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Transforming Growth Factor-β1 Protein, Proliferation and Apoptosis of Oval Cells in Acetylaminofluorene-Induced Rat Liver Regeneration

Administering of 2-acetylaminofluorene (2-AAF) before a two-thirds partial hepatectomy (PHx) results in suppression of hepatocyte proliferation and stimulation of oval cell proliferation. The objectives of this study was to examine the oval cell behaviour and associated transforming growth factor-β1 (TGF-β1) protein expression by combining 2-AAF with selective hepatic damage caused by PHx. We also studied the temporal relationship between TGF-β1 expression, and proliferation and apoptosis of oval cells. Oval cells emerged from the portal areas and became more numerous with time fanning out into the periportal and midzonal hepatic parenchyma. Both smooth muscle actin (SMA) and TGF-β1 immunostain revealed that TGF-β1-positive cells were SMA-positive hepatic stellate cells (HSCs). Coinciding with the proliferation of oval cells, an increase expression of TGF-β1 produced by SMA-positive HSCs was observed, thereafter apoptosis of oval cells reached its peak. This result implicated that TGF-β1 produced by HSCs is intimately associated with proliferation and apoptosis of oval cells, and plays a role in the cessation of oval cell activation and remodeling of liver parenchyma in 2-AAF induced liver regeneration.

Key Words: *Transforming growth factor beta; Cells, oval; Apoptosis; Immunohistochemistry*

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INTRODUCTION

The customary wave of hepatocyte regeneration, which occurs in the rat liver after two-thirds partial hepatectomy (PHx), can be abolished by oral administration of carcinogen 2-acetylaminofluorene (2-AAF). Instead, regeneration is achieved through the proliferation and differentiation of oval cells (1). Thