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Regulation of pancreatic stellate cell function *in vitro*: biological and molecular effects of all-*trans* retinoic acid

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Abstract

Pancreatic stellate cells (PSCs) are essentially involved in the development of pancreatic fibrosis, a constant feature of chronic pancreatitis and pancreatic cancer. Profibrogenic mediators, such as ethanol metabolites and cytokines, induce a PSC activation process that involves proliferation, enhanced production of extracellular matrix proteins and a phenotypic transition towards myofibroblasts which includes a loss of the characteristic retinoid-containing fat droplets. Here, we have analysed how exogenous all-*trans* retinoic acid (ATRA) affects activation of rat PSCs induced by sustained culture. Bromodeoxyuridine-incorporation assays indicated an ATRA-dependent inhibition of DNA synthesis. In contrast, ATRA did not affect expression of α-smooth muscle actin, a protein typical for myofibroblasts. Quantification of [^{3}H]proline incorporation revealed a diminished collagen production in ATRA-treated PSCs. Furthermore, zymography experiments showed that supernatants of ATRA-exposed PSC cultures contained higher levels of matrix metalloproteinase-9 but not of matrix metalloproteinase-2 than untreated controls. At the level of intracellular signalling, ATRA had no effect on extracellular signal-regulated kinase activation after incubation of PSCs with the mitogen platelet-derived growth factor (PDGF). In addition, PDGF-induced DNA binding of activator protein-1 (AP-1) transcription factors was not inhibited by ATRA treatment. Luciferase reporter gene assays, however, revealed an ATRA-dependent transrepression of AP-1 in PDGF-stimulated PSCs. Together, the results indicate that exogenous ATRA displays inhibitory effects on PSC proliferation and collagen synthesis but does not block phenotypic transition towards myofibroblasts. We hypothesise that inhibition of AP-1 signalling may be involved in the mediation of biological effects of ATRA on PSCs.

Keywords: Pancreatic stellate cells; Myofibroblasts; Proliferation; Collagen synthesis; All-*trans* retinoic acid; AP-1

1. Introduction

In the development of pancreatic fibrosis, a characteristic feature of chronic pancreatitis and pancreatic cancer [1,2], PSCs play a crucial role [3–5]. Profibrogenic mediators, such as oxidative stress, ethanol metabolites and cytokines, stimulate fibrogenesis through the induction of PSC activation; a process that involves enhanced cell proliferation, a phenotypic transition towards myofibroblasts as well as production of increased amounts of collagens (types I and III) and other extracellular matrix (ECM) proteins [3–8]. Cytokines promoting PSC activation include the mitogen PDGF and the autocrine stimulator of ECM synthesis transforming growth factor-β (TGF-β) [4,9,10]. In a previous study, we have shown that at the intracellular level extracellular signal-regulated kinases (ERKs) are essentially involved in the transduction of mitogenic signals [11]. The precise molecular mechanisms which underlie the activation process, however, remain to be deciphered.

Like their hepatic counterparts, the hepatic stellate cells (HSCs), PSCs store retinoids in fat droplets [3,6,12]. Activation and phenotypic transition of HSCs and PSCs towards myofibroblasts correlate with a strong decrease of

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Abbreviations: PSC, pancreatic stellate cell; ECM, extracellular matrix; PDGF, platelet-derived growth factor; ERK, extracellular signal-regulated kinase; HSC, hepatic stellate cell; RA, retinoic acid; ATRA, all-*trans* retinoic acid; α-SMA, α-smooth muscle actin; MMP, matrix metalloproteinase; AP-1, activator protein-1; FCS, fetal calf serum; BrdU, 5-bromo-2′-deoxyuridine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Luc, luciferase; EMSA, electrophoretic mobility shift assays; SEM, standard error of the mean.

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cellular Vitamin A reserves [3,6,13]. Retinoids are well-established regulators of proliferation and differentiation of various types of cells [14], raising the question whether Vitamin A depletion may be not just a phenomenon that accompanies activation of stellate cells but plays a direct role in this process. Indeed, studies with cultured HSCs have indicated inhibitory effects of exogenous biological active Vitamin A metabolites, such as retinoic acid (RA) derivatives, on cell proliferation [15,16] and collagen type I synthesis [17,18]. Still, the precise biological effects of the various retinoids on HSCs are incompletely known. For example, conflicting data have been reported regarding the influence of the most studied RA derivative, ATRA, on HSC growth [15,16,18]. More studies are also required to test the hypothesis that HSC activation could be associated with diminished RA responsiveness and signalling [19]. The biological functions and molecular effects of retinoids in pancreatic stellate cells are largely unknown.

