Retinoic acid suppresses the response to platelet-derived growth factor in human hepatic Ito-cell-like myofibroblasts: a post-receptor mechanism independent of *raf/fos/jun/egr* activation

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Activated Ito-cell-like myofibroblasts proliferate *in vivo* during human liver injury and subsequent fibrogenesis. To examine the associated regulatory mechanisms, human liver myofibroblasts were characterized after culture purification from mixed liver-cell isolates obtained from perfused normal human livers. The cells resembled rat Ito-cell-derived myofibroblasts expressing desmin and α-smooth-muscle actin filaments as well as the interstitial collagens type I and III. [^{3}H]Thymidine incorporation was inducible with platelet-derived growth factor (PDGF) and was suppressible with retinoic acid (RAc) in a concentration-dependent fashion. RAc suppression did not alter PDGF α- or β-receptor abundance or activation. In addition, RAc functioned via a pathway distal or independent of cytolasmic *raf* activation (i.e. phosphorylation, kinase function and perinuclear translocation) and nuclear *fos*, *jun* and *egr* expression, as these steps were similarly unaffected by RAc treatment. Since normal Ito cells contain abundant amounts of vitamin A which is lost during activation, these data suggest that retinoids could contribute to the maintenance of the quiescent non-proliferative state by suppressing mitogenesis at a post-cytokine receptor step distal from or independent of *fos/jun/egr* [e.g. via changes in activator protein-1 (AP-1) binding].

INTRODUCTION

The factors which potentially regulate the hepatic fibrogenic process during the development of cirrhosis are poorly understood. Although the common inciting events leading to chronic liver disease are generally attributed to viruses, alcohol exposure and autoimmune phenomena, the forces which lead to the subsequent cellular alterations resulting in the general histologic pattern of extracellular-matrix overproduction, disarray of the space of Disse and nodule formation have not been well defined. Rat models of hepatic fibrosis, as well as studies *in vitro* utilizing rat Ito cells and their derived myofibroblast counterparts, have begun to delineate some of these potential regulatory forces [1–4]. Retinoids and prostaglandin compounds have recently been suggested as potential modulators (i.e. suppressors) of the rat fibrogenic process [5–10]. Studies *in vitro* suggest this may relate to suppression of cytokine responsiveness at post-receptor levels [9,10]. Although analogies have been drawn between the rat and human systems, direct comparisons between the two cellular systems have not been made. Previous morphological studies have suggested, however, that during experimental and clinical hepatic fibrosis there is an alteration in the non-parenchymal sinusoidal Ito-cell fraction, with a loss of prominent vitamin A lipid droplets and the 'transformation' to an apparent activated myofibroblast phenotype [11–14]. This circumstantial type of evidence would support the notion that alterations in the handling of intracellular vitamin A occur in both species during the cirrhotic process.

The ability to test directly the hypothesis that the human hepatic myofibroblast is similar to its rat counterpart and is similarly sensitive to retinoid suppression has not been available until the development of techniques to obtain the appropriate human cells. Recent refinement of isolation techniques has permitted the isolation of human lipocytes (Ito cells) and the maintenance of these cells in primary culture [15,16]. The data obtained from these cells to date suggest they are remarkably similar to their rat counterparts with regard to staining characteristics and collagen-synthetic phenotype [15,16]. These new techniques are similar to the approaches used to purify rat Ito cells and require sequential perfusion with collagenase and Pronase as well as differential centrifugation to isolate the lipocyte fraction [15,16]. This generally results in the elimination and destruction of the dominant hepatocyte fraction, but results in lipocytes of high purity and viability. After several days of primary culture, lipocytes are transformed, resemble the activated myofibroblast seen during the fibrotic process *in vivo*, and maintain this similar phenotype through several passages in tissue culture [15,16].

To obtain a similar fraction of 'activated' human lipocytes, the present work developed a simplified technique to select for lipocytes contaminating routine human hepatocyte-enriched cultures [17]. This type of contamination has been explored in the analogous rat hepatocyte system, where extensive techniques were required to eliminate low levels of lipocyte undergrowth [18]. The different growth requirements and optimal conditions previously noted for rat hepatocytes compared with rat lipocytes were used in the present study to permit lipocyte overgrowth and loss of viable hepatocytes [17,18]. The enriched activated lipocyte or myofibroblast cultures were then further purified. The present study characterized the early-passaged human hepatic myofibroblast and has specifically contrasted it with the known data on the similar rat cell. As previously suggested in studies of their primary culture predecessors, the human cells were phenotypically similar to rat-derived cells. The cells displayed marked sensitivity to retinoid suppression of platelet-derived growth factor (PDGF)-induced mitogenesis, and this similarity to rat cells was further pursued mechanistically by examining several levels of the PDGF activation cascade. The data to be presented support the contention that retinoic acid (RAc) suppresses myofibroblast activation at a post-receptor nuclear level. The

Abbreviations used: PDGF, platelet-derived growth factor; RAc, retinoic acid; FCS, fetal-calf serum.
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potential significance of these findings with respect to regulation of Ito-cell activation during cirrhosis is discussed.

