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Research Article

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Induction of β-Platelet-derived Growth Factor Receptor in Rat Hepatic Lipocytes during Cellular Activation In Vivo and in Culture

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Abstract

A consistent response to liver injury is the activation of resident mesenchymal cells known as lipocytes (Ito, fat-storing cells) into a proliferating cell type. In cultured lipocytes, platelet-derived growth factor (PDGF) is the most potent proliferative cytokine, but requires the activation-dependent expression of its receptor protein (Friedman, S. L., and M. J. P. Arthur. 1989. *J. Clin. Invest.* 84:1780–1785); the role of PDGF receptor (PDGFR) in liver injury is unknown. We have examined PDGFR gene expression in freshly isolated lipocytes during liver injury and correlated these findings with a culture model of cellular activation. Whereas lipocytes from normal rats had no detectable transcript for the β-PDGFR subunit, this mRNA was induced within 1 h after a dose of carbon tetrachloride (CCl_4). In contrast, α subunit mRNA was detected in normal cells, but was unchanged after liver injury. Similar results were observed in lipocytes from bile duct-obstructed rats, although β-PDGFR induction was less marked. By immunoblot, induction of β-PDGFR protein in lipocytes isolated from CCl_4-treated animals correlated with mRNA increases. In contrast to lipocytes, endothelial cells from normal liver expressed low levels of α- and β-receptor subunit mRNA, which did not increase with injury. Using a β-PDGFR antibody, receptor protein could be identified within fibrotic septa in CCl_4-treated animals in regions where cells expressed proliferating cell nuclear antigen (PCNA). In cultured lipocytes activated by growth on uncoated plastic, β-PDGFR transcripts appeared within 3 d after plating, which coincided with the onset of cellular proliferation. In contrast, quiescent cells in suspension culture had no detectable β-PDGFR mRNA. These results indicate that β-PDGF receptor induction by lipocytes is an early event during hepatic injury *in vivo* and in primary culture. (*J. Clin. Invest.* 1994. 94:1563–1569.)

Key words: hepatic fibrosis • liver injury • Ito cells • fat-storing cells • proliferation

Introduction

The early cellular response to hepatic injury has been well-studied in animal models and human disease. In CCl_4-induced liver injury, for example, early infiltration by mononuclear cells is followed by proliferation of mesenchymal cells, development of fibrotic septa, and ultimately formation of nodules encased by dense scar tissue (1–3). Accumulating evidence indicates that the septal mesenchymal cells are activated lipocytes, cells which in normal liver express desmin (2) and are the primary site for storing hepatic vitamin A (4). After injury, lipocytes are the major site of extracellular matrix gene expression (5,6), and are thought to play a central role in formation of the hepatic scar (for review see reference 7).

A major element of the injury response by lipocytes is their activation into myofibroblast-like cells. This phenotypic transformation has been well characterized in situ, and includes new expression of smooth muscle α actin (2), enlargement of the secretory apparatus (8), increased pericellular matrix (9), and proliferation (10). Mechanisms underlying the proliferative component of lipocytes activation have been partly elucidated using primary culture models. Cells isolated from normal rats are initially nonproliferative when grown on uncoated plastic; cell replication accompanies progressive culturing on plastic (11,12). Proliferation can be accelerated by exposing cells to conditioned medium from hepatic macrophages (13); this model is particularly relevant to the in vivo response, because, as noted, macrophage infiltration always precedes mesenchymal cell proliferation in parenchymal injury (1).

In culture studies of lipocyte proliferation, platelet-derived growth factor (PDGF) has been identified as the principal mitogen (13, 14). Responsiveness to PDGF requires expression of a specific dimeric transmembrane receptor (PDGFR)[1], which is composed of either αα, αβ, or ββ subunits (15). Indeed, in cultured lipocytes activated by exposure to conditioned medium from macrophages, proliferation is preceded by induction of PDGF receptor protein as assayed by immunoblot (13).

While these culture studies have suggested that PDGF receptor induction is an important component of lipocyte activation, proof has been lacking that a similar event occurs in vivo. Additionally, the cellular distribution and receptor phenotype of PDGF receptor have not been studied in normal and injured liver. Evidence from analogous models of wound healing, especially in kidney (16) and skin (17), suggest that in vivo expression of PDGF receptor could also be an important event in hepatic injury, and, moreover, might offer a potential target for therapeutic intervention.

In this study we have characterized PDGF receptor expression in two mechanistically distinct models of liver injury in vivo, both in situ and by examining freshly isolated cell populations, and have correlated these findings with culture models of lipocyte activation. The results indicate that after injury in vivo

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1. *Abbreviations used in this paper:* PDGFR, platelet-derived growth factor receptor.

