Effects of Retinol and Hepatocyte-Conditioned Medium on Cultured Rat Hepatic Stellate Cells

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Abstract. Hepatic stellate cells (HSC) become activated in liver injury, proliferating and secreting components of connective tissue. Activated HSC lose their native retinol and fat storing capacity. Signals from hepatocytes and/or Kupffer cells injured (eg, by iron overload) may contribute to the so-called activated HSC phenotype. Primary rat HSC cultures were treated with retinol to determine if this could produce a quiescent cell for controlled in vitro studies of activation. Retinol resulted in suppressed DNA synthesis in proliferating HSC, a reorganization of actin filaments, and a return of fat storage. However, it did not suppress the expression of fibrogenic genes such as those for collagens type I and IV, and TGF-β1. Furthermore, retinol-treated cells may increase expression of these genes in response to conditioned medium from hepatocyte cultures. The effect is especially apparent for collagen type I mRNA, and with conditioned medium from iron-loaded hepatocytes. Thus, retinol may be a two-edged sword in iron overload, potentially suppressing HSC proliferation on the one hand, and sensitizing a fibrogenic pattern of gene expression on the other. Factors influencing this balance merit further study. (received 24 March 2003; accepted 30 March 2003)

Keywords: hepatic stellate cell, hepatocyte, Kupffer cell, fibrosis, iron overload, α-actin, collagen

Introduction

Hepatic stellate cells (HSC) are present throughout the liver, in contact with hepatocytes and endothelial cells and located primarily in Disse’s space. In the past two decades HSC have become recognized as significant contributors to initiation, propagation, and resolution of hepatic fibrosis [1]. In their quiescent state in uninjured liver, they function primarily as a fat-storing cell, and are prominent in retinol storage and metabolism [2]. Involvement of HSC in fibrosis begins with the conversion or “activation” of quiescent, vitamin A-storing HSC into a proliferative, fat-depleted phenotype responsible for enhanced extracellular matrix turnover [3, 4]. Because these activated cells express α-smooth muscle (α-SM) actin as well as connective tissue proteins such as interstitial collagens, they are often referred to as showing a myofibroblast phenotype [3]. In this regard, they resemble transdifferentiated renal mesangial cells and pulmonary and cardiac interstitial fibroblasts that are thought to contribute to fibrosis of chronic disease in their respective organs [5-7].

Vertebrates are dependent on the food supply as a source of retinoids and precursor carotenes, and can store large amounts of absorbed retinol in the liver, mainly as retinyl esters. Eighty percent of hepatic retinoids are in the HSC [8]. The functions of hepatic retinol are attributed to the ability of its oxidation products, all-trans and 9-cis retinoic acids, to regulate gene expression via nuclear receptors [2]. Retinol can induce the fat-storing phenotype and hyperplasia in HSC [9]. Thus, one view is that growth inhibition by retinoids may be able to affect HSC distribution between quiescent and proliferative populations and thus affect fibrogenesis. Borojevic and coworkers [10,11] demonstrated that induction of the lipocytic phenotype by retinol in a
murine HSC line is accompanied by changes in the expression of genes responsible for retinol metabolism. These changes are also accompanied by rearrangement of the actin cytoskeleton and of vimentin and desmin intermediate filaments. An association of actin and vimentin filaments around fat droplets in adipocytes has been noted, suggesting a similar relationship between fat storage and intermediate filaments may obtain in HSC. Thus, retinol-dependent cytoskeletal reorganization may be one indicator of HSC phenotype.

Iron overload, originating as either a primary disorder (genetic hemochromatosis) or secondarily from multiple blood transfusions used to treat anemias such as thalassemia, is among the major causes of hepatic fibrosis. Iron accumulating in the liver at elevated plasma concentrations is fibrogenic, and increased HSC activation accompanies iron overload. Because HSC are interspersed throughout the liver in close proximity to hepatocytes, endothelial cells, and resident hepatic macrophages, activation of HSC need not be a direct effect of iron-induced injury, but may be modulated by extracellular cytokines derived from these neighbouring cells. In keeping with this idea, we found that medium conditioned by hepatocytes stimulated DNA synthesis in proliferating cultures of HSC, while heat treatment, a process that activated TGF-β, reversed this effect. Iron loading the hepatocytes produced conditioned medium that supported increased expression of collagen type I after heat treatment.

