Retinoic acid and transforming growth factor β differentially inhibit platelet-derived-growth-factor-induced Ito-cell activation

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Sinusoidal Ito cells (stellate or fat-storing cells) undergo excessive cellular proliferation before the establishment and progression of hepatic fibrosis and cirrhosis. Retinoic acid and transforming growth factor β (TGF β) both inhibit Ito-cell [3 H]thymidine incorporation in serum-containing media. Serum-induced mitogenicity was dependent on platelet-derived growth factor (PDGF). Additionally, pre-treatment of Ito cells with retinoic acid and TGF β blocked PDGF-induced cell proliferation. TGF β , but not retinoic acid, diminished PDGF-receptor and smooth-muscle α -actin abundance.

INTRODUCTION

Recent intense interest has focused on the molecular factors which regulate cell proliferation. Although this remains a central issue in cell biology, an understanding of the regulatory factors is also critical for therapeutic interventions in the various pathobiological conditions associated with excessive cellular proliferation. During hepatic cirrhosis, experimental and clinical morphologic studies have suggested that an 'activated' non-parenchymal hepatic sinusoidal Ito cell or a related myofibroblast increases in number and eventually surrounds the developing hepatic nodules, which are infiltrated with interstitial collagen bundles (Ballardini *et al.*, 1983; Minato *et al.*, 1983; Takahara *et al.*, 1988).

This activation/transformation process is characterized by an alteration in the Ito cell phenotype, with the expression of smooth-muscle α -actin and the PDGF receptor (Friedman & Arthur, 1989; Ramadori *et al.*, 1989; Rockey *et al.*, 1990). Recent studies involving non-hepatic cells suggest that smooth-muscle α -actin expression occurs during the transformation to an 'activated' pathological phenotype which is associated with increased cellular proliferation and collagen production (Skalli *et al.*, 1989). The expression of the PDGF receptor may be causally related to the aforementioned cellular proliferation, since recent models *in vivo* of injury and fibrosis have found increases in PDGF and implicated a central role for this mesenchymal mitogen (Chen & Rabinovitch, 1989; Pierce *et al.*, 1989a,b).

The factors which specifically regulate Ito-cell proliferation are poorly understood, but recent evidence suggests that both endogenous and exogenous vitamin A and TGF β may play regulatory roles (Shiratori *et al.*, 1987; Russell *et al.*, 1988; Davis, 1988; Davis & Vucic, 1988, 1989; Davis *et al.*, 1990; Meyer *et al.*, 1990). The current study explores modulation by retinoic acid and TGF β of the Ito-cell mitogenic response to PDGF and their associated alterations in smooth-muscle α -actin and PDGF-receptor abundance and activation.

MATERIALS AND METHODS

Chemicals

Retinoic acid used in the cell-culture experiments (final concn.

1 μ M) was prepared as stock 1 mM solutions in 100 % ethanol and stored under nitrogen in brown bottles at -20°C for less than 1 week. Control cultures treated with an equivalent volume of vehicle alone (final concn. 0.1 %) were indistinguishable from untreated cells. TGF β used in cell-culture experiments (1 ng/ml) and in e.l.i.s.a. was generously provided by Dr. Anita Roberts (NIH) (Sporn *et al.*, 1987). It was stored at -70°C as a stock solution in 4 mM-HCl/BSA (1 mg/ml) at a concentration of 20 ng/ml and was used within 1 week after defrosting. EGF, PDGF-AB and goat polyclonal anti-PDGF-AB were obtained from Collaborative Research. PDGF-BB was obtained from Amgen.

Cells and cell culture

Hepatic Ito cells were isolated from Sprague–Dawley male rats (> 500 g) by previously described methods (Davis *et al.*, 1987). The cells were subsequently subcultured (two passages) on tissue-culture flasks pre-coated with calf type I collagen as previously described (Davis, 1988; Davis *et al.*, 1987). Experimental manipulations were performed with cells maintained on either pre-coated 24-well plates or 25 cm² plates. Passaged Swiss 3T3 cells (A.T.C.C. 173; passages 30–34) were studied under similar conditions on uncoated tissue-culture plastic.