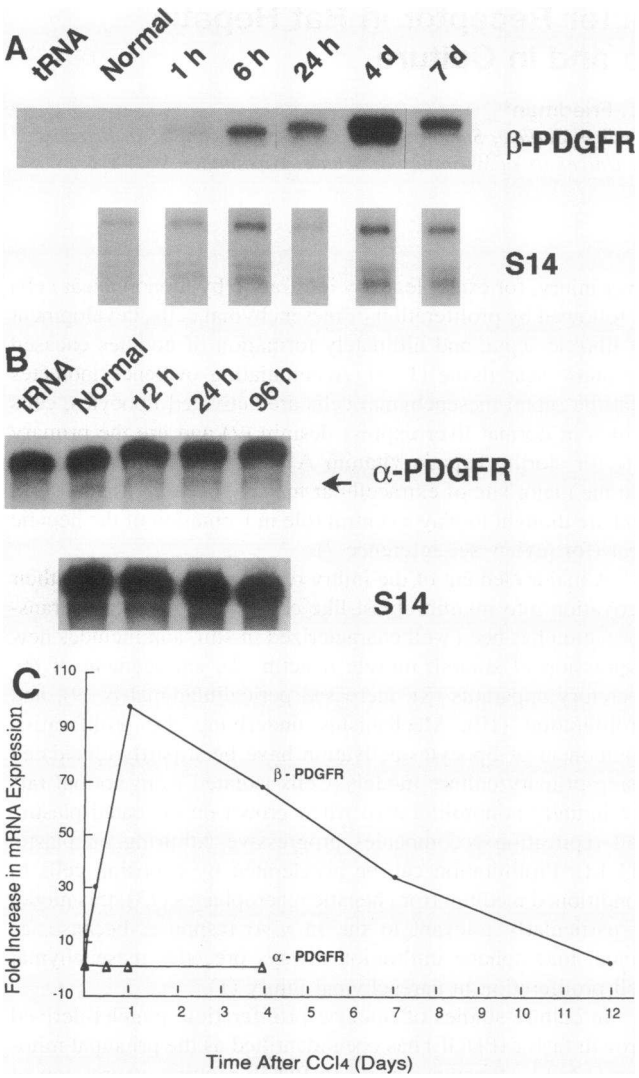

Figure 1. In vivo expression of α- and β-PDGFR mRNA by rat lipocytes after CCl_4. (*A* and *B*) Freshly isolated lipocytes from either untreated rats or after a single dose of CCl_4 were analyzed for expression of β- and α-isoforms of PDGFR by RNAse protection. Protected fragments of 458 bp for the β-isoform and 466 bp for the α-isoform are evident; for the α-isoform the arrow indicates the specific band. Each sample contained 0.5 μg mRNA; corresponding expression of S14 mRNA is shown below each sample. *C* depicts relative receptor mRNA expression normalized for S14 in the same isolate, with cells from normal liver assigned a value of 1.

