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Original Research Paper

SEQUENTIAL QUANTITATIVE ANALYSIS OF OVAL CELL PROLIFERATION IN THIOACETAMIDE TREATED RATS

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ABSTRACT

Oval cells are the progeny of facultative stem cells found in the periportal areas in response to liver injury in experimental animals and humans. The aims of this study were to describe the morphological, stereological and morphometric features of oval cells, and to compare them with those of bile duct cells and hepatocytes. It was also aimed to study the fate of oval cells by morphological and morphometric criteria. Rats were given thioacetamide to induce liver injury. The livers from experimental and control groups were processed routinely and stereological and morphometric analysis assessed using a computer image analysis system. Eight morphometric parameters were assessed in oval cells, bile duct cells and hepatocytes from control and experimental rats. Mitoses were observed in both oval cells and hepatocytes. Stereological area fraction analysis indicated that necrosis reached its maximum extent at 30 hours followed by regeneration and almost complete restoration of liver cell parenchyma at 132 hours. Oval cell proliferation reached a peak at 48-52 hours but was not apparent at 132 hours. Morphometric findings have shown increases in the nuclear diameter and nuclear area of oval cells with changes in the roundness and contours ratios of the nuclear membrane. It is concluded from this study that in thioacetamide treated rats, the liver responds to injury by bile ductal proliferation in the periportal areas which, accompanied by hepatocyte regeneration, leads to restoration of the hepatic parenchyma. At a subcellular morphological level the nuclei of oval cells showed a progressive change to a hepatocyte phenotype from that of a normal biliary cell, suggesting the differentiation of these cells into hepatocytes.

Keywords: bile ducts, liver regeneration, oval cells, stem cell, thioacetamide, toxicopathology.

INTRODUCTION

Oval cells are multipotential cells having the capacity to differentiate into hepatocytes, bile duct cell (Evarts *et al.,* 1987) and intestinal type epithelium (Tatematsu *et al.,* 1985). They are thought to represent the progeny of hepatic stem cells and so in some instances to be a precursor for hepatic tumours (Hixon *et al.,* 1990; Alison *et al.,* 1997a, b). Isolated oval cells in culture can be induced to differentiate into both hepatocyte like cells and biliary type cells (Gemain *et al.,* 1998). These cells are called oval cells because of their characteristic shape (Farber, 1956). Oval cell proliferation is seen in the livers of animals after submassive liver necrosis (Grisham and Porta, 1964; Lesch *et al.,* 1970; Evarts *et al.,* 1987; Lemire *et al.,* 1991; Lenzi *et al.,* 1992; Bennoun *et al.,* 1993; Dabeva and Shafritz, 1993) or after partial hepatectomy when hepatocyte regeneration is compromised (Sarraf *et al.,* 1994; Alison *et al.,* 1996; Alison *et al.,* 1997a, b). They have been described in a variety of animal models employing hepatotoxins (Lemire *et al.,* 1991; Lenzi *et al.,* 1992; Bennoun *et al.,* 1993; Dabeva and Shafritz*,* 1993; Sarraf *et al.,* 1994; Alison *et al.,* 1996; Alison *et al.,* 1997a, b).

Oval cells have been described in viral hepatitis, (Hsia *et al.,* 1992) focal nodular hyperplasia, (Roskams *et al.,* 1996) small cell hepatoblastoma in infants and children (Ruck *et al.,* 1996), hepatitis B virus associated hepatocellular carcinoma (Hsia *et al.,* 1992) and in alcoholic liver disease (Ray *et al.,* 1993). Although the origin and localisation of oval cells remains controversial (Desmet *et al.,* 1995), it is generally believed that they are related to terminal biliary ductules, the so-called canals of Hering (Lemire *et al.,* 1991; Germain *et al.,* 1998).

In this study we describe the morphological and stereological analysis of the liver in control rats and rats with thioacetamide-induced hepatic necrosis. Our aim was to compare the morphometric features of oval cells with those of hepatocytes and bile duct cells in relation to the time after the induction of hepatic necrosis. This is the first report of oval cell differentiation into hepatocytes after thioacetamide induced liver injury.

