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Immunohistochemical and Ultrastructural Studies on Oval Cells During Hepatic Carcinogenesis and Fibrosis in Rats

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Abstract: The development and proliferation of oval cells in experimental hepatocarcinogenesis induced by N-nitrosodiethylnitrosamine (NDEA) followed by carbon tetrachloride (CCl_4) and in experimental hepatic fibrosis induced by CCl_4 were compared. Forty adult male Sprague-Dawe-ley rats were subjected to two experiments: 1) in hepatocarcinogenesis experiment, rats were treated with a single intraperitoneal injection of NDEA (200 mg/kg body weight), then, weekly, with subcutaneous injections of CCl_4 (3 ml/ kg body weight/week) for 6 weeks and; 2) in hepatic fibrosis experiment, rats were treated with intraperitoneal injection of CCl_4 (2 ml/kg body weight) twice weekly for 3 months. Oval cells in the liver were identified by light microscopy, immunohistochemical expression of cytokeratin 19 and electron microscopy. In the hepatocarcinogenesis model, there was massive proliferation of oval cells, originated from the periportal area, forming ductules in between hepatocytes. In ultrathin sections, stellate cells were adjacent to the ductules. The ductules were surrounded by prominent basement membrane. In hepatic the fibrosis model, few oval cells were limited to the periportal area and not extended to the hepatic parenchyma. In conclusion, oval cells proliferate more intensively with formation of ductules in the early stages of the hepatocarcinogenesis model while, limited oval cell proliferation was observed in hepatic fibrosis model.

Key words: N-nitrosodiethylnitrosamine · Carbon tetrachloride · Hepatocarcinogenesis · Oval cells · Cytokeratin 19

INTRODUCTION

Oval cells are hepatic stem cells that proliferate in the portal area following injury in the liver at the early stages of hepatocarcinogenesis [1]. Oval cells are considered bipotential precursors as they can differentiate into either bile duct cells or hepatocytes [2]. Immunohistochemically, oval cells resemble both hepatocytes and bile duct cells since they expressed alpha-fetoprotein (AFP), cytokeratins 7 and 19 (CK7, CK19) and Oval cell marker antibody (OV-6) [3, 4]. Oval cells are considered the source of stem cells for liver regeneration [5, 6]. Oval cell proliferation initiates liver regeneration in 2-N-acetylaminofluorene-partial hepatectomy (2-AAF-PH) models [7]. Recent study indicated that oval cells may be valuable in therapeutic liver regeneration following liver transplantation [8]. Several models for oval cells activation were described and oval cell proliferation was induced by 2-AAF-PH model [9], 2-AAF/CCl_4 [10], choline deficiency, an ethionine-supplemented diet [11], dipin and partial hepatectomy [12], acetaminophen [13], D-galactosamine [14] and 2,4-dichlorophenoxyacetic acid [15].

N-nitrosodiethylnitrosamine (NDEA), is a hepatic carcinogen that induces neoplasms similar to human neoplasms [16]. Oval cell proliferation can be induced by exposure to NDEA in mice [1]. NDEA associated with high trans-fat diet induced a cancer derived from oval cells in the liver of mice [17].

The proliferation of oval cells was demonstrated in liver biopsies from patients suffering from hepatitis B and C. The numbers of oval cells are correlated to the grade and stage of hepatitis [18]. Oval cells are thought to be the cell of origin in 28-50% of human hepatocellular carcinomas (subclass A HCC) [19]. Oval cells bear the worst expectation for the development of hepatocellular

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carcinoma [18, 19]. Experimentally, oval cells proliferated during experimental liver brosis without pretreatment with a known inhibitor of hepatocyte proliferation [20].

This study was performed to compare the development and the proliferation of oval cells in experimental hepatocarcinogenesis model induced by N-nitrosodiethylnitrosamine (NDEA) followed by carbon tetrachloride (CCl_4) to those in experimental hepatic fibrosis model induced by CCl_4 alone.

MATERIALS AND METHODS

Experimental Design: This study was conducted in accordance with the principles of the Animal Research Ethical Committee of the Faculty of Veterinary Medicine, Assiut University, Egypt. Forty Sprague-Dawe-ley adult male rats, weighing 150-170 g, were purchased from Experimental Animal House, Faculty of Medicine, Assiut University. The animals were housed in stainless steel cages (five rats per cage), under controlled temperature (23°C) and humidity conditions and 12h dark/light cycles. The rats were divided into 2 experimental groups.