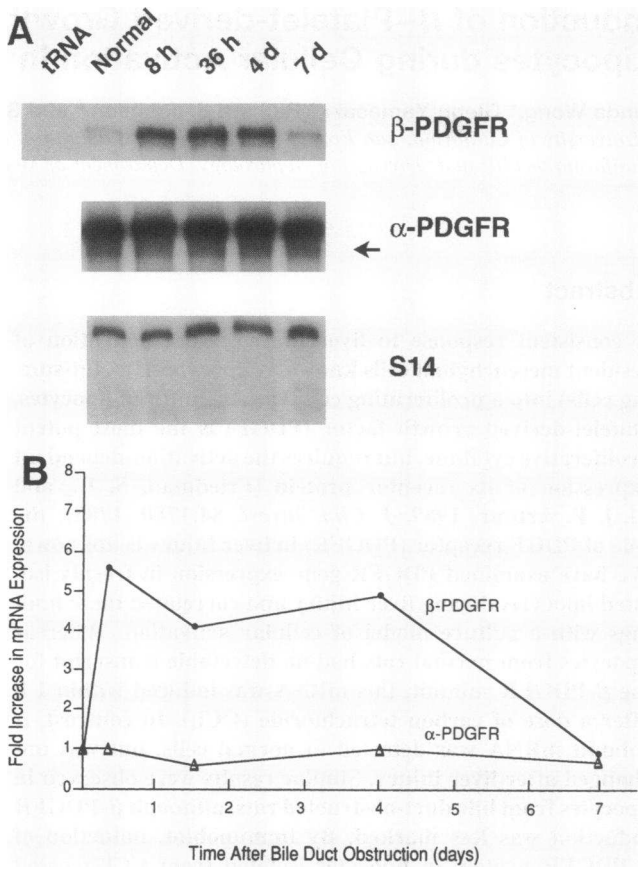

Figure 2. In vivo expression of α and β-PDGFR mRNA by rat lipocytes after bile duct obstruction. (*A*) Freshly isolated lipocytes from either normal animals or after bile duct ligation were analyzed by RNAse protection for expression of β- and α-PDGFR mRNA. Samples contained 5 μg total RNA; corresponding S14 mRNA expression is shown. (*B*) Relative PDGFR mRNA expression normalized for S14 expression.

in rats, induction of β- but not α-PDGF receptor mRNA occurs rapidly, and is accompanied by evidence of receptor protein in situ. Moreover, receptor gene induction is confined to lipocytes and is not observed in the other prominent mesenchymal cell type, sinusoidal endothelial cells. In lipocyte culture models cellular proliferation is also closely tied to the appearance of β-PDGF receptor mRNA.

Methods

In vivo models of liver injury

Hepatic fibrosis was induced by either bile duct ligation or carbon tetrachloride (CCl_4) administration. Bile duct ligation and scission were performed under ether anesthesia (18). For all studies except immunostaining, a single dose of corn oil:CCl_4 (1:1) (1 μl/g body wt.) was administered by gavage to ether-anesthetized animals (19); for immunostaining at day 8, animals received doses of CCl_4 on days 1 and 5. Rats were maintained on standard chow and allowed access to food and water ad libitum.

Cell isolation and culture

Lipocytes were isolated from Sprague–Dawley retired breeder rats by pronase/collagenase perfusion and Stractan gradient centrifugation as described previously (20). Endothelial cells, collected from the bottom two interfaces of the gradient (8–12%, 12–15%), were purified further by centrifugal elutriation at 2,500 rpm at a flow rate of 18 ml/min. (J2-21 centrifuge with JE 6-B rotor and Sanderson chamber; Beckman Instruments, Inc., Fullerton, CA). Purity of isolates was ~ 95% and was assessed by direct cell counting using anti-desmin as a marker for lipocytes, and DiI-conjugated acetoacetylated low-density lipoprotein for endothelial cells. Lipocytes were also identified by the vitamin A autofluorescence under ultraviolet epiillumination. Yields per animal were $30–40 \times 10^6$ cells for lipocytes and $10–20 \times 10^6$ for endothelial cells.

Experiments were performed using either freshly isolated cells or cells in primary culture. For culture studies, lipocytes were suspended in medium 199 OR with 10% horse and 10% calf serum at a concentration of 10^6 cells per ml. Cells were maintained in either suspension culture in teflon-coated containers (Savillex Corp., Minnetonka, MN)

or in monolayer culture on uncoated plastic plates (Lux Scientific, Naperville, IL). Our earlier studies have shown that lipocytes in suspension culture are nonproliferative and negative for smooth muscle α actin; activation can be induced if cells are transferred to monolayer culture (21). All cultures were incubated at 37°C in 100% humidity, 95% O_2, and 5% CO_2, without agitation. Medium was changed every 48 h.

PDGF receptor immunoblot

Immunoblot of lipocyte extracts was performed using a polyclonal antibody to rat β-PDGF receptor subunit generated against a mixture of synthetic peptides of the human and mouse β subunit, as previously described (22). Cells were isolated from either normal or CCl_4-treated animals, and maintained in suspension culture for 24 h in 20% serum (21) before lysis directly into electrophoresis buffer (3% SDS, 62.5 mM Tris-HCl, 10% glycerol, and 2% β-mercaptoethanol, pH 6.8). Protein content (75 μg/lane) was normalized by Bio Rad protein assay (Bio-Rad Laboratories, Richmond, CA) before 8% SDS-polyacrylamide gel electrophoresis and overnight transfer to nylon. The primary antibody was used at a dilution of 1:1,500, and secondary antibody (goat anti-rabbit conjugated to horseradish peroxidase) at 1:2,000.

PDGF receptor mRNA expression

Expression of mRNA for α and β-PDGF receptors was measured in freshly isolated in vivo or cultured cells by RNAse protection assay using rat-specific cRNA probes.