HSC proliferating in culture exemplify the activated phenotype. Because animal studies of iron overload do not completely recapitulate the extent of fibrotic liver disease in humans, a model to study activation of quiescent stellate cells in vitro would be desirable. The present study was undertaken to characterize further the response of HSC in primary culture to retinol as a potential source of quiescent cells for studying iron-mediated hepatotoxicity. We found that retinol suppresses DNA synthesis, causes rearrangement of F-actin, and restores fat storage in these cells, but does not suppress the expression of fibrogenic genes. In the presence of hepatocyte-conditioned medium it may actually enhance the latter.

**Methods**

**Hepatic stellate cell isolation.** Cells were isolated from male retired-breeder Sprague-Dawley rats according to the method of Friedman and Roll. Briefly, livers were perfused progressively with Ca²⁺, Mg²⁺-free Hank’s balanced salt solution (HBSS), Ham’s F12/DMEM (1:1; Life Technologies, Burlington, ON) containing 0.2% (w/v) pronase (Roche Molecular Biochemicals, Laval, PQ), and Ham’s F12/DMEM containing 0.05% (w/v) collagenase Type IV (Sigma, St. Louis, MO). The liver was then shaken for 30 min at 37°C in Ham’s F12/DMEM containing 0.02% pronase and 0.001% (w/v) bovine pancreatic DNAase I (Roche).

HSC, purified by centrifugation on an arabinoglycan density gradient (Larcoll; Sigma), were plated on 6-well tissue culture dishes at a density of 2 x 10⁵ cells/cm² and maintained in medium 199 containing Hank’s salts (Life Technologies), 10 mM HEPES (pH 7.3), 12 mM bicarbonate, 12 mM glucose, 4 mU/ml insulin (Eli Lilly, Toronto, ON), 1 µM corticosterone (Sigma), 10% (v/v) FBS, 10% (v/v) horse serum, and penicillin/streptomycin/amphotericin B (Life Technologies).

HSC homogeneity was confirmed by their morphology, the presence of Oil Red O-staining lipid vacuoles (Fig. 1a), and vitamin A autofluorescence (data not shown). Cells were passaged and adapted to growth in low glucose-DMEM containing 10% FBS, and penicillin/streptomycin/amphotericin B after “activation” (approximately 14 days in culture). All retinol studies were performed on cells between passages 2 and 6 and used dilutions from a 20 mM stock solution of retinol (Sigma) in 100% ethanol, which was stored in the dark (-20°C, under a N₂ atmosphere). Control cells were treated with equivalent volumes of ethanol.

**Histochemical studies.** To monitor the establishment of a lipid storing phenotype after treatment with retinol, HSC were routinely stained with Oil Red O. Briefly, cultures grown on round 12 mm glass coverslips in 24-well dishes were rinsed 3 times with PBS and fixed for 15 min with freshly prepared 2.5% glutaraldehyde. The fixative was removed and the cells were rinsed several times with distilled water.
and then with 60% (v/v) isopropanol. Three volumes of a saturated stock solution of Oil Red O in isopropanol were diluted with two volumes of distilled water and allowed to stand for 15 min prior to filtration (Whatman #1 filter paper). The filtered solution was added immediately and allowed to incubate for 15 min. Cells were then rinsed several times in 60% isopropanol and counterstained with Harris’ hematoxilin for three min. Hematoxilin was removed and warm tap water was added for 1 min and then excess dye was removed with distilled water to complete the staining process. Coverslips were inverted on glass slides in mounting medium (8% (w/v) polyvinyl chloride, 0.2% (w/v) diazabicyclo[2.2.2]octane (Sigma) in 0.2 M Tris-HCL buffer, pH 8.5).

