transfection by using a GFP reporter plasmid (Clontech). We found them to vary markedly in their efficacy for hepatic SECs. Only Lipofectamine 2000 (GIBCO/BRL) produced a workable level of transfection without major toxicity. On day 3 or day 6 of culture, $\approx 10 \times 10^6$ cells were transfected with 5 μl of Lipofectamine 2000 and 5 μg of plasmid DNA in 35-mm tissue culture dishes. Minigene constructs were transfected together with a GFP reporter plasmid for estimating transfection efficiency. Cells were harvested at 48 h posttransfection. Total RNA was isolated with TRIzol (Invitrogen) by using the protocol provided by the supplier.

Assay of Active TGFβ. Secretion of the active cytokine was assessed by coculturing the cells of interest with a reporter cell line, mink lung epithelial cells that had been stably transfected with a construct consisting of the TGFβ-responsive portion of the plasminogen activator inhibitor 1 (PAI-1) promoter, coupled to luciferase (TMEL). The reporter cells have the TGFβ receptor but do not secrete the cytokine. Cocultures were maintained for 24 h and then lysed and assayed for luciferase as described (16).

RT-PCR Amplification. The amount of RNA template was adjusted for 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) activity from the cotransfected cells. One-step RT-PCR was performed with a Qiagen RT-PCR kit; primers were pSV5j and pSV3j as described (7). The integrity of the RNA was verified by amplifying GAPDH in the same samples. RT-PCR products were electrophoresed in 1.8% agarose native gels and detected by ethidium bromide staining. The ratio $EIIIA^+/EIIIA^-$ was determined by densitometry. The observed ratios were validated by quantitative PCR of the individual transcripts by using the following primers and probes. Primers were GGTTGCCTTGCACGATGAT (forward) and TGAGTGAACTTCAGGTCAGTTGGT (reverse). The probe was 5′-FAM-CCTGATTGGAACCCAGTCCACAGCTA TT-BHQ1-3. The primers span the 5′ and 3′ boundaries of the minigene, overlapping the α-globin exon, and thus will not anneal to endogenous Fn mRNA. Total minigene mRNA was calculated as the sum of $EIIIA^+$ and $EIIIA^-$ mRNA.

Results

Liver Histology from CBDL Rats. In preliminary studies, pairs of animals underwent liver biopsy and SEC isolation at 2, 4, 6, 12, and 24 h after CBDL. At all time points, the liver biopsy showed portal infiltration of lymphocytes. Nine hours after CBDL, spotty necrosis associated with small infarctions was present and, after 12 h, early fibrosis, which increased thereafter (not shown). The appearance of fibrosis coincided with up-regulation of EIIIA-Fn expression (4). It was found also that cells harvested

earlier than 12 h after the injury had a reduced ability to attach and survive in culture. Therefore, for the studies reported here, rats were euthanized for SEC preparation at 24 h after CBDL.

Cell Growth Pattern of SEC from Normal and CBDL Rat Liver and With or Without TGFβ Soluble Receptor. Freshly isolated normal SEC, cultured in medium with 10% FBS and on a substratum of type I collagen, attached with high efficiency (≈90%) and spread over the initial 24 h. Over the period 24–48 h in culture, a proportion of the cells detached from the substratum; this cell loss was minimized by a plating density that resulted in confluent cultures after 24 h of incubation. After 3 days, proliferation was evident, producing a net gain in cells per culture by 72 h. Vascular endothelial growth factor was necessary for stability of the culture as well as cell proliferation and in our hands was superior to conditioned hepatocyte medium (14). Without this factor, cells from either normal or CBDL rat liver survived in primary culture for only 1 week or 5 days, respectively. Blockade of TGFβ with a soluble receptor antagonist also stimulated the proliferation of primary SECs in culture (data not shown).