Rat α- and β-PDGF receptor cDNAs

β-PDGF receptor. A 458-bp rat cDNA fragment was cloned by "homology" polymerase chain reaction (PCR), using degenerate primers based on the published human β-PDGF receptor amino acid sequence (aa 588–746) (23). Primer sequences were constructed to include EcoRI and XbaI restriction sites: forward - 5' CTCCC TCTAGA CCITA(TC)-GA(TC)(AT)(GC)IACITGGGA 3' ; reverse - 5' CTCCC GAATTC TC(AG)TC(CT)TTI(CG)(TA)CAT(AG)TCCAT(AG)TA 3'. 1 μg cDNA derived from activated lipocytes was used as a template, prepared as follows: total RNA was isolated from 6–10 d cultured rat cells (~ 100 $\times$ 10^6 cells). mRNA was extracted using a commercial kit (PolyATract mRNA Isolation System; Promega, Madison, WI) from 250 μg total RNA. First strand cDNA was synthesized using Avian Myeloblastosis Virus reverse transcriptase and oligo dT primers (Promega). PCR was conducted in a 50 μl reaction using a thermal cycler (Ericomp, San Diego, CA): 1 cycle (94°C $\times$ 4 min); 30 cycles (95°C $\times$ 45 s, 45°C for 45 s, 72°C $\times$ 60 s); 1 cycle (72°C $\times$ 10 min). The product was visualized by electrophoresis through 1.5% low-melting agarose containing ethidium bromide and purified using phenol/chloroform. The β-PDGF receptor cDNA was cloned into pGEM-4 (Promega) and sequenced by the dideoxy chain termination method (24) (Sequenase Version 2.0; United States Biochemical Corp., Cleveland, OH). After these cloning studies were completed, a partial cDNA for the rat β-PDGFR sequence which incorporated this region was entered into Genbank by others (accession No. Z14119, unpublished).

α-PDGF receptor. A 466-bp BamHI fragment of the published cDNA sequence for the rat α-PDGF receptor (25) (provided by Dr. Daniel Bowen Pope, University of Washington) was subcloned into pGEM-4. The plasmid was linearized with BstXI for use as a template for riboprobe synthesis.

RNAse protection assay

RNAse protection assay was performed exactly as described by Maher and McGuire (6), using 1.0 μg of linearized plasmid template DNA to synthesize a rat specific cRNA probe. Either 0.5 μg of mRNA or 5 μg of total RNA (specified in Figure Legends) was used in the overnight hybridization with the probe (60°C). mRNA was directly isolated using Invitrogen Micro-Fast Tract® kit (Invitrogen, San Diego, CA) according to manufacturer's specifications; total RNA was extracted and purified according to Chomczynski and Sacchi (26).

All autoradiographs were quantitated by laser densitometry. Data from in vivo experiments were normalized for expression of the ribosomal S14 protein mRNA (27). For culture experiments equal RNA amounts were assured by measuring OD_{260} and inspection of 28 and 18 S ribosomal bands on agarose/formaldehyde electrophoresis.

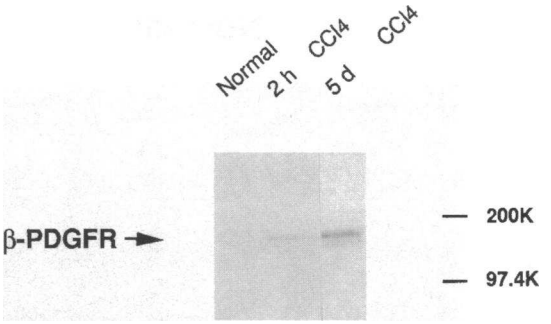

Figure 3. Immunoblot of β-PDGF receptor in lipocytes activated in vivo. Lipocytes were isolated from a normal rat and from animals treated with CCl_4 either 2 h or 5 d before isolation. After 24 h of suspension culture to allow receptor recovery, cell extracts were analyzed for β-PDGFR expression as described in Methods. Increased expression of the 180-kD receptor is detectable within 2 h of CCl_4, and clearly visible in cells from an animal treated 5 d earlier (the location of the molecular weight standards is shown in the right hand margin).

Immunohistochemistry of rat liver

For immunostaining, liver fragments were excised and fixed in Methyl Carnoy's solution; 3-μm sections were examined. Tissue staining was performed using an indirect avidin/biotin immunoperoxidase method exactly as previously described (22). Primary antibody was either a polyclonal IgG to the β subunit of PDGFR (9 μg/ml) (22), a murine monoclonal IgM to proliferating cell nuclear antigen, (PCNA) (American Biotech, Plantation, FL), or a murine monoclonal IgG (ED-1) to monocytes-macrophages (Bioproducts for Science, Inc., Indianapolis, IN) (22). Tissue sections were also incubated with the following negative control primary antibodies: rabbit non-immune IgG (for β-PDGFR) or an irrelevant murine monoclonal antibody (for ED-1 and PCNA).