MATERIALS AND METHODS

EXPERIMENTAL PROCEDURE

Male Sprague-Dawley rats weighing 180-230g were used. Thioacetamide (TAA) 150 mg/kg was administered to the treatment group by gavage after an overnight fast. All animals were given 2 ml 10% glucose subcutaneously and then allowed free access to food and water. A second dose (100 mg/kg) of TAA was given after 24 hours with further 2 ml subcutaneous 10% glucose. Rats were placed in an incubator, the temperature of which was maintained at 25°C until killed at intervals (number shown in brackets) of 26(4), 30(4), 48(4), 52(2), 68(6), 96(4) and 132(4) hours after the last dose of TAA. Control rats were gavaged with water. The livers from 28 thioacetamide treated and 10 controls were available for histological examination. Liver samples were fixed in buffered 4% formaldehyde and processed routinely. At least three slices from three different lobes were taken from each animal. Paraffin sections were cut at 5 μm thickness and stained with hematoxylin and eosin.

STEREOLOGICAL ANALYSIS

The area fraction (A_A) of parenchymal area, oval cell area, necrotic area (central vein area in controls) and portal tract area were estimated using a 68.55 point parallel Weibel grid (final magnification ×750, objective 10; Labophot 2 Nikon microscope). The parallel Weibel grid was used assuming that the liver tissue was isotropic and the changes in experimental animals and controls were uniform and randomly orientated, and that sampling was similar in controls and experimental animals. At least 500 points covering parenchymal area, area of necrosis and oval cell area and portal tract areas were counted in each liver. In this study the A_A was estimated using the basic stereological relation

$$A_A = N_c/(N_c+N_o)$$

Where N_c is the mean number of points that hits the parameter (i.e. necrosis) and N_o is the mean number of points per unit area that hits the remaining structures.

MORPHOMETRIC MEASUREMENTS

The area measurements for nuclei and nucleoli were performed using a commercially available interactive video overlay measuring system (Prodit version 5.2 BMA, The Netherlands). With this system the microscopical image is recorded by a video camera and displayed on the computer screen with ×100 objective (Nikon Labophot 2 microscope); total magnification was ×7000. The outline of the nucleus was demarcated followed by the outline of nucleoli. At least 50 nuclei from each cell type were analysed.

MORPHOMETRIC VARIABLES

The following parameters were used in this study:

Area is the total area of the nucleus (area 1) and nucleolus (area 2). If there were several nucleoli, there areas were summed.

Diameter is the diameter of the nucleus or nucleolus.

Axis ratio is derived from the ratio of long axis to short axis of the nucleus or nucleolus.

Contour ratio - when the nucleus is circular the contour ratio = 1.

Roundness - nuclear roundness is a shape factor of the nucleus. It is equal to 1 in ideal conditions: when a circle.

Area ratio = mean area of nucleus / mean area of nucleoli.

All measurements were in micrometer for length and micrometer squared for area.

STATISTICS

Results were expressed as mean and standard error of the mean (SEM). Difference between groups was equated using two tailed probability (SPSS for Windows 6.1). The level of significance was set to $p < 0.05$. The analysis of variance of nuclear roundness and nuclear contour was also obtained using the same computer program.

To test the intraobserver reproducibility, the nuclear area of oval cells, hepatocytes (treated and controls) and bile duct cells were each measured ten times. The co-efficient of variation (CV) for nuclear area was calculated for each type of cell. CV for oval

cells was 3.25, for hepatocytes (treated) was 2.78, for bile duct cells was 2.74 and for hepatocytes (control) was 2.04.

RESULTS

MORPHOLOGICAL CHANGES

Fig. 1 shows necrosis of hepatocytes and bile ductule proliferation at 26 hours after the second dose of TAA. Hepatic necrosis was most marked in zone 3 (liver acinus of Rappaport 1958) which led to collapse of the parenchyma; neutrophil polymorph infiltration accompanied by a few mononuclear cells was common. Necrosis extended to involve part of zone 2 in some animals. Proliferation of biliary cells was seen in the periportal space and this increased the number of bile duct profiles. Oval cells formed rows, clusters and duct-like structures which invaded the hepatic lobule.