Cell proliferation

Ito cells or Swiss 3T3 cells (groups of two to four parallel wells) were cultured on 24-well plates under sub-confluent (Ito cells) or confluent (Swiss 3T3 cells) conditions in serum-free (Swiss 3T3 cells) media or media containing 0.4 % fetal-calf serum (Ito cells) with or without retinoic acid (or an equivalent amount of ethanol diluent) or TGF β for 24–48 h. In experiments evaluating the effects of varying the serum concentration on Ito-cell proliferation, parallel experiments were done as above with the use of a range of serum concentrations (0.4–10 %). When stimulation by PDGF or EGF was assessed, the medium was replaced with various concentrations of the cytokines (with or without retinoic acid or TGF β) diluted directly in the tissue-culture media, and the incubation was continued for an additional 24 h. To assess the potential contribution of PDGF to serum-induced mitogenesis, medium containing 5 % (v/v) fetal-calf serum was

Abbreviations used: TGF, transforming growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; BrdU, bromodeoxyuridine.

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incubated with goat polyclonal anti-PDGF-AB IgG (Collaborative Research) at a concentration of 0.05 mg of IgG/ml (capable of neutralizing 5 ng of PDGF) or an equivalent amount of normal goat serum for 30 min at 37 °C, and was then used directly as tissue-culture medium for Ito-cell incubation. The degree of cell proliferation was assessed after either a final 2 h (Swiss 3T3 cells) or 16 h (Ito cells) labelling period with [³H]thymidine by the method of McGowan *et al.* (1981). This method correlated well with previous studies involving serial cell counts (Davis, 1988; Davis & Vucic, 1988). In addition, parallel cultures were exposed to similar conditions in the presence of hydroxyurea to determine the contribution of DNA repair to apparent DNA synthesis (McGowan *et al.*, 1981). This latter contribution was generally < 5 % of the total counts.

To assess further the alterations in cell proliferation, labelling of nuclei *in situ* was performed by using the bromodeoxyuridine (BrdU) labelling method of Muir *et al.* (1990), with minor changes. This technique has a high correlation with other measures of cell proliferation *in vitro* and *in vivo* (Humes & Thompson, 1989). In brief, quiescent Ito cells were exposed to retinoic acid, TGF β or diluent alone for 24 h, followed by PDGF-BB (16 ng/ml) or 10 % fetal-calf serum in the presence of 2.5 μ M-BrdU for an additional 24 h. The cells were then washed and fixed in 75 % (v/v) ethanol and exposed to 2 M-HCl at 37 °C for 15 min as described by Muir *et al.* (1990). BrdU-labelled nuclei were detected immunohistochemically by using a rat monoclonal anti-BrdU (Accurate Scientific) antibody, followed by a biotinylated rabbit anti-rat IgG secondary antibody (Vector) and the avidin-biotin-peroxidase complex (Vector). The final brown/black reaction product was produced by using a mixture of diaminobenzidine (0.4 mg/ml), H₂O₂ (0.01 %), nickel ammonium sulphate (0.016 %) and CoCl₂ (0.02 %) (Harms *et al.*, 1987). Light nuclear counterstaining was performed with dilute Toluidine Blue (Muir *et al.*, 1990).

Modulation of TGF β production

Confluent monolayers of early-passage Ito cells were incubated in tissue-culture media containing 0.4 % fetal-calf serum for 48 h. During the final 24 h, the cells were exposed to PDGF-AB (8 ng/ml) or an equivalent amount of Dulbecco's minimal essential medium (DMEM) diluent. The medium was subsequently collected and stored at -70 °C prior to assay. TGF β was quantified by e.l.i.s.a. as described in detail previously and expressed as pg/well (Davis *et al.*, 1990). The rabbit polyclonal anti-TGF β antiserum used in the development of the e.l.i.s.a. was kindly provided by Dr. A. B. Roberts and Dr. D. Danielpour (NIH) (Danielpour *et al.*, 1989). Parallel curves were routinely obtained for the standard curve of TGF β and serial dilutions of the Ito-cell culture media.