MATERIALS AND METHODS

Chemicals

RAc (1 mM in 100 % ethanol) was freshly prepared immediately before use as previously described [7]. Control cultures treated with equivalent volumes of ethanol vehicle (final concn. < 0.1 %) were indistinguishable from untreated cells. PDGF-BB was obtained from Amgen and GIBCO–BRL.

Cell isolation and culture

Segments of normal human liver obtained at the time of organ preparation before transplantation were used for cell preparations. Liver cell isolation was performed as previously described for baboon liver [17]. After collagenase perfusion, the cells were sedimented twice at 50 ***g*** for 5 min, adjusted to approx. 1×10^6 cells/ml in Williams' Medium E supplemented with 10 mM Hepes/KOH + 10 % fetal-calf serum (FCS) and plated on flasks coated with Primaria (Falcon). After 48 h of culture, the medium was changed either (depending on the desire to maintain either hepatocytes or mesenchymal cells) to a serum-free formulation, previously described for hepatocyte culture, or to Dulbecco's modified Eagle's medium containing 10 % calf serum/10 % FCS (Ito medium) [7,17]. The latter Ito medium was changed daily, and during the subsequent 5–10 days of culture there was gradual loss of hepatocytes coincident with the prominent appearance of stellate-like cells. To enrich these latter cells and to decrease the percentage of remaining viable hepatocytes, the mixed cell cultures were trypsin-treated (0.5 % trypsin/0.02 % EDTA) when the stellate cells (which had lost most of their lipid droplets) appeared to occupy over 50–60 % of the available surface area. The released cells were centrifuged (50 *g* for 2 min), and the pellet and the supernatant were separately cultured. The pellet contained a mixture of non-viable cells as well as predominantly hepatocytes and rare stellate cells (< 20 %). The supernatant fraction yielded a homogeneous stellate-cell population which was subsequently passaged and maintained on type I collagen flasks as previously described for rat Ito cells [10]. As this method was primarily an outgrowth technique, precise yields cannot be quantified. Each mixed liver cell flask contained approx. $(0.5\text{–}1) \times 10^6$ mesenchymal cells/75 cm^2 before centrifugation to remove parenchymal cells. The number of flasks originally obtained from the liver segment varied considerably (10–30 flasks) and was partially dependent on segment size and adequacy of enzyme perfusion. The cells were split at a 1:3 ratio and were characterized at passage 1–3. Some cells were also studied at later passage (passage 5–6) as noted separately. The present work includes passaged cells from three separate human donors.

Morphological and phenotypic characterization

The early liver cell cultures (before trypsin treatment) as well as all passaged cells were routinely viewed under phase-contrast and Hoffman optical microscopy. The passaged cells (subcultured on 24-well type I collagen-coated plates) were further characterized immunohistochemically with regard to collagen-synthetic phenotype and Ito-cell-like features (desmin and α-smooth-muscle actin staining). Staining was performed as described below by using rabbit polyclonal antisera to calf procollagen I or III, rabbit polyclonal antiserum to chicken desmin (courtesy of Dr. J. Madri, Yale University), or mouse monoclonal antiserum to α-smooth-muscle actin (Sigma) [19]. Cells were initially fixed in 10 % formalin/0.2 % Triton X-100. Control wells either excluded the primary antibody or contained equal amounts of normal rabbit serum or mouse IgG.

Cell proliferation

Cells (groups of 4 parallel wells) were cultured on 24-well plates under sub-confluent conditions in 0.4 % FCS with or without RAc for 18 h. The medium was then replaced with fresh medium containing PDGF (10 ng/ml) with or without RAc; 8 h later, the cultures were pulsed with [^{3}H]thymidine and incubated for an additional 16 h. The degree of labelling was previously found to correlate with changes in cell number as well as bromodeoxyuridine nuclear labelling *in situ* in the analogous rat cell culture system [8,10].