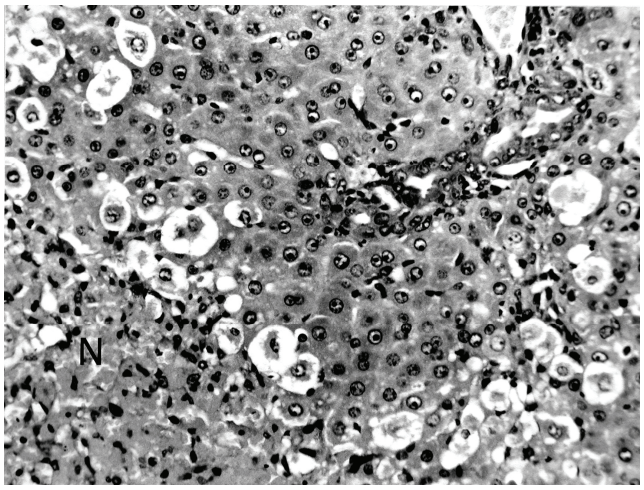

Fig. 1. *Morphological changes in the liver at 26 hours after TAA treatment. There is focal necrosis (N) with inflammatory cellular infiltrate in zones 2 and 3 with hyperplasia of bile ductules in zone 1. Haematoxylin and eosin stain (H&E) ×200.*

Oval cells exhibited a high nuclear/cytoplasmic ratio, prominent nucleoli and euchromatic chromatin. They were recognised by their characteristic slightly basophilic cytoplasm (Fig. 2), but they became more eosinophilic with time as the cells appeared to undergo differentiation towards hepatocytes, with the nuclear/cytoplasmic ratio becoming lower

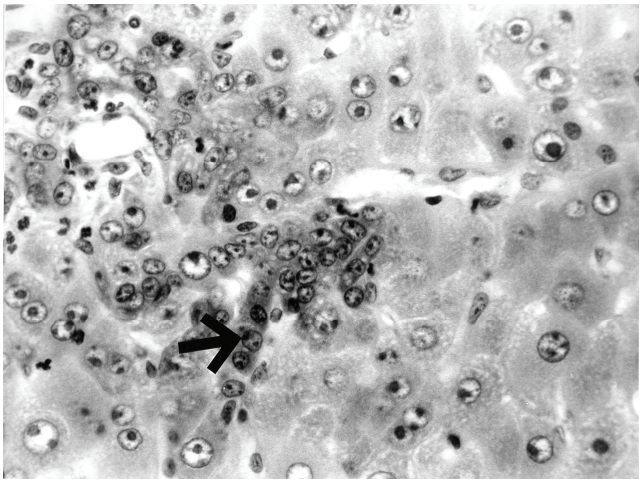

Fig. 2. *At 52 hours after TAA proliferation of oval cells in the portal tract extends into the parenchyma forming rows and duct-like structures (arrow). H&E, ×400. Note the high nuclear/cytoplasmic ratio, basophilic cytoplasm, oval shape of nuclei and their irregular contour in comparison with hepatocytes.*

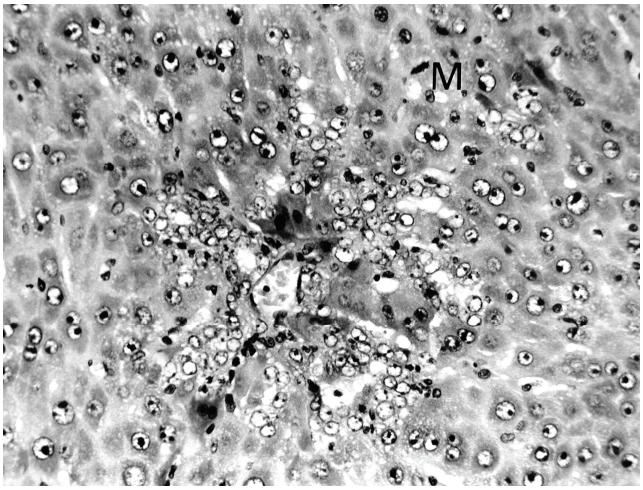

Fig. 3. *At 68 hours after TAA the oval cells clustered around the portal tracts are much more hepatocyte like, with a more eosinophilic cytoplasm and a lower nuclear/cytoplasmic ratio than seen before. Mitoses (M) were also evident in hepatocytes. H&E, ×200.*

The liver from control rats showed no oval cell proliferation. Mitoses were observed in oval cells and hepatocytes (Fig. 3) and were most prominent between 48-68 hours.

At 132 hours after TAA most oval cells had disappeared to be replaced by groups of small hepatocytes around the portal tracts (Fig. 4).