Vimentin and α-SM actin were visualized using immunofluorescence as described previously [26]. HSC cultures grown on glass coverslips were fixed with 4% (w/v) paraformaldehyde, and permeabilized for 5 min with 0.5% (v/v) Triton X-100, 1 mM EGTA, 4% (w/v) polyethylene glycol (PEG 8000) in 0.1 M Pipes buffer, pH 6.9, and blocked with 0.2% (w/v) bovine serum albumin (BSA) in PBS. Non-specific IgG binding sites were blocked with 5% (v/v) non-immune sheep serum in BSA/PBS for 30 min and appropriate dilutions of monoclonal primary antibody to vimentin or α-SM actin (Sigma) were added and incubated for 60 min at room temperature. Three washes with BSA/PBS preceded the addition of FITC-labeled anti-sheep IgG secondary antibody (DAKO, Mississauga, ON). Total F-actin was stained using rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) as described previously [27].

**Hepatocyte isolation and culture.** Hepatocytes were isolated from male Sprague-Dawley rats (275-300 g) using a two-stage non-recirculating perfusion method described previously [22]. Livers were perfused for 5 min via the portal vein with Ca2+-, Mg2+-free Hank’s balanced salt solution (HBSS), containing 5.6 mM glucose, 10 mM HEPES (pH 7.3), 0.5 mM EGTA, and 0.67% (w/v) BSA (fraction V; Roche Molecular Biochemicals), followed by buffered William’s medium E (Life Technologies) with antibiotics and 0.25 mg/ml collagenase Type IV (435 U/mg; Sigma). The minced liver was passed through 2 layers of cheese-cloth and hepatocytes were resuspended in William’s medium E and filtered through nylon (62 mesh). After centrifugation for 4 min at 45 × g, hepatocytes were resuspended in growth medium and filtered through 2 layers of nylon. The final cell pellet was resuspended in a small volume of growth medium.

Hepatocytes were added to 100 mm dishes coated with collagen film (Vitrogen 100; Collagen Corp., Palo Alto, CA) at 2 × 10⁵ cells·cm⁻² density. After 3 hr, unattached cells were removed and fresh growth medium was applied. Iron loading was initiated in 2-day old cultures by the addition of ferric ammonium citrate (FAC) to the growth medium to a final iron concentration of 20 µg/ml, and continued for 3 days as described previously [28]. Conditioned medium (CM) was collected from hepatocytes using α-MEM supplemented with 0.2% FBS in order to minimize confounding effects from factors present in serum. Growth medium was removed, the monolayer was washed with PBS, and then incubated with α-MEM containing 0.2% (v/v) FBS for 6-8 h with several changes. Fresh α-MEM/0.2% FBS was added and conditioned for 24 h. Supernatant fractions designated CM were immediately frozen and stored at -80°C.

**Kupffer cell isolation and culture.** Kupffer cells were prepared during HSC isolation by a two-stage process described by Friedman and Roll [25]. Briefly, in the first step Kupffer cells were harvested from the 1.080/1.11 g/ml density interface of the same gradient used for HSC isolation. Cells were washed once in 1:1 Ham’s F-12/DMEM and plated at a density of 2 × 10⁶ cells/cm² on collagen coated plates in DMEM containing 10% FBS and penicillin/streptomycin/amphotericin B. After 20 min the non-adherent cells were discarded and adherent Kupffer cells were cultured in low-glucose DMEM, containing 10% FBS and antibiotics. In the second stage, 24 hr later, medium was removed and cells were washed once with PBS and subjected to a brief trypsin treatment (2 min at 37°C with 0.05% (w/v) trypsin/EDTA). Kupffer cells were characterized by their ability to take up latex beads while being virtually free of HSC. Control and iron-
loaded Kupffer cells were prepared by incubation in the absence and presence, respectively, of 20 μg/ml Fe for 3 days. CM was isolated from these cultures using 0.2% FBS in α-MEM as described above for hepatocytes. In several preparations, the phagocytic nature of the cultures was confirmed by staining with Latex beads, which were taken up by 95 to 97% of the cells (data not shown).