Here, we show that the Vitamin A metabolite ATRA reduced the growth rate of stellate cells isolated from rat pancreas as well as collagen production but did not inhibit induction of α-smooth muscle actin (α-SMA) expression in the course of primary culture. Furthermore, ATRA stimulated expression of matrix metalloproteinase (MMP)-9. At the level of signal transduction, ATRA displayed characteristics of a transrepressor of AP-1, a transcription factor family (composed of Fos and Jun proteins) with complex functions in the regulation of cell growth, differentiation and survival [20]. In contrast, AP-1 DNA binding activity and ERK 1/2 activation were not affected by ATRA.

2. Materials and methods

2.1. Reagents

Nitrocellulose, the enhanced chemiluminescence (ECL) Plus kit, peroxidase-labelled antibodies and radiochemicals were purchased from Amersham Biosciences, the phospho-ERK 1/2 (Thr202/Tyr204 of human ERK 1) antibody from New England BioLabs, the MMP-9 antibody and the gelatinase zymography standard from Chemicon International, the antibody to the ERK 1/2 protein and antibodies used for supershift analysis from Santa Cruz Biotechnologies, and polynucleotide kinase as well as poly(dI-dC) from Roche Diagnostics. Media and supplements for cell culture were obtained from Life Technologies, rat PDGF-BB from R&D Systems and rat tail collagen from Tebu. ATRA, ascorbate, β-aminoproprionitrile (BAPN), the α-SMA antibody and standard laboratory chemicals were from Sigma-Aldrich.

2.2. Cell culture and ATRA treatment

Stellate cells were isolated from the pancreas of male LEW.1W inbred rats by collagenase digestion of the organ followed by Nycodenz density gradient centrifugation as previously published [11,21]. PSCs were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids (dilution of a 100× stock solution), 100 U/mL penicillin and 100 μg/mL streptomycin at 37° in a 5% CO_2 humidified atmosphere. All experiments were performed with cells growing in primary culture (>95% pure PSC cultures as assessed by light, phase contrast, fluorescence and electron microscopy), or depending on the experimental settings, with cells of the first passage. If replating of the cells was required, PSCs were passaged on day 7 of primary culture.

ATRA (dissolved in DMSO as a 20 mM stock solution and stored in the dark at −80°) was diluted immediately prior to use. All manipulations of solutions and cell cultures were carried out in subdued light. ATRA-containing media were changed every 48 hr. Corresponding control cultures were always treated with the same final concentration of DMSO (0.1% or less).

2.3. Cell proliferation assay

Cell proliferation was assessed through the quantification of 5-bromo-2′-deoxyuridine (BrdU) incorporation into newly synthesised DNA, using the BrdU labelling and detection enzyme-linked immunosorbent assay kit (Roche Diagnostics). Therefore, cells plated in 96-well plates were grown for 2 days in the presence of ATRA or its vehicle DMSO (control) as indicated. After 3 hr of labelling, BrdU uptake was measured according to the manufacturer's instructions.

2.4. Immunoblotting

Protein extracts of PSCs growing in 6-well plates (pretreated as indicated) were prepared and adjusted to identical protein concentrations as previously described [11]. After protein separation (15 μg per sample) by SDS–PAGE and blotting onto nitrocellulose filters, membranes were blocked with 1% BSA and exposed to the indicated protein-specific antibodies overnight at 4°. Next, blots were incubated for 2 hr at room temperature with a horseradish peroxidase-labelled anti-rabbit or anti-mouse Ig antibody, before they were developed using the ECL Plus kit. For reprobing with additional antibodies, blots were stripped by incubation in stripping buffer (62.5 mM Tris–HCl, pH 6.7, 2% SDS, 100 mM 2-mercaptoethanol) at 50° for 30 min.

2.5. Measurement of collagen synthesis

Collagen synthesis was assessed through the quantification of [^{3}H]proline incorporation into acetic acid-soluble proteins as described by Becker *et al.* [22]. Therefore, PSCs growing in 12-well plates were exposed for 3 days to

ATRA or its vehicle DMSO as indicated. During the last 24 hr, medium also contained 2.5 μCi/mL [^{3}H]proline (48 Ci/mmol), 50 μg/mL ascorbate and 50 μg/mL BAPN. [^{3}H]Proline incorporation was stopped by the addition of 50 μL/mL 10 N acetic acid to each well. After an overnight incubation at 4°, culture supernatants were transferred to microcentrifuge tubes, mixed with 100 μL/mL FCS, 5 μg/mL rat tail collagen as well as 250 μL/mL 25% NaCl dissolved in 0.5 N acetic acid and incubated at 4° for 30 min. Afterwards, protein precipitates received by centrifugation (30 min at 10,000 *g*) were washed two times with 5% NaCl, before the pellet was dissolved in 0.5 N acetic acid. [^{3}H]Proline incorporation was measured by liquid scintillation counting. Raw data were normalised on the basis of absolute cell counts determined by trypan blue staining of PSCs cultured in parallel under identical conditions (presence or absence of ATRA), except that no [^{3}H]proline was present during the last 24 hr of incubation.

