Curcumin-induced recovery from hepatic injury involves induction of apoptosis of activated hepatic stellate cells

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Hepatic stellate cells (HSCs) undergo activation and transdifferentiation to myofibroblast like cells in liver injury, leading to liver fibrosis. During recovery from injury, activated HSCs may either revert back to quiescent state or undergo apoptosis or both. In the present study, we have examined whether recovery from hepatic injury involves apoptosis of activated HSCs and tested whether curcumin (the yellow pigment from Curcuma longa Linn.) promotes recovery from hepatic injury by inducing apoptosis of these cells. Hepatic injury was induced by CCl₄ and apoptosis was studied in HSCs isolated from liver by MTT assay, DNA fragmentation, and DAPI and annexin staining. Hepatic recovery was assessed by measuring hepatic marker activities, such as serum GOT, GPT and protein. Hepatic recovery occurred within 4 weeks after inducing injury in untreated control, whereas curcumin treatment caused hepatic recovery within 2 weeks, as evidenced by the reduction of hepatic marker activities to near normal levels. HSCs isolated from liver of animals treated with curcumin showed maximum apoptotic marker activities in 2nd week, whereas in HSCs from untreated control recovering from injury, maximum apoptosis was observed in 4th week. Induction of apoptosis in vivo during hepatic recovery was also suggested by increase in caspase-3 activity. Treatment of isolated HSCs in culture with curcumin caused apoptosis during later stages confirming that curcumin induced apoptosis of activated HSCs and not in unactivated quiescent HSCs. These results suggested that hepatoprotective effect of curcumin causing recovery from injury involved apoptosis of activated HSCs.

Keywords: Hepatic stellate cells, Apoptosis, Curcumin, Hepatic fibrosis, Retinol

Hepatic fibrosis occurs as a wound-healing process after different forms of chronic hepatic injury, including viral infection, drug-induced hepatitis and sustained alcohol abuse. During the process of fibrosis, production of extracellular matrix (ECM) exceeds its degradation in the liver. Without effective treatment at an early stage, reversible hepatic fibrosis progresses to irreversible cirrhosis. During hepatic injury, hepatic stellate cells (HSCs), which normally reside in the space of Disse in a quiescent, non-proliferative state become active and undergo profound phenotypic changes, including enhanced cell proliferation, de novo expression of α-smooth muscle actin (α-SMA) and excessive production of ECM, including fibril forming collagens, fibronectin and proteoglycans, resulting in the formation of septa in chronically damaged liver.

Although liver fibrosis and cirrhosis have been earlier considered irreversible, report indicates that hepatic fibrosis can be reversed. During recovery from experimental liver injury, the number of activated stellate cells decreases and tissue integrity is restored. The activated stellate cells may either revert back to quiescent stage in an IL-10 dependent autocrine negative feedback signal or may undergo apoptosis. Inhibition of HSC proliferation and induction of HSC apoptosis have been proposed as strategies for the elimination of activated HSC for the prevention and treatment of hepatic fibrosis. However, evidence in support of this is not adequate.

The oxidative stress plays a crucial role in the activation of HSC during liver injury. Based on this, a number of naturally occurring antioxidant substances have been tested for their hepatoprotective action. Curcumin, a yellow pigment present in the turmeric (the rhizome of the plant Curcuma longa Linn.), is a commonly used spice and has been shown to be a potent antioxidant and exhibits a number of...
pharmacological activities. It acts as an anti-inflammatory agent\textsuperscript{13}, inhibit pulmonary fibrosis\textsuperscript{14,15} and is an effective topical microbicide\textsuperscript{16}. Studies have shown that its chemopreventive action might be due to its ability to induce apoptosis\textsuperscript{17-19}. Although curcumin has shown hepatoprotective effect in experimental animals, its mechanism of action is not yet clear. Curcumin induces apoptosis of HSC, activated after multiple passages in culture in vitro in a peroxisomal proliferation activated receptor (PPAR) dependent process\textsuperscript{20}. But, it is not known whether its hepatoprotective effect involves apoptosis of activated stellate cells in vivo in injured liver tissue. In the present study, we have investigated whether curcumin-induced recovery from hepatic injury in rats involves apoptosis of activated HSCs in vivo.