Transfection of Cell Lines and Primary SECs. The transfection efficiency was assessed by using a GFP reporter plasmid, and for several cell lines, it was similar to published reports: PY4-1, 50%; PAC-1, 66%; HUVE, 45%; Huh7, 52%; and HeLa, 60%. Transfection of primary SECs was substantially less efficient than of cell lines, regardless of the method used. With Lipofectamine 2000, for cells from normal liver, transfection efficiency at day 3 of primary culture was 5%; for cells from normal liver on day 6 of culture, 14.5%; for cells from normal liver on day 3 in the presence of sTBr, 5.5%; for cells from injured liver on day 3, 3.7%; and for cells from injured liver on day 3 of culture with sTBr, 3.6%.

FN Splicing by Cell Lines. The minigene FN splicing reporter construct was tested initially in two epithelial cell lines, one derived from liver (Huh-7) and one non-liver (HeLa). It was used also in an endothelium-derived cell line (PY4-1) and one from smooth muscle (PAC-1). The two epithelial lines yielded similar results (Fig. 2). With a nonspecific promoter (α-globin), the EIIIA$^+$/EIIIA$^-$ mRNA ratio was <1, whereas with the wild-type FN promoter, the ratio was >1, consistent with an effect of the promoter on splicing activity. An FN promoter mutated in the CRE binding site (*mutFn*) resulted in an increased EIIIA$^+$/EIIIA$^-$ RNA ratio relative to that with the wild-type promoter, again in line with previous studies (7). For the two nonepithelial lines, minigene mRNA with EIIIA$^+$ included was the predominant species regardless of promoter, although the native and mutated Fn promoters were more active in this regard than the α-globin promoter (Fig. 2).

FN Splicing by SECs in Primary Culture. For transfecting primary SECs from normal rats, we chose day 3 as a time when the cultures were stable and retained the Fn phenotype of normal cells (15). At later time points, the cells spontaneously display features of the "injury" phenotype, including increased expression of the EIIIA$^+$ splice isoform. The increase in total Fn mRNA from the minigene for each promoter was assessed by RT-PCR, in SECs isolated from normal liver or from liver injured by CBDL. Liver injury results in a 6-fold increase in total Fn mRNA when the native Fn promoter is driving minigene transcription (Fig. 3). Only a minor increase is seen with the α-globin or the mutated Fn promoter. Addition of sTBr to the culture of cells from the injured liver completely blocks the increase in total Fn mRNA. Overall, the data mirror the change in endogenous Fn mRNA expression after CBDL (4, 15), validating the minigene as a probe of Fn transcription.

In Fig. 4, the EIIIA$^+$/EIIIA$^-$ ratio is shown for normal

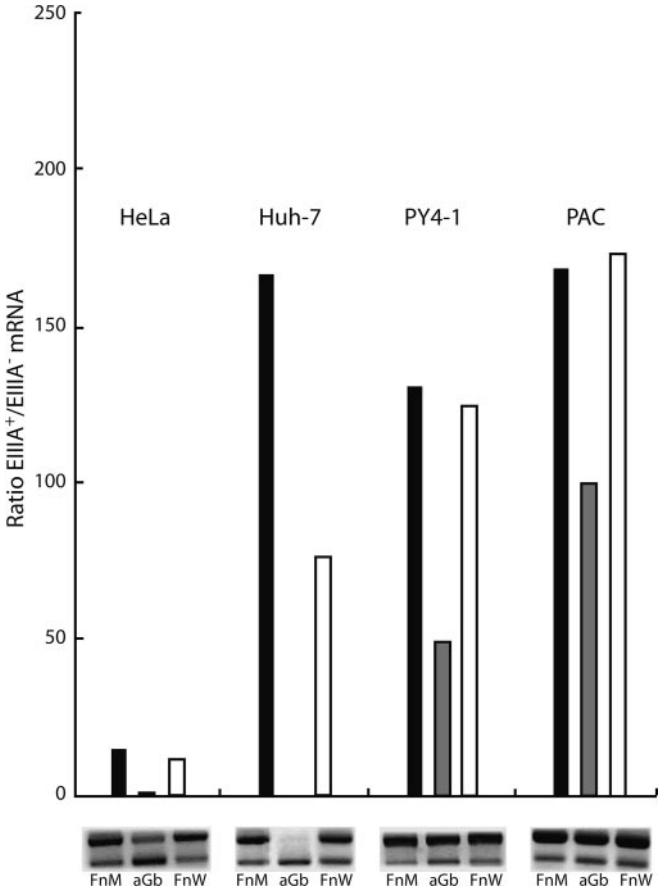

Fig. 2. Fn splicing by cell lines. Minigene reporter constructs were transfected into the cell lines shown, and splicing was determined as described in *Methods*. FnW, minigene driven by the wild-type Fn promoter; FnM, minigene driven by the mutated Fn promoter; aGb, α-globin promoter. A representative result from three studies is shown.

