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9,13-di-*cis*-Retinoic acid induces the production of tPA and activation of latent TGF-β via RARα in a human liver stellate cell line, LI90

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Abstract We studied the mechanism by which 9,13-di-*cis*-retinoic acid (9,13dcRA), a novel and endogenous stereoisomer of all-*trans*-RA, induces TGF-β formation in a human liver stellate cell line, LI90. 9,13dcRA induced the expression of RARα and RARβ, enhanced the production of tissue-type plasminogen activator (tPA), thereby, surface plasmin levels, and induced the activation of latent TGF-β. Similar effects were obtained with RARα-selective retinoid, but not with RARβ- or RARγ-selective retinoid, and the induction was inhibited by RARα-selective antagonist. These results suggest that 9,13dcRA up-regulates tPA expression, resulting in the formation of TGF-β by LI90 cells, at least in part, via induction and activation of RARα.

Key words: 9,13-di-*cis*-Retinoic acid; RARα; tPA; TGF-β; Proteolytic activation; Liver stellate cell

1. Introduction

Transforming growth factor-β (TGF-β) is a major cytokine implicated in the pathogenesis of liver fibrosis/cirrhosis [1,2] In addition to its fibrogenic effect, TGF-β suppresses liver regeneration [3] and the function of hepatocytes [4]. Since TGF-β is mainly expressed in hepatic stellate cells (SC) in the damaged liver [5,6], it would be of importance to elucidate the mechanism by which TGF-β is induced in SCs. TGF-β, composed of three subtypes (TGF-β1, -β2 and -β3), is secreted in a biologically latent form that must be activated before it can bind to its cell surface receptor and exert biological activities [7,8]. Activation releases the 25-kDa active TGF-β molecule from latency associated peptide (LAP) and its associated binding protein (latent TGF-β binding protein: LTBP). Plasmin-mediated activation is best characterized and occurs in several cell culture systems [8]. Activation occurs at the cell surface by plasmin generated from serum plasminogen by the action of plasminogen activator (PA) [9,10].

Retinoic acids (RA) are active metabolites of retinol (vitamin A) and exert profound effects on the cell growth and differentiation [11]. The biological effects of RA are mediated all or in part by two families of nuclear receptors, RA receptors (RAR) and retinoid X receptors (RXR) [12]. The *RAR* and *RXR* gene family each comprises three subtypes named α, β and γ. These nuclear receptors are ligand-dependent transcription factors that bind to *cis*-acting DNA sequences called RA responsive elements (RARE) or retinoid X responsive elements in the promoter region of the target genes [12]. To recognize RARE, RARs usually form a heterodimer with RXRs [12]. It has been reported that in many cell types, RA enhances the production of PA [11]. In bovine endothelial cells, elevation of surface PA/plasmin levels by RA [13] causes the formation of active TGF-β, and this TGF-β subsequently mediates some of the RA effects on endothelial cells [14,15].

Taken together, these findings suggest that RAs might induce the formation of TGF-β in liver SCs, resulting in the progression of fibrogenesis. Most recently, we have found that a significant amount of 9,13-di-*cis*-RA (9,13dcRA), a novel stereo-isomer of all-*trans*-RA, is generated in the fibrotic liver in rat models (M. Okuno et al., unpublished observation). In the present study, we have investigated the molecular mechanism by which 9,13dcRA enhances tissue-type PA (tPA) expression and subsequently activates latent TGF-β in liver stellate cells, employing the culture of a human SC cell line, LI90 [16], as an in vitro model system.

2. Materials and methods

2.1. Retinoids

9,13dcRA was kindly supplied from Eisai Co. (Tokyo, Japan). The pan-RAR-selective retinoid (Ch55), the RARα-selective retinoid (Am580), and the RARβ-selective antagonist (LE135) were synthesized and characterized as described previously [17–21]. The RARβ-selective retinoid (CD2019) and RARγ-selective retinoid (CD437) were kindly provided by Dr. S. Michel (CIRD/GALDERMA, Sophia Antipolis, France) [22,23]. The RARα-selective antagonist Ro41-5253 was a generous gift from Dr. M. Klaus (F. Hoffmann-La Roche, Basel, Switzerland) [24]. Stock solution of retinoids was made in ethanol and serially diluted into culture medium to yield a final ethanol concentration of 0.05%.