PDGF receptor abundance

Cell cultures initially maintained in media containing 10 % FCS were changed to media with 0.4 % FCS with or without RAc (1 μM) for 18–24 h. When the acute effects of PDGF were assessed, PDGF was added directly to the tissue culture medium for the last 15 min at a final concentration of 30 ng/ml. The medium was then removed, and the cells were washed twice in iced PBS and then scraped directly into RIPA (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 % Triton X-100, 0.5 % deoxycholate, 0.1 % SDS, 1 mM sodium orthovanadate, 2 mM EDTA, 10 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 300 μg/ml phenylmethanesulphonyl fluoride) buffer, and centrifuged at 12000 ***g*** for 15 min to remove insoluble cell material. Protein concentrations were determined on the cleared cell lysates by using the Bio-Rad protein-determination kit, and the samples were heated at 95 °C for 3 min and either used directly for Western blotting or stored in sub-samples at −70 °C. Samples initially solubilized in RIPA were later mixed with 4 × solubilization buffer (4 % SDS/10 % glycerol/50 mM Tris/HCl, pH 6.8) and re-heated at 95 °C for 3 min before gel loading. Samples of equivalent protein concentrations were run on an SDS/7.5 %-polyacrylamide gel and then electroblotted on to polyvinylidene difluoride membranes (Immobilon P; Millipore, Bedford, MA, U.S.A.). As previously described [10], the membranes were initially blocked with PBS + 0.03 % Tween-20 at 37 °C and then stained with India ink to confirm equivalent transfer and to identify molecular-mass standards (BRL). The blots were then blocked in Blotto (5 % dried milk, 0.01 % antifoam, 0.5 % Tween-20, 0.2 % NaN_3) for 1 h and probed (4 °C for 18 h or room temperature for 1 h) with either rabbit polyclonal antiserum to PDGF human α- or β-receptor (courtesy of Dr. J. Escobedo and L. Williams, University of California, San Francisco) or rabbit polyclonal antiserum to phosphotyrosine (East Acres Biologicals, Southbridge, MA, U.S.A.; courtesy of Dr. J. Avruch, Massachusetts General Hospital) [20]. Similar blots were probed with a monoclonal antiserum to human α-smooth-muscle actin (Sigma) as previously described [10]. The blots were then washed several times with 0.05 % Tween-20, and were then probed with peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (Amersham) followed by the ECL Amersham method. Autoradiograms were developed by using Kodak XAR-5 film and intensifying screens as needed.

Assessment of PDGF-induced cascade

To consider some of the distal events which follow PDGF-receptor activation, two potential signal transducers were

examined: (*a*) activation and translocation of the cytoplasmic proto-oncogene c-*raf*, and (*b*) nuclear expression of the Fos, Jun and Egr-1 proteins. On the basis of previous studies, these transducers are sequentially linked and critically involved in the cascade at progressively more distal or nuclear levels [21,22].

(*a*) Raf activation

The c-Raf serine/threonine kinase activation which occurs upon exposure to PDGF is due to tyrosine, serine and threonine phosphorylation and may be detected as a change in the electrophoretic migration pattern seen on an SDS/7.5%-acrylamide gel [22]. Cell lysates were processed in RIPA as described above and were then immunoprecipitated overnight at 4 °C by using monoclonal antibody to human PBB1 Raf purified from mouse ascites with a Protein G column [22,23]. The characteristic 72 kDa Raf protein identified by the monospecific antibody is completed blocked by preincubation with the Raf protein [23]. Immunoprecipitation was performed with Protein A–agarose beads prebound with a horse anti-mouse IgG (Vector) linker. The Raf antigen–antibody complexes were subsequently washed twice in RIPA, eluted with 4 × solubilization buffer, heated to 95 °C for 3 min, and then centrifuged at 6500 ***g*** for 4 min to remove the beads. The samples were then either frozen at −70 °C or loaded directly on an SDS/7.5%-acrylamide gel. The gel was electroblotted as described above and the transferred proteins were probed with either the PBB1 Raf monoclonal antibody or a rabbit polyclonal Raf antibody [22,23]. After activation, the Raf kinase then phosphorylates a series of proteins ranging from 55 to 170 kDa [22]. Raf kinase function was assessed by using the Raf-bound Protein A–agarose immunoprecipitates obtained after a 3 h incubation as previously described [22,23]. In brief, the complexes were washed twice in RIPA, once in kinase buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM $MnCl_2$, 10 mM $MgCl_2$, 2 mM dithiothreitol) and incubated for 10 min at room temperature in kinase buffer with [^{32}P]ATP (10 μCi/reaction) and histone 1 protein (24 μg/reaction; Worthington) as an exogenous substrate for phosphorylation. The reaction was terminated by adding 4 × solubilization buffer, heated to 95 °C for 3 min, and processed as described above. Eluates were loaded on an SDS/12.5%-acrylamide gel, and the gel containing the resolved proteins was subsequently fixed, stained, dried, and exposed to Kodak XAR-5 film and intensifying screens at −70 °C.

The activated Raf protein has been strongly implicated as a cytoplasic transmitter leading to nuclear transcriptional events and is required for serum-induced initiation of mitogenesis in some cell-culture systems [22]. The cytoplasm-to-nucleus signal transmission may in part relate to the physical translocation of the Raf protein, which has been observed in some cell lines [21]. The Raf cytoplasm-to-perinuclear translocation was assessed during cell culture in response to PDGF stimulation with or without RAc pretreatment. This enabled an assessment of the capacity of the activated Raf kinase (as demonstrated above) to shift its localization physically, which might be important in the further downstream transmission of the PDGF cascade. Cells were cultured in 24-well plates and made quiescent with overnight culture in 0.4% FCS as described above and then exposed to PDGF (30 ng/ml) for 15 min. The cells were then washed twice with iced PBS, fixed in methanol for 10 min, washed sequentially in PBS followed by PBS + 1% BSA (PBS/BSA), and blocked in normal horse serum (1:10 dilution in PBS/BSA) for 10 min. All incubations were performed at room temperature. The wells were then incubated with PBB1 antibody (5 μg/well) diluted in PBS/BSA for 1 h. Control wells either omitted the primary antibody or used equal amounts of purified mouse IgG. After three washes with PBS/BSA, the wells were incubated with a biotinylated horse anti-mouse secondary antibody (Vector) for 1 h. After three additional PBS/BSA washes, the wells were incubated with the avidin–biotin–peroxidase complex (Vector) for 45 min and the final brown/black reaction product was produced by using diaminobenzidine as previously described [10]. The cells were viewed under Hoffman optics on an inverted Olympus microscope and photographed with Technical Pan (Kodak) film.