endothelial cells transfected with the three reporter plasmids. Of note, the level of Fn-EIIIA$^+$ expression in the presence of the native or mutated Fn promoter was 3- to 6-fold greater than that seen with the irrelevant α-globin promoter; also there was no relationship between Fn-EIIIA$^+$ expression and total minigene Fn mRNA. These observations are consistent with the known transition from low EIIIA expression by freshly isolated normal endothelial cells to high expression in culture-adapted endothelial cells. Also, the lack of correlation with total Fn expression confirms that promoter effects on splicing are independent of transcriptional activity (7). The EIIIA$^+$/EIIIA$^-$ ratio was >1 regardless of promoter, although a degree of promoter specificity was present, as in the endothelium-derived PY4-1 cell line.

Contrasting data were obtained from endothelial cells that had been harvested from the injured liver (bile duct ligation, 24 h prior) and studied in parallel. For all three reporter plasmids, the EIIIA$^+$/EIIIA$^-$ ratio was similar and strikingly increased over that exhibited by endothelial cells from normal liver (Fig. 5). Again, there was no correlation with total minigene Fn mRNA (Fig. 3).

To test whether the changes in splicing exhibited by endothelial cells from injured liver were regulated by TGFβ, we introduced a competitive antagonist of the TGFβ receptor into cultures (sTBr, or "soluble receptor"). In normal endothelial cells cultured for 3 days, the antagonist had a minor effect on both total Fn mRNA from the reporter plasmid (data not shown) and splicing (Fig. 6). When endothelial cells from the injured

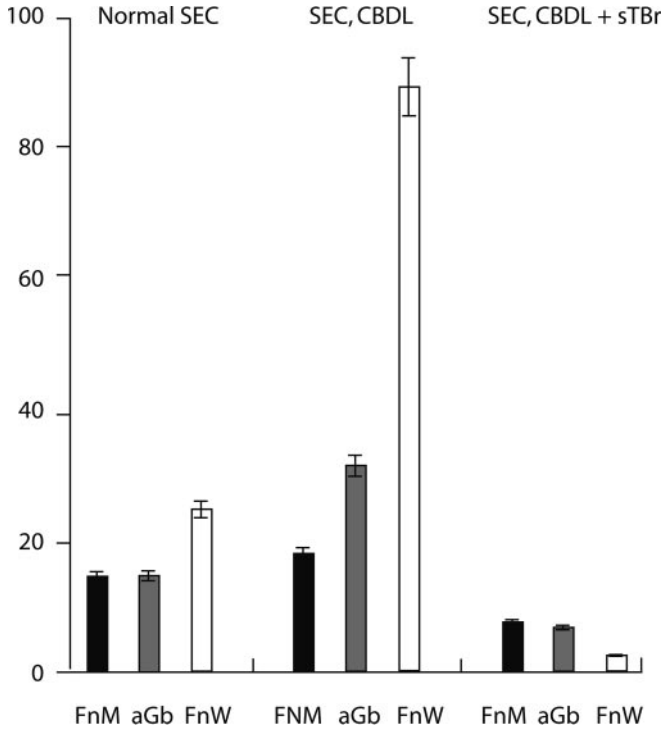

Fig. 3. Total Fn mRNA from minigenes driven by different promoters. Cells in primary culture were transfected with the indicated constructs on day 1 of culture, then harvested for RNA on day 3, as described in *Methods*. Quantitative PCR was used to assess expression of minigen EIIIA$^+$ and EIIIA$^-$ Fn mRNA, respectively, with specific primers and probes (see *Methods*). Their sum is represented as total Fn mRNA. The data are from three independent experiments; the error bars represent SEM. Abbreviations as in Fig. 2. sTBr, soluble competitive inhibitor of the TGFβ type 2 receptor.