2.2. Cell culture

LI90 cells were maintained in DMEM (Gibco, Gaithersburg, MD) containing 10% FCS (Gibco). The cultures were rinsed with phosphate-buffered saline (PBS) and incubated in serum-free DMEM containing 0.2% BSA (DMEM–BSA) with either 0.05% ethanol or 1 μM each retinoid for 12 h. After the incubation, the medium was collected, centrifuged to remove cell debris, and used for TGF-β assays. For zymography conditioned medium was prepared with serum-free DMEM without BSA. The remaining cells were washed with PBS, and used for either RNA isolation or assays of cellular PA and surface plasmin levels.

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RA, retinoic acid; 9,13dcRA, 9,13-di-*cis*-retinoic acid; RAR, retinoic acid receptor; SC, stellate cell; TGF-β, transforming growth factor-β; tPA, tissue-type plasminogen activator

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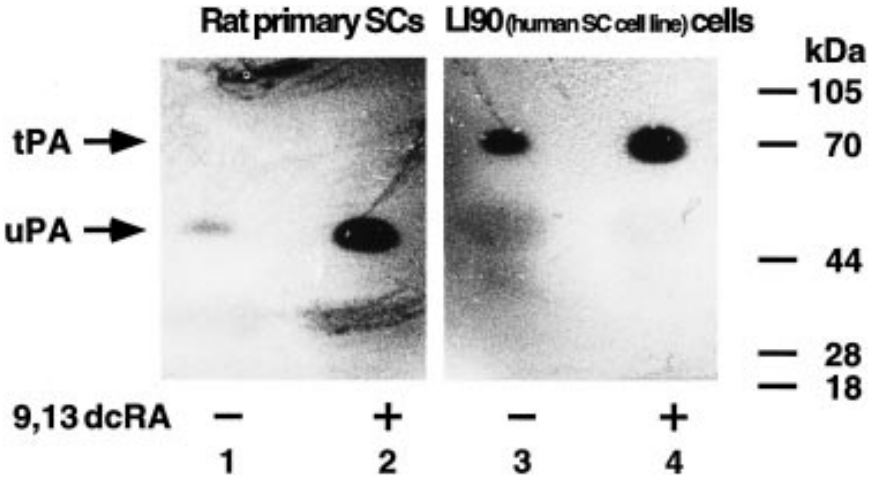

Fig. 1. Augmentation of uPA secretion from the rat primary SC cultures and tPA secretion from the cultures of human SC line, LI90 by 9,13dcRA. Confluent rat primary SCs or LI90 cells were incubated either with vehicle (control) or with 1 μM 9,13dcRA in serum-free DMEM for 12 h. The conditioned medium was collected, concentrated 100-fold on Microcon concentrator (Amicon) and subjected to SDS-PAGE with a 10% resolving gel. The gel was washed, applied onto fibrin-agar gel containing plasminogen and incubated until uPA- or tPA-derived lysis bands were detected. Lane 1, control rat SCs; lane 2, 9,13dcRA-treated rat SCs; lane 3, control LI90 cells; lane 4,9,13dcRA-treated LI90 cells.

2.3. Zymography

Zymography was carried out as described previously [25].

2.4. Assay of cellular PA and cell surface plasmin

The levels of cellular PA and surface plasmin were measured using the chromogenic substrate S-2403, as described previously [25]. Protein concentration was measured by BCA (Pierce, Rockford, IL) assay using BSA as the standard.

