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Hepatology Research 33 (2005) 225–233
HEPATOLOGY RESEARCH
http://www.elsevier.com/locate/hepres

Rat hepatic stellate cells become retinoid unresponsive during activation

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Received 20 June 2005; received in revised form 17 August 2005; accepted 25 August 2005
Available online 25 October 2005

Abstract

Hepatic stellate cells (HSC) play an essential role in fibrogenesis. Many stimuli cause HSC to activate, lose their Vitamin A and produce collagen. It is unclear whether Vitamin A loss causes activation, potentiates it or is simply an event in the cascade of activation changes.

We determine if exogenous retinoids prevent the activation of freshly isolated rat HSC activated by plating on plastic. We also determine if retinoids: (1) reverse HSC activation; (2) maintain/restore HSC intracellular retinoid levels; (3) maintain expression of HSC nuclear receptors for retinoic acid (RAR) in HSC that are becoming activated or are chronically activated.

Markers of activation in freshly isolated HSC were decreased by either retinol or retinoic acid without increases in HSC retinoid concentration. mRNA levels for RAR-α, RAR-β and RAR-γ, the nuclear receptors for retinoic acid, decreased during activation of freshly isolated HSC even with retinoid supplementation. RAR-α, RAR-β and RAR-γ mRNA and RAR-β protein was undetectable in chronically activated HSC and remained absent after retinoic acid supplementation. Activation markers in chronically activated HSC were only slightly decreased after retinoid exposure.

We conclude that exposure of HSC to extracellular retinoids diminishes some markers of activation but does not prevent HSC activation.

Keywords: Retinol; Retinoid; Fibrogenesis; Nuclear receptors

1. Introduction

Hepatic stellate cells (HSC) play an essential role in the fibrogenesis seen following a variety of hepatic insults. Current dogma is that HSC (formerly known as Ito cells, Vitamin A storing cells or lipocytes) are responsible for the majority of production of the extracellular matrix seen in hepatic fibrosis. In their native state in healthy liver, HSC are in a dormant or quiescent state, characterized by storage of large amounts of Vitamin A, little or no proliferation and a round cell morphology. Under a multitude of stimuli, HSC become activated and lose their Vitamin A, change morphology to a fibroblastic shape and begin to produce collagen (mainly types I and IV). One the most intriguing stimuli that cause this activation is the exposure of HSC to particular components of the extracellular matrix. For instance if HSC are isolated from liver in a quiescent state and plated on glass or plastic, they rapidly (over 2–5 days) lose their Vitamin A and subsequently become activated [1–3].

The loss of Vitamin A during activation of HSC has been noted for many years [4–7]. However, it is unclear whether Vitamin A loss causes activation, potentiates it or is simply an event in the cascade of changes that occur during activation. Vitamin A signaling controls a variety of biochemical and nuclear processes [5,8–11]. Previous work has demonstrated two families of nuclear receptors for retinoic acid (the most physiologically active derivative of Vitamin A): retinoic acid receptors (RAR's) and retinoid X receptors (RXR's). Ohata et al. [8] demonstrated that HSC from cholestatic rats had suppressed expression of both RAR-β and RXR-α. It has

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doi:10.1016/j.hepres.2005.08.007

also been shown that different forms of retinoic acid affect HSC differentially. All *trans*-retinoic acid inhibited procollagens types I, III and IV while 9-*cis* retinoic acid increased procollagen mRNA levels. These authors also showed that effects of different forms of retinoic acid were time dependent with inhibition of HSC proliferation occurring maximally after 120 h of exposure to 9-*cis* retinoic acid. Finally all *trans*-retinoic acid lowered mRNA levels for a multitude of ECM proteins. Matrix metalloproteinase 1 (MMP-1) is down-regulated by retinoic acid although this effect is dependent on which cell type is being investigated [12]. It is possible that even a modest lack of ligand (e.g., retinoic acid) could result in a significant down-regulation of HSC RAR's and interfere with retinoid signal transduction, leading to cell proliferation and transformation of HSC to an activated state.

In this paper, we determine if exogenous retinoids (either in the form of retinol or retinoic acid) would prevent or minimize the activation of freshly isolated rat HSC. We also determine if media supplementation with retinoids would (1) return chronically activated HSC to the quiescent state; (2) maintain/restore HSC intracellular retinoid levels; (3) maintain expression of HSC RAR's in rat HSC that were becoming activated or were chronically activated.