2.6. Zymography

Freshly isolated PSCs were grown for 7 days in primary culture in the presence or absence of ATRA as indicated. Then, the cells were washed free of FCS and cultured for 8 hr in serum-free culture medium (ATRA treatment continued). Afterwards, the supernatants were collected and mixed 3:1 with zymography sample buffer (250 mM Tris–HCl, pH 6.8, 10% SDS, 4% sucrose, 0.1% bromophenol blue). To consider the different growth rates of ATRA-exposed and untreated cells, sample volumes were adjusted according to the protein concentrations (determined as previously described [11]) in total cell lysates. Zymography was performed by modification of a procedure described by Tyagi *et al*. [23]. Therefore, samples and gelatinase zymography standard were loaded onto 8% SDS–polyacrylamide gels supplemented with 1% gelatine. After electrophoresis, the gels were soaked in 2.5% Triton X-100 with gentle shaking (30 min at room temperature; one change of detergent solution), before they were incubated overnight at 37° in substrate buffer (50 mM Tris–HCl, pH 8.0, 5 mM $CaCl_2$). Finally, lysis bands were visualised using a silver staining kit (Bio-Rad), and, after drying of the gel, quantitated by densitometric analysis (Image Station; Kodak).

2.7. Transient-transfection assays

For luciferase (Luc) reporter assays, the AP-1-Luc vector (Clontech) was used, which contains the luciferase gene driven by the TATA box of the thymidine kinase promoter and an AP-1-dependent enhancer element. Plasmid DNA was purified by using Qiagen columns (Qiagen Inc.). Prior to transfection, PSCs were washed free of FCS and cultured for 4 hr in serum-free culture medium. Afterwards, cells were harvested by trypsination and resuspended in plain IMDM at 1×10^7 cells/mL. Afterwards, cells and DNA (10 μg per transfection) were preincubated at 37° for 15 min, followed by electroporation with an electroporator (350 V, 975 μF) (Bio-Rad). Ten minutes after transfection, the cells were diluted in complete culture medium, seeded onto 24-well plates in equal densities and treated with PDGF and/or ATRA as indicated. Sixteen hours later, non-adherent (mostly dead) cells were removed by medium aspiration, and luciferase activity in the attached cells was assayed using the Constant Light Signal kit (Roche Diagnostics). Therefore, lysis buffer (containing all assay reagents) was added directly to the cell monolayer, and luciferase activity was quantitated using a luminometer (Berthold Technologies). For each independent transfection, data were expressed as percent of untreated controls (no PDGF, ATRA vehicle DMSO only).

The growth rates of ATRA-, PDGF- and DMSO-treated cells were analysed by cell counting (after trypan blue staining) and found to be not significantly different after the incubation period of only 16 hr.

2.8. Electrophoretic mobility shift assays (EMSA)

PSCs growing in 6-well culture plates and pretreated by ATRA exposure (2 days) as indicated were cultured for 12 hr in serum-free medium (ATRA treatment continued). Afterwards, the cells were stimulated with PDGF (10 ng/mL) for the indicated periods of time. Nuclear extracts of PSCs were prepared essentially as previously described [11] and stored in aliquots at −80°.

EMSA experiments were performed as previously described [11]. Briefly, nuclear proteins corresponding to 10^5 cells were incubated with a [γ^{32}P]-end-labelled double-stranded oligonucleotide probe (BioTez; labelling with polynucleotide kinase) containing a consensus sequence (bold) for the binding of AP-1 proteins (5′-CGCTTGA**TGACTCA**GCCGATC-3′). The binding reactions (30 min at room temperature) were performed in a total volume of 20 μL in the following buffer: 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 0.1% NP40, 1 mg/mL BSA, 100 μg/mL poly(dI-dC). For identification of DNA-bound proteins by supershift analysis, 1 μg of the indicated antibody was added afterwards, and the incubation continued for additional 20 min. Samples were analysed by electrophoretic separation on a 6% non-denaturating polyacrylamide gel. Dried gels were exposed to X-ray film.

2.9. Statistical analysis

Results are expressed as means ± SEM for the indicated number of separate cultures per experimental protocol. Statistical significance was checked using Wilcoxon's rank sum test. $P < 0.05$ was considered to be statistically significant.