(*b*) Nuclear proto-oncogene expression

To determine whether the PDGF-initiated cascade ultimately produced nuclear events generally associated with the initiation of mitogenesis and the G_0-to-G_1 shift, the enhanced expression of the nuclear Fos, Jun and Egr-1 proteins was determined immunocytochemically after FCS or PDGF stimulation with or without RAc. The nuclear expression of these transcriptional inducers is generally associated with the rapid and transient transcription of their respective genes and would imply that the PDGF cascade has reached the nuclear level [20,22,24]. Immunocytochemical staining was performed by the same technique described above with minor modifications. Cell fixation was achieved with either methanol alone for 10 min (Fos and Jun staining) or methanol/acetone (1:1, v/v) for 10 min (Egr staining). Primary antibody staining utilized a rabbit polyclonal antiserum to Fos or c-Jun (3 and 2 μg/well respectively; Oncogene Science) for 1 h at room temperature, or a rabbit polyclonal antiserum to Egr-1 (R5232) (1:500; courtesy of V. Sukhatme, University of Chicago) for 1 h at 37 °C [25]. Control staining included comparable amounts of rabbit IgG.

Statistical analysis

Differences between the means of various sub-groups was assessed by Student's t test by using the Statworks statistical package.

RESULTS AND DISCUSSION

Morphological phenotypic characterization

Non-parenchymal cells were found adjacent to hepatocytes in liver cell cultures maintained in serum-containing media (Figure 1a). The spherical hepatocytes with contracted cytoplasm appear brighter under phase microscopy and are readily distinguished from the flatter stellate-shaped cells with their greater cytoplasm/nucleus ratio. These latter cells resemble primary cultures of human and rat hepatic Ito cells or lipocytes, as well as passaged cultures of the rat Ito cell/myofibroblast [3,4,7,15,16,26–28]. Stellate-cell growth in these mixed cultures may relate to a hepatocyte paracrine factor [29]. The passaged stellate-cell number/T75 flask of confluent cells was similar to that for rat Ito cells, $(675 \pm 130) \times 10^3$ cells ($n = 8$). The characteristic appearance at confluence is shown in Figure 1(b). To enhance the visibility of cytoplasmic features, most of the immunohistochemical studies were performed with the cells at a sub-confluent density, which maximized cell spreading. Immunostaining of the passaged human cells revealed that most (> 80%) displayed desmin and α-smooth-muscle actin cytoplasmic filaments as well as procollagen I protein cytoplasmic granules. Procollagen III reactivity was much less than procollagen I reactivity, which is consistent with quantitative data from passaged rat Ito-cell/myofibroblasts, showing type I collagen dominance [27]. Since these early-passaged cells share

