Hepatic oval cells activated by hepatocyte apoptosis in diethylnitrosamine-induced rat liver cirrhosis

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ABSTRACT

الأهداف: دراسة فيما إن كانت خلايا الكبد البيضاوية نشيطة في ديثالنتيروسمين (DEN) الذي يحرض تشمع الكبد و عرض آليته.

الطريقة: تم تحريض تشمع الكبد في الفئران العدد=8 بواسطة حقن داخل الصفاق أسبوعياً بجرعة مقدارها 50mg/kg لوزن الجسم لمدة 12 أسبوع، ثم فترة فشل لمدة 2 أسبوع. تلقى 5 فئران في مجموعة التحكم سواغ منقبض متساوي الحجم. تم فحص أنسجة الكبد، وتعريف خلايا الكبد الميتة وكميتها بواسطة مقايسة TUNEL. اكتشفت الخلايا البيضاوية بالبقعة الكيميائية النسيجية المناعية لكيناز البيروفات، (M2PK)، و الستيكراتين (CK19). أجريت الدراسة في مستشفى رنمين – جامعة وهان – هوبى – الصين خلال الفترة من فبراير حتى ديسمبر 2009م.

النتائج: تطور تشمع الكبد في الفئران التي تمت معالجتها باستخدام ديثالنتيروسمين (DEN). أظهرت مقايسة TUNEL، والنسيجية أعداد كبيرة لخلايا الكبد خضعت للموت الخلوي. كان مؤشر موت الخلية للفئران المعالجة باستخدام ديثالنتيروسمين (0.15 ± 0.75) (DEN) أعلى من الفئران في مجموعة التحكم الطبيعية (0.05 ± 0.10). كان ظهور كلا من CK19 و M2PK معتدلاً في تشمع كبد الفئران، وتوقف ظهور أو تكون حبال صغيرة في الكبد، وكان ظهورها صعباً في أنسجة الكبد للفئران الطبيعية في مجموعة التحكم.

خاتمة: في الفئران المعالجة ديثالنتيروسمين (DEN) لتحريض تشمع الكبد، كانت الخلايا البيضاوية نشيطة واستجابت للتكاثر، قد ترتبط الآلية مع كثرة موت خلايا الكبد في النموذج.

Objectives: To investigate whether hepatic oval cells are activated in diethylnitrosamine (DEN)-induced rat liver cirrhosis, and to explore its mechanism.

Methods: Liver cirrhosis was induced in rats (n=8) by weekly intraperitoneal injections of DEN at a dose of 50mg/kg body weight for 12 weeks followed by a 2-week wash out period. Rats (n=5) that received isovolumic vehicle served as the control group. Liver pathology was examined. Apoptotic hepatocytes were identified and quantified by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay. Oval cells were detected using immunohistochemical staining for pyruvate kinase type M2 (M2PK) and cytokeratin 19 (CK19). The work was carried out at Renmin Hospital of Wuhan University, Wuhan, Hubei, China from February to December 2009.

Results: Liver cirrhosis developed in rats subjected to DEN administration. The TUNEL and morphology assay showed that a substantial number of hepatocytes underwent apoptosis. The apoptotic index in rats subjected to DEN administration (0.75 ± 0.15) was much higher than normal control rats (0.10 ± 0.05). Both CK19 and M2PK were moderately expressed in the rat liver cirrhosis, and the expression was dispersed or forming small cords in the liver; but the expression was hardly detected in the liver tissue of normal control rats.

Conclusion: In the DEN-induced rat liver cirrhosis, oval cells are activated and stimulated to proliferation, the mechanism of which may be related to substantial hepatocyte apoptosis in the model.