**DNA synthesis.** In studies involving conditioned medium, HSC plated in 24-well dishes were rendered quiescent by growing to 75% confluence and then switching to α-MEM containing 0.2% FBS for 48 hr. DNA synthesis was stimulated by addition of 5% (v/v) NuSerum IV (Becton Dickinson, Bedford, MA) to α-MEM/0.2% FBS (control unconditioned medium) or to CM prepared from rat hepatocyte cultures as described above. After 18 hr, 1 μCi of methyl-[3H]-thymidine (6.7 Ci/mmol; ICN, Irvine, CA) in α-MEM/0.2% FBS was added to each well for a further 6 hr. At 24 hr the medium was discarded, the cells were washed twice with ice-cold PBS, washed with ice-cold 5% TCA, and processed for scintillation counting. In other studies where the effects of retinol were examined (Figs. 3, 4), DNA labeling was for 24 hr.

**Northern blot analysis.** Quiescent HSC as above were treated for 24 hr with either: (a) unconditioned medium (α-MEM/0.2% FBS), (b) unconditioned medium containing 5% NuSerum (control), or (c) CM from control or iron-loaded hepatocytes supplemented with 5% NuSerum. Total RNA was extracted from cell monolayers using Trizol (InVitrogen, Burlington, ON) according to the manufacturer’s instructions and resolved by agarose-gel electrophoresis as previously described [29]. Probes for transforming growth factor-β1 (TGF-β1), collagen type I, collagen type IV, plasminogen activator inhibitor-1 (PAI-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs, and 18S rRNA were labeled with [32P]-dCTP by a random-primed DNA labeling kit (Roche) as before [29], and their hybridization was quantitated by densitometry, using the abundance of 18S rRNA to correct for variations in RNA loading.

**Statistical methods.** Results were expressed as mean ± SD and differences among groups of observations were determined by ANOVA followed by Tukey’s post hoc test. Paired data were analyzed by t test.

**Results**

**Histological effects of retinol.** It is well established that HSC in culture lose their fat stores and acquire a proliferating phenotype [1]. Abundant Oil Red O-positive fat droplets in 4-day old cultures of HSC (Fig. 1A) progressively decline and are absent after 2 passages (Fig. 1B). Exposure of these cells to 50 μM retinol for 10 days results in the reappearance of fat droplets (Fig. 1C), indicating that pathways for retinol uptake and storage remain intact after establishment of the activated phenotype in culture.

After 2 passages in culture, HSC possess characteristics associated with a smooth muscle phenotype, including a well-developed network of α-SM actin and vimentin filaments (Fig. 2). This is in keeping with the myofibroblast phenotype described for these cells in other studies [3,30]. The pattern of these cytoskeletal elements was unaffected by retinol treatment. In contrast, retinol treatment caused some redistribution of total filamentous (F-) actin as determined by phalloidin staining. In untreated cells, total F-actin was distributed throughout the cell in a pattern indistinguishable from α-SM actin (Fig. 2C). With retinol treatment, the distribution of F-actin assumed a prominent peripheral component (Fig. 2D). There was no evidence of an association of F-actin with the fat droplets distributed throughout the cytoplasm of retinol-treated cells.

**Effect of retinol on DNA synthesis.** Consistent with other cells of smooth muscle and/or myofibroblast phenotype, DNA synthesis was suppressed in serum-deprived HSC cultures held in 0.2% FBS for 48 hr, and reappeared after stimulation with 5% serum (data not shown), suggesting induction of, and subsequent release from, quiescence. In keeping with these observations, serum-deprived HSC established a basal level of DNA synthesis that was simulated over 4-fold by addition of 5% serum [(468 ± 154)% with serum vs (100 ± 64)% in serum-
starved control; Fig. 3]. However, when HSC were treated with 10 µM retinol for 10 days prior to serum deprivation, DNA synthesis was nearly completely eliminated [(9.0 ± 0.9)% of controls not exposed to retinol] and was not restored by a return to 5% serum (Fig. 3). These results indicate that retinol suppresses the proliferation of HSC even after the addition of serum. Indeed, retinol treatment alone, in the absence of serum deprivation, decreased DNA synthesis in HSC cultures. It did so in a time- and concentration-dependent manner (Fig. 4), with significant effects as early as 2 days at 20 µM (p <0.01) and at 1 µM at 10 days (p <0.05), in complete medium.