Materials and Methods

Materials

Eagles minimal essential medium (MEM), penicillin, streptomycin, percoll, nycodenz, collagenase, curcumin, trypan blue, retinol, 3-(4, 5 dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT), 4’,6-diamidine-2’-phenylindole dihydrochloride (DAPI), annexin V staining kit and caspase-3 assay kit were purchased from Sigma Chemical Co., USA. Tissue culture plates were obtained from NUNC, Denmark. Radioactive isotopes, such as \textsuperscript{35}S methionine and \textsuperscript{35}S sulphate used for metabolic labeling were purchased from BARC, Mumbai. All the chemicals and reagents used were of high quality analytical grade.

Selection of animals and experimental protocol for in vivo study

Male albino rats (Sprague Dawley strain, 150-200 g body wt) bred and reared in the animal house of the Department of Biochemistry were used for the study. They were provided standard laboratory diet and water \textit{ad libitum}. The study was approved by the Institutional Animal Ethics Committee.

Hepatic injury was induced in animals by administering CCl\textsubscript{4} orally by intubation in groundnut oil (1% v/v). Control rats were given the same amount of groundnut oil. After 2 weeks of CCl\textsubscript{4} administration, hepatic injury was assessed by monitoring the changes in the activities of liver function enzymes, such as serum GOT and GPT, total protein and the ratio of albumin to globulin (A/G ratio). The animals were divided into two groups: Group I were given 0.5 ml of curcumin at a dosage of 5 mg/100 g body wt and the Group II served as control. The hepatic recovery in these groups was monitored for subsequent four weeks and compared with the normal untreated animals.

Isolation and culture of HSCs

HSCs were isolated from rat liver by collagenase perfusion\textsuperscript{21,22}. Briefly, the rat to be perfused was anesthetized with ether, slit opened the abdomen and a fine catheter was inserted through the incision made in the portal vein. After washing out the blood by preperfusion, the liver was perfused with collagenase solution for 15 min. All these operations were carried out at 37°C. Liver capsule was peeled off and the cells were gently dispersed into MEM. After washing with MEM, the parenchymal cells were separated by a nycodenz gradient centrifugation. From the non-parenchymal cell suspension thus obtained, HSCs were separated using a percoll gradient centrifugation\textsuperscript{22}.

The viability of cell preparation was checked by trypan blue exclusion and only preparations having more than 90% viability were used for in vitro experiments. HSCs were suspended in MEM containing penicillin (100 mg/L), streptomycin (100 mg/L) and NaHCO\textsubscript{3} (2.2 g/L). Cells were seeded on culture dishes previously coated with collagen I (50 µg/ml) and were incubated at 37°C in a Forma CO\textsubscript{2} incubator at 95% air and 5% CO\textsubscript{2} atmosphere and allowed to attach for 4-5 h. The medium and unattached cells were removed, fresh medium was added and maintained in culture for different time intervals. Protein was estimated by the method of Lowry et al\textsuperscript{23}.

Uptake of retinol

The cells were maintained in culture in retinol containing Eagles MEM. Retinol acetate dissolved in alcohol and reconstituted with 10% rat serum, was added to the MEM such that the final concentration of alcohol was less than 1%. At specific time intervals, the medium was collected, the cells were harvested and retinol was extracted from both the cell layer and medium. An aliquot of the sample was mixed with 500 µl ethanolic KOH (10%), kept at 37°C for 3 h. HPLC grade \textit{n}-hexane (400 µl) was then added, vortexed and centrifuged at 3000 rpm for 5 min. The hexane layer was pooled, concentrated and estimated for the amount of retinol\textsuperscript{24}.