2.5. Assay of TGF-β

Active TGF-β was assayed by the inhibition of [^{3}H]thymidine incorporation by CCL-64 mink lung epithelial cells as described previously [14].

2.6. Northern blot analysis

Total RNA was extracted from LI90 cells using the acid guanidinium thiocyanate–phenol–chloroform extraction method [26]. Northern blotting was performed as described previously [14]. The membranes were hybridized with cDNA probes for either RARα, -β or -γ (all gifts from Dr. P. Chambon, INSERM, Université Louis Pasteur, Strasbourg, France), tPA (ATCC), and TGF-β1, -β2 or -β3 (all gifts from Dr. H.L. Moses, Vanderbilt University, Nashvill, TN), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Autoradiography was performed using a Fujix BAS 2000 Bio-imaging analyzer (Fuji Photo-Film, Tokyo, Japan).

2.7. Statistics

Each value in figures represents the average ± S.D. (n=3). Unless noted, each similar experiment was repeated three times, representative results being shown. Significance was determined by Dunnett's t test.

3. Results and discussion

To see the effect of 9,13dcRA on the production of PA by hepatic SCs, we first compared the amount of PA present in the culture medium derived from two different kinds of hepatic SCs, between untreated cells and 9,13dcRA-treated cells (Fig. 1). Rat primary SCs produced and secreted predominantly urokinase-type PA (uPA) (lane 1), whereas human SC cell line, LI90 cells, produced and secreted tPA as a dominant species (lane 3). 9,13dcRA enhanced both uPA levels in rat primary SCs and tPA levels in human LI90 cells (lanes 2 and 4, respectively). uPA has a highly strict species specificity and there is no cross-reactivity between probes derived from

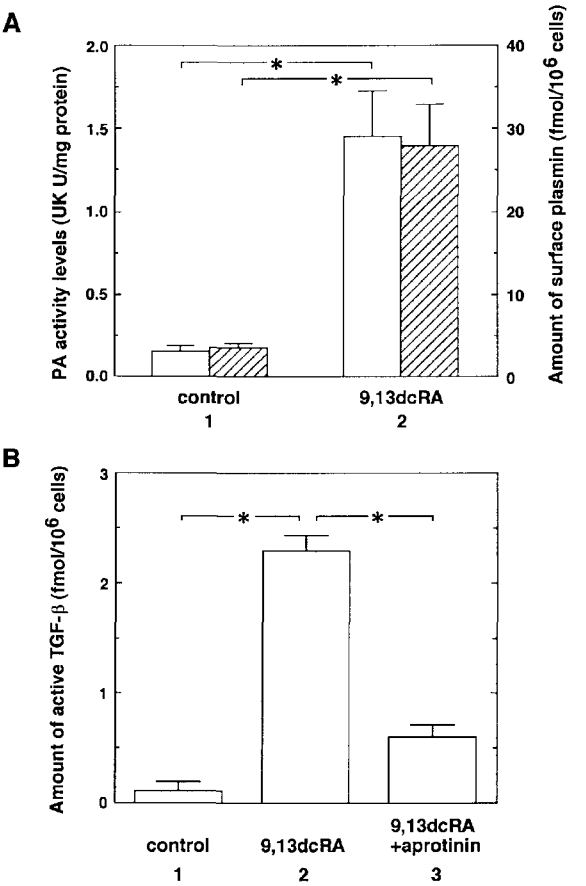

Fig. 2. Enhancement of LI90 cell PA/plasmin and TGF-β levels by 9,13dcRA. A: After treatment of LI90 cells with 1 μM 9,13dcRA for 12 h in DMEM-BSA, either cellular PA (open column) or surface plasmin (slashed column) was recovered from the cultures and both activity levels were determined. B: The culture medium was collected and the levels of active TGF-β in the medium were measured as described in Section 2. Sample 1, control cells; sample 2, 9,13dcRA-treated cells; sample 3, 9,13dcRA-treated cells in the presence of 50 μg/ml aprotinin. $^*P < 0.01$.