Hepatocarcinogenesis Experiment: In this experiment, 20 rats were divided into two groups (A1=10, A2=10). In group A1 rats received a single intraperitoneal injection of NDEA (200 mg/ kg body weight) (Sigma- Aldrich, Germany). After one week, rats received subcutaneous injections of Ccl_4 (3 ml/ kg body weight /week) (Sigma- Aldrich, Germany), weekly, for 6 weeks [21]. Rats of group A2 were used as controls and received single intraperitoneal injection of normal saline and subcutaneous injections of corn oil, weekly, for 6 weeks.

Hepatic Fibrosis Experiment: In this model, 20 rats were divided into two groups (B1=10, B2=10). In group B1, rats received intraperitoneal injection of CCl_4 (2 ml/kg body weight) twice a week for 3 months as described before [22]. Rats of group B2 were used as controls and received intraperitoneal injections of corn oil, twice a week, for 3 months.

In both experiments, all rats were sacrificed after 3 months. Rats were anesthetized with chloroform before being euthanized.

Histopathological Examination: Tissue samples were collected from the liver of all animals, fixed in 10% buffered formalin, embedded in paraffin, stained with hematoxylin and eosin and Van Gieson staining [23]. Stained sections were examined under a light microscope (Olympus CX31, Japan) and photographed using digital camera (Olympus, Camedia C-5060).

Immunohistochemisty: Expression of CK19 by using (CK19) kit (Biocare Medical, Concord, CA, 94520, USA) was evaluated by immunohistochemistry of formalin fixed, paraffin embedded section [24]. After deparaffinization and rehydration with ethanol, slides were washed with distilled water 3 times for 2 min each. Slides were transferred to a microwave-proof container and cover with citrate buffer and heat for 10 minutes then allow slides to cool in the citrate buffer for approximately 35 minutes. Slides were rinsed three times with 1X Tris-buffered saline (TBS) for 3 minutes each. Slides were incubated with 3% H_2O_2 solution (diluted in distilled water) for 10 minutes. Slides were rinsed three times with 1X TBS for 3 minutes each. Then were incubated for 10 minutes with 5% normal blocking serum in 1X TBS to block non-specific binding. The sections were subsequently incubated overnight at 4 with primary antibody diluted in 1X TBS. Slides were rinsed three times with 1X TBS for 3 minutes each. Sufficient peroxidase labeled polymer was applied and incubated for 30 minutes. Slides were rinsed three times with 1X TBS for 3 minutes each. The substrate was prepared by mixing one drop of liquid 3,3'-Diaminobenzidine (DAB) plus chromogen immediately with 1 ml of substrate buffer prior to use. The substrate was applied carefully and incubated for 5-10 minutes till a brown color developed. Sections were gently rinsed with sufficient distilled water. Sections were counterstained with hematoxylin.

Computerized Morphometrical Analysis: Number of oval cells was evaluated by counting the number of cells per 40X power field in 10 fields (per case) in HE and immune slides. The morphometrical analysis was done by Research Microscope type Axiostar Plus made by Zeiss transmitted light bright field examinations upgradeable to professional digital image analysis system (Carl Zeiss Axiovision Product Suite DVD 30).

Ultrastructural Analysis: Samples of liver (0.1 mm3) were fixed in glutaraldehyde 5% and in cacodylate buffer (0.1 M, pH 7.2) for 3 times of 20 minutes. After dehydration the samples were embedded in Epon. Ultrathin sections were contrasted with uranyl acetate and lead nitrate and the slides were examined using a transmission electron microscope (Jeol, CX11) at 80 kv (Electron Microscope Unit, Assiut University).

Statistical Analysis: Statistical analysis was carried out by analysis of variance and the results were compared using Student's t test. All statistical tests were performed using Graphpad Software Package. Values are expressed as mean ± SEM.

RESULTS

Histopathology: Incidence of the lesions in hepatocarcinogenesis and fibrosis models is summarized in Table (1). In hepatocarcinogenesis model (group A1), injections of NDEA followed by CCl_4 induced massive proliferation of oval cells. Oval cells appeared as small cells with oval nuclei and scanty cytoplasm. These cells originated from the periportal area and extended in between the hepatocytes (Fig.1a, b), forming structures that resembled bile ducts with occasional central lumina (Fig. 1c). Some ducts were dilated and formed multiple cyst-like structures lined by flattened epithelium (Fig. 1d). Oval cells were also around preneoplastic foci composed by swollen and vacuolized hepatocytes with prominent nucleoli (Fig. 1e). In CCl_4 group (group B1), portal fibrosiswas observed (Fig. 2a). The connective tissue was identified by Van Gieson staining (Fig. 2b). Careful examination of tissue sections revealed few oval cells in the periportal area that did not extend in between the hepatic parenchyma (Fig. 2c).