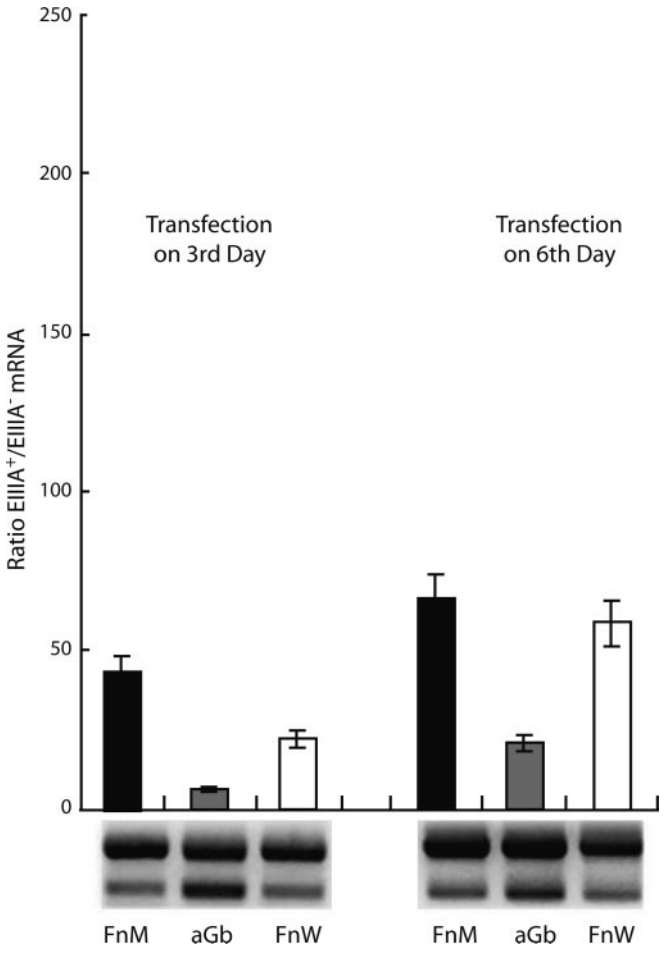

Fig. 4. Fn splicing by primary SECs from rat liver. Minigenes with the indicated promoters (see Fig. 2) were transfected into primary endothelial cells on day 3 or day 6 of culture. Mean values ± SEM from three independent experiments are shown.

liver were incubated with the antagonist, total minigene Fn mRNA fell dramatically (Fig. 3) and splicing became promoter-dependent, as in endothelial cells from normal liver.

We noted that splicing by Huh-7 or HeLa cells resembled that of the normal state, whereas splicing in PAC-1 or PY4-1 was similar to that of the endothelial cells from injured liver. We added sTBr to cultures transfected with the reporter plasmids, finding that the inhibitor had no effect in Huh-7 but in PAC-1 cells caused a partial, but significant, shift in the splice pattern toward that of normal endothelial cells. Consistent with these results, we found that production of active TGFβ itself was undetectable in Huh-7 cells but was present in cultured PAC-1 cells (data not shown).

Discussion

Fn containing the EIIIA domain has been found in several tissues responding to injury including skin (3), kidney (17), lung (18), and liver (4, 19) and may be common to all epithelial repair. In the liver, EIIIA-Fn seems to cue stellate cells to assume a myofibroblastic phenotype (4, 20). Ongoing stimulation of stellate cells results in exuberant fibrogenesis, which ultimately compromises the overall structure of the tissue, leading to liver failure. Because EIIIA-Fn is relatively specific to the injury setting, it is attractive as a target of therapeutic intervention in chronic and functionally deleterious fibrogenesis. Insight into its splicing could open up new therapeutic possibilities for progressive fibrosis.