Production of matrix proteins

Collagen production by HSCs was studied by metabolic labeling of the cells with \textsuperscript{35}S-methionine
and estimated as collagenase susceptible activity\textsuperscript{25}. Production of glycosaminoglycans was studied by metabolic labeling of cells with \textsuperscript{35}S sulphate\textsuperscript{25}.

**Cell proliferation assay — [\textsuperscript{3}H]-Thymidine uptake**

The isolated cells were maintained in culture supplemented with \textsuperscript{3}H-thymidine (5 µCi/ml) for different time intervals. The culture medium was removed, the cell layer was washed with PBS and the proteins were precipitated with 5% TCA. The \textsuperscript{3}H-radioactivity incorporated into DNA was measured using a liquid scintillation counter.

**MTT assay**

The effect of curcumin on growth of HSC was studied by MTT assay\textsuperscript{26}. For this, 100 µl of MTT (5 mg/ml in PBS) was added to cells and incubated at 37°C for 1 h. Medium was removed, the cell layer was washed with PBS and lysed in triplex solution (10% SDS, 5% isobutanol – HCl 12 mM/L). The suspension was centrifuged at 2500 rpm for 10 min after incubation for 2 h and the absorbance of extracted solution was read using a micro plate reader at a test wavelength of 570 nm. The inhibition of cell growth was calculated by the equation:

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\text{Growth inhibitory rate} = \left[ 1 - \frac{\text{Ab at 570 nm treated}}{\text{Ab at 570 nm control}} \right] \times 100\%
\]

**DNA fragmentation – Colorimetric assay**

Cells were washed with cold PBS and lysed with 10 mM Tris, pH 7.5 containing 1 mM EDTA and 0.2% Triton-X 100. Fragmented and intact DNA were separated by centrifugation at 18,800 × g at 4°C, and the pelleted DNA was resuspended in the same original volume of lysis buffer. 100 µl of each sample was mixed with 100 µl diphenylamine reagent and incubated at 37°C for 18 h. The mixture was then transferred to a multiwell plate and the absorbance at 600 nm was measured and the percentage of fragmented DNA was calculated\textsuperscript{27}.

**DAPI staining**

DAPI staining was performed according to the protocol of Yim et al\textsuperscript{28}. Cells were fixed by incubation in 4% paraformaldehyde for 30 min. Following washing with PBS, the cells were incubated in 1 µg/ml DAPI solution for 30 min in dark. The dye was removed, washed the cell layer with PBS and the cells were then observed under a fluorescent microscope (Leica DFC 280, Germany).

**Annexin V Cy 3 staining**

Apoptotic cells were detected using apoptosis detection kit Annexin V Cy3 (APO-AC, Sigma Chemical Co., USA) by the procedure of the manufacturer. 6-Carboxy fluorescein (6-CF) and annexin V Cy 3 (Ann. Cy3) were observed as green and red fluorescence respectively. Live cells were stained with 6-CF (green), whereas the cells starting the apoptotic process stained both Ann. V Cy 3 and 6-CF (red and green).

**Assay of caspase-3**

Caspase-3 was assayed using a kit purchased from Sigma Chemical Co., USA and following the protocol provided by the manufacturer. Briefly, the liver tissue extracts (10% w/v) prepared in the lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT) provided in the kit was incubated with the peptide conjugate-substrate DEVD-p-nitroanilide (pNA) at 37°C for 90 min. Intensity of the coloured product p-nitroaniline was read by an automated microplate reader at 405 nm.

**Statistical analysis**

Results were expressed as mean ± SE. The data were analyzed by one-way ANOVA, followed by Duncan analysis using SPSS. Differences were considered statistically significant at \( p < 0.05 \).