Immunohistochemistry: Immunohistochemical analysis revealed the massive expression of CK19 by oval cells in hepatocarcinogenesis model (Fig. 3a). In hepatic fibrosis model, weak expression of CK19 was observed (Fig. 3b).

Morphometrical Analysis: The total number of the oval cells and their mean in hepatocarcinogenesis model and hepatic fibrosis model in both HE and immunohistochemistry were shown in Table (2). Oval cells were significantly increased ($P< 0.001$) in hepatocarcinogenesis model-in both HE and CK19 when compared with hepatic fibrosis model.

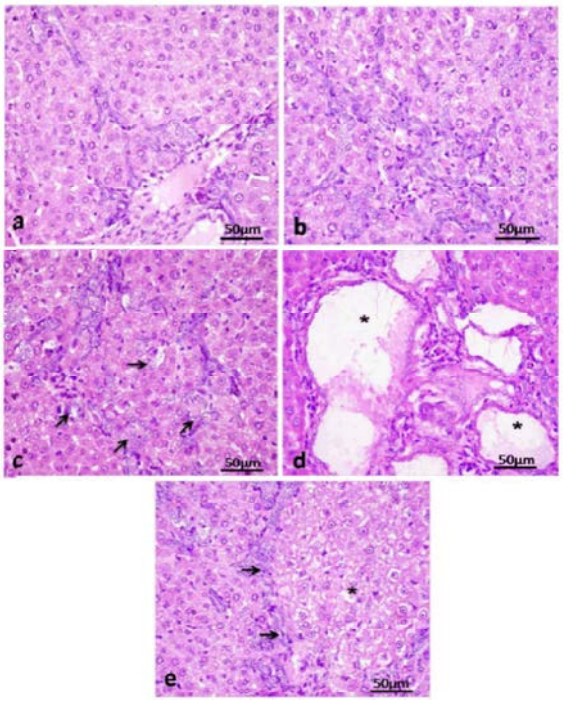

Fig. 1

Liver from rats of group A1 showing (hepatocarcinogenesis model); (a,b) Proliferation of oval cells in the portal area and extended in between the hepatocytes. (c) Development of lumen and formation of ductules. (d) Some ductules formed by oval cells are dilated forming multiple cysts lined by flattenend epithelium (asterisks). (e) The oval cells (arrows) surrounding a preneoplastic foci (asterisk) in which the hepatocytes are enlarged and vacuolated with prominent nucleoli. HE stain.

Table 1: Histopathological lesions in the liver of rats the hepatocarcinogenesis model and fibrosis model.

Lesion	(Hepatocarcinogenesis group)	(Fibrosis group)
-Proliferation of oval cells in the portal area	+++	++
-Oval cells extended between hepatocytes	+++	-
-Oval cells development of lumen and formation of ductules	+++	-
-Preneoplastic foci	+++	_
-Portal fibrosis	-	+++

-No lesion, + Lesion found in 1-3 rats, ++ Lesion found in 4-6 rats and +++ Lesion found in 7-10 rats.

Table 2: Computerized morphometrical analysis of oval cell proliferation in the liver of rats of hepatocarcinogenesis model and fibrosis model.

Lesion	(Hepatocarcinogenesis group)	(Fibrosis group)
-Proliferation of oval cells by HE	391±79.88 ***	64.00±14.47
- Proliferation of oval cells by CK19	188±18.3 ***	32.00±6.11

Mean±SE, *** extremely significant (P< 0.001).