The present studies have extended the analysis of Fn splicing from model cell lines to native liver SECs. We have contrasted cells from normal resting liver and liver that is responding to injury. By transfecting a reporter minigene, we have been able to characterize EIIIA-Fn splicing activity directly in these two states and examine its regulation. Two main findings have emerged: (*i*) Fn splicing is promoter-dependent for endothelial cells from normal liver, but not for cells isolated from the injured liver; and (*ii*) the splicing pattern exhibited by cells from the injured liver reverts to normal in the presence of a competitive blocker of the TGFβ type 2 receptor. An additional point is that endothelial cells converting spontaneously to an injury phenotype in culture maintain promoter-dependent splicing while increasing their total Fn expression. In this regard, they resemble the cell lines that have been used in prior studies to assess Fn splicing (7) but differ from SECs reacting to injury *in vivo*. We have shown also, in a survey of cell lines representing epithelia, smooth muscle, or endothelial cells, that promoters regulate Fn splicing to a varying degree. When the α-globin promoter was compared with the native Fn promoter, the difference in splicing was much more striking for the two epithelial lines (Huh-7 and HeLa) than for the nonepithelial lines (PY4-1 and PAC). The basis for this variation remains to be determined. The promoter constructs used in these studies include the SV40 enhancer, which contains two 72-bp repeats. The latter may alter splicing of the EIIIA domain by increasing the processing speed of RNA polymerase II (21). Other variables include the intracellular level of SR (serine-arginine)-rich factors that participate in spliceosomes: those directly implicated in Fn splicing include SF2/ASF

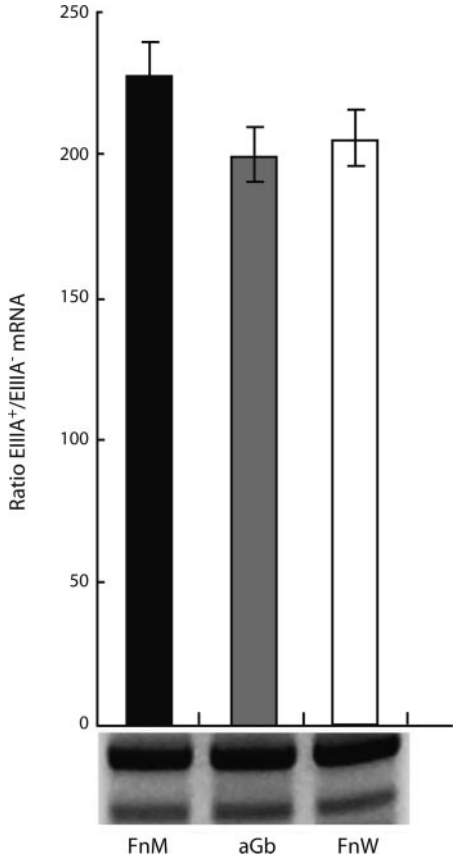

Fig. 5. Fn splicing by primary SECs from injured rat liver. Animals were subjected to CBDL 24 h before cell isolation. The primary cells were studied on day 3 of culture by transfection of minigenes with the promoters indicated in Fig. 2. Mean values ± SEM from three independent experiments are shown.