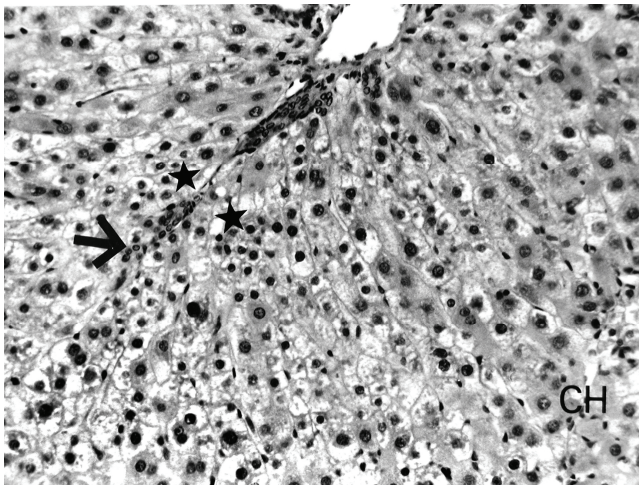

Fig. 4. *At 132 hours after TAA there are groups of hepatocytes with uniform small nuclei congregated around the portal tracts, presumably derived from oval cells. The size of these nuclei contrasts vividly with those of centrilobular hepatocytes (CH). Note a few remaining oval cells (arrow). H&E, ×200.*

STEREOLOGICAL FINDINGS

Fig. 5 shows the time-dependent changes after TAA in the area of the liver occupied by viable parenchyma, necrosis, oval cells and portal tract areas.

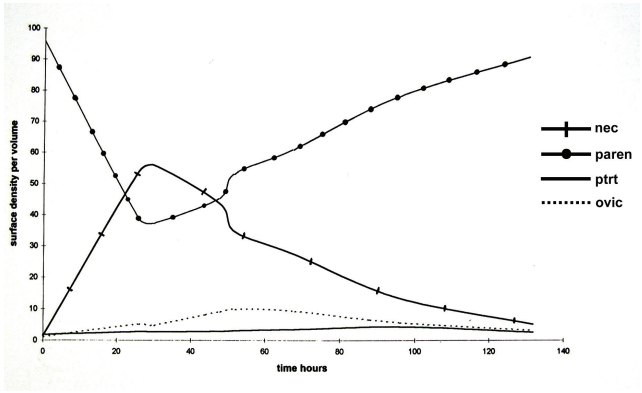

Fig. 5. *A computer drawn graph showing area fraction A_A. The changes in the liver occupied by necrosis (nec), parenchyma (paren), portal tract area (ptrt) and oval cell area (ovlc) assessed by stereology (point counting). Necrosis reached a maximum level at 30 hours and then declined. Restoration of the parenchyma began at 30 hours and occupied an increasing volume of the liver thereafter. Oval cells occupied the highest proportion of the liver at 48-52 hours.*

Over the period of observation (26-132 hours) the area of centrilobular necrosis increased to reach a maximum at 30h. The area occupied by oval cells increased to reach a maximum level at 48-52h and then declined. This decline coincided with an increase in the proportion of the liver occupied by parenchymal cells.

MORPHOMETRIC FINDINGS

Changes in the nuclei: Figs. 6a and 6b show the changes in nuclear areas and diameters of oval cells and hepatocytes. There was an increase in nuclear areas of oval cells and hepatocytes but the oval cell nuclear area always remained smaller than that of hepatocytes. At 132 hours, the nuclear area of hepatocytes decreased due to the increased number of small hepatocytes in zone 1 (Fig. 4).

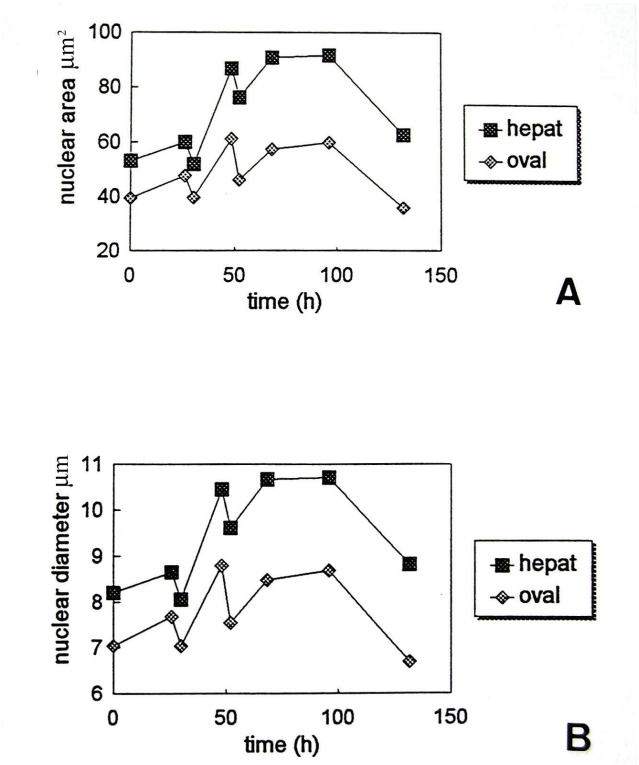

Fig. 6. *(A) The morphometric changes in nuclear areas and (B) nuclear diameters of hepatocytes and oval cells with time after TAA.*

Fig. 7 shows the changes of the axis ratio of both types of nuclei. Minimal changes were observed in the axis ratio of hepatocytes, however a significant decline in the axis ratio of oval cells was observed between 26h (1.66) to 96h (1.49) $p < 0.0002$.

