Effect of lipid on proliferation and activation of rat hepatic stellate cells (I)

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Abstract

AIM To study the effect of lipid (triglyceride and very low-density lipoprotein, VLDL) on proliferation and activation of rat hepatic stellate cells (HSC).

METHODS HSC were isolated and cultured from liver of Wistar rats by in situperfusion with pronase and collagenase and density gradient centrifugation with Nycodenz. HSC proliferation was examined with MTT colorimetric assay.

RESULT Triglyceride of 12.5 mg/L had a promoting effect on proliferation of HSC ($P<0.05$), 25, 50, 100 and 200 mg/L had no effects ($P>0.05$), but 400 mg/L had an inhibiting effect ($P<0.01$). VLDL of 6.25 and 12.5 mg/L had no effect on proliferation of HSC ($P>0.05$), but increased concentration of VLDL could promote the HSC proliferation ($P<0.05$).

CONCLUSION Lipid had an effect on proliferation of HSC. Triglyceride and VLDL may promote HSC proliferation and may be associated with fatty liver and hepatic fibrogenesis.

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Received 1998-09-07

INTRODUCTION

Despite the early controversy over the primary cellular source of extracellular matrix proteins in liver fibrosis, compelling *in vitro* and *in vivo* experimental evidence is now available to implicate activated hepatic stellate cells (HSC) in the pathogenetic role[1-3]. Mechanisms by which collagen-producing cells are activated under pathological conditions remain unknown and continue to be a topic of research interest[1,2,4,5]. Although the role of hepatocytes in lipid metabolism and transportation have been discussed intensively, that of HSC was not known More and more studies showed that HSC took part in lipid metabolism and transportation[6], their abnormality was related to the pathogenesis of fatty liver and liver fibrosis[7]. In order to seek a possible explanation for the role of lipid in activation of HSC in liver fibrogenesis, we observed the effects of triglyceride and very low-density lipoprotein (VLDL) on proliferation of rat HSC.

MATERIALS AND METHODS

Animals

Male Wistar rats, weighing 400g-450g were fed ad libitum with standard rodent chow.

Preparaton of HSC

HSCs were prepared by the methods of Friedman *et al*[8] and Baroni *et al*[9] with slight modifications. The rats were anesthetized with intraperitoneal pentobarbital (30 mg/kg). The liver was perfused in situ through the portal vein with 500ml of calcium-free Gey's balanced salt solution for 10min at a flow rate of 40ml/min-50ml/min and then enzymatically digested with perfusate containing 0.05% collgenase (Sigma) and 0.1% pronase E (Merck) for 10min-15min. After being removed, the liver was cut into small pieces and incubated in 50ml fresh GBSS-BSA containing 0.05% collagenase and 0.1% pronase E and stirred at 37℃ for 30min. After passing through gauze, cell suspension was centrifuged at 500×g for 7 min, the supernatant discarded at the cells washed twice further with Dulbeco's modified Eagle's medium (DMEM) (Gibco). The cell suspension was mixed with 18% (W/V) of Nycodenz (Sigma) in GBSS without NaCl. The gradient was centrifuged at 1450

×g for 17min at 4℃. The white, diffuse, flufly band in the lower region of the DMEM layer just above the Nycodenz cushion, which contained highly enriched HSC, was gently aspirated, diluted in about 30ml DMEM and centrifuged at 450×g for 7min at 4℃. The cell pellet was suspended in the incubation medium and seeded in the culture flasks with DMEM containing 20% fetal calf serum. Sterile condition was maintained during the entire isolation and purification procedures. Cell viability was assessed by trypan blue exclusion, and cell counting were conducted in a hemocytometer.

Culture and determintion of HSC[8,9]
Purified HSC suspended in DMEM containing HEPES (15mmol), penicillin (100U/ml), streptomycin (100 mg/ml) and fetal calf serum (20% V/V) were seeded at a density of 1×10^5 cells/cm^2 in the culture flasks. The medium was changed 20 to 24 hours after plating, and every 3 to 4 days thereafter. Cells were grown at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air. To evaluate the purity of the cultures, HSC at day 2 or 3 and 7 after plating were tested by immunohistochemistry staining for desmin, lysozyme and factor VIII-related antigen, and by ultraviolet excited fluorescence microscopy at the length of 328 nm.

Proliferation of HSC
HSC proliferation was studied by colorimetric MTT [3 - (4, 5-dimethylthiazol - 2 - yl) - 2, 5-diphenyl tetrazolium bromide] (Sigma) assay[10]. HSC (1×10^5 cells/100 μl) were cultured in multiwell tissue culture plates (96 well/plate) (Corning, New York, NY, USA) for 2 days and 48hrs in the presence or absence of triglyceride and VLDL. Twenty μl of MTT solution (5mg/L) was added to all wells of an assay, and plates were incubated at 37℃ for 4hrs. Then 100μl dimethyl sulfoxide was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After 20min-30min at room temperature to ensure that all crystals were dissolved, the plates were read on a E-Liza Mat-300 reader, using a test wavelength of 570nm, a reference wavelength of 630nm. Plates were normally read within 1hr of adding the dimethyl sulfoxide. All samples were analyzed in pentaplicate.