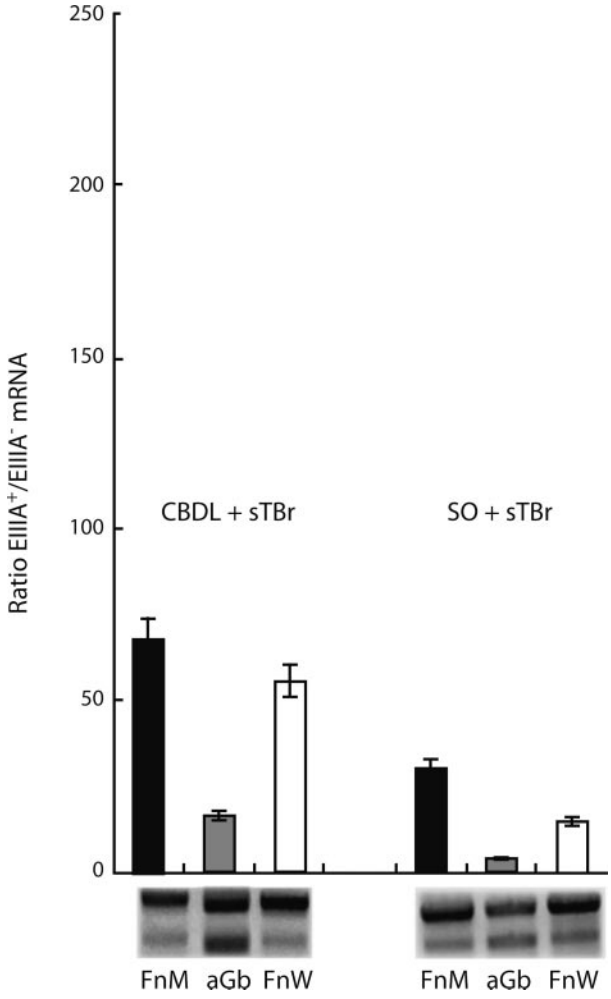

Fig. 6. Effect of TGFβ receptor blockade on Fn splicing by SECs from sham-operated (SO, right) or injured (CBDL, left) liver. The sTBr was introduced at the time of cell plating, and the cells were transfected on day 3 of culture. The minigene constructs were those described in Fig. 2. The results are shown as mean ± SEM (n = 3).

and 9G8 (22). Interspecies differences in these factors may play a role.

TGFβ has a multifaceted role in the injury response. We postulate that it is an autocrine regulator of hepatic SECs, which is produced constitutively in its latent form. In injury, changes in the pericellular milieu result in activation of latent TGFβ (23, 24). The mediators of this change are speculative at present, but a number of candidates exist (25). Thus, TGFβ seems to have modest effects in the normal state. By contrast, as we show here, in acute liver injury, it modulates endothelial function, specifically Fn splicing.

The cytokine also plays a major role in the initial fibrogenic response to injury, not only in liver but in numerous other tissues (26). The most dramatic change in total TGFβ mRNA after liver injury occurs in the perisinusoidal stellate cell population. The latter are perisinusoidal cells that are in direct contact with the sinusoidal endothelium (24). In parallel, stellate cells undergo activation, initiating a remodeling of the subendothelial matrix. Endothelial cells respond with morphological changes, such as loss of fenestrae. The latter event is governed in part by the composition of the pericellular matrix (27), leading to the question whether changes in Fn splicing could be due to altered signal input from matrix receptors. The present experiments exclude this possibility as the principal mechanism. In the first place, normal endothelial cells showed no overall change in splicing profile while adapting to an ''injury'' matrix environment (collagen-coated culture plastic). Also, a TGFβ receptor antagonist introduced in culture reversed the injury-associated splicing profile of endothelial cells isolated from CBDL liver. This is strong evidence for a direct effect of endogenous (autocrine) TGFβ on endothelial Fn splicing.

TGFβ signaling could impact on the Fn promoter, the spliceosome, or both, consistent with the postulated participation of the promoter in splicing regulation (7). None of the promoters used contains a consensus TGFβ response element (*Smad*-binding CAGAC). Moreover, it seems unlikely that the increase in the $EIIIA^+/EIIIA^-$ ratio seen in cells from injured liver could be due to a direct effect on the promoter, because the splicing profile of cells from injured liver was similar regardless of promoter. The results with this model therefore point to the possibility that TGFβ signaling intermediates or products of genes modulated by this cytokine participate in the spliceosome. Whether this would occur through binding to the promoter or independent of the promoter is unknown. Whatever their molecular identity, these factors have the interesting property in injury of overriding the influence of promoter structure on the splicing activity that occurs in normal SECs. Its dependence on TGFβ points again to antagonists of this cytokine as potential agents for modulating the unwanted consequences of wound repair.

The Genomics Core of the University of California at San Francisco Comprehensive Cancer Center (Dr. D. Ginzinger) provided quantitative

PCR. This work was supported in part by National Science Council Grant NSC 93-2314-B-182A-148 (to M.-L.C.), Fogarty International Award 5R03TW00717 (to A.R.K. and D.M.B.), and National Institutes of Health Grants R32DK31198 (to D.M.B.) and P30DK26743 (to the University of California at San Francisco Liver Center). A.R.K. is an International Research Scholar of the Howard Hughes Medical Institute.

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