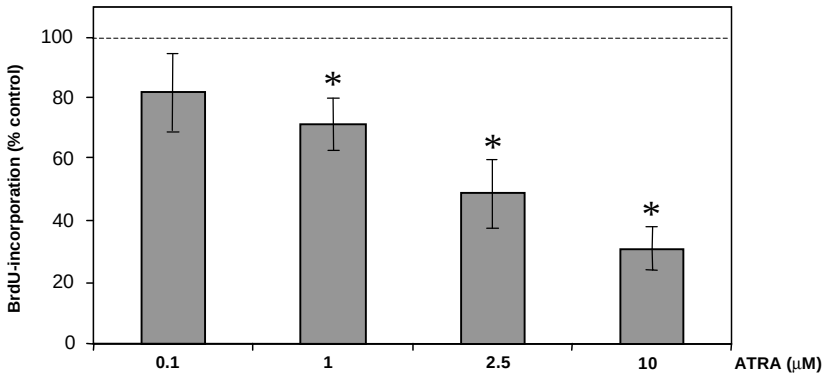

Fig. 1. ATRA inhibits PSC proliferation. PSCs growing in primary culture were harvested, replated at equal seeding densities in 96-well plates and treated with ATRA at the indicated concentrations for 2 days, the time when the fastest growing cultures were almost confluent. Cell proliferation was assessed with the BrdU DNA-incorporation assay. One hundred percent cell proliferation corresponds to PSCs exposed to DMSO only. Data from six separate cultures were used to calculate mean values and SEM. $^{*}P < 0.05$ vs. DMSO-treated control cultures.

3. Results

3.1. Effects of ATRA on PSC proliferation and induction of α-SMA expression

To study the effect of ATRA on PSC growth, cell proliferation after exposure to ATRA concentrations that did not affect cell viability (analysed by trypan blue staining) was assessed using the BrdU DNA-incorporation assay (Fig. 1). ATRA significantly inhibited PSC proliferation in the concentration range 1–10 μM.

Transition of PSCs growing in primary culture towards a myofibroblastic phenotype was monitored by analysing the expression of the α-SMA protein. As previously shown [11], PSCs on day 10 after isolation and seeding express much higher levels of α-SMA than cells on day 3, indicating progression of the activation process (Fig. 2, lanes 1 and 5). ATRA at 0.1–10 μM did not prevent the increase of α-SMA expression (lanes 2–4). Furthermore, ATRA did not cause a reduction of α-SMA protein levels when already activated cells (day 10 after isolation) were exposed to the retinoid (data not shown).

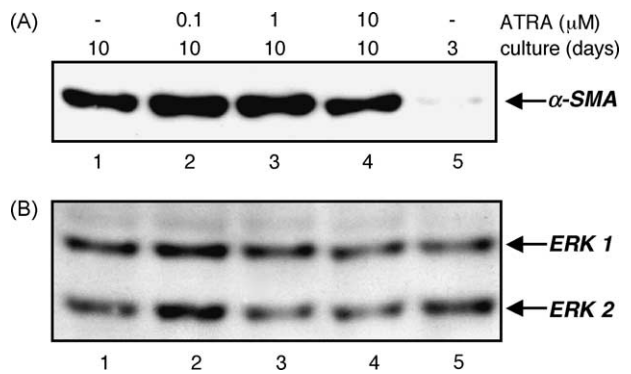

Fig. 2. ATRA does not inhibit expression of α-SMA in PSCs. Freshly isolated PSCs were grown in primary culture in the presence of ATRA or its vehicle DMSO (control cultures) as indicated, and cell lysates (normalised for protein concentration) were resolved by 8% SDS–PAGE. (A) Expression of α-SMA was assayed by immunoblotting. Results are representative of three independent experiments. (B) To control loading, the blot was stripped and reprobed with an anti-ERK 1/2 protein-specific antibody.

3.2. Inhibition of collagen synthesis and maintenance of MMP-9 expression in ATRA-treated PCSs

The effect of ATRA on collagen synthesis in PSCs was analysed through the quantification of [^{3}H]proline incorporation into newly synthesised acid-soluble proteins. Figure 3 shows that ATRA inhibited [^{3}H]proline incorporation in a dose-dependent manner (maximal effect: 40% decrease at 10 μM), suggesting a reduction of collagen synthesis.

We also studied the effects of ATRA on the expression of MMP-2 (gelatinase A) and MMP-9 (gelatinase B), two members of the MMP family of matrix-degrading proteolytic enzymes [24] that have previously been shown to be secreted by PSCs [25,26]. Zymography experiments (Fig. 4A) indicated the presence of much more MMP-9 enzyme in the supernatants of ATRA-exposed PSC cultures (day 7 after isolation) than in corresponding controls (compare lane 2 with lanes 3 and 4). In contrast, MMP-2 secretion was not affected by ATRA treatment. Densitometric quantification of lysis band intensities confirmed these results (Fig. 4B). To further analyse MMP-9 expression, immunoblots were performed. The results indicate a decrease of the MMP-9 protein level in PSCs in the course of primary culture (Fig. 4C, lanes 1 and 3). In ATRA-treated PSCs (lanes 2 and 4), MMP-9 expression remained high.