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Liver cirrhosis is a common disease that poses a threat to public health, and is characterized by fibrosis and nodular regeneration. It can be regarded as a premalignant state as more than 80% of hepatocellular carcinomas develop in a cirrhotic liver.[1] However, the mechanism by which liver cirrhosis leads to hepatocellular carcinoma remains unknown. Emerging evidence suggests that hepatocellular carcinoma may arise from hepatic progenitor cells, called "oval cells."[2,3] Oval cells are bipotential stem cells able to differentiate into hepatocytes and biliary epithelial cells, and express phenotypic markers of both these cells, including pyruvate kinase type M2 (M2PK), cytokeratin 19 (CK19), and A6.[4] Studies have shown that inhibition of oval cell responses may prevent the onset of liver tumorigenesis in a choline deficient, ethionine supplemented (CDE)-fed mice model of carcinogenic liver injury.[5-7] Oval cells proliferate under certain conditions, in which replication of hepatocytes is inhibited by chronic disease or develop premature senescence with impaired ability to generate.[8] In the present study, we examined the presence of proliferating oval cells in a diethylnitrosamine (DEN)-induced rat liver cirrhosis model by immunohistochemistry, and explored its mechanism.

Methods. ***Animal model and treatment.*** Male Wistar rats, 8 weeks old and weighing around 200 g, were obtained from the Experimental Animal Center of Wuhan University, and kept under specific pathogen-free conditions. After acclimation for 6-7 days, the animals received intraperitoneal injections of DEN (Sigma Chemical Co., St. Louis, MO, USA) at a dose of 50 mg/kg body weight (n=8), or isovolumic vehicle (n=5) once a week for 12 weeks. The animals were sacrificed by decapitation 2 weeks after the last intraperitoneal injection. Liver tissue samples were harvested, and fixed in 10% buffered formalin and embedded in paraffin. The study was approved by the Experimental Animal Committee of Renmin Hospital of Wuhan University, and was carried out at Renmin Hospital of Wuhan University, Wuhan, Hubei, China from February to December 2009.

Histological evaluation. Rat liver samples, with approximate size 1.0 × 0.5 × 0.3 cm^3, were processed for light microscopy as described previously.[9] The processing consisted of fixing the specimens in 10% formaldehyde for 12-24 hours, embedding them in paraffin, slicing sections of 5 µm thickness, and staining the sections with hematoxylin and eosin. A pathologist evaluated the sections.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay. A TUNEL assay was performed using a commercial kit (Calbiochem TdT-FragEL™ DNA Fragmentation Detection Kit, PromoKine, Cambridge, MA, USA). After deparaffinization in xylene and hydration through a graded series of alcohol, the tissue sections were permeabilized with proteinase K (2 mg/ml, 1:100 in 10mM Tris, pH 8) solution for 20 minutes at room temperature, and then rinsed with 1 × Tris-buffered saline (TBS). Endogenous peroxidase was quenched with 30% hydrogen peroxide (1:10 in methanol) for 5 minutes. Sections were again rinsed with 1 × TBS and incubated with terminal deoxynucleotidyl transferase (TdT) equilibration buffer for 10-30 minutes. The sections were then incubated with the labeling reaction mixture consisting of TdT and biotinylated nucleotides for 90 minutes at room temperature. The reaction was terminated with stop buffer. Following a rinse with TBS, the sections were blocked with blocking buffer for 10 minutes. Labeled DNA fragments were visualized by adding streptavidin-horseradish peroxidase conjugate, incubating for 30 minutes and developing with diaminobenzidine. The sections were counterstained with hematoxylin. Apoptotic cells containing labeled DNA fragments were identified by dark brown stain over the nuclei as visualized under a light microscope. The apoptotic index (AI) was expressed as the number of positively stained cells per 100 hepatocytes.