**Results**

**Curcumin promotes recovery from CCl4 induced liver injury**

To study the recovery from experimental hepatic injury, rats in which hepatic injury was induced by CCl\textsubscript{4} were treated with curcumin. Untreated rats recovering from injury served as control. Recovery was assessed by monitoring hepatic marker activities, such as serum GOT, GPT, A/G ratio at regular intervals after inducing injury and the results are shown in Fig. 1. There was a steady decrease in the serum levels of GOT, GPT and increase in A/G ratio on treatment with CCl\textsubscript{4} which attained near normal level in untreated controls within 4 weeks, indicating hepatic recovery. But, in curcumin treated animals near normal physiological levels of GOT, GPT and A/G ratio were attained in 2 weeks after treatment, unlike in untreated controls. The results indicated that curcumin promoted a faster recovery from hepatic injury.

**Changes in HSC specific activity: Retinol storage capacity**

One of the main biological features of HSC is its ability to store retinol. The retinol content in activated
transdifferentiated HSCs in injured liver was significantly less than in HSCs from normal liver. The changes in the HSC specific activity during recovery from CCl₄ induced injury were studied by determining the retinol content in HSCs, isolated at regular intervals from the livers of curcumin treated and untreated recovering animals and the results are presented in Fig. 2. The HSCs isolated from animals treated with CCl₄ showed a significant decrease in the total retinol content, compared to normal control, whereas those isolated from animals treated with curcumin after inducing liver injury had significantly higher retinol content in the 2nd week than the untreated normally recovering group.

Changes in apoptotic marker activities

To study whether hepatic recovery on curcumin treatment involved the apoptosis of activated HSCs in vivo, the apoptotic marker activities were studied at regular intervals in HSCs isolated from rats recovering from injury and the results are given in Fig. 3. The growth inhibitory effect was studied by MTT assay. The results in Fig. 3A showed that maximum growth inhibition was in the case of HSCs treated with curcumin in the 2nd and 3rd weeks, whereas in the case of normal recovery, significant growth inhibition was observed only after 4th week. The results of fragmentation of the DNA was studied by colorimetric estimation and are shown in Fig. 3B. In the case of curcumin treated animals, the percentage of fragmentation of DNA in HSCs was maximum in the 2nd and 3rd week of recovery and no fragmented DNA was found in the 4th week. But, in the case of untreated normally recovering animals, considerable fragmentation of intact DNA occurred only after the 4th week.

The in vivo apoptotic effect of curcumin was also studied by DAPI staining and the results are shown in Fig. 4. Considerable nuclear staining of apoptotic bodies was observed in HSCs from animals treated with curcumin in the 2nd and 3rd week, but in the case of HSCs from untreated normally recovering group, it occurred only after the 4th week. In the case of curcumin treated group, the nuclear fragmentation and membrane blebbing associated with apoptosis was not found from the 4th week onwards, indicating the clearance of apoptotic HSCs. The in vivo apoptotic effect of curcumin was further studied by determining the activity of caspase-3 in HSCs isolated from these groups of
Fig. 3—Changes in apoptotic specific marker activities [(A): Growth inhibitory rate by MTT assay [Hepatic injury was induced and the animals were allowed to recover as described in legend to Fig. 1. HSCs isolated from curcumin treated and untreated recovering animals at regular intervals were treated with MTT solution and the growth inhibitory rate was determined as described in the text. Values given are the average of six experiments ± SE. *p < 0.05, when compared with that of 1st week]; and (B): DNA fragmentation by colorimetric assay [Hepatic injury was induced and the animals were allowed to recover as described in legends to Fig 1. HSCs were isolated from curcumin treated and untreated recovering animals at regular intervals and DNAs were isolated and subjected to colorimetric estimation for the determination of fragmented DNA as described in the text. Values given are the average of six experiments ± SE. *p < 0.05, when compared with that of 1st week]

Fig. 4—DAPI staining [Hepatic injury was induced and the animals were allowed to recover as described in legend to Fig. 1. HSCs were isolated from curcumin treated and untreated recovering animals at regular intervals and subjected to DAPI staining as described in the text. In the normally recovered animals considerable changes in the nucleus associated with apoptosis found only after the 4th week (indicated by white arrows), but in the case of curcumin treated animals, such changes were more in 2nd and 3rd week (indicated by white arrows) and completely absent in the 4th week. Magnification 40X. Numbers indicate duration of recovery in weeks]