Effect of conditioned medium on DNA synthesis. In vivo, HSC are surrounded by other cell types (hepatocytes, Kupffer cells, etc.) whose secreted cytokines and growth factors may modulate DNA synthesis by paracrine mechanisms [21]. Previously we described a model in which the medium conditioned by 24 hr exposure to rat hepatocyte cultures in 0.2% serum (conditioned medium, CM) significantly augmented serum-stimulated DNA synthesis in HSC cultures that had been rendered quiescent by 48 hr treatment with 0.2% serum [22]. In the present study, we examined the effects of CM from both hepatocytes and Kupffer cells on retinol-treated HSC. In addition, CM was prepared from cultures of hepatocytes and Kupffer cells that had been treated with iron-supplemented media for 3 days prior to collection of CM. CM from neither control nor iron-loaded hepatocytes significantly affected DNA synthesis by HSC (Table 1). HSC treated for 10 days with retinol exhibited a decline in DNA synthesis to 33 ± 6% of control, in keeping with the retinol-induced suppression of DNA synthesis described above. These reduced levels were also unaffected by CM from either control or iron-treated hepatocytes. DNA synthesis suppressed by retinol was also unaffected by CM from control and iron-treated Kupffer cells (Table 1). These studies suggest that retinol suppresses proliferation (ie, DNA synthesis) of HSC by mechanisms that resist growth factors in serum or in media conditioned by other hepatic cell types.

Fig. 1. Histochemical staining of rat hepatic stellate cells (HSC) with Oil Red O. Fat droplets stain red. A) 4-day old primary culture of rat HSC; B) “activated” rat HSC after 2 passages; C) HSC incubated with 50 µM retinol for 10 days. (magnification x 400)
Fig. 2. Immunohistochemical staining of rat HSC. Third-passage untreated HSC (panels A, C, and E) and HSC treated with 50 µM retinol for 10 days (panels B, D, and F) were fixed and stained for α-smooth muscle actin (panels A and B), F-actin (panels C and D), or vimentin (panels E and F).
Table 1. Effect of conditioned medium (CM) from control and iron-loaded rat hepatocytes and Kupffer cells on DNA synthesis in rat HSC.

<table>
<thead>
<tr>
<th>Source of CM*</th>
<th>HSC retinol treatment †</th>
<th>Additions</th>
<th>DNA synthesis‡ (% control)</th>
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<tbody>
<tr>
<td>Hepatocytes</td>
<td>-</td>
<td>None</td>
<td>100 ± 16</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>None</td>
<td>33 ± 6.3</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Control CM</td>
<td>119 ± 17</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Control CM</td>
<td>37 ± 16</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Fe-loaded CM</td>
<td>68 ± 63</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Fe-loaded CM</td>
<td>29 ± 9</td>
</tr>
<tr>
<td>Kupffer cells</td>
<td>-</td>
<td>None</td>
<td>100 ± 3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>None</td>
<td>5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Control CM</td>
<td>89 ± 11</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Control CM</td>
<td>11 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Fe-loaded CM</td>
<td>77 ± 30</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Fe-loaded CM</td>
<td>5 ± 0.7</td>
</tr>
</tbody>
</table>

*Data are the mean ± SD of triplicate determinations of [3H]-thymidine incorporation into HSC DNA expressed as percent of untreated control.
† Rat hepatocytes or Kupffer cells were maintained for 3 days in the absence (Control) or presence (Fe-loaded) of growth medium supplemented with 20 µg/ml iron as ferric ammonium citrate prior to collection of conditioned medium (CM) as described in Methods.
‡ Rat HSC were grown for 9 days in the presence or absence of 10 µM retinol. An additional 48 hr treatment with 0.2% serum (± 10 µM retinol) preceded addition of conditioned medium.