Immunohistochemical staining for CK19 and M2PK. Three µm sections were prepared from rat paraffin embedded tissues. Immunohistochemical staining was performed with the method described previously with modifications.[9] After deparaffinization in xylene, the sections were hydrated through a graded series of alcohol, and then incubated in a citrate buffer (pH 6.0) in a microwave oven for antigen retrieval. Endogenous peroxidase was quenched with 3% hydrogen peroxide in phosphate buffered saline (PBS) for 10 minutes. Nonspecific binding sites were blocked by incubating the sections in 10% normal goat serum for 10-15 minutes. Sections were then incubated with the primary antibodies, mouse monoclonal anti-CK19 antibody ([1:50], ProMab Biotechnologies, Richmond, CA, USA) or rabbit polyclonal anti-M2-PK antibody ([1:250] CST Inc., MA, USA) overnight at 4°C, followed by incubation with the second antibody, HRP (horse radish peroxide)-Polymer anti-mouse/rabbit IgG (Fuzhou Maixin Biotechnology Development Co., Ltd, Fuzhou, China) at room temperature for 20 minutes. The peroxidase activity was visualized with a color reaction using diaminobenzidine, and the sections were counterstained with hematoxylin. Sections incubated with PBS instead of the primary antibody were used as negative controls. A pathologist evaluated immunostaining.

Statistical analysis. Results were presented as mean ± standard deviation (SD) unless otherwise indicated. Student t test was used to analyze the differences between the 2 groups. All the statistical analyses were performed with the Statistical Package for Social Sciences Version 13.0 (SPSS Inc, Chicago, IL, USA). A *p*-value less than 0.05 was considered statistically significant.

Results. ***Pathological changes of the liver in rats subjected to DEN administration.*** Liver cirrhosis developed in all rats subjected to DEN administration, which showed disorderly hepatocyte cords, fatty degeneration, and collagen deposition extending from central veins or portal tracts with pseudolobuli formation (**Figure 1a**). However, no hepatocellular carcinoma nodule was detected either on macroscopic or microscopic examination; and no obvious hepatocyte necrosis or infiltration of lymphocytes was observed. In rats subjected to isovolumic vehicle administration, liver tissue samples showed normal lobular architecture with central veins and radiating hepatic cords with irregular sinusoids, and a normal distribution of collagen with a variable amount in portal tracts and a thin rim around central veins (**Figure 1b**).

Hepatocyte apoptosis in rat liver after administration of DEN. In the present study, TdT-FragEL™ DNA fragmentation detection assay was performed to evaluate hepatocyte apoptosis in liver sections. A dark brown stain over the nuclei was an indication of apoptotic cells. Numerous positive staining was observed in rats subjected to DEN administration (**Figure 2a**). As expected, a few TUNEL-positive cells were detected in the liver of normal control rats (**Figure 2b**). These TUNEL-positive staining cells had sharply delineated masses or crescents of condensed chromatin, the features of which are consistent with apoptotic cell death. The AI in rats subjected to DEN administration (0.75 ± 0.15) was much higher than control rats (0.10 ± 0.05) (p=0.0009).

Expressions of CK19 and M2PK in the liver of rats subjected to DEN administration. Hepatic oval cells were detected using immunohistochemical staining for CK19 and M2PK in the cytoplasm. Only faint immunoreactive staining was detected in the liver from normal control rats for CK19 (**Figure 3a**). However, in rats subjected to DEN administration, moderate staining for CK19 was observed, and the staining was dispersed or forming small cords (**Figure 3b**). For M2PK, no or faint staining was detected in the liver tissue of control rats (**Figure 3c**); however, moderate staining was observed in rats subjected to DEN administration, and the expression pattern was similar to CK19 (**Figure 3d**).

Discussion. Diethylnitrosamine-induced liver injury in rats is a reproducible and valuable animal model for studying human liver diseases. Diethylnitrosamine is a potent hepatotoxin, which causes liver injury by covalent binding and methylation of nucleic acids and proteins in hepatocytes.[10] It can cause liver cirrhosis and subsequent hepatocellular carcinoma.[11,12] In the present study, DEN-induced liver cirrhosis was observed in rats, which showed disorderly hepatocyte cords, marked collagen deposition with pseudolobuli formation. The liver histology is consistent with characteristic features of cirrhosis. In the rat model, DEN also caused fatty degeneration, but no hepatocellular carcinoma nodule was found either at macroscopic or microscopic examination. Cell death occurs by at least 2 distinct processes, necrosis and apoptosis.[13] In the DEN-induced rat liver cirrhosis model, we found that a substantial number of hepatocytes underwent apoptosis identified by TUNEL assay. Quantitative analysis showed that the AI in rats subjected to DEN administration was much higher than control rats. The result is consistent with other studies that DEN can induce apoptosis of liver cells, which may be involved in its hepatotoxic injury.[14]