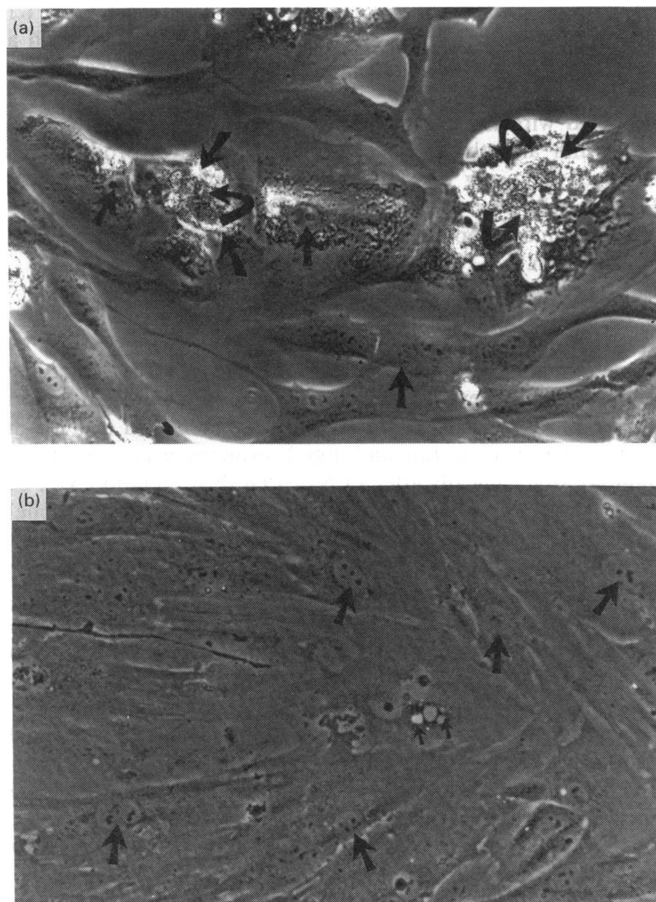

Figure 1 Myofibroblast morphology in culture

Primary (**a**) or passaged human hepatic myofibroblasts (**b**) were grown on collagen-coated flasks or multiwells and were photographed without fixation. (**a**) Myofibroblasts (straight arrows) are shown in a mixed primary cell culture adjacent to deteriorating hepatocytes (small curved arrows). Note the myofibroblast's characteristic prominent nucleoli and dense perinuclear cytoplasmic aggregates. (**b**) Passaged myofibroblasts at confluence following trypsin/EDTA release and differential centrifugation removal of contaminating parenchymal cells. Note nuclei with characteristic prominent nucleoli (straight arrows) and occasional cytoplasmic vacuoles (small arrows). Phase contrast: final magnification × 225.

many of the commonly described features of the rat Ito cell/ myofibroblast, it was important to determine whether similar factors regulate mitogenesis in both the human and rat systems [8,10,30].

Regulation of cell proliferation

As shown in Table 1, the early-passaged human myofibroblasts are responsive to PDGF-BB, as previously reported for rat Ito cells as well as numerous other mesenchymal cell types [10,28,30]. It has previously been demonstrated that retinol or RAc can suppress serum- and PDGF-induced mitogenesis in the rat-derived Ito cell [8,31]. Although the precise mechanism of the retinoid suppressive effect is not clear, this responsiveness was thought to be a central feature in Ito-cell mitogenesis and relevant to the overall fibrogenic process [2,4,7,8,31]. The human cells were found to be similarly sensitive to RAc suppression. Pre-treatment with RAc, using the 1–100 nM RAc dose range used in numerous other studies [7,32], produced a concentration-dependent inhibition of the PDGF stimulus (Table 1). This experiment performed in quadruplicate culture was representative of the retinoid inhibition seen in cells obtained from each of the three human liver donors. The RAc doses did not cause any apparent change in cell morphology or viability. In addition, RAc treatment had no effect on total RNA synthesis or the abundance of the type I collagen mRNA transcript, suggesting that the anti-proliferative effect did not represent a global suppression of most cell functions. No accumulation of intracytoplasmic lipid droplets was apparent. This may be due to the detection limits and sensitivity of phase-contrast microscopy or the inability of the cells to convert RAc into retinol, a precursor of retinyl esters [31]. RAc also caused a 45 % decrease in FCS-induced mitogenesis (results not shown).

Table 1 PDGF-induced DNA synthesis

Subconfluent passaged human myofibroblasts were made quiescent in media containing 0.4% FCS and then exposed to PDGF-BB (10 ng/ml) for 24 h. Proliferation was assessed via [^{3}H]thymidine uptake during the final 16 h. RAc (or ethanol vehicle) was added as indicated during the initial 24 h pre-treatment culture in 0.4% media containing FCS and during the addition of PDGF. Results are * $P < 0.002$; $F = 10.3$.

PDGF	RAc (nM)	[^{3}H]Thymidine incorporation (c.p.m./well)
–	–	4670 ± 1360*
+	–	9271 ± 1740*
+	100	3560 ± 670*
+	10	5140 ± 1190
+	1	7000 ± 2500*

The proliferation data demonstrate marked similarities to the reported studies involving primary and passaged rat Ito cells. The rat studies demonstrated that retinoid inhibition of [^{3}H]thymidine incorporation directly mirrored nuclear thymidine incorporation *in situ* as well as reversible changes in cell number [7,8]. In addition, when later-passaged human cells were used in the present study (after passage 5), the retinoid sensitivity was lost. Similar loss of retinoid responsiveness was noted in later-passage rat cells, suggesting the likely requirement of persistent expression of retinoid receptors and/or response elements which may wane with increasing length of culture [8]. It also implies that a non-specific toxic retinoid effect is less likely. These studies collectively suggest that the passaged human cell type corresponds well to the predicted behaviour of an activated Ito cell/ myofibroblast. Since an understanding of the mechanism(s) of activation or repression of activation should have ultimate clinical significance as well, we chose to explore several levels of the PDGF activation cascade in the presence of the RAc repressor.