Statistical analysis
All data were expressed as mean ±SD. Statistical differences were assessed by the standard t test and P values of < 0.05 were judged to be statistically significant.
RESULTS
The yeild, viability and purity of HSC

The yield of HSC ranged from 5×10^6 to 1×10^7 cells per liver. HSC displayed fading green-blue fluorescence with fluorescence microscopy at a wave length of 328nm. After the first washing at 24hr, the HSC purity in culture exceeded by 95% as assessed by phase contrast microscopy and ultraviolet excited fluorescence microscopy. Viability of HSC assessed by trypan blue exclusion exceeded by 95%. HSC positive for desmin by immunohistochemistry in primary culture was above 90%, and in subculture above 95%. Lysozyme and factor VIII-related antigen were negative for HSC.

Effect of triglyceride on proliferation of rat HSC
Trigylceride was directly administered to HSC at concentrations of 12.5, 25, 50, 100, 200 and 400mg/L, respectively. The HSC proliferation was measured with colorimetric MTT assay. HSC proliferation in the presence of triglyceride is shown in Table 1. Compared with the contrast (0.1395± 0.0276), 12.5 mg/L of triglyceride had a promoting effect on proliferation of HSC ($P<$ 0.05), 25, 50, 100 and 200mg/L had no effects ($P >$0.05), but 400mg/L had an inhibiting effect ($P <$0.01).

Table 1   The effects of triglyceride on HSC proliferation

Group	Concentration (mg/L)	Value of OD
Triglyceride	400	0.0990±0.0163[b]
	200	0.1226±0.0138
	100	0.1212±0.0275
	50	0.1450±0.0264
	25	0.1637±0.0243
	12.5	0.1894±0.0316[a]
Normal control		0.1395±0.0276

[a]$P<0.05$, [b]$P<0.01$ *vs* normal control.

Effect of VLDL on proliferation of rat HSC
VLDL was directly administered to HSC at concentrations of 6.25, 12.5, 25, 50 and 100 mg/L of VLDL, respectively. The HSC proliferation was measured with colorimetric MTT assay. HSC proliferation is shown in Table 2. Compared with the contrast (0.1395 ± 0.0276), 6.25 and 12.5mg/L of VLDL had no effect on proliferation of HSC ($P > 0.05$), but 50 and 100mg/L of VLDL could promote HSC proliferation ($P < 0.05$ or $P < 0.01$).
Table 2 The effects of VLDL on HSC proliferation

Group	Concentration (mg/L)	Value of OD
VLDL	100	0.2202±0.0284[b]
	50	0.1964±0.0287[b]
	25	0.1834±0.0498[a]
	12.5	0.1642±0.0269
	6.25	0.1583±0.0314
Normal control		0.1395±0.0276

[a]$P<0.05$,[b]$P<0.01$ *vs* normal control.

DISCUSSION

There is now overwhelming evidence that HSC, which reside in the space of Disse, are the principal effectors of hepatic fibrogenesis. However, in contrast to most other conditions, it is far from clear whether the characteristic phenotypic transformation, proliferation and fibrogenesis by HSC is always a response to liver cell injury/ or inflammation.

HSCs were demonstrated to present lipoprotein receptor in their surface, contained a large amount of fat droplets in their cytoplasm, and synthesized and secreted apolipoproteins. HSCs were in direct contact with plasma in the Disse's space, accessable to both chylomicron and VLDL triglyceride, and do not need to transport the enzyme to the adjacent endothelium. All these suggested that HSC and hepatocytes were necessary for lipid mobilization and transportation[1,2]. Their abnormality was related to the disturbance of lipid mobilization and transportation which made triglyceride concentrate in the liver, inducing fatty liver and liver fibrosis[12]. Reeves *et al*'s study[7] showed that the mechanism of HSC activation/ proliferation and subsequent fibrogenesis related to alcohol may be unrelated to necroinflammation, and that HSC activation occured in the absence of hepatitis in alcoholic liver disease and correlated with the severity of steatosis. However, isolated fatty liver without hepatitis occurs more frequently than does steatohepatitis. Thus it is not clear whether the accumulation of fat in the liver is responsible for inflammation or whether inflammation evoked by some stimulus caused cell dysfunction that resulted in steatosis[12]. Vicente *et al*[11] studied the lipid metabolism during in vitro induction of the lipocyte phenotype in HSC. HSC can produce and bind lipoprotein lipase to their own surface, using it potentially for processing exogenous lipids in the acute phase of lipocyte induction, when the accumulation of lipids was accelerated. In the our study, we found that triglyceride and VLDL promoted HSC proliferation at certain concentrations. Some animal studies also confirmed that steatosis and collagen content in experimental liver cirrhosis are affected by dietary monounsaturared and polyunsaturated fatty acids[12,13]. The great amount of lipid in diet produced fatty liver and liver fibrosis more easily. These experimental evidence indicated that the infiltration of lipid increased the production of fibrous tissue in the liver. The earliest event in the development of fibrosis appears to be activation of HSC by such factors as lipid peroxides[14,15]. This caused proliferation of HSC and initiation of fibrogenic cascade in the liver. In addition, the investigation showed that high concentration of triglyceride (400mg/L) had an inhibiting effect on HSC proliferation. It was deduced that high concentration of triglyceride might have a toxic effect on HSC. The precise role of triglyceride and VLDL in the HSC proliferation and activation needs to be addressed in futher studies.

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