Fig. 5—Assay of caspase 3 [Hepatic injury was induced and the animals were allowed to recover as described in legend to Fig. 1. HSCs were isolated from curcumin treated and untreated recovering animals at regular intervals and cell extracts were prepared in the lysis buffer (250 mM HEPES, pH 7.4 containing 25 mM CHAPS and 25 mM DTT) and the caspase-3 activity was measured as described in the text and expressed as μ moles of p-nitroaniline formed/min/mg protein. Values given are average of six experiments ± SE. *p < 0.05, when compared with that of first week. In the case of normal recovery no significant activity was detected in the 1st, 2nd and 3rd weeks]
animals and the results are given in Fig. 5. The maximum caspase-3 activity was found in HSCs isolated from curcumin treated groups in the 2\textsuperscript{nd} and 3\textsuperscript{rd} week.

**Effect of curcumin in inducing apoptosis of HSCs in vitro**

The apoptotic marker activities were studied by MTT assay, DNA fragmentation DAPI staining and annexin V staining. To evaluate the effect of curcumin on cell growth, HSCs maintained in culture were treated with curcumin and the growth inhibitory effect was measured. The results are shown in Fig. 6A. During the initial period of culture, treatment with curcumin did not show any difference in the MTT dye level from that of the untreated control. But at later time intervals, during 72 h, when the HSCs underwent transdifferentation, exposure to curcumin caused a decrease in the level of MTT dye compared to untreated control, indicating a growth inhibitory effect. Effect of changes in concentration of curcumin was studied by using 5–50 µM concentrations. As about 60% inhibition of growth was produced by 20 µM of curcumin, in all further experiments, this concentration was used.

To find out whether the growth inhibition caused by curcumin was due to apoptosis, the intact and fragmented DNA were isolated from HSCs and assessed for the extent of fragmentation by calorimetric method. A significant increase in low molecular weight fraction of DNA was observed in cells treated with curcumin during the later stages of culture, compared to early time intervals (Fig. 6B), suggesting that significant fragmentation of DNA occurred in cells treated with curcumin at later stages, when HSCs underwent transdifferentiation.
The morphological changes associated with curcumin induced apoptosis of HSCs were examined by fluorescence microscopy following treatment with DAPI and the results are shown in Fig. 6C. The assay revealed the presence of significant nuclear membrane breakage and presence of perinuclear apoptotic bodies upon treatment with curcumin in the 72 h of culture confirming the induction of apoptosis in activated HSCs.

During apoptosis, the phosphatidyl serine (PS) is translocated from the cytoplasmic face of plasma membrane to the cell surface and annexin V has a strong affinity for PS and, therefore, it is used as a probe for detecting apoptosis. The HSCs treated with curcumin as well as control cells during the early and later stages of culture were treated with Ann. Cy 3 and 6-CF. The results are given in Fig. 6D. The percentage of annexin positive apoptotic cells was higher in cultures treated with curcumin during the later stages of culture than untreated controls. During the first day of culture, very few cells treated with curcumin stained for annexin.

Discussion

In normal liver, HSCs in the space of Disse are maintained in a quiescent, non-fibrogenic phenotype and are in contact with a complex ECM. Activation of HSCs is a key pathobiocchemical event in fibrogenesis. Full competency fibrogenesis is reached by stimulation of proliferation, phenotypic transition from retinoid storing to ECM secreting cell type, enhanced expression of almost all matrix proteins and acquisition of contractility. Analysis of markers of hepatic functions, such as serum levels of GOT, GPT and A/G ratio showed that on withdrawing injury causing agents, the liver underwent recovery. This was also evidenced by decrease in the matrix components and histological changes (data not shown). During recovery, the activated HSCs underwent apoptosis, as evidenced by apoptotic marker activities such as DNA fragmentation, annexin positivity and nuclear membrane changes.

