Original Article
The role of C/EBP-α expression in human liver and liver fibrosis and its relationship with autophagy

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Abstract: Aim: To investigate the expression of CCAAT enhancer binding protein-α (C/EBP-α) in normal human liver and liver fibrosis and its probable association with autophagy. Methods: Double label immunohistochemistry was used to detect the location of C/EBP-α in hepatocytes and hepatic stellate cells (HSCs). The expression of C/EBP-α, Atg5, and Atg6 was also evaluated by immunohistochemistry in paraffin sections of human liver. HSC-T6 cells were treated with rapamycin and 3-methyladenine (3MA) to induce or inhibit autophagy, and the expression of C/EBP-α protein was detected by Western blotting. Results: Double label immunohistochemistry showed that C/EBP-α was predominantly located in hepatocytes and that its expression was significantly decreased in fibrosis compared with normal liver. Atg5 expression was increased in fibrosis but was located primarily in liver septa and peri-vascular areas, which was consistent with the distribution of HSCs. In contrast, Atg6 was not expressed in normal or fibrotic liver. Treatment of HSC-T6 cells in culture with rapamycin or 3MA decreased or increased C/EBP-α expression, respectively, as shown by Western blotting. Conclusion: C/EBP-α was primarily expressed in hepatocytes in normal liver, but its expression decreased significantly in liver fibrosis. Autophagy might play a role in liver fibrosis through its association with C/EBP-α, but this hypothesis warrants further investigation.

Keywords: C/EBP-α, hepatocytes, hepatic stellate cells, autophagy

Introduction

Liver fibrosis and its end-stage disease, liver cirrhosis, are major world health problems. A better understanding of the pathogenesis of liver fibrosis is crucial for the development of treatments that reverse its progression. For instance, the activation of hepatic stellate cells (HSCs) plays a pivotal role in the process of liver fibrosis. Activated HSCs are major cellular targets for preventing the progression of liver fibrosis.

The differentiation of HSCs is similar to that of adipocytes [1]. Consequently, adipocyte-specific genes might also coordinate the regulation of alterations in HSCs. The CCATT/enhancer binding protein (C/EBP) family plays a key role in the differentiation of adipocytes, and C/EBP-alpha (C/EBP-α) is known to control the maturation and cellular growth of preadipocytes [2-5].

In our previous research, we found that C/EBP-α induces apoptosis in HSCs but only slightly influences hepatocytes in vitro and murine liver function in vivo. However, C/EBP-α expression in human liver and liver fibrosis remains unknown. In addition, previous studies determined that the activation of caspase-9 is the main event in apoptosis in vitro. However, a caspase-independent pathway, such as autophagy, paraptosis, or mitotic catastrophe, might be involved in liver fibrosis [6]. Because of the role of autophagy in normal adipocyte differentiation [7-9], we hypothesized that autophagy is associated with C/EBP-α in liver fibrosis.

In this report, we first investigated the expression and localization of C/EBP-α in normal human liver and liver fibrosis by double label immunohistochemistry. The expression of autophagy-related 5 (Atg5) and 6 (Atg6) was also measured in normal human liver and liver fibrosis by immunohistochemistry. Finally, the association of C/EBP-α and autophagy was demonstrated using a rat hepatic stellate cell line (HSC-T6 cells).
C/EBP-α expression in liver fibrosis and its relationship with autophagy

Materials and methods

Materials

Formalin-fixed, paraffin-embedded liver tissue sections from 30 patients with pathologically demonstrated hepatic fibrosis between 2014 and 2015 at Beijing University, Shenzhen Hospital, were selected for this study. Normal liver specimens were also obtained from patients who underwent hepatectomy due to trauma. An antibody against C/EBP-α was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Double label immunohistochemistry kits and an antibody against α-SMA were obtained from Maixin Biotechnology Company (Maixin, Fuzhou, China). Antibodies against Atg5 (clone EPR1755-2) and Atg6 (clone EPR1733Y) were obtained from Abcam Biotechnology (Cambridge, UK). Rapamycin and 3MA were purchased from Sigma-Aldrich (USA).

Figure 1. Double label immunohistochemistry of C/EBP-α and CK8/18 or α-SMA.