Table 2. Effect of retinol on gene expression of HSC.

<table>
<thead>
<tr>
<th>mRNA (%) control HSC*</th>
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<tbody>
<tr>
<td>TGF-β1</td>
</tr>
<tr>
<td>COL I</td>
</tr>
<tr>
<td>COL IV</td>
</tr>
<tr>
<td>PAI-1</td>
</tr>
<tr>
<td>GAPDH</td>
</tr>
<tr>
<td>α-SMA</td>
</tr>
<tr>
<td>177 ± 70</td>
</tr>
<tr>
<td>153 ± 53</td>
</tr>
<tr>
<td>150 ± 62</td>
</tr>
<tr>
<td>159 ± 75</td>
</tr>
<tr>
<td>152 ± 76</td>
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<tr>
<td>189 ± 81</td>
</tr>
</tbody>
</table>

*HSC were treated for 10 days with 10 µM retinol and their mRNA levels expressed as percent of mRNA in control HSC prior to treatment (day 0). Data are the mean ± SD of 3 to 6 separate Northern blots of total RNA probed for transforming growth factor β1 (TGF-β1), collagen type I (COL I), collagen type IV (COL IV), plasminogen activator inhibitor 1 (PAI-1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and α-SM actin (α-SMA). In each case mRNA levels were corrected for RNA loading using 18S ribosomal RNA.
Fibrogenic gene expression in HSC. To determine if the persistent inhibition of DNA synthesis by retinol was accompanied by changes in expression of genes associated with accumulation of fibrous tissue, we examined mRNA levels of genes associated with promotion, development, and maintenance of fibrosis. Transcripts for TGF-β1, collagen type I, collagen type IV, and PAI-1 were increased some 50-60% by treating HSC with 10 µM retinol for 10 days, but these increases failed to reach statistical significance and were paralleled by levels of mRNA for GAPDH and α-SM actin transcripts (Table 2), indicating that if increased macromolecular synthesis does accompany growth suppression by retinol, it has no specificity for fibrogenic components.

Addition of CM increased collagen type I mRNA in absence of retinol treatment. Consistent with our previous studies [22], retinol treatment itself increased the mRNA levels of collagen type I, and to a lesser extent of collagen type IV and PAI-1, in response to serum stimulation of serum-starved HSC (Fig. 5). This effect of retinol was enhanced in CM from iron-loaded cells. An increase in TGF-β1 transcript was observed only in retinol-treated cells exposed to medium conditioned by iron-loaded hepatocytes.

Discussion

Previously, an immortalized mouse liver myofibroblast cell line, GRX, was shown to acquire retinol from the medium and establish a quiescent fat-storing phenotype [10,11]. Our study shows that activated rat HSC displaying the myofibroblast phenotype in primary culture can also respond to retinol by shifting to a quiescent fat-storing phenotype. Retinol is converted into retinyl esters for storage in lipid droplets while the metabolically active form, retinoic acid, is synthesized intracellularly by oxidation of retinol [2]. Similar fat droplets were observed when retinol acetate was used in our studies (J.G. Parkes and D.M. Templeton, unpublished observations), suggesting that primary HSC cultures can metabolize and store either form. Even though the concentrations of retinol used in this study were greater than normally found in vivo (2 µM [11]), the extent of restored fat deposition was less than in freshly isolated quiescent HSC (Fig. 1A vs 1C), possibly due to the shorter exposure times used in vitro, and/or the composition of the culture medium.
Despite the suppression of DNA synthesis and appearance of fat droplets in these cells, equating this to the resting state of pre-activated HSC is not warranted. Retinol-treated HSC retain a well-developed network of α-SM actin, a characteristic of activated myofibroblasts. The absence of any change in expression of α-SM actin transcripts by retinol (Table 2) supports the notion that simply forming fat storage droplets does not revert activated myofibroblasts. That gene expression of TGF-β1, collagen types I and IV, and PAI-1 are also not decreased is in keeping with the view that retinoid-induced lipocytes are an alternate state of activated fibrogenic myofibroblasts [31]. Mermelstein et al [12] have observed that cytoskeletal reorganization preceeds conversion of GPX cells into either myofibroblasts or retinol-induced lipocytes, and have concluded that HSC are originally pluripotent, resting perivascular cells capable of activation to 2 alternative states by distinct molecular programs, depending on exogenous stimuli. Interestingly, although α-SM actin was apparently unaffected by retinol in the present study, total filamentous actin was reorganized to an appearance of more prominent peripheral stress fibers. This may reflect the expression of β-actin known to occur in these cells [13], and may indicate microfilament specialization.