PDGF receptor abundance and activation

The PDGF receptor phenotype was initially examined in the human cells and contrasted with the passaged rat Ito cell previously described [10,28]. As shown in Figures 2(a) and 2(b), the human cell type differs from the rat Ito cell in that it contains abundant quantities of both the PDGF α-receptor and the PDGF β-receptor, whereas the rat cell contains predominantly the PDGF β-receptor. The slight difference in electrophoretic mobility between the human and rat PDGF β-receptors may relate to previously described differences in glycosylation patterns which occur during receptor processing [20]. When the human cells were exposed to 1 μM RAc, there was no significant change

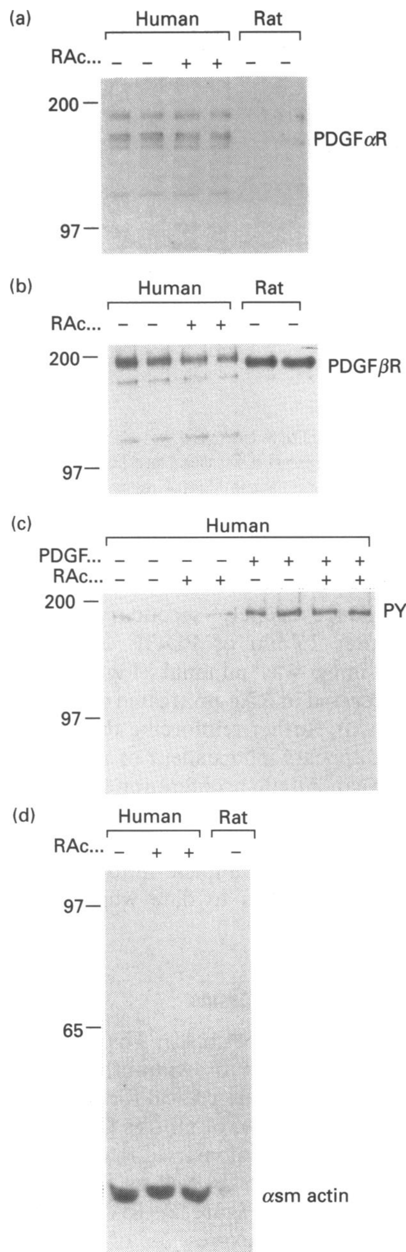

Figure 2 RAc modulation of 'activation' markers

Quiescent human myofibroblasts or passaged rat Ito cells with or without PDGF-BB (30 ng/ml for 15 min) were extracted in RIPA buffer. Lysates were normalized for comparable amounts of cell protein, further solubilized into Laemmli loading buffer and separated on an SDS/7.5%-polyacrylamide gel, transblotted and subsequently probed with (**a**) polyclonal PDGF α-receptor antiserum (PDGFαR), (**b**) polyclonal PDGF β-receptor antiserum (PDGFβR), (**c**) polyclonal phosphotyrosine (PY) antiserum, or (**d**) monoclonal α-smooth-muscle (αsm) actin antiserum. Molecular-mass markers (kDa) are indicated on the left of each gel.

in the abundance of either receptor type. This is consistent with previous studies involving the rat PDGF β-receptor [10]. To assess the receptor's functional capacity for activation after ligand binding with or without RAc, tyrosine phosphorylation of the receptor *in vivo* was assessed via phosphotyrosine immunoblotting (Figure 2c). A single band corresponding to the PDGF receptor was found after a 15 min exposure to PDGF and the relative intensity of this band was similar in the presence or

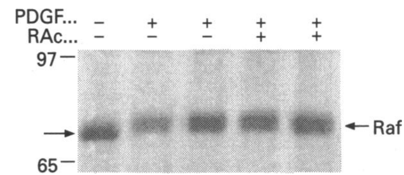

Figure 3 Raf activation

Human myofibroblasts were exposed to PDGF-BB (30 ng/ml for 15 min), solubilized in RIPA, and after protein normalization the lysates were immunoprecipitated with monoclonal PBB1 Raf antiserum pre-bound to horse anti-mouse IgG linked protein A–agarose beads for 18 h at 4 °C. The samples were resolved on a SDS/7.5% polyacrylamide gel, transblotted and probed with a polyclonal raf antiserum. Molecular-mass markers are indicated on the left side of the gel. PDGF induces characteristic shift in electrophoretic mobility in the presence or absence of RAc (indicated by large arrow at right) as compared with baseline Raf protein (indicated by smaller arrow at left).

absence of RAc, suggesting that receptor activation and tyrosine kinase activity were not the alterations responsible for RAc repression of mitogenesis. Since α-smooth-muscle actin was demonstrated immunohistochemically in these cells and has been suggested as a marker of an activated myofibroblast, similar blots were probed with a monoclonal antibody to α-smooth-muscle actin (Figure 2d) [33,34]. There was no change in the abundance of this marker in the presence of RAc, as previously reported for rat cells [10]. Furthermore, despite equal loading of human and rat cell lysates, the human cells appear to contain significantly greater amounts of this actin isoform. The faint actin immunoreactive band seen in the rat lane was clearly intensified with longer incubation exposures (results not shown). These differences do not appear to be secondary to unequal antigen-antibody affinities, as other studies suggest that this antibody stains intracellular human and rat actin equally well [34]. The significance of these inter-species differences is unclear, but may relate to differences in contractile function, as recent results demonstrate that both rat and human Ito cells have the capacity to contract in response to agonist stimulation [16,35].