Liver cirrhosis is the main risk factor for the development of hepatocellular carcinoma.[1] However, the mechanism by which liver cirrhosis leads to hepatocellular carcinoma has not been clarified completely. Emerging evidence suggests that hepatocellular carcinoma may arise from hepatic oval cells,[2,3] which can differentiate into hepatocytes and biliary epithelial cells, and express characteristic markers, including CK19, M2PK and A6.[4] Several studies have shown that inhibition of oval cell responses with tumor necrosis factor (TNF) receptor type 1 knockout, or pharmaceutical agents such as a peroxisome proliferator activated receptor γ (PPARγ) agonist, ciglitazone, and a cyclooxygenase 2 inhibitor, SC-236, may prevent the onset of liver tumorigenesis in a CDE-fed mice model of carcinogenic liver injury.[5-7] In the present DEN-induced rat liver cirrhosis model, we found that both CK19 and M2PK were moderately expressed in the liver, whereas neither of them was detected in the liver tissue of normal control rats. The M2PK is a reliable marker for oval cells, and it does not stain ductal cells and is not expressed in adult hepatocytes.[15] The CK19 is less specific as a marker for oval cells because it stains biliary epithelium in the adult liver.[16] In the present study, we combined these 2 markers to identify oval cells reliably.

The results suggested that liver cirrhosis induced by DEN harbors activated and proliferating oval cells, which may be a reason why liver cirrhosis leads to hepatocellular carcinoma. However, the mechanism to stimulate oval cell proliferation is not fully understood. Hepatic oval cell activation and proliferation have been

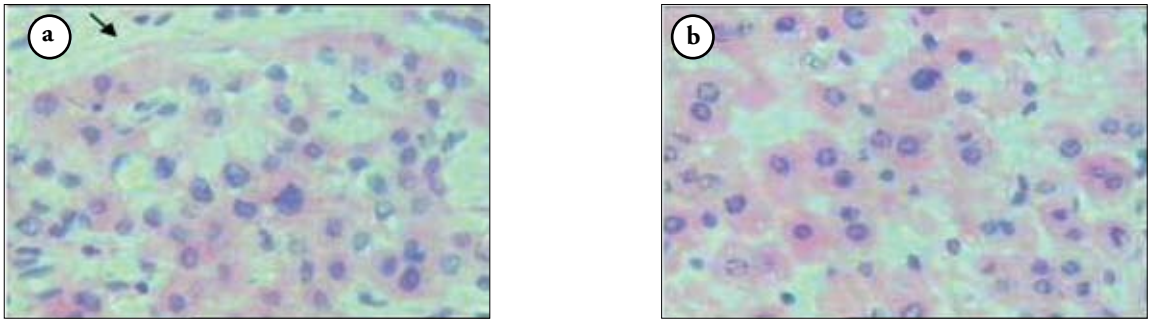

Figure 1 - Pathological examination of the liver in rats. **a)** The liver in the rat subjected to diethylnitrosamine administration showed disorderly hepatocyte cords, fatty degeneration, and collagen deposition with pseudolobuli formation (arrow). **b)** The liver in the rat subjected to isovolumic vehicle administration showed normal lobular architecture. Hematoxylin and eosin stain × 200.