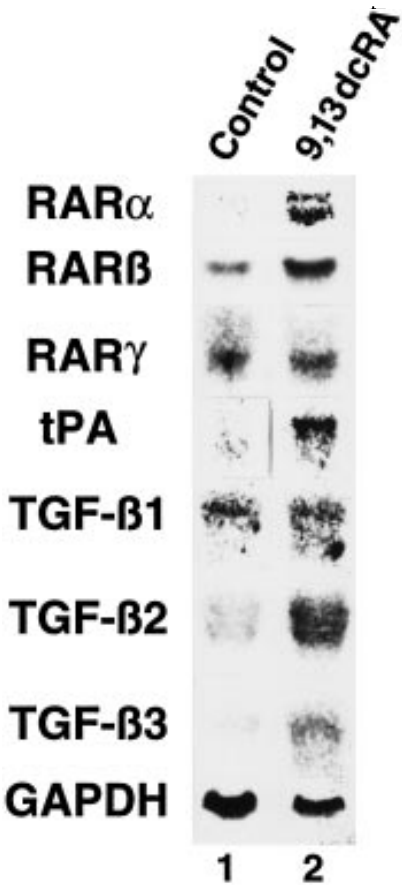

Fig. 3. Changes in the mRNA levels of RARα, -β, -γ, tPA and TGF-β1, -β2, -β3 following the exposure of LI90 cells to 9,13dcRA. Confluent LI90 cell cultures were incubated either with vehicle (control; lane 1) or with 1 μM 9,13dcRA (lane 2) in DMEM-BSA for 12 h. Total RNA was isolated from each cell sample, fractionated through 1% agarose–formaldehyde gels, transferred to nylon membranes and hybridized with ^{3}P-labeled probe for RARα, -β, -γ, tPA, TGF-β1, -β2, -β3 or GAPDH. The radioactivity of each band was detected on an imaging analyzer.
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different species. In contrast, tPA does not have such a species specificity. Because we could not obtain a probe for rat uPA, we decided to analyze the mechanism of 9,13dcRA-induced tPA expression and potential formation of TGF-β by LI90 cells. As seen in Fig. 2, 9,13dcRA enhanced both cellular PA/plasmin (panel A) and TGF-β (panel B) levels in LI90 cell cultures, and the induction of TGF-β was suggested to be dependent upon plasmin since an inhibitor of plasmin, aprotinin, alleviated 9,13dcRA-induced formation of TGF-β (panel B, lane 3), as in the case in endothelial cells [14]. This suggests that 9,13dcRA-treatment elaborated proteolytic activation of latent TGF-β in human SCs via up-regulation of tPA production. Because we treated LI90 cells in the serum-free medium after rinsing the cultures with PBS, the source of surface plasmin is thought to be serum plasminogen bound to the cell surface during subculture [10]. Fig. 3 shows the result of changes induced in the mRNA levels of several related genes following the exposure of LI90 cells to 9,13dcRA. Untreated LI90 cells expressed nominal levels of RARβ and RARγ, but very low level of RARα. Upon treatment with 9,13dcRA, the expression of RARα and tPA increased simul-