raf activation

The cytoplasmic *raf* proto-oncogene plays a key role in transmitting surface receptor (i.e. PDGF receptor) and cytoplasmic activation signals to the nucleus during the initiation of cellular proliferation [20,21,36]. Through the use of transfections involving constitutively activated *raf* constructs, it has been demonstrated in numerous cell types that *raf* activation alone is sufficient to activate the large repertoire of nuclear transcription activators and initiate DNA synthesis and cell proliferation [22]. In addition, studies to date suggest that *raf* activation is an obligate integral upstream component of the activation cascade induced by proximal PDGF and serum agonists as well as by the *src* cytoplasmic tyrosine kinase [21,22]. The capacity of PDGF to activate *raf* in Ito cells with or without RAc was therefore examined. This activation was studied at the three levels associated with *raf* activation, as it is likely that all three components are necessary for signal transmission [22]. These levels include phosphorylation of the Raf protein, Raf's kinase capacity, and Raf's ability to translocate. Figure 3 demonstrates that PDGF induced the characteristic shift in Raf's electrophoretic mobility (attributed to its transient phosphorylation) after 15 min of PDGF exposure with or without RAc. Immunoprecipitated Raf displayed kinase activity by phosphorylating both endogenous proteins (120, 55 and 33 kDa)

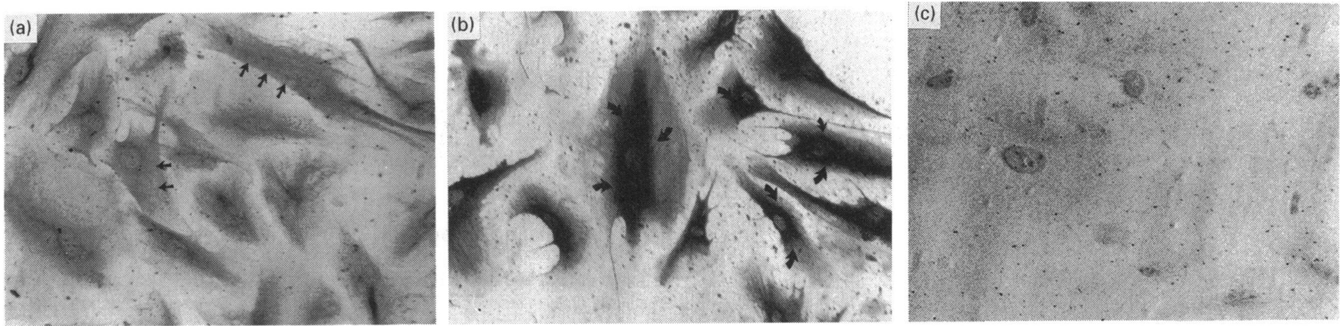

Figure 4 Raf perinuclear translocation

The Raf protein was immunolocalized by using the PBB1 Raf antiserum (5 μg/well) after initial methanol (10 min) fixation. Antibody localization was via biotinylated secondary antibody, avidin–biotin–peroxidase complex and diaminobenzidine. Phase contrast: final magnification × 140. (**a**) Baseline raf distribution in quiescent sub-confluent myofibroblasts maintained in 0.4% serum containing media. Note diffuse cytoplasmic localization (arrows). (**b**) Peri-nuclear raf re-distribution following PDGF-BB × 15 minutes (arrows). Similar redistribution was observed in cultures pretreated with retinoic acid. (**c**) Quiescent cells stained with non-immune mouse IgG (5 μg/well).

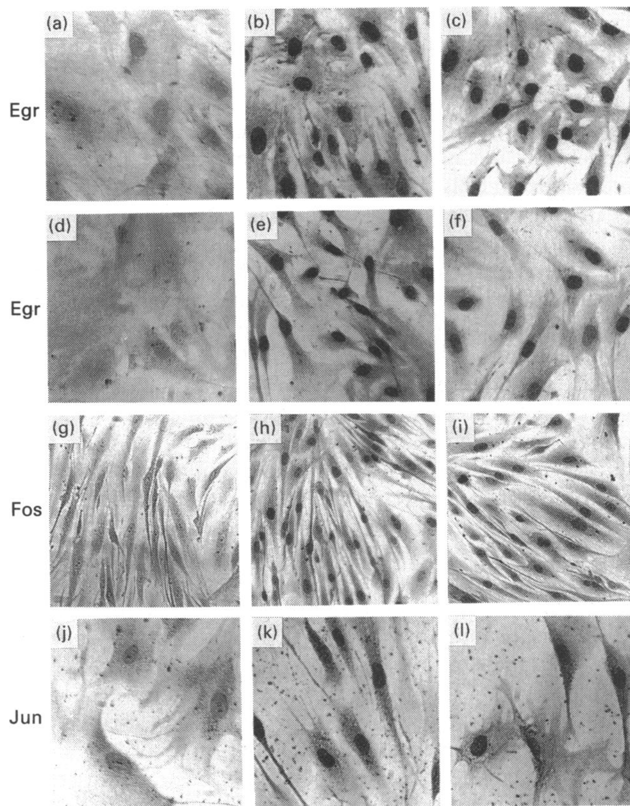

Figure 5 Proto-oncogene nuclear localization

The Egr, Fos and Jun proteins were immunolocalized to the nucleus as indicated 90 min after stimulation with FCS (10%) (**b**), FCS + RAc (**c**), PDGF-BB (**e**, **h**, **k**), or PDGF-BB + RAc (**f**, **i**, **l**). In (**a**), quiescent cells were stained with normal rabbit serum. In (**d**), (**g**) and (**j**), quiescent cells were stained with the corresponding antibodies as indicated on the left side of the figure. Antibody localization was via biotinylated secondary antibody, avidin-biotin–peroxidase complex and diaminobenzidine. (Relative cell density was high in Fos stained groups.) Phase contrast: final magnification × 225.