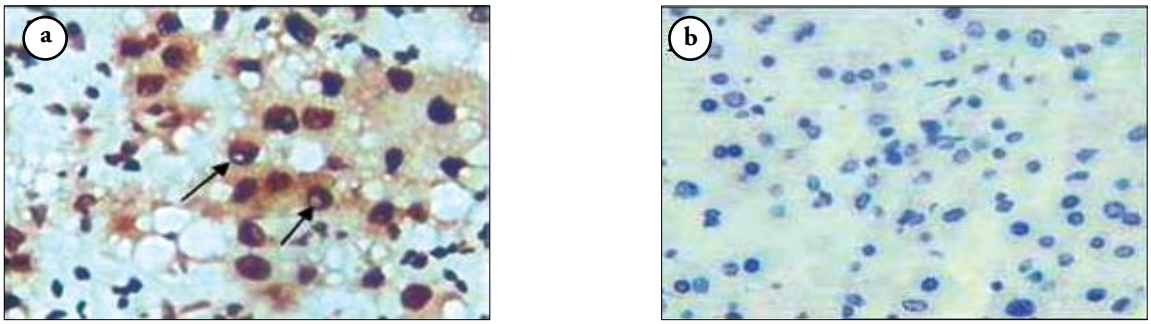

Figure 2 - The TUNEL staining in liver sections. **a)** Numerous positive staining (arrows) was observed in the rat subjected to diethylnitrosamine administration; **b)** however, little staining was detected in the liver of normal control rat. Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling stain × 200.

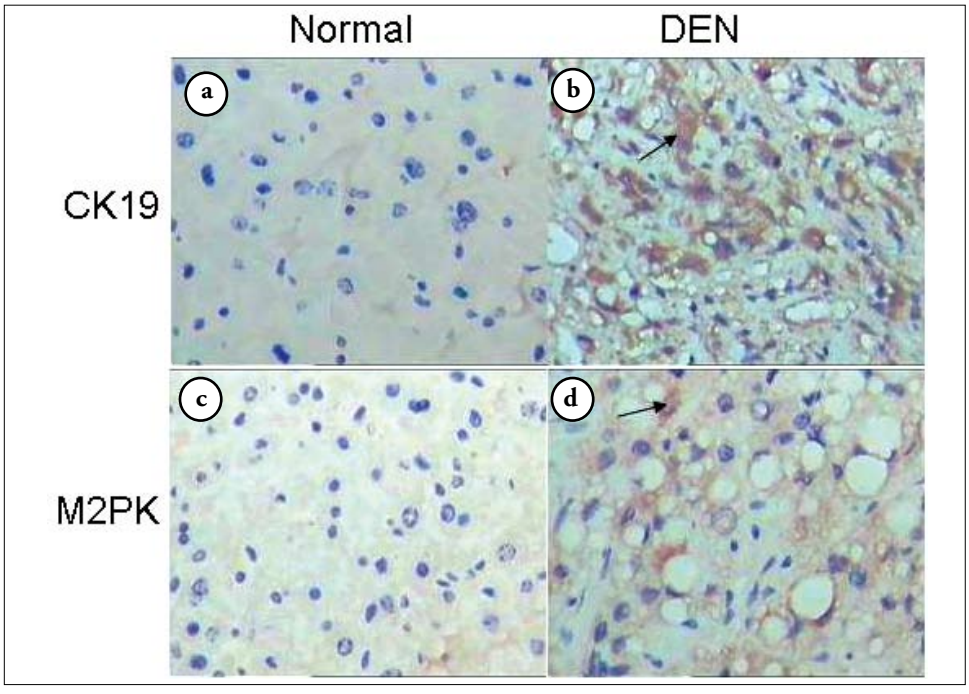

Figure 3 - Expressions of CK19 and M2PK in the liver. Only faint staining for **a)** CK19 and **c)** M2PK was detected in the liver from the control rat. However, the rat subjected to diethylnitrosamine administration showed moderately staining (arrows) for **b)** CK19 and **d)** M2PK. Immunohistochemical stain × 200.

observed under certain conditions, in which replication of hepatocytes is blocked.[8] As mentioned above, in the DEN-induced rat liver cirrhosis model, a substantial number of hepatocytes underwent apoptosis, which might stimulate oval cell proliferation. Apoptosis is a programmed cell death and may result in reduction of hepatocytes. However, further studies are needed to confirm the causative link between apoptosis of hepatocytes and activation of oval cells.

In conclusion, in the DEN-induced rat liver cirrhosis, oval cells are activated and proliferate, which is associated with increased risk of development of hepatocellular carcinoma. The mechanism to stimulate oval cell proliferation may be related to substantial hepatocyte apoptosis in the model.

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