The suppression of DNA synthesis by retinol treatment is noteworthy. Retinoids are considered anticarcinogenic due in part to their ability to inhibit cell proliferation in animal models of certain stages of hepatocarcinogenesis [32]. Hepatocytes and Kupffer cells may both play paracrine roles in HSC activation in vivo [21]. We previously found that hepatocyte-conditioned medium augmented the proliferative response of HSC to serum [22]. Therefore, the ability of retinol treatment to suppress DNA synthesis in response to conditioned medium from both Kupffer cell and hepatocyte cultures, with or without iron loading, suggests that retinol may have a role to play in damping HSC proliferation in liver injury during iron overload. As freshly isolated HSC have variable retinoid content [33], HSC populations may exist in vivo with variable proliferative response. However, it remains to be determined how antiproliferative properties of retinol are coordinated with the growth-regulatory mechanisms employed by cytokines and growth factors, and any model that relates retinol content and the ability of a fibrogenic agent to activate HSC will be complex, as this process involves numerous transcription factors [34].

HSC are the main producers in liver of collagen for extracellular matrix synthesis and of enzymes that remodel matrix, such as tissue inhibitor of metalloproteinase-1 and matrix-metalloproteinase-2 [35]. Chronic iron overload activates HSC in hereditary hemochromatosis in man [18] and increases collagen synthesis in rat models of iron overload [20], indicating a role for iron in HSC activation and/or gene expression. How this occurs remains controversial. Suggestions that reactive aldehydes produced by iron-catalyzed lipid peroxidation are key intermediates arise from observations that antioxidants abrogate the stimulation of collagen synthesis by iron [36,37]. On the other hand, iron enhances collagen synthesis in HSC without detectable changes in aldehyde formation [38], suggesting that reactive oxygen intermediates are not required. Co-culture and conditioned media studies emphasize a synergistic relation between HSC and other cell types such as hepatocytes [39-41] and Kupffer cells [42-44]. Iron may affect each cell type differently resulting in alterations in secreted growth factors and cytokines. In keeping with this suggestion, previous studies showed that media conditioned by iron-treated hepatocytes contained TGF-β and increased collagen type I mRNA in activated HSC cultures [22]. In the present work, retinol exposure itself increased collagen type I mRNA and appeared to contribute to a general increase in fibrogenic gene expression, particularly in the presence of medium conditioned by iron-loaded hepatocytes. This is consistent with increased secretion of latent TGF-β in cells exposed to conditioned medium from iron-loaded cells, noted previously [22]. Retinol treatment may facilitate the activation of this latent form, resulting in increased expression of the TGF-β-inducible genes, collagen type I, collagen type IV, PAI-1, and TGF-β-1 itself.

Thus, adequate retinol levels may protect the liver from HSC proliferation, but may also independently enhance a fibrogenic response, particularly in the presence of iron-loaded hepato-
cytes. Primary cultures of iron-loaded hepatocytes and retinol-treated HSC may provide an in vitro system for exploring this balance.

Acknowledgement

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References