Tissue sections were photographed with a Nikon Microphot® microscope using Kodak T-Max film (ASA 100) (Eastman Kodak Co., Rochester, NY).

Cell proliferation assay

Cells from normal rats were plated on uncoated plastic in 24-well dishes and assayed for the incorporation of [^{3}H] thymidine exactly as described previously (11). Data were normalized for cell number.

Results

In lipocytes from normal liver, β-PDGFR mRNA was undetectable. A transcript was apparent within 1 h after CCl_4, which increased to maximal at 24 h (Fig. 1, *A–C*); there was a greater than 100-fold relative increase between 1 and 24 h. Receptor mRNA levels returned to normal within 12 d after a single dose.

To assess whether induction of the β-PDGFR was common to different forms of liver injury, we examined its expression after ligation of the common bile duct, a form of liver injury mechanistically distinct from CCl_4. Receptor mRNA induction was rapid (within 8 h) and sustained for at least 4 d, although to a much lesser extent than in CCl_4 (~ 5.5-fold maximal increase) (Fig. 2, *A–C*).

In contrast to the β isoform, expression of α-PDGFR mRNA was apparent in lipocytes from normal liver, but was unchanged after either CCl_4 or bile duct ligation (Figs. 1 and 2).

Two approaches were used to correlate induction of β-

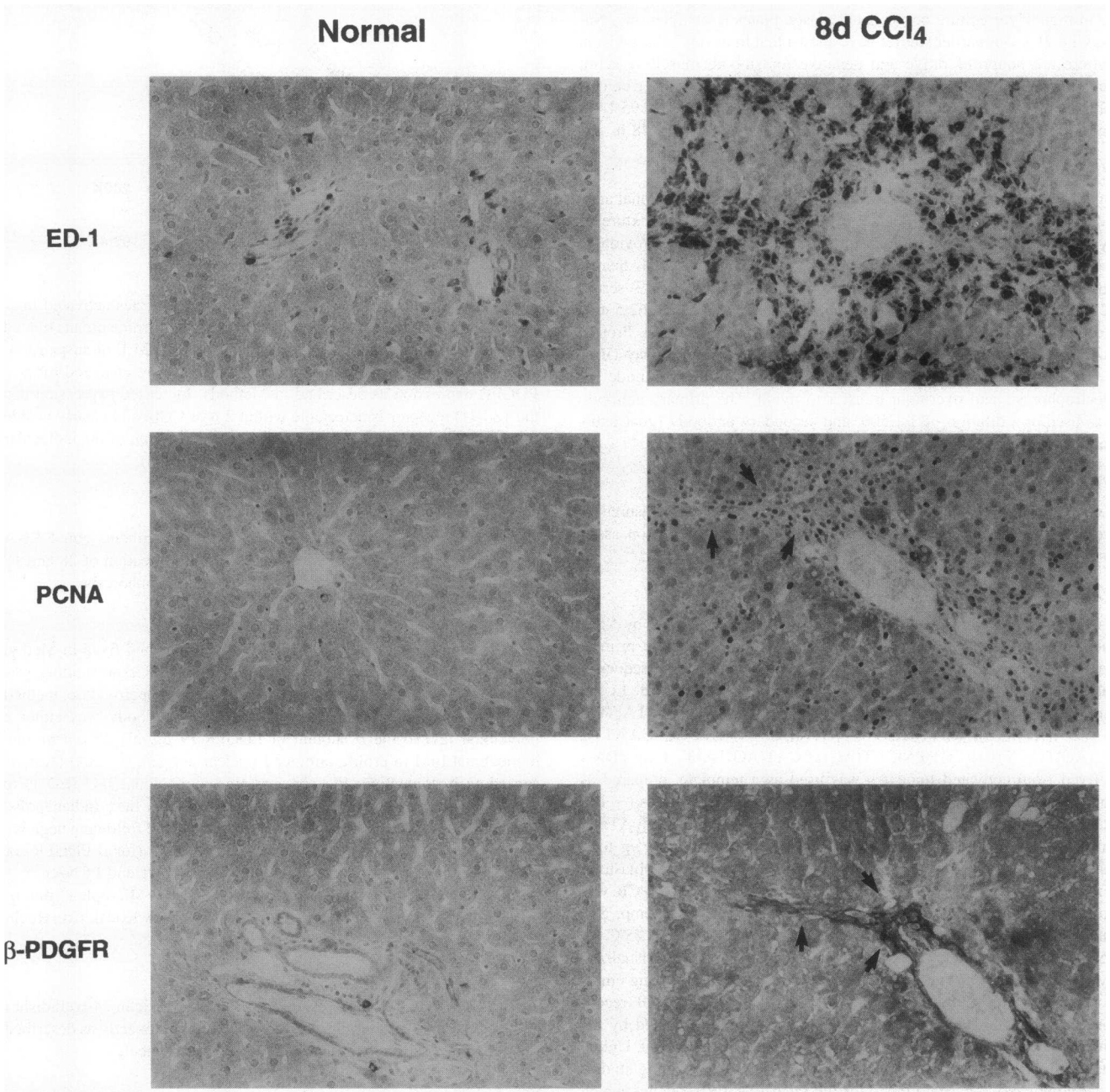

Figure 4. Immunostaining of rat liver following CCl_4 administration. Liver from either normal rats or animals treated with two doses of CCl_4 on days 1 and 5 before harvest on day 8 were examined for expression of ED-1, proliferating cell nuclear antigen (PCNA) and β-PDGFR by immunostaining. Scattered ED-1 positive cells (*darkly stained cells*) are apparent in normal liver. At 8 d, marked mononuclear cell infiltration in forming septa is apparent (*dark cells*). Similarly, in normal liver few cells expressing PCNA are seen, but positive cells are