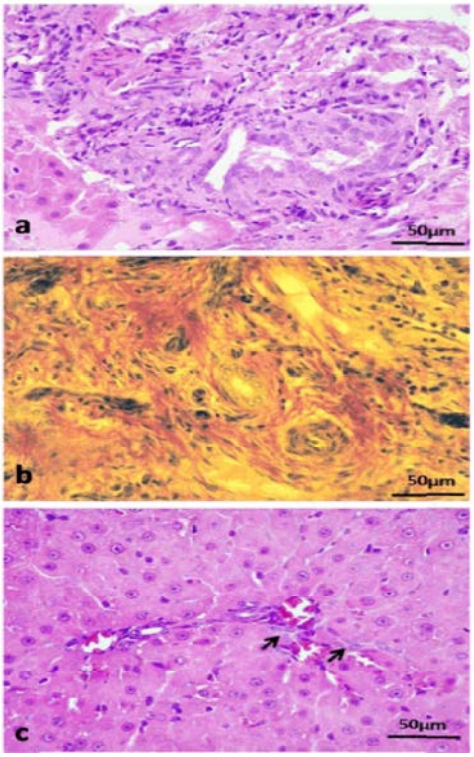

Fig. 2

Liver from rats of group B1 showing (fibrosis model); (a) Portal fibrosis, HE; (b) Portal fibrosis, Van Gieson staining; (c) Few oval cells limited to the periportal area and not extended between the hepatic parenchyma (arrows), HE stain.

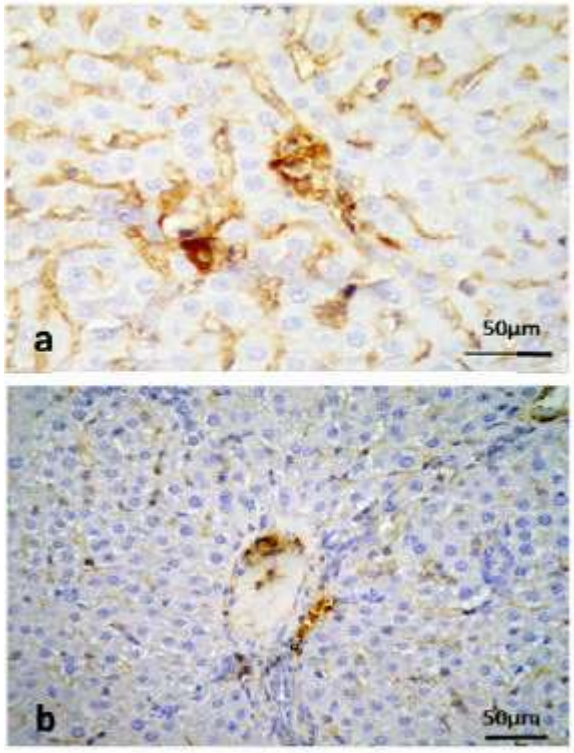

Fig. 3

Liver from rats showing immunohistochemical expression of CK19 within oval cells. (a) Massive distribution of CK oval cells in between the hepatocytes in liver sections obtained from group A1. (b) Weak expression of CK oval cells in liver sections obtained from rats of group B1. CK19 immunohistochemical staining.

Ultrastructure of Oval Cells: Ultrastructural analysis revealed massive oval cell proliferation in the livers of the hepatocarcinogenesis experiment. Oval cells had irregular oval nuclei with scanty cytoplasm. Stellate cells were adjacent to the oval cells (Fig. 4a). Oval cells forming small ductules were within the hepatic parenchyma and caused squeezing the sinusoids. These ductules were surrounded by apparent basement membrane. Some oval cells contained prominent nucleoli and resembled hepatocytes (Fig. 4b). Other ductules were lined by hypertrophied oval cells with a scanty cytoplasm and large round to oval nuclei (Fig. 4c). Few oval cells were detected in the ultrathin sections of the liver of fibrosis model (Fig. 5).

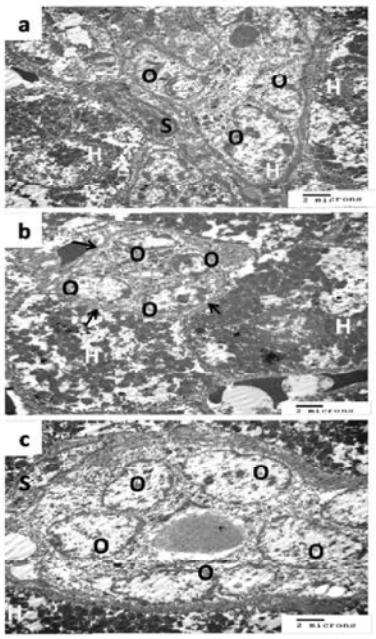

Fig. 4

Electron micrograph from liver of group A1 showing; (a) Oval cells with their more or less ovoid nuclei in between the hepatocyte. Stellate cell in adjacent to the oval cells. (b) Ductule formed by 4 oval cells , two of them have prominent nucleoli and resemble hepatocyts. Prominent basement membrane surrounding the ductules (arrows). (c) Ductule lined by hypertrophied oval cells. Notice, stellate cell in adjacent to the ductule H, hepatocyts; O, oval cells; S, stellate cells. X 2900.