as well as exogenously added histone 1 equally well with or without RAc (results not shown). The diffuse cytoplasmic distribution of Raf in quiescent cell culture (Figure 4a) was observed to change acutely secondary to its perinuclear relocalization after 15 min of PDGF exposure (Figure 4b). Background staining was minimal (Figure 4c). This similar translocation occurred in RAc-pretreated cells exposed to PDGF (results not shown), further reinforcing the findings that RAc's repressive effect appears independent of *raf* function. Since raf activation alone can initiate proliferation in the absence of other upstream stimulants, the data collectively suggest that RAc is functioning either at a distal/nuclear level or via a parallel pathway required for the complete mitogenic response. There have been no other studies to date which have shown RAc alterations in *raf* function.

Nuclear proto-oncogene expression

PDGF or serum induction of nuclear Fos, Jun and Egr proteins was examined in cells with or without RAc. Previous work suggest that these proteins are critical for the mitogenic process and the shift to the G_1 phase of mitosis [22,25,26]. In addition, transfection experiments demonstrate that *raf* activation alone can induce their expression as well as initiating proliferation [21,22,26]. As shown in Figure 5, RAc did not cause any apparent decrease in the expression of these transcriptional mediators. In other cell types, the induction of the proto-oncogenes within their nuclear site of action correlated highly with radiolabelling studies as well as RNA-transcription data, collectively implying that new mRNA transcription and protein synthesis *de novo* were initiated following *raf* activation [20,22,24,25]. Subtle changes in the nuclear protein levels may have escaped detection. In addition, alterations in the nuclear proteins' differential phosphorylations and dephosphorylations associated with the activated state were not examined. However, the simultaneous demonstration of all three major proteins in > 95% of the cells suggests that the cytoplasm-originated signal had at least reached a nuclear level. This further corroborates the idea that *raf* activation as well as other pre-nuclear cascade steps required for the stimulation of these nuclear proteins were unlikely to have been altered by RAc exposure. Although it is possible that RAc's effect is mediated by a more proximal parallel pathway required for mitogenesis, most RAc studies to date imply that its mode of action is at a nuclear level [32]. The current work's implications of a post-*fos* and post-*jun* mechanism of action is in fact consistent with a recent study utilizing a cell line transfected with the α, β or γ RAR (retinoic acid receptor)

nuclear receptors [32]. It was demonstrated that retinoic acid suppressed AP-1 (activator protein-1) responsive genes via an α-RAR interaction with the Jun protein, which prevented Jun binding to the AP-1 promoter site [30]. Although that study did not specifically examine cellular proliferation, *fos* and *jun* have been strongly implicated in mitogenic signalling independent of their effects on AP-1 binding [20,22]. Preliminary studies in the cultured rat Ito cell have demonstrated the presence of the α-RAR, but there are no published studies to date on the human cellular counterpart [37]. However, conclusions drawn by extrapolation to different retinoid-responsive cell types may be inaccurate. Recent work with malignant cell lines has found very variable effects on *fos* and *myc* expression in response to RAc treatment [38,39]. The apparent lack of a RAc effect on *fos* expression in the present study highlights the complexities of the retinoid-mediated response and the co-modulators, which are likely present in a cell-specific manner and may differ in neoplastic and non-neoplastic cells.

In summary, the present work has characterized a potential human hepatic fibrogenic effector cell and considered one system of mitogenic stimulation and repression which has been suggested by numerous studies to be relevant during injury and fibrosis *in vivo* [1–4,11,12]. Though the human cells are PDGF-responsive, they differ from their rat counterparts in the expression of abundant amounts of both the α- and β-receptors, which could have potential relevance *in vivo* as it relates to the regulation of Ito-cell activation, since several studies have shown differential modulation of the α- and the β-receptors [40–43]. Recent work has indicated differential Ca^{2+} responses in cells expressing the α- rather than the β-receptors as well as interactions between the two receptor subtypes [40,41]. The human cells herein were shown to be sensitive to retinoid suppression, which again emphasizes their similarity to the rat cell and further implies that retinoids in general may be relevant in the regulation of this cell and the activation process which occurs during fibrogenesis. The RAc suppressive effect was shown to occur via either a parallel pathway unrelated to *raf* activation and the induction of *fos*, *jun* and *egr*, or to involve a more distal mechanism. More detailed examinations of these pathways will await successful transfection-type experiments, which are currently in progress.

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