readily observed within 8 d (*darkened nuclei*). In normal liver, β-PDGFR is evident only around large arterial walls, but becomes detectable within 8 d of CCl_4 in septa which contain PCNA-positive cells (the same region is highlighted with arrows in parallel tissue sections stained for PCNA and β-PDGFR). There was no background immunoreactivity at all time points using appropriate negative control antibodies (not shown).

PDGFR mRNA with expression of the receptor. First, in cells isolated from normal or CCl_4-treated rats then maintained in brief suspension culture, increased receptor protein was apparent within 2 h after CCl_4, and markedly increased 5 d after dosing (Fig. 3); cells were placed in suspension culture to allow for reappearance of receptors cleaved by proteases during isolation. Second, in situ expression was identified in liver sections from normal and CCl_4-treated rats immunostained with a β-PDGFR antibody. Receptor immunoreactivity was apparent in fibrotic septa at day 8 after two doses of CCl_4 in regions of PCNA positivity (Fig. 4). This distribution corresponds to α smooth muscle-expressing activated lipocytes (2, 28, 29) and was accompanied by a marked increase in macrophage infiltration as determined by staining with the macrophage marker ED-1 (Fig. 4).

To determine the cellular specificity of PDGFR mRNA expression in vivo, we asayed α- and β-PDGFR gene expression in sinusoidal endothelial cells from normal and bile-duct–li-

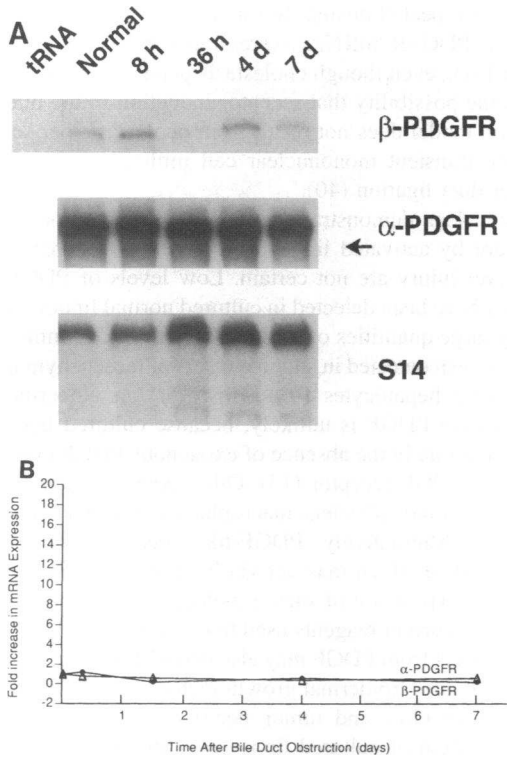

Figure 5. In vivo expression of α and β-PDGFR by sinusoidal endothelial cells after bile duct obstruction. (*A*) Endothelial cells from normal rats or following bile duct obstruction were analyzed by RNAse protection for β and α isoforms. 5 μg total RNA was used per sample, with corresponding S14 mRNA expression shown for each sample. (*B*) Relative changes and time course of expression.