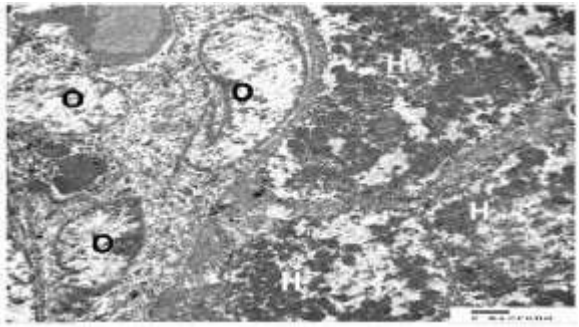

Fig. 5

Electron micrograph from liver of group B1 showing oval cells with their ovoid nuclei without formation of ductules. H, hepatocyts; O, oval cells. X 2900.

DISCUSSION

The present study investigates the development and proliferation of oval cells in both experimental hepatocarcinogenesis induced by NDEA followed by CCl_4 and experimental liver fibrosis induced by CCl_4 alone without pretreatment with a carcinogen. The demonstration of oval cell was done using light microscope, appropiate immunohistochemial staining of CK19 and ultrastructural analysis.

In the present study, there was massive oval cells proliferation following injection of NDEA and CCl_4 (hepatocarcinogenesis model). Oval cell proliferation was observed following exposure to NDEA [17, 25]. On the other hand, few oval cells proliferated following injection of CCl_4 alone without pretreatment with a carcinogen and the proliferations didn't extend between the hepatocytes. These results were in consistent with a previous study which found limited oval cell proliferation following injection of CCl_4 alone and also added that CCl_4 injection following 2-AAF induced higher oval cell proliferation [26]. This may be attributed to the observation that the damage of the periportal zone reduced oval cell proliferation [27]. On the other hand, oval cell proliferation was demonstrated during hepatic cirrhosis without pretreatment with a hepatocyte inhibitor [20]. Previous study indicated that there was a relation between liver disease severity and the increase in the number of oval cells which was associated with increased chance for development of hepatocellular carcinoma in chronic liver disease [28].

Different models for oval cell activation were used. Although, 2-AAF-PH model is the most extensively used model for oval cell activation [27, 29], it induced high mortality rate because of partial hepatocetomy procedure [30]. In the NDEA and CCl_4 model, the proliferating oval cells formed bile ductular like structures. These ductules were found among the hepatic parenchyma. Similarly, morphological analysis of 2-AAF-PH model showed that oval cells always proliferate in the form of elongated and tortous ductules which are suggested to be extensions of the pre-existing canals of Hering [31]. Many authors concluded that oval cell proliferate in the form of ductules during carcinogenesis and biliary obstruction [32, 33] and during regeneration of liver during periportal necrosis after treatment with hepatocyte proliferations inhibitors [26].

The relationship between the oval cells and the preneoplastic foci is not well understood. In our results we observed massive proliferation of oval cells surrounding these foci. It was reported that the progenitor cells responsible for the formation of nodules and hepatomas are carcinogen-altered parenchymal hepatocytes [34].

Using transmission elecrton microscope, oval cells having more or less oval nuclei with scanty cytoplasmThe ultrastructural characteristics of oval cells are similar to biliary epithelial cells. This result was previously described by several authors [12, 35]. A stellate cell was located in adjacent to the ductules formed

by oval cells. This result is in agreement with a previous study which demonstrates stellate cells close to these ductules in which the processes of the stellate cells penetrate the basement membrane and form direct cell-cell contact with the oval cells [31]. Tsamandas *et al*. [20] suggested that hepatic stellate cells and Kupffer cells produced cytokines or other factors may be required to stimulate the development and proliferation of oval cells.

The ductules formed by oval cells were surrounded by basement memrane. Similar results were described by [31]. Oval cells communicate with the adjacent hepatocytes by desmosomes. These findings are in agreement with a previous study that studied or investigated the connections between oval cells and hepatocyts [36].

In conclusion, the administration of NDEA and CCl_4 induced progressive oval cell proliferation with formation of ductules within the hepatic parenchyma. Administration of CCl_4 alone induced limited oval cell proliferation which didn't extend between the hepatocytes.

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