Assessment of PDGF-receptor abundance and phosphorylation

Sub-confluent cultures of Ito cells or Swiss 3T3 cells on 25 cm² flasks (precoated with type I collagen for Ito-cell culture) were pre-treated for 24 h with retinoic acid, TGF β or a diluent control as described above in the presence of media containing 0.4 % serum. When the acute effects of PDGF exposure were assessed, the cell medium was replaced with fresh medium (lacking retinoic acid or TGF β) containing PDGF-BB (8 ng/ml) for the final 15 min. The medium was then removed and the cells were scraped directly into solubilization buffer (4 % SDS/10 % glycerol/50 mM-Tris/HCl, pH 6.8), heated at 95 °C for 3 min and frozen at -70 °C. Samples were mixed with dithiothreitol (final concn. 10 mM), heated to 95 °C and subjected to electrophoresis and electroblotted on to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA, U.S.A.). The membranes were then washed and blocked with phosphate-

buffered saline + 0.03 % Tween-20 at 37 °C and subsequently were probed (4 °C for 18 h) with either polyclonal PDGF α - or β -receptor antiserum (courtesy of Dr. J. Escobedo and Dr. L. Williams, U.C.S.F.), monoclonal anti-phosphotyrosine antiserum (ICN), or monoclonal antiserum to smooth-muscle α -actin (Sigma) (Morrison *et al.*, 1988). After several washes with 0.05 % Tween-20, the blots were probed with [¹²⁵I]-Protein A (ICN; sp. radioactivity 1.8 μ Ci/ μ l). Autoradiograms were developed by using Kodak XAR-5 film and intensifying screens at -80 °C.

Statistical analysis

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

RESULTS AND DISCUSSION

PDGF modulation of Ito-cell proliferation

Ito-cell proliferation *in vitro* has generally been observed in culture conditions which contain either 10 % or 20 % serum (Shiratori *et al.*, 1987; Davis, 1988; Davis & Vucic, 1988; Friedman & Arthur, 1989). Initial studies confirmed that cell proliferation as assessed by [³H]thymidine incorporation (c.p.m.; mean $\pm$ S.D., *n* = 4) may be stimulated by very low serum concentrations: 0.4 % serum, 1049 $\pm$ 43; 1 % serum, 1345 $\pm$ 68; 2.5 % serum, 1480 $\pm$ 56; 5.0 % serum, 2427 $\pm$ 260; 10 % serum, 3585 $\pm$ 161. Fetal-calf serum demonstrated concentration-dependent mitogenic activity which was most evident at serum concentrations greater than 2.5 %. At these higher serum concentrations, > 95 % of [³H]thymidine incorporation was due to DNA synthesis *de novo*, as opposed to DNA repair as assessed by parallel assays with hydroxyurea (results not shown) (McGowan *et al.*, 1981). Although serum is likely to contain a variety of mitogenic peptides, several studies have implied that PDGF is likely to be a significant component (Bowen-Pope *et al.*, 1989). In support of this proposal, the present studies revealed Ito-cell proliferation to be significantly attenuated in cultures grown in 5 % fetal-calf serum pre-treated with a polyclonal goat anti-PDGF-AB antiserum (fold increase in [³H]thymidine versus a 0.4 %-serum control; mean $\pm$ S.D., *n* = 4): 5 % serum, 4.2 $\pm$ 0.8; 5 % serum + anti-PDGF, 2.2 $\pm$ 0.3 (*P* < 0.005). Parallel studies conducted with an equivalent volume of pre-immune goat serum showed no inhibitory effect (results not shown). To confirm directly the mitogenic capacity of PDGF, Ito cells made quiescent by prolonged (i.e. 48 h) culture in 0.4 % serum were exposed to various concentrations of PDGF-AB for 24 h, and [³H]thymidine incorporation was determined. As reported for numerous other cell types, PDGF-AB in the 5-15 ng/ml concentration range stimulated Ito-cell proliferation (fold increase in [³H]thymidine versus 0.4 %-serum control; mean $\pm$ S.D., *n* = 4): PDGF 5 ng/ml, 1.77 $\pm$ 0.3; PDGF 12.5 ng/ml, 3.2 $\pm$ 0.2 (*P* < 0.001) (Bowen-Pope *et al.*, 1989). In parallel studies, Ito cells were also stimulated by similar concentrations of PDGF-BB (results not shown).