Treatment with curcumin of animals in which hepatic injury was induced by CCl₄ caused a faster recovery from injury, as evidenced by decrease in the activities of GOT, GPT and increase in A/G ratio in serum within 2 weeks of treatment, whereas in untreated controls, the recovery took longer time. Normally, HSCs which remain quiescent are the major storage site of retinol in liver. Their pathogenic relevance in fibrosis relies on their ability to be activated to a synthetic phenotype containing much less vitamin A. The retinol content of HSCs was also found to be normal after 2 weeks in the case of curcumin treatment.

Results presented above indicated that curcumin induced apoptosis of activated HSCs, as evidenced by the expression of apoptosis marker activities both in vivo and in vitro. Alteration in function of HSCs on induction of hepatic injury was indicated by a decrease in total retinol content in HSCs. Administration of curcumin at a dose of 5 mg/100g body wt to the animals in CCl₄ induced liver injury was effective in inducing considerable apoptosis of activated HSCs. The apoptotic effect was revealed by MTT assay, DNA fragmentation and DAPI staining. Increase in the activity of caspase-3 in liver of curcumin-treated animals provided further evidence for induction of apoptosis. Earlier studies from our laboratory and from others indicated that curcumin at this dose had no toxic effects. Treatment of HSCs maintained in culture with curcumin caused apoptosis only in activated stages and not in early stages, suggesting that curcumin was more effective in inducing apoptosis of activated HSCs.

Different cellular mechanisms have been suggested to explain the effects of curcumin in various cell systems. In human renal Caki cells, curcumin causes a dose-dependent apoptotic effect, which is preceded by the sequential dephosphorylation of Akt, downregulation of the anti apoptotic Bcl-2, Bcl-Xₐ and IAP proteins, release of cytochrome c and activation of caspase-3. Curcumin is also reported to inhibit the activation of transcription factors NF-κB and AP-1 and the activity of c-jun-N-terminal kinase and protein tyrosine kinase. Modulation of the activity of these factors might be linked with the initiation of apoptotic signal.

It is also reported that curcumin requires the activation of PPARγ to induce apoptosis in passaged HSCs in rats. We also found that treatment of in vitro activated HSCs in culture with PPARγ antagonist causes reversal of the effect of curcumin in inducing apoptosis (data not shown), suggesting that curcumin induced apoptosis in activated HSCs involves PPARγ-dependant pathway. But, how the curcumin causes activation of PPARγ is not clear, particularly because the HSCs are maintained in a serum-free medium in the absence of any added ligands or activators, unlike passaged HSCs, which are maintained in serum supplemented medium.
Results of the present study indicate that curcumin-induced apoptotic signaling pathway involves the activation of caspase-3 in vivo. However, further studies are required to establish the intermediate activators that lead to activation of caspase-3 in activated HSCs. Further, it is not clear why curcumin causes induction of apoptosis in activated HSCs and not in unactivated HSCs or other hepatic cells like hepatocytes.

Although a direct relation between the induction of apoptosis of activated HSCs and recovery from injury could not be established, analysis of marker activities and histological data suggested that curcumin promoted hepatic recovery. Apart from restoring the marker activities, such as serum GOT, GPT and protein profile to normal level in 1-2 weeks after treatment with curcumin, the HSC isolated from curcumin-treated animals also stored near normal levels of retinol and showed significant ability to take up retinol, like normal HSCs, suggesting that curcumin treatment markedly improved hepatic function. This was consistent with the data reported from other laboratories on the hepatoprotective effect of curcumin. It has been suggested that the antioxidant property of curcumin is responsible for its hepatoprotective effect. It may protect free radical-mediated damage of parenchyma. Curcumin is also reported to inhibit collagen deposition in CCl₄-induced injury in rats and may serve as an antifibrotic agent. Our results indicated that curcumin by inducing apoptosis of the activated HSCs could promote the hepatic recovery.

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