Our results show that HSC exposed to exogenous retinoids (supplied as 1 and 100 μM retinol or 1 μM retinoic acid) did not demonstrate toxicity based on desmin levels, cell morphology, trypan blue exclusion or fluorescent dye exclusion. Markers of activation in freshly isolated HSC were significantly decreased in HSC exposed to either retinol or retinoic acid for 7 days but had a similar decrease in the intracellular retinoid concentration compared to unsupplemented HSC. Nuclear expression of the retinoic acid receptors decreased dramatically during activation of freshly isolated HSC even with supplementation of retinoids. RAR-α, RAR-β and RAR-γ mRNA and RAR-β protein was undetectable in chronically activated cells and remained absent even after supplementation with retinoic acid. Markers of activation (α-SMA expression and cell proliferation) in chronically activated HSC were slightly but not significantly decreased after exposure to retinoids for 7 days but did not decrease to levels seen in freshly isolated quiescent HSC.

2. Materials and methods

2.1. Sources of materials

Retinol, retinal, retinoic acid, retinol acetate and retinol palmitate were obtained from Sigma (St. Louis, MO). Ham's DMEM was purchased from Gibco (Grand Island, NY). HPLC solvents (acetonitrile, methanol and dichloromethane) were purchased from Sigma. ^{3}H-thymidine (specific activity 6.7 Ci/mmole) was from New England Nuclear (Boston, MA). Mouse monoclonal antibody against the nuclear retinoic acid receptor beta (RAR-β) was from Affinity Bioreagents, Inc. (product number MA1-811). Secondary IgG anti-mouse antibody conjugated with biotin was purchased from Sigma. A monoclonal hamster antibody to rat α-SMA was purchased from Pharmingen (San Diego, CA). Desmin levels were detected using monoclonal antibody to rat desmin (Sigma) [3]. Secondary anti-hamster antibodies were from Sigma. Primers used to measure RAR-α, RAR-β, RAR-γ, 18S and GAPDH mRNA by real time reverse transcriptase polymerase chain reaction (RT-PCR) were purchased from Invitrogen (Carlsbad, CA). All other reagents were of the highest grade commercially available.

2.2. Hepatic stellate cell isolation

Hepatic stellate cells (HSC) were isolated from male Sprague–Dawley rats by a density gradient centrifugation technique [13,3]. All HSC were maintained in Ham's DMEM supplemented with 10% fetal calf serum containing penicillin (100 U/ml) and streptomycin (100 μg/ml).

2.3. Experimental design

Freshly isolated HSC were harvested and plated on 6 well plastic culture dishes, 2×10^6 HSC/well, in Ham's DMEM supplemented with 10% fetal calf serum with or without retinoids (retinol 1 or 100 μM; retinoic acid 1 μM). Retinoids were solubilized in 100% ethanol before being added to HSC media. Chronically activated HSC were passed (10–20 passages) on uncoated plastic dishes in Ham's DMEM with 10% fetal calf serum and remained in an activated state [13]. These passaged HSC were phenotypically similar to chronically activated HSC that had not been passaged as has been shown by other investigators [13,1,14,15]. On day 0, these chronically activated HSC were harvested and replated on 6 well plastic culture dishes, 2×10^6 HSC/well, in Ham's DMEM with the retinoid doses as above.

Morphology was assessed by phase contrast microscopy and viability by trypan blue exclusion and fluorescent dye exclusion (Live/Dead^{TM} assay, Molecular Probes, Eugene, OR). HSC samples for α-SMA, desmin and total protein were harvested with lysis buffer [16]. Cell samples for retinoid content were removed with trypsin into fresh Ham's DMEM. HSC were harvested at day 0, 3, 5, 7 and, in some cases, day 10. Control HSC, both freshly isolated and passaged, were plated under identical conditions except they were not exposed to retinoids but were exposed to ETOH vehicle.

2.4. Measurement of intracellular retinoid content

Retinoic acid, retinol and retinol palmitate levels were determined by reverse phase HPLC using a C18 (5 μm) column. As suggested by Ramm [1] and Blomhoff [17] the mobile phase was acetonitrile:methanol:dichloromethane (70:15:15, v/v/v) at a flow rate of 1.8 ml/min. UV absorbance was detected at 325 nm using a Waters HPLC model 501 (Waters, Milford, MA). After HSC were harvested, the cells were denatured with an equal volume of absolute ethanol,