Modulation of the Ito-cell mitogenic response

Previous work had suggested that Ito-cell proliferation and type I collagen gene expression may be markedly blunted by exposure to retinol or retinoic acid (Davis & Vucic, 1988; Davis *et al.*, 1990). To explore further the potential modulation of PDGF mitogenicity by retinoic acid, quiescent Ito cells (in 0.4 % serum) were exposed to PDGF as described above in the presence of 1 μ M-retinoic acid. When retinoic acid was added simultaneously with PDGF-AB, the degree of stimulation of [³H]thymidine incorporation was similar to cultures treated with PDGF alone [2.3 $\pm$ 0.2 (PDGF) versus 2.4 $\pm$ 0.5 (PDGF and retinoic

Fig. 1. Nuclear labelling *in situ* with BrdU

(a) Quiescent Ito cells in 24-well culture plates were treated for 24 h with 2.5 μ M-BrdU containing: (1) 0.4 % fetal-calf serum (FCS); (2) 10 % FCS; (3) 0.4 % FCS + PDGF-BB; (4) 0.4 % FCS + PDGF-BB + retinoic acid (RA); (5) 0.4 % FCS + PDGF-BB + TGFβ. Large arrows indicate labelled nuclei; small arrows indicate unlabelled nuclei (final magnification $\times 800$; phase contrast). (b) Ito cells treated as above were counted (three wells/condition) and expressed as percentage of labelled nuclei. A total of 600–800 cells was counted for each condition and the number of stained nuclei is expressed as a percentage: * $P < 0.001$.

acid) (fold increase versus control)]. However, when Ito cells were pre-treated with retinoic acid for 24 h and subsequently exposed to PDGF and retinoic acid, the mitogenesis was significantly inhibited [2.3 ± 0.2 (PDGF) versus 0.5 ± 0.05 (PDGF and retinoic acid pre-treated) ($P < 0.001$) ($n = 4$)]. These same observations were made when PDGF was used (results not shown). Parallel studies were conducted to examine EGF-induced mitogenicity. This growth factor was considered in part because its signal-transduction mechanism(s) are generally considered to be similar to those for PDGF (Yarden & Ullrich, 1988). In addition, the accumulated evidence suggests that it shares the same cell surface receptor as TGF- α , a cytokine which has previously been shown to stimulate Ito-cell proliferation and which has been strongly implicated as an important growth stimulant *in vivo* during hepatic regeneration (Mead & Nelson, 1989). EGF stimulation of Ito-cell proliferation was blunted by simultaneous administration of EGF and retinoic acid [5.8 ± 0.8 (EGF) versus 3.5 ± 0.5 (EGF and retinoic acid) (fold increase versus control) ($P < 0.001$) ($n = 4$)], but was markedly decreased by a 24 h pre-treatment period with retinoic acid [5.8 ± 0.8 (EGF) versus 1.5 ± 0.1 (EGF and retinoic acid pre-treated) ($P < 0.001$)]. This observation is of interest, since studies involving different cell systems have suggested that retinoids potentiate EGF responses by mechanism(s) involving up-regulation of the EGF receptor (Thompson *et al.*, 1988; Thompson & Rosner, 1989). However, the present studies demonstrate the same general pattern in Ito cells with EGF and PDGF, notably with respect to retinoic acid-induced modulation of their mitogenic responses. These observations indirectly suggest that retinoic acid may operate at a common point distal to the early signal-transduction events.