3.3. Effects of ATRA on AP-1 and ERK signalling

AP-1 transcription factors are among the key mediators of activation signals in hepatic and pancreatic stellate cells [11,27],[1] and they have previously been suggested as important targets of ATRA signalling [28–31]. To analyse

[1] B. Fitzner and R. Jaster, unpublished data.

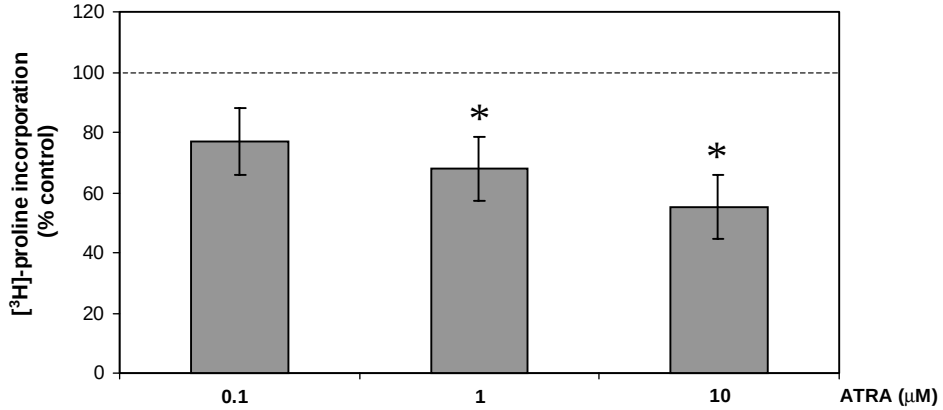

Fig. 3. [^{3}H]Proline incorporation is reduced in ATRA-treated PSCs. PSCs growing in 12-well plates (one passage) were exposed for 3 days to ATRA as indicated. Collagen synthesis was analysed through the quantification of [^{3}H]proline incorporation into acetic acid-soluble proteins, and raw data were normalised for differences in cell growth rates as described under "Section 2". One hundred percent [^{3}H]proline incorporation corresponds to PSCs exposed to DMSO only. Values are expressed as mean (±SEM) of six independent experiments. $^*P < 0.05$ vs. DMSO-treated control cultures.

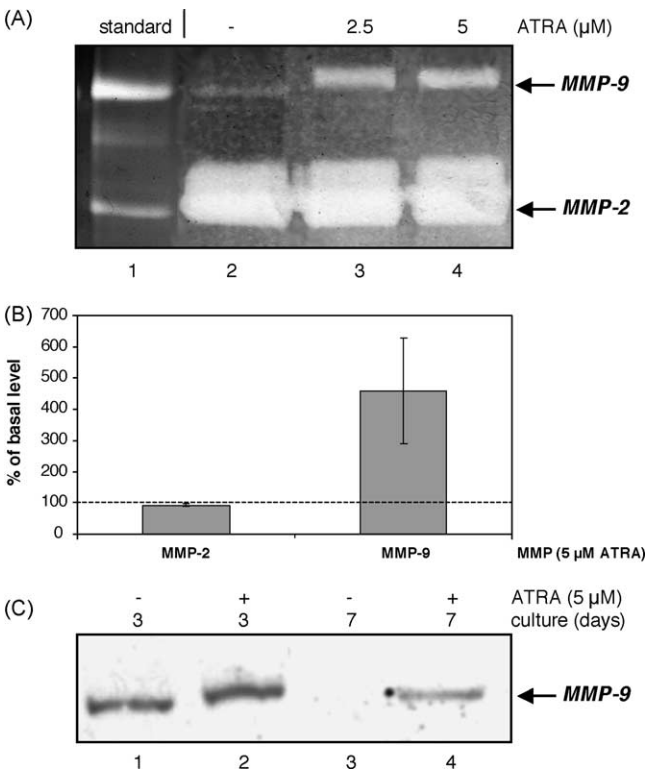

Fig. 4. Stimulation of MMP-9 expression by ATRA treatment of PSCs. Freshly isolated PSCs were grown in primary culture in the presence of ATRA or its vehicle DMSO (control cells) as indicated. (A) Cells on day 7 after isolation were cultured for 8 hr under serum-free conditions (ATRA treatment continued). Afterwards, supernatants were harvested, adjusted in their volumes according to the protein concentrations of the corresponding cell lysates and subjected to zymography as described under "Section 2". In lane 1, a gelatinase A and B zymography standard (250 pg) was loaded. (B) MMP-2 and MMP-9 lysis band intensities were quantitated by scanning densitometry. One hundred percent intensity corresponds to DMSO-treated control cultures. Data of three independent experiments were used to calculate averages and SEM. (C) Cell lysates (normalised for protein concentration) were resolved by 8% SDS–PAGE, and expression of MMP-9 was assayed by immunoblotting.

if ATRA interferes with AP-1 activation in pancreatic stellate cells, luciferase reporter gene assays with the AP-1-Luc plasmid were performed. As shown in Fig. 5 (column 2), the mitogen PDGF, as expected, induced an enhanced expression of AP-1-Luc in PSCs. ATRA had little effect on the low basal luciferase expression level (column 1) but, at 1–10 μM, efficiently blocked the PDGF-dependent increase (columns 3 and 4).