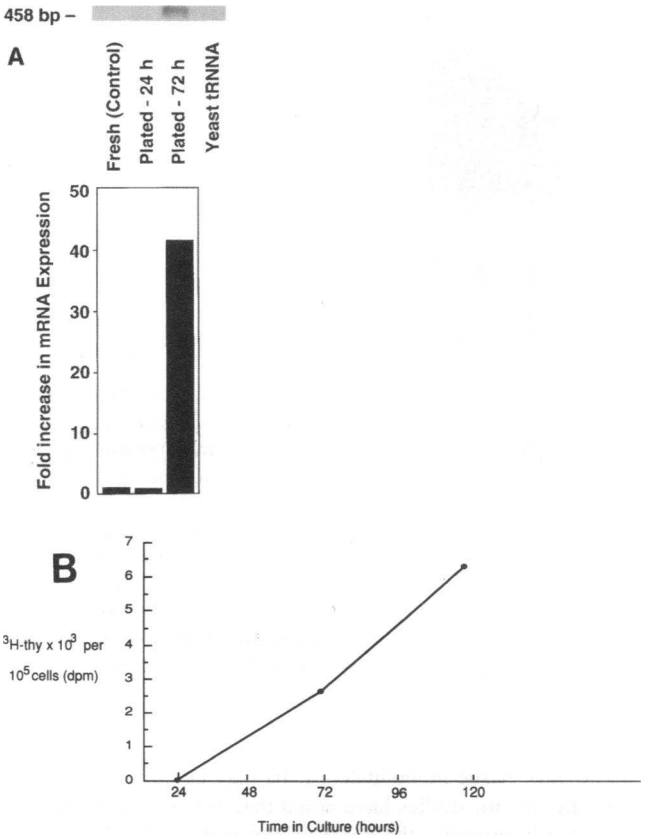

Figure 6. Expression of β-PDGFR mRNA and cellular proliferation by lipocytes from normal rat liver at isolation or in monolayer culture. (*A*) 5 μg of total RNA from the same lipocyte isolate was examined for β-PDGFR mRNA expression at the time of isolation, and 24 and 72 h after plating on uncoated plastic in serum-containing medium. The presence of equal quantities of RNA in each sample was confirmed by absorbance at OD_{260} and intensity of 28S and 18S ribosomal bands on formaldehyde/agarose electrophoresis (not shown). Relative mRNA expression is graphically depicted, with samples from freshly isolated cells assigned a value of 1. (*B*) The time course of cellular proliferation in cells maintained in monolayer culture, as assessed by incorporation of [^{3}H] thymidine. Data points represent mean value of triplicate determinations normalized for cell number.

gated animals. Low levels of α- and β-PDGFR transcripts were present in cells from normal animals, but neither was induced after liver injury (Fig. 5).

Assessment of β-PDGFR mRNA levels under culture conditions which we have previously associated with cellular replication confirmed that a proliferative phenotype correlated with induction of receptor mRNA. Specifically, cells grown on uncoated plastic expressed receptor mRNA at 72 h after plating, coincident with onset of cellular proliferation as assessed by incorporation of [^{3}H] thymidine (Fig. 6). In contrast, cells maintained in a quiescent phenotype by suspension culture remained spherical and nonproliferative, with undetectable β-PDGFR mRNA (Fig. 7).

Discussion

In this study we demonstrate that acute liver injury in rats is accompanied by induction of β-PDGFR mRNA and protein. Receptor gene induction is associated with in situ cell proliferation, and is restricted to hepatic lipocytes and not sinusoidal endothelia, the other potential PDGFR-expressing cell type in liver parenchyma. We further show that β-PDGFR mRNA is also induced under culture conditions in which PDGF or serum-dependent lipocyte proliferation occurs (8, 11, 14).

Induction of PDGF receptor has not previously been documented in liver injury in vivo, although upregulation of PDGFR appears to be a major means of amplifying the proliferative response of mesenchymal cells in several other forms of tissue injury. In particular, arterial injury (30), renal injury (22), tissue rejection (31), and synovitis (32) all are associated with in situ expression of PDGFR. In each of these tissues the β isoform is induced. Predominant expression of the β isoform is also consistent with previous observations in cultured rat lipocytes (33, 34). In contrast to the β subunit, expression of the α subunit mRNA is detectable in normal cells, but is not increased with injury. Although the specific band for α-PDGFR mRNA using RNAse protection was subtle, it nonetheless likely reflects a genuine signal, because we have also detected α-PDGFR mRNA in lipocytes by reverse transcriptase-polymerase chain reaction (S. Friedman, unpublished results). Despite this apparent mRNA expression, however, significant expression of functional α receptor by lipocytes is unlikely based on the lack of responsiveness of culture-activated lipocytes to PDGF AA (33, 34).