Figure 2. Immunohistochemical analysis of the expression of C/EBP-α, Atg5 and Atg6 in normal liver and liver fibrosis (magnification, ×400).
Double label immunohistochemistry of C/EBP-α and α-SMA or CK8/18

The procedure was performed according to the manufacturer’s instructions. Briefly, deparaffinized tissue sections were immunostained first with α-SMA or CK8/18 antibodies and visualized by BCIP/NBT/alkaline phosphatase staining. The sections were washed, incubated with the antibody against C/EBP-α and visualized by AEC/peroxidase staining.

Immunohistochemistry of liver tissue sections

Deparaffinized tissue sections (3 µm thick) were incubated in 0.01 M citrate buffer (pH 6.0) at 95°C for 20 minutes for antigen retrieval, followed by immunohistochemical staining for C/EBP-α, α-SMA, Atg5, or Atg6 and visualization using 3,3′-diaminobenzidine (DAB). Immunohistochemistry was performed on an autostainer (BenchMark XT, Roche) using DAB as the chromogen and Mayer’s hematoxylin as the counterstain.

Western blot analysis

HSC-T6 cells were incubated in the presence of 600 µM rapamycin for 8 hours or 10 mM 3MA for 6 hours. After collecting the cells, they were washed in cold phosphate-buffered saline (PBS), lysed in ice-cold RIPA buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 10 µl/ml protease inhibitor cocktail) for 45 min on ice. Lysates were cleared by centrifugation at 13,000 g for 30 min, and the total protein concentration was determined using a BCA protein assay kit. The extracted cellular proteins were denatured in sodium dodecyl sulfate (SDS)-containing sample buffer, separated in a 10-12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Protein bands were visualized using an ECL assay kit. Antibodies against C/EBP-α and β-actin were used, with β-actin serving as an equal loading control.

Statistical analysis

Statistical analysis was performed using SPSS software version 11.0. The χ² test was used to compare the percentage of C/EBP-α expression in normal liver and liver fibrosis. A P-value less than 0.05 was considered statistically significant.
Results

C/EBP-α was primarily located in hepatocytes, not in HSCs, in normal liver and liver fibrosis based on double label immunohistochemistry

Based on CK8/18 and C/EBP-α double label immunohistochemistry, we found that C/EBP-α was mainly expressed in the nuclei of hepatocytes in normal livers. Because HSCs are quiescent in normal liver and because α-SMA labels activated HSCs, α-SMA was not detected in normal liver. In liver fibrosis, the expression of C/EBP-α was reduced in hepatocytes; however, C/EBP-α was not expressed in α-SMA-positive cells (Figure 1).

C/EBP-α was primarily located in hepatocytes in normal liver and its expression is reduced in liver fibrosis based on immunohistochemistry

In normal liver, the expression of C/EBP-α was abundant, but in liver fibrosis, it was almost absent. However, the expression of Atg5 was increased in liver fibrosis compared with normal liver; Atg5 was primarily expressed in the nuclei of cells around fibrotic septae, which was in agreement with the localization of α-SMA-positive cells (Figure 2).

Rapamycin, which induces autophagy, reduced C/EBP-α expression

To investigate whether C/EBP-α was involved in autophagy, HSC-T6 cells were incubated in the presence of 600 μM rapamycin for 8 hours, and Western blot analysis of cell lysates was performed to detect the expression of C/EBP-α. Compared with untreated cells, C/EBP-α expression was decreased in treated cells (P<0.05; Figure 3A).

The autophagy inhibitor 3MA increased C/EBP-α expression

To investigate whether C/EBP-α was involved in autophagy, HSC-T6 cells were treated with 10 mM 3MA for 6 h, and Western blot analysis was performed on cell lysates to detect the expression of C/EBP-α. Compared with untreated cells, C/EBP-α expression was increased in treated cells (P<0.05; Figure 3B).

Discussion

Liver fibrosis results from chronic damage to the liver. Prolonged liver injury triggers the activation of HSCs, which produce extracellular matrix components. HSC activation plays a pivotal role in the process of liver fibrosis and represents an appealing target for anti-fibrotic therapy.