We have previously shown that, in PSCs, PDGF induces the formation of an AP-1/DNA complex containing Fos and Jun family proteins [11]. In contrast to AP-1-Luc expression, DNA binding of AP-1 in response to PDGF stimulation was not affected by ATRA treatment of PSCs (Fig. 6A, compare lanes 1–3 with 4–6). Furthermore, a supershift analysis using antibodies to c-Fos, c-Jun, JunB and JunD (Fig. 6B) indicated the presence of these proteins in the AP-1 complexes but revealed no differences between control cultures (lanes 1–5) and ATRA-exposed cells (lanes 6–10), suggesting an identical complex composition. In addition, ATRA did not inhibit PDGF-induced phosphorylation of ERK 1 and 2 (Fig. 7, compare lanes 1–3 with 4–6). ERK enzymes are involved in the regulation of AP-1 activity through the induction of c-*fos* expression [32].

4. Discussion

The molecular principles of pancreatic stellate cell activation, a key step in the development of pancreatic fibrosis, are incompletely understood. One of the characteristics of the activation process is a progressive decrease in the size and number of retinoid-containing fat droplets that proceeds in parallel to the transition of PSCs towards a myofibroblastic phenotype [3,6]. It is therefore tempting to speculate that RAs, which exert their effects on target cells through binding to RA receptors (α, β and γ RARs) and

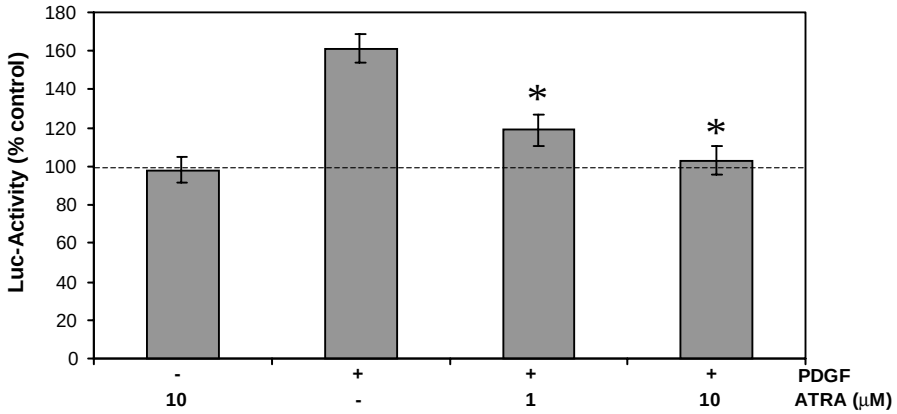

Fig. 5. ATRA acts as a transrepressor of AP-1 in PSCs. PSCs, cultured for 4 hr under serum-free conditions, were transfected with AP-1-Luc, divided into aliquots, seeded onto 24-well plates and treated with PDGF-BB (10 ng/mL) and ATRA as indicated. Luciferase activity in the adherent cells was assayed 16 hr after transfection. One hundred percent luciferase activity corresponds to PSCs exposed to the ATRA solvent DMSO only. Results of six independent transfections were used to calculate mean values and SEM. $^{*}P < 0.05$ vs. cells treated with DMSO and PDGF (no ATRA).

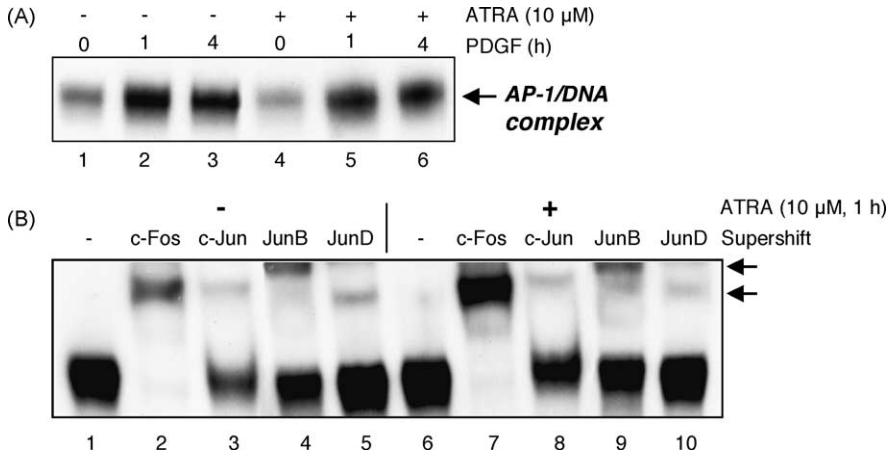

Fig. 6. ATRA does not affect PDGF-induced DNA binding of AP-1 in PSCs. PSCs (one passage) pretreated by ATRA exposure for 2 days as indicated were cultured for 12 hr in serum-free medium (ATRA treatment continued), before they were stimulated with PDGF-BB (10 ng/mL) for the indicated periods of time. (A) Nuclear extracts were subjected to EMSA analysis using a [^{32}P]-labelled oligonucleotide probe with an AP-1 motif. (B) Supershift analysis was performed by incubating the binding reactions with the indicated antibodies. The positions of complexes shifted by antibody binding are pointed out by arrows.