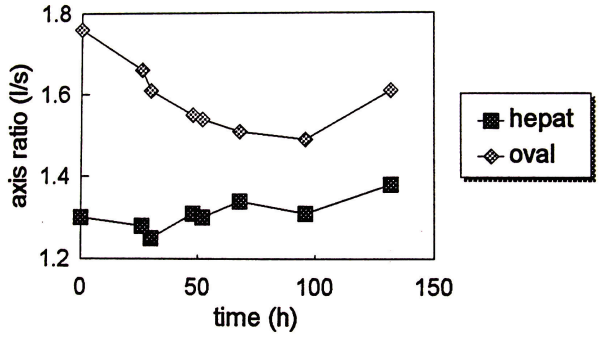

Fig. 7. *The axis ration (long/short) of oval cell and hepatocyte nuclei with time after TAA.*

Fig. 8 shows the changes in the nuclear roundness with their variance from the mean. Nuclear roundness of oval cells changed to reach a minimum level at 96 hours; the small variance in the roundness indicates that these changes were genuine.

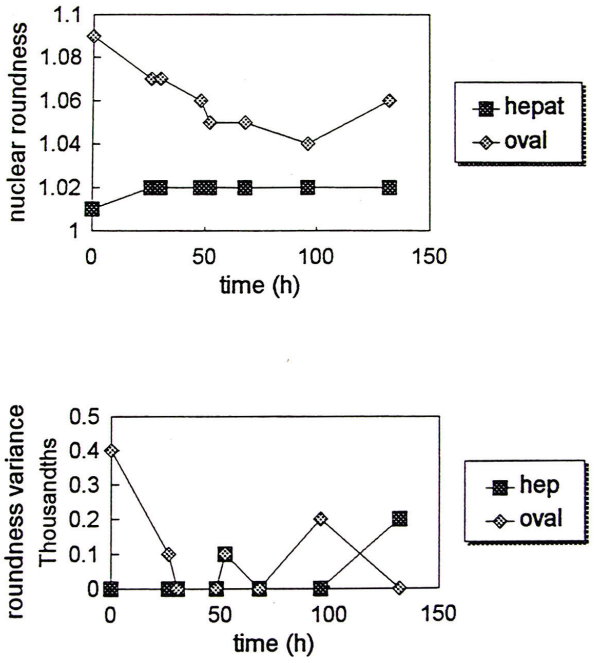

Fig. 8. *Nuclear roundness with the variance of oval cells and hepatocyte nuclei with time after TAA.*

The changes in the nuclear contour of oval cells with their variance were similar to those of the nuclear roundness (Fig. 9).

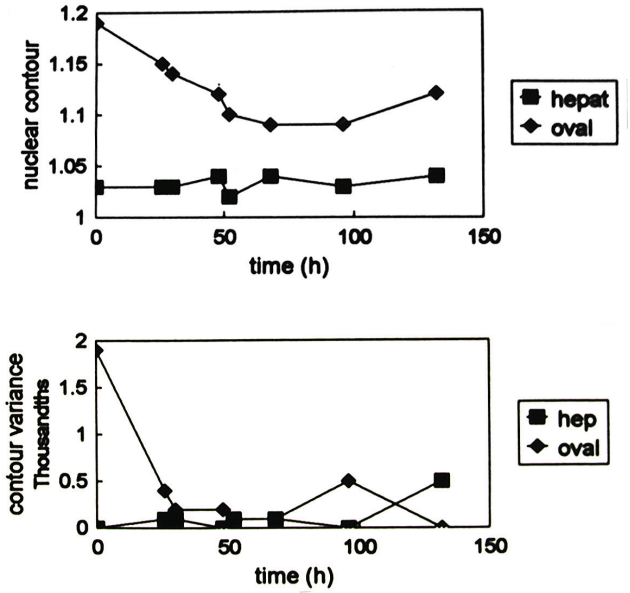

Fig. 9. *Nuclear contour with the variance of oval cells and hepatocyte nuclei with time after TAA.*