Our previous research found that C/EBP-α could induce apoptosis in HSCs, with little effect on hepatocytes in mice [10]. However, C/EBP-α expression in human normal liver and liver fibrosis has not been studied. Using formalin-fixed, paraffin-embedded liver tissue sections from 30 patients with pathologically demonstrated hepatic fibrosis, double label immunohistochemistry for C/EBP-α and α-SMA or CK8/18 was used to determine their expression in HSCs and hepatocytes. CK8/18 is expressed in hepatocytes, and α-SMA is mainly expressed in activated HSCs; thus, we found that C/EBP-α is mainly expressed in hepatocytes in normal liver. Because HSCs are quiescent in normal liver, these cells did not express α-SMA. In contrast, HSCs were activated in liver fibrosis, but C/EBP-α was not expressed in HSCs, which is consistent with our previous findings in mice [10].

Our previous research found that the expression of C/EBP-α was reduced in liver fibrosis, but this decline was not statistically significant by double label immunohistochemistry. Double label immunohistochemistry might have produced a darker background, which may have rendered our results more difficult to interpret. To further investigate this issue, immunohistochemistry was performed to detect C/EBP-α expression using an immunohistochemistry autostainer, and it was found that C/EBP-α is primarily expressed in normal liver but is dramatically down-regulated in hepatic fibrosis.

Our previous research also determined that caspase-independent pathways might be involved in liver fibrosis. To the best of our knowledge, several models of caspase-independent cell death programs have been proposed, such as autophagy, parapotosis, and mitotic catastrophe [11].

Autophagy is a dynamic and fundamental process that has been reported to play roles in development, differentiation, cell repair, and immunologic defense as well as in protein quality control. It is generally accepted that the dynamic autophagic process is mainly execut-
ed by a series of proteins encoded by autophagy-related genes (ATGs) [12].

The ATG system is critical for completion of the autophagosome and resembles the ubiquitination system. The atg5 gene encodes an acceptor protein, Atg5, for the ubiquitin-like protein Atg12 [13]. The function of atg5 is important for adipogenesis, suggesting an involvement of autophagy in adipogenesis. Consistent with this notion, pharmacologic inhibition of autophagy by chloroquine blocks adipogenesis in a cellular model [14]. The differentiation of HSCs is similar to that of adipocytes [7]. Therefore, adipocyte-specific genes might also coordinately regulate alterations in HSCs. Our previous research found that C/EBP-α is involved in the regulation of HSCs, so we wondered whether Atg5 is involved in the regulation of HSCs. In this study, we investigated the expression of Atg5 in normal liver and liver fibrosis. To the best of our knowledge, we are the first to demonstrate that Atg5 is over-expressed in liver fibrosis by immunohistochemistry.

Beclin-1 was first identified as a Bcl-2-binding protein that is structurally similar to Atg6 in yeast and evolutionarily conserved among various species [16]. The Beclin-1 locus frequently undergoes monoallelic deletion in various human malignancies, including brain, ovarian, prostate, and breast cancers [17]. However, in this study, Atg6 was not detected in normal liver and liver fibrosis, and it appears that Atg6 does not play a role in liver fibrosis.

HSC-T6 cells, an immortalized rat hepatic stellate cell line, have been widely used as an in vitro assay system to study the mechanism of action of anti-fibrotic agents. This cell model is particularly valuable for studies of retinoid metabolism based on their similar retinoid phenotype as primary cells [18]. As we had used these cells previously, we chose HSC-T6 cells as a model system for HSCs. In this study, the autophagy inducer rapamycin and the autophagy inhibitor 3MA were used to induce or inhibit autophagy in HSC-T6 cells, respectively, and the protein expression of C/EBP-α was measured by Western blot analysis. The protein expression of C/EBP-α was decreased by rapamycin and increased by 3MA (both P<0.05). These data suggest that the expression of C/EBP-α might be controlled by autophagy, but an understanding of its mechanism will require further investigation.

In summary, our results strongly suggest that C/EBP-α is expressed primarily in hepatocytes in normal liver but that its expression is decreased in liver fibrosis. The expression of C/EBP-α might be associated with autophagy, but the underlying mechanism remains unknown. To the best of our knowledge, we are the first to investigate the existence of an association of C/EBP-α with autophagy, and we suggest that this association may be essential to our understanding of hepatic fibrosis.

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Disclosure of conflict of interest

None.

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