Increasing evidence suggests that many cytokines, including TGFβ, PDGF and EGF, are likely to be involved in the pathogenesis of tissue injury, repair and/or fibrosis, but an

understanding of their interactions in incomplete (Braun *et al.*, 1988; Russell *et al.*, 1988). Previous work has shown that TGFβ up-regulates Ito-cell type I collagen production and causes marked inhibition of Ito-cell proliferation when the cells were maintained on either an interstitial matrix of type I collagen or the basement-membrane-like matrix of type IV collagen (Davis, 1988). Since a type I collagen matrix is likely to predominate *in vivo* during hepatic fibrogenesis when 'activated' Ito cells or myofibroblasts might be exposed to fibrogenic cytokines, Ito cells maintained on a type I collagen matrix were exposed to PDGF-AB in the presence of TGFβ. Although simultaneous treatment with PDGF and TGFβ modestly decreased the observed mitogenic response, {(fold increase in [3 H]thymidine versus 0.4 % -serum control); mean $\pm$ s.d. 40 ± 2 (PDGF) versus 27.1 ± 5 (PDGF and TGFβ) ($P < 0.006$)}, a 24 h pre-treatment with TGFβ before PDGF-BB exposure caused > 95 % decrease in [3 H]thymidine incorporation [40 ± 2 (PDGF) versus 3.4 ± 1.7 (PDGF and TGFβ pretreated) ($P < 0.001$) ($n = 4$)]. This anti-proliferative effect of TGFβ has been demonstrated *in vivo* during hepatic regeneration (Russell *et al.*, 1988).

Visual confirmation of the [3 H]thymidine-incorporation experiments was obtained by the use of BrdU labelling of Ito-cell nuclei, as previously described (Muir *et al.*, 1990). As shown in Fig. 1, baseline BrdU incorporation was increased by adding either PDGF-BB or 10 % fetal-calf serum, but the former effect was abolished by treatment with either retinoic acid or TGFβ. Parallel cultures which lacked BrdU failed to demonstrate any positive nuclear staining (results not shown).

Since preliminary work in other cell systems suggested that the abundance of TGFβ mRNA can be transiently elevated by PDGF exposure, the potential modulation of TGFβ production by PDGF was considered (Pierce *et al.*, 1989a,b). It was found (Fig. 2) that a 24 h exposure of quiescent Ito cells (in 0.4 % serum) to PDGF-AB (12.5 ng/ml) resulted in a 25 % increase

Fig. 2. $TGF\beta$ /PDGF modulations

Ito cells were made quiescent in 0.4% fetal-calf serum and then exposed to PDGF-AB (12.5 ng/ml) for 24 h. The amount of secreted $TGF\beta$ (b) was quantified by e.l.i.s.a. by using serial dilutions of the tissue-culture media which were parallel to the $TGF\beta$ standard curve (a). Each data point represents the mean $\pm$ S.D. of four separate cultures (* $P < 0.006$).

Fig. 3. Western-blot analysis of Ito-cell activation markers

Ito cells were solubilized, subjected to SDS/PAGE, electroblotted on to polyvinylidene difluoride membranes, probed with either (a) polyclonal anti-PDGF β -receptor (PDGF β -R) antiserum or (b) monoclonal anti-(smooth-muscle α -actin) antiserum and then probed with ^{125}I -Protein A. Quiescent Ito cells were cultured in the presence of 0.4% fetal-calf serum alone or with either 1 μ M-retinoic acid or $TGF\beta$ (1 ng/ml) for 24 h. (a) PDGF β receptor: lane 1, control; lane 2, retinoic acid; lane 3, $TGF\beta$. (b) Smooth-muscle α -actin: lane 1, control; lane 2, $TGF\beta$; lane 3, retinoic acid. These same blot patterns were obtained in three separate sets of experiments.

in the amount of secreted $TGF\beta$ protein [26 ± 0.5 versus 33 ± 2 pg/well (control versus PDGF treated); ($n = 4$) ($P < 0.006$)]. Recent studies quantifying $TGF\beta$ via receptor-competition assays confirm the Ito cell's ability to produce latent $TGF\beta$, which is biologically active after acid activation (Meyer *et al.*, 1990). This observation suggests the possibility that Ito cells have the capacity to up-regulate $TGF\beta$ production in response to mitogenic peptides. The biological relevance of this observation remains speculative, but evidence from other systems involving mesenchymal-cell proliferation suggests that the temporal pattern of PDGF and $TGF\beta$ accumulation may contribute to the formation of the cirrhotic nodule by both initiating and limiting mitogenesis as well as regulating the excessive production of interstitial collagen (Pierce *et al.*, 1989a,b).