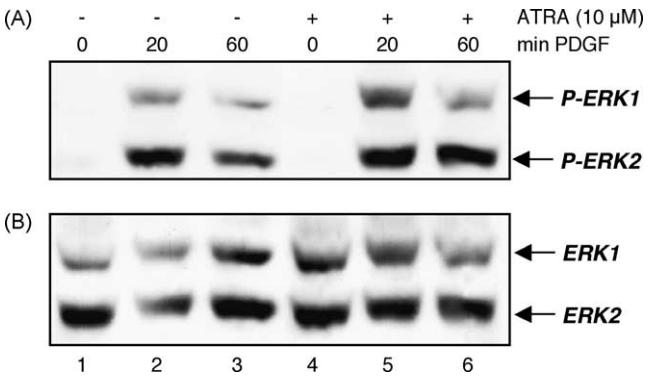

Fig. 7. ATRA does not inhibit PDGF-induced ERK 1/2 phosphorylation in PSCs. PSCs (one passage) pretreated by ATRA exposure for 2 days as indicated were starved from serum for 12 hr (ATRA treatment continued), before they were stimulated with PDGF-BB (10 ng/mL) for the indicated periods of time. Cell lysates were resolved by 8% SDS–PAGE. (A) Phospho-ERK (P-ERK) 1/2 levels were assayed by immunoblotting. (B) To control loading, the phospho-ERK blot was stripped and reprobed with an anti-ERK 1/2 protein-specific antibody.

retinoid X receptors (α, β and γ RXRs) [33] in the nucleus, are regulatory factors in maintenance of PSCs in their native, quiescent state.

In the current study, we have addressed the question how treatment of PSC cultures with the biological active Vitamin A metabolite ATRA affects the activation process both at the cellular and molecular level. Our data indicate that ATRA-exposed PSCs proliferate at a lower rate and synthesise less collagen than untreated controls. On the other hand, exogenous ATRA did not prevent the increase in α-SMA expression in the course of primary culture, suggesting that development of a myofibroblastic phenotype was not interrupted by the retinoid supply.

Studies on the effects of retinoids in cultured hepatic stellate cells, a closely related cell type playing a key role in the development of liver fibrosis, have also shown that retinoids are not only stored in these cells but have complex biological functions. Thus, activated HSCs were found to proliferate at a lower rate in response to retinoid treatment

[15,16,34], and a reduction in type I collagen synthesis was detected [17]. Interestingly, a reduced cell proliferation and a diminished ECM synthesis were also observed when HSCs were isolated from rats pretreated with retinyl palmitate [35]. Hellemans *et al*. [18] directly compared the biological effects of two RA derivatives, all-*trans* and 9-*cis* RA, on HSCs and found that only ATRA lowered the mRNA levels of various ECM proteins (procollagen I, III and IV, fibronectin and laminin), while 9-*cis* RA efficiently inhibited the growth of activated HSCs. ATRA displayed a clear antiproliferative effect only on HSCs in early primary culture (day 2 after isolation) but not on 12-day-old recultured cells; a finding conflicting with earlier reports [15,16]. Our data indicate that PSCs recultured early in the course of activation induced by sustained culture (day 7 after isolation) still show a significantly reduced growth rate in response to ATRA treatment.

In a recent study, Huang *et al*. [34] observed a RA-dependent decrease of α-SMA expression in activated HSCs stimulated with TGF-β, while our data revealed that ATRA had no effect on α-SMA protein levels in primary cultured PSCs. Here, further studies will be necessary to analyse whether these contradictory results are due to the specific design of the experiments, or reflect differences between HSCs and PSCs.

In summary, our results regarding the biological effects of exogenous ATRA on PSCs suggest that this Vitamin A metabolite displays characteristics of a partial antagonist of the activation process; a conclusion that is also supported by the results of our further investigations at the molecular level: we found that ATRA acts as a transrepressor of AP-1. In a previous study, we have shown that the strong PSC mitogen PDGF is also a potent activator of AP-1 transcription factors [11]. In the course of PSC activation induced by sustained culture, formation of AP-1/DNA complexes is an early event that precedes enhanced expression of α-SMA (see footnote 1). Together, these data suggest an important role of AP-1 in the induction of PSC activation. Furthermore, AP-1 proteins have also been implicated in transcriptional regulation of HSC activation [27]. Targeting AP-1 may therefore account, at least in part, for the biological effects of ATRA on PSCs.