Factors directly responsible for the induction of β-PDGFR in our and related models are not yet established, but the accu-

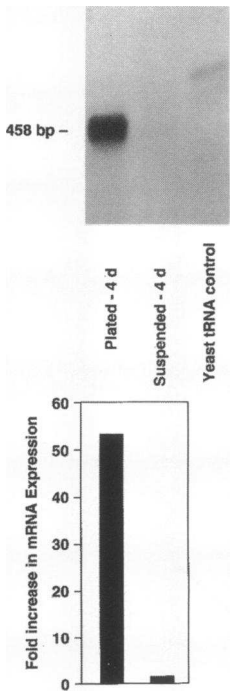

Figure 7. Expression of β-PDGFR mRNA in cultured lipocytes; plated vs suspension culture. Lipocytes from the same isolate were either plated in monolayer culture on plastic or grown in suspension culture, both in the presence of serum. 5 μg from each isolate (quantitated as described in Fig. 5 legend) were analyzed by RNAse protection. Autoradiograph and relative increases depicted graphically are shown, with the sample from plated cells assigned a value of 1.

mulation of tissue mononuclear cells may be an important prerequisite. In situ studies have noted that in liver this infiltration consistently precedes the onset of mesenchymal cell proliferation (1, 3). Moreover, in our earlier study using cultured lipocytes, receptor expression was accelerated by exposure to macrophage-conditioned medium (13). Nonetheless, results from other studies (11, 12, 35) suggest that macrophage products are not essential for induction of PDGF-mediated proliferation in cultured lipocytes, and that serum-dependent proliferation will occur once cells have spread. A similar observation has been made in smooth muscle cells (36). The importance of cell spreading for cellular activation and β-PDGFR induction is underscored by the lack of cell proliferation (21) or β-PDGFR gene expression in cells maintained in suspension culture, similar to an earlier report of PDGFR downregulation in cell lines during suspension (37).

We confined our experiments to time points within 2 wk after injury, because our primary interest is in mechanisms of early lipocyte activation. Accumulated evidence indicates that lipocyte activation is a complex cascade of cellular events involving alterations in both structural and regulatory genes (38). Therapeutic efforts to control lipocyte activation will likely be most meaningful if directed towards these early changes, when activation is reversible.

While injury induced by either CCl_4 or bile duct ligation was associated with β-PDGFR mRNA induction, the relative increase in gene expression in CCl_4 disease was more marked than in bile duct obstruction. The basis for this difference is not known, but could be related to the extent of necrosis and inflammation. Carbon tetrachloride results in extensive parenchymal injury due to membrane disruption, with a marked inflammatory response (1, 39). In contrast, parenchymal injury and inflammation are present but more subtle in rats with bile duct obstruction (40). On the other hand, the return to normal of β-PDGFR mRNA expression within 1 wk after a single dose of CCl_4 is consistent with the known reversibility of this lesion in the absence of repeated dosing. Surprisingly, even after bile duct ligation, β-PDGFR mRNA expression returned to near normal within 1 wk, even though cholestasis persists. This finding reinforces the possibility that receptor induction in the bile duct obstruction model does not result from cholestasis per se, but rather from transient mononuclear cell infiltration, which peaks 3 d after duct ligation (40).

Despite the clear demonstration of PDGFR induction in vivo and culture by activated lipocytes, the cellular source(s) of PDGF in liver injury are not certain. Low levels of PDGF A-chain mRNA have been detected in cultured normal lipocytes when assaying large quantities of RNA (34), and PDGF immunoreactivity has been detected in situ in clusters of mesenchymal cells and necrotic hepatocytes (41). However, an autocrine source for bioactive PDGF is unlikely, because cultured lipocytes fail to proliferate in the absence of exogenous PDGF even when expressing PDGF receptor (13). Other potential sources for PDGF in vivo include platelets, macrophages, and sinusoidal endothelial cells. Alternatively, PDGF-like species have recently been described which may act via binding to the PDGF receptor (42, 43); expression of such a molecule in liver might elude detection by current reagents used to detect native PDGF. Other mitogens apart from PDGF may also drive lipocyte proliferation in tissue injury. Epidermal growth factor (14, 44), fibroblast growth factor (14), and tumor necrosis factor (45) all stimulate proliferation of cultured lipocytes, although they are less potent than PDGF (14).

Acknowledgments

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