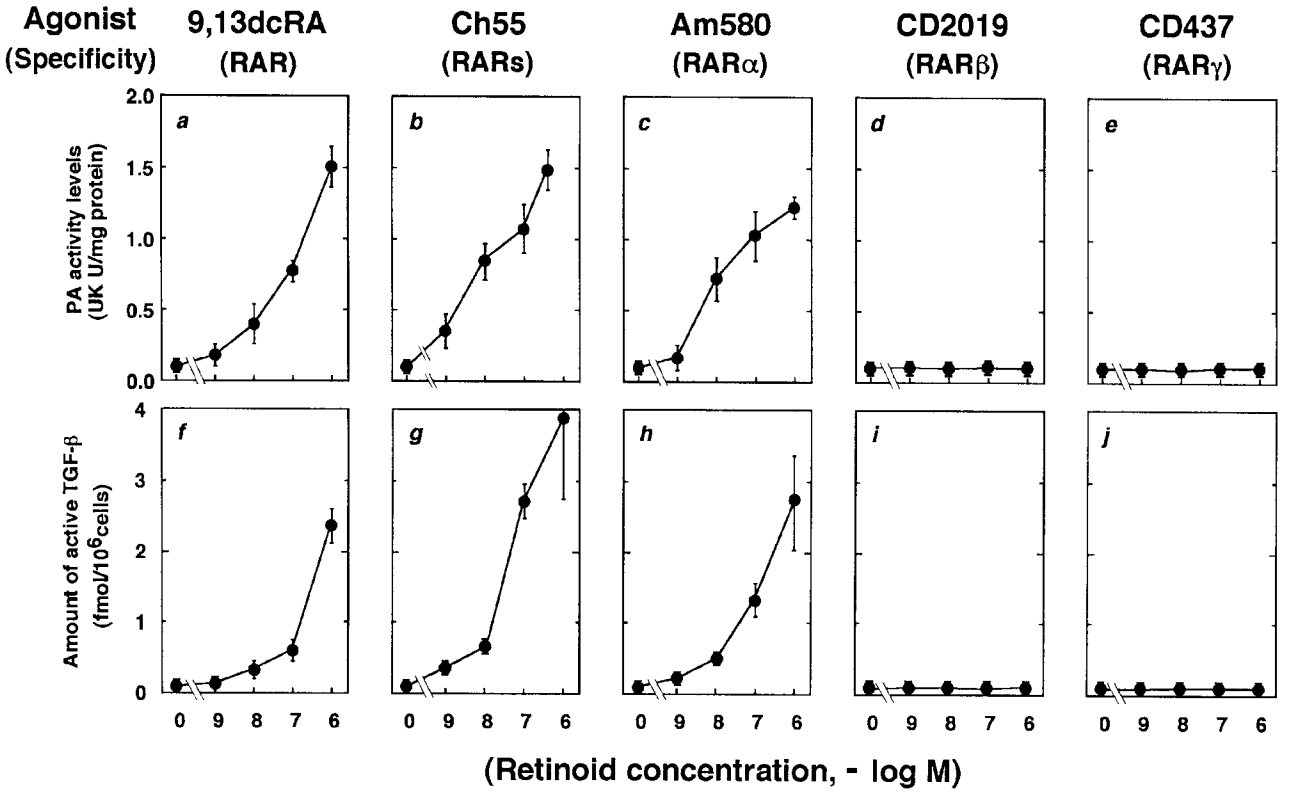

Fig. 4. Enhancement of cellular PA levels and induction of TGF-β formation by RARα-selective retinoid in LI90 cell cultures. After confluent LI90 cell cultures were incubated in DMEM-BSA for 12 h with various concentrations of 9,13dcRA or each subtype-specific retinoid, cellular PA activities (panels a–e) and active TGF-β levels in the medium (panels f–j) were measured as before.

taneously, suggesting the possible involvement of RARα in the up-regulation of tPA expression. The expression of RARβ was also enhanced by 9,13dcRA, but the expression of RARγ was minimally affected. TGF-β2 and -β3 expressions were also increased by 9,13dcRA treatment, supporting the potential linkage between the enhanced tPA production and the induction of TGF-β in LI90 cells.