How exactly ATRA and its intracellular receptors inhibit AP-1 function in PSCs remains to be addressed in further studies. Our data indicate that neither PDGF-stimulated DNA binding of AP-1, nor mitogen-induced activation of ERK 1 and 2 (upstream regulators of c-*fos* expression) were blocked by ATRA pretreatment of PSCs. These results are in agreement with a study of Suzukawa and Colburn [31] which suggests that, in mouse epidermal JB6 cells, RA directly inhibits transcriptional activity of activated AP-1 dimers (containing JunB and/or the Fos family protein Fra-1) without affecting their DNA binding. However, alternative mechanisms, such as RA-mediated inhibition of c-Jun N-terminal kinase [29,36], an upstream activator of AP-1, have been proposed. Contradictory results have also been published regarding the presence [37] or absence [31,38] of inhibitory ATRA effects on ERK activation. Together, these data render it likely that the specific cellular background determines, at least in part, through which pathways RA inhibits AP-1 signalling.

Although our data indicate a correlation between the biological effects of ATRA and transrepression of AP-1, they are no proof that targeting this transcription factor complex indeed plays a decisive role in retinoid action in PSCs, and they do not exclude the existence of alternative mechanisms which we have not studied so far. For example, downregulation of human kinesin-related protein HsEg5 expression has previously been shown to be an important principle underlying the inhibitory effect of ATRA on pancreatic carcinoma cell growth [39].

PSCs affect ECM metabolism not only by synthesising ECM proteins but also by modulating matrix degradation. Our data indicate that PSCs secrete the gelatinases MMP-2 and MMP-9, confirming recently published results of Philipps *et al*. [25]. In the course of primary PSC culture, expression of the collagenase MMP-9 decreased, suggesting that PSC activation is not only accompanied by an increased collagen production but may also lead to a diminished collagen degradation. In ATRA-exposed cells, expression of the MMP-9 protein remained high, and more active MMP-9 enzyme could be detected in culture supernatants. Interestingly, the ATRA effect on gelatinase expression was specific for MMP-9 because secretion of MMP-2 was not affected. Studies using other types of cells have shown similar [40,41] but also conflicting [42,43] results regarding the effects of RA on MMP-9 expression, indicating again the existence of cell type-specific differences in the response to RA treatment. In ATRA-exposed PSC cultures, maintenance of MMP-9 secretion may synergistically with suppression of collagen synthesis lead to diminished levels of extracellular collagen; a hypothesis that needs to be tested in further studies.

Interestingly, AP-1 transcription factors have been implicated in the activation of the MMP-9 promoter [44,45]. In the course of primary PSC culture, the early formation of AP-1/DNA complexes mentioned above is followed by a continuous decline when transition towards a myofibroblastic phenotype proceeds (see footnote 1), suggesting the involvement of AP-1 in the maintenance of MMP-9 expression in PSCs. The stimulatory effects of ATRA on MMP-9 expression and activity, however, cannot be explained by our data regarding AP-1 activation because ATRA acts as a transrepressor, not activator of AP-1. Two possible explanation that have to tested further are conceivable: firstly, ATRA directly stimulates MMP-9 promoter activity through AP-1-independent pathways and, secondly, the effect of ATRA on MMP-9 expression in PSCs is indirect and the consequence of phenotypic changes in response to ATRA treatment.

Further studies are also required to decipher how ATRA inhibits collagen synthesis in PSCs. In pilot experiments,

we found that at least the mRNA level of α1 (I) collagen was not downregulated by ATRA exposure,[2] suggesting that ATRA inhibits collagen synthesis at the post-transcriptional level. Alternatively, or in addition, ATRA may specifically reduce the transcription of other collagen genes expressed in PSCs.

Experimental studies concerning the influence of retinoids on the development of hepatic fibrosis *in vivo* have provided conflicting results, suggesting either inhibitory [46], lacking [47] or even worsening [48] effects which may depend on the precise experimental settings. Furthermore, there are case reports suggesting a possible link between ATRA treatment of patients with promyelocytic leukaemia and induction of acute pancreatitis [49,50]. It seems therefore unlikely that the inhibitory effects of retinoids on PSC activation can be exploited for therapeutical purposes. Nevertheless, a systematic analysis of retinoid effects on pancreatic fibrosis, using well-defined animal models of chronic pancreatitis, such as dibutyltin dichloride-treated rats [51], would be valuable because it could contribute to our understanding of the complex functions of retinoids in the regulation of PSC activation and ECM metabolism in the pancreas.

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