DISCUSSION

TAA is a chemical substance that selectively induces death in parenchymal cells of the liver. TAA metabolites bind to hepatocyte proteins with the formation of acetylimidolysine derivatives (Dyroff and Neal, 1981). In this animal model TAA elicits a zonal lesion mainly affecting zone 3 of the hepatic lobule (perivenous region). The periportal zone and portal tract areas are not adversely affected by TAA but become the site of regenerative changes. The histopathological changes observed in the liver after TAA injury were similar to those described after other injurious insults (Lemire *et al.,* 1991; Hsia *et al.,* 1992; Bennoun *et al.,* 1993; Dabeva and Shafritz, 1993; Sarraf *et al.,* 1994). The earliest changes were characterised by hyperplasia of bile ducts followed by proliferation of oval cells, mitoses were observed in hepatocytes located in zone 1 and 2.

In the present study we have used a basicaly stereological method to study the sequential cell changes in the liver of these treated animals. This method yields accurate estimation of the area fraction

A_A of all compartments in the tissue section. Our findings of a gradual increase in the oval cell population up to 52 hours with a progressive decrease thereafter, together with a steady increase in parenchymal area, support the role of oval cells in liver regeneration. Of course, the increase in parenchymal area is also attributable to concomitant hepatocyte regeneration. No differentiation of oval cells into cholangiolytic lesions was noted, and very little oval cell apoptoses suggesting neither mechanism accounted for the disappearance of oval cells.

We have also used a morphometric analysis to delineate major and minor characteristic features of the nuclei and nucleoli of hepatocytes and compared them with those of oval cells. Hepatocytes in treated and control groups have a higher nuclear area with a fairly uniform rounded nuclei (nuclear roundness approaches 1). In comparison oval cells have an irregular nuclear outline with high axis ratio and both nuclear roundness and contour ratios were greater than 1.

In the current study, there was an increase in both nucleolar and nuclear areas of TAA treated hepatocytes compared to control animals. Similar changes have been observed by other authors using a different animal model (Dabeva and Shafritz, 1993).

Unlike in hepatocytes, the area of oval cell nuclei increased and so did the nucleolar area. Changes in nuclear roundness and nuclear contour ratios were also seen in oval cells, and the analysis of variance showed that the changes observed in the mean values were statistically significant.

Our results have shown that activation and morphological transformation of progenitor cells takes several days (up to 96 hours). Changes in the function and the differentiation of progenitor cells to hepatocytes have been studied by several authors (De Vos and Desmet, 1992; Dabeva and Shafritz, 1993; Roskams *et al.,* 1996). The similarity of oval cells to both bile duct cells and hepatocytes is seen in their characteristic immunohistochemical reactivity to cytokeratin antibodies. Activated progenitor cells stained strongly with monoclonal antibodies raised against cytokeratins 8 and 19 (Sarraf *et al.,* 1994). In normal liver, both bile duct cells and hepatocytes express cytokeratins 8 and 18, whereas cytokeratins 7 and 19 are exclusively expressed by bile duct cells (Eyken *et al.,* 1987). Following the fate of ductular cells by labelling them with tritiated thymidine, Lemire *et al,* (1991), demonstrated a precursor-product relationship between bile ductular cells and both oval cells and small hepatocytes. Grisham (1980) found that hepatocytes in culture are non-clonogenic and suggested that the liver epithelial cell lines that display some hepatocyte-like features *in vitro* originate from stem cells. Grisham also introduced the term facultative for these cells indicating that they are only activated after hepatic necrosis when hepatocyte replication is compromised. The finding of proliferative oval cells in our experimental model is in agreement with those of (Grisham, 1980).

Progenitor stem cells are called oval cells because of their characteristic shape (Farber, 1956) a fact confirmed by electron microscopy (Sarraf *et al.*, 1994). Our morphometric results revealed a change from oval-like cells with irregular elongated nuclei to hepatocyte-like cells with more regular and rounded nuclei.

We conclude that in TAA treated rats, pericentral necrosis is restored by activation of proliferation of bile duct-like cells and partially by mitotic activity in hepatocytes. Stem cells (oval cells) proliferate and differentiate into hepatocytes and acquire larger nuclei. However, the nuclei of these cells did not reach the same size as pre-existing hepatocyte nuclei in this animal model.

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