Next, in order to test whether RARα played an important role in 9,13dcRA-induced tPA expression and subsequent TGF-β formation by LI90 cells, we examined the effects of RAR-subtype specific agonists as well as antagonists. Fig. 4 shows the result of agonists. LI90 cells were treated with various concentrations of 9,13dcRA or each RAR subtype-selective retinoid, and both cellular PA activity and active TGF-β levels in the medium were measured. Among retinoids tested, 9,13dcRA, pan-RAR-selective Ch55 and RARα-selective Am580 enhanced both PA (panels a–c, respectively) and TGF-β (panels f–h, respectively) levels. We cannot explain why, roughly estimated, about 10 times higher concentrations of these retinoids were required for induction of TGF-β compared with those required for enhancement of PA levels. In contrast, no induction was observed in both PA and TGF-β levels with RARβ-selective retinoid (CD2019; panels d and i) or RARγ-selective retinoid (CD437; panels e and j). These results suggest that activation of RARα may mediate 9,13dcRA-induced tPA production and thus TGF-β formation. This was confirmed by testing the effect of subtype specific antagonist (Fig. 5). Both 9,13dcRA- and Ch55-induced up-regulations were blocked significantly by the co-addition with RARα-selective antagonist, Ro41-5253, and weakly with

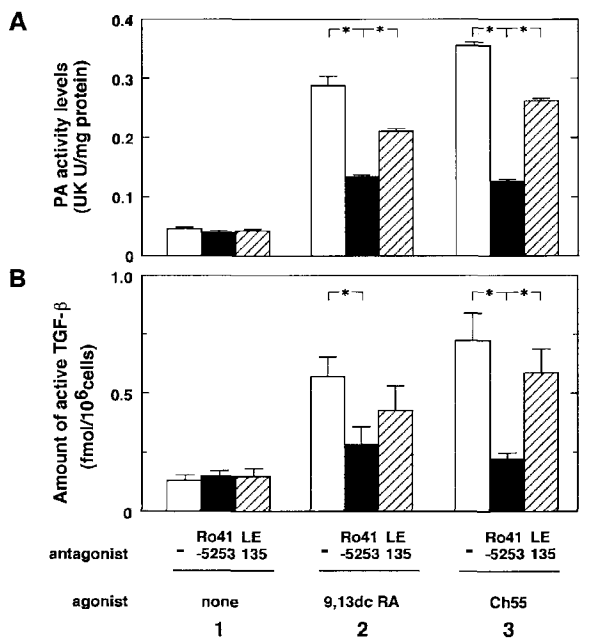

Fig. 5. Effect of subtype-selective retinoid antagonists on the induction by 9,13dcRA of cellular PA and active TGF-β in LI90 cells. Confluent LI90 cell cultures were incubated in DMEM-BSA for 12 h with vehicle (none; sample 1), 0.01 μM 9,13dcRA (sample 2) or the same concentration of Ch55 (sample 3), in the absence (open columns) and presence of 1 μM RARα-selective antagonist, Ro41-5253 (closed columns), or RARβ-selective antagonist, LE135 (slashed columns). Cellular PA activities (panel A) and active TGF-β levels in the medium (panel B) were measured as before. $^{*}P < 0.01$.