To consider further the cell-specific nature of the observed modulations of PDGF responsiveness, Swiss 3T3 cells grown on tissue-culture plastic were also pretreated with either retinoic acid or $TGF\beta$, and [3H]thymidine incorporation was determined. Since their inherent proliferative capacity is much greater than that of Ito cells, the cells were initially maintained for 24 h in serum-free media in the absence or presence of retinoic acid or $TGF\beta$. The cells were then exposed to PDGF-BB for 24 h in serum-free media, and during the final 2 h of incubation [3H]thymidine was added. $TGF\beta$ pre-treatment markedly decreased [3H]thymidine incorporation in the presence of PDGF-BB [PDGF-BB 81 ± 8 versus $TGF\beta$ /PDGF-BB 16 ± 3 (fold change versus hydroxyurea control) (mean $\pm$ S.D., $n = 4$) ($P < 0.001$)],

whereas retinoic acid pre-treatment had no apparent effect [retinoic acid/PDGF-BB 85 ± 9 (fold change versus hydroxyurea control)]. Ito cells have previously been shown to contain high concentrations of cytoplasmic retinoid-binding proteins, and it is possible that differences in the expression of cytoplasmic or nuclear retinoic acid-binding proteins underlie the observed differences between these two cell types (Blomhoff *et al.*, 1985). These studies raise the possibility that retinoic acid may function by modulating common factors which are critical in the overall Ito-cell mitogenic response, and re-emphasize its importance as an appropriate tool for further dissecting the cytokine mitogenic

process. This work also emphasizes the functional differences between Ito cells and widely studied fibroblast cell lines, and suggests that the numerous biological observations made with these latter cell types may be difficult to extrapolate to Ito-cell behaviour.

Alterations in Ito-cell activation

Since accumulating evidence suggests that the pathologically activated Ito cell is characterized by smooth-muscle α -actin expression and PDGF-receptor expression, it was considered that these two central features might be altered by the various pre-treatments. As shown in Fig. 3(a), retinoic acid treatment caused no change in the abundance of the PDGF β receptor, whereas TGF β treatment decreased its abundance. Densitometric scanning of blots from three similar experiments adjusted for differences in protein loading confirmed this observation [(relative units) control $99 \pm 4^*$; TGF β $58 \pm 7^*$; retinoic acid 102 ± 20 ($*P < 0.001$)]. Western blotting failed to detect an Ito-cell PDGF- α receptor, though Swiss 3T3 controls demonstrated a band in the reported (185 kDa) molecular mass for this receptor (results not shown). It is possible that Ito cells express low levels of the PDGF- α receptor below the limits of detection for this Western-blotting technique, and this would be consistent with the limited Ito-cell mitogenic response to PDGF-AA (which requires a PDGF- α receptor) versus either PDGF-AB or PDGF-BB (Pinzani *et al.*, 1990). Fig. 3(b) similarly demonstrated that retinoic acid caused no change in the abundance of the smooth-muscle α -actin activation marker, whereas TGF β caused a slight decrease in its abundance.

Although other studies have noted alterations in PDGF-receptor abundance primarily through receptor-binding studies, it is unclear whether the TGF β -induced decrease in PDGF β -receptor protein and the modest decrease in α -actin protein observed in the present study are sufficient to account for its marked inhibition of Ito-cell proliferation after PDGF-induced activation (Gronwald *et al.*, 1989). The relative affinity of the PDGF receptor was not assessed in the current study, owing to inconsistencies observed in PDGF binding during collagen-coated-plate assays.

Further studies are needed to determine whether the proximal events surrounding secondary messenger activation are altered or whether the dominant effects of retinoic acid and TGF β relate to other distal or nuclear-associated events.

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