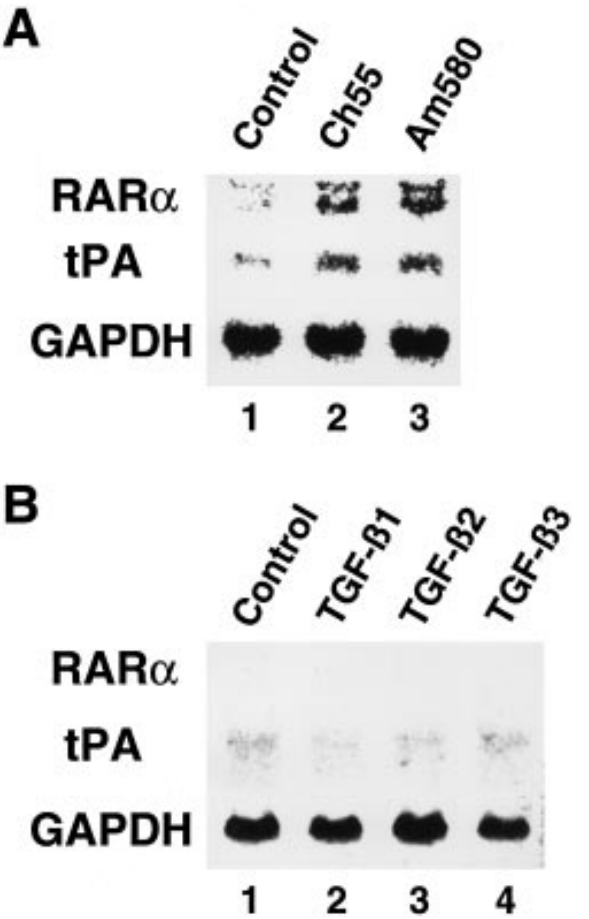

Fig. 6. Effects of RARα agonists and TGF-β on the mRNA levels of RARα and tPA in LI90 cells. After confluent LI90 cell cultures were incubated in DMEM-BSA for 12 h either with (A) vehicle (control; lane 1), 1 μM Ch55 (lane 2) or the same concentration of Am580 (lane 3), or (B) 1 ng/ml each of TGF-β subtypes (lanes 2–4), the levels of RARα and tPA mRNAs were determined by Northern blotting as before. Each similar experiment was repeated twice and the representative results are shown.

RARβ-selective antagonist, LE135. The PA and TGF-β levels were decreased by approximately 60∼70% with Ro41-5253 and by approximately 25∼30% with LE135. It is reported that all-*trans*-RA up-regulates tPA expression through the interaction of RAR with RARE locating at 7 kb up-stream in the tPA promoter [27]. Together with the present results, it is suggested that 9,13dcRA may enhance tPA production and thereby induce the activation of latent TGF-β in LI90 cells, at least in part (∼60%) through the induction and activation of RARα as the first step, and, therefore, both Ch55 and Am580 should also induce the expression of the *RARα* gene. This was confirmed by Northern blotting for RARα mRNA in cultures of either Ch55- or Am580-treated LI90 cells. As seen in Fig. 6, panel A, both agonists induced the expression of RARα mRNA and tPA mRNA. The induction by Am580 implied that untreated LI90 cells expressed as small amount of RARα as was not fully detectable by northern blotting. The result in Fig. 5 also suggests that 30∼40% of the induction by 9,13dcRA was independent upon RARα. It is reported that RARα is expressed in both quiescent and transformed SCs in vivo, whereas RARβ expression is down-regulated during the transformation in the cultures and in the fibrotic liver [28].

Taken together with the findings that both RARβ- and RARγ-selective retinoids failed to induce tPA and TGF-β in SCs and that RARβ-selective antagonist did not significantly block 9,13dcRA-induced up-regulation, it is suggested that RARβ and RARγ seemed to have relatively less effects on tPA induction and subsequent TGF-β formation. We need to examine the effect of a RARγ-selective antagonist, if we could obtain it. Finally, in order to see whether the effects of 9,13dcRA on RARα and tPA were partly mediated by increased TGF-β or not, RARα and tPA mRNA expressions were determined following the exposure of LI90 cell cultures to active TGF-β1, -β2, and -β3 for 12 h. The results are shown in Fig. 6, panel B. Any subtypes of TGF-βs did not induce these genes, suggesting that the induction was not mediated by resultant TGF-β.

The present study suggests that 9,13dcRA, a novel endogenous RA discovered in the fibrotic liver, may provoke in human SCs the enhancement of tPA and thus surface plasmin levels, resulting in the formation of TGF-β, and that RARα may play an important role in this phenomenon. We are now examining if TGF-β, generated upon stimulation with 9,13dcRA, promotes fibrogenesis, i.e. stimulation of collagen production. Our study might give a clue to a therapy against liver fibrosis, in which TGF-β plays important roles. If the production and activation of latent TGF-β are regulated by endogenous RA mainly through RARα, the inhibition of the transcriptional activation via RARα might be a strategy for the therapy of the disease. The use of a retinoid antagonist for the future therapy is now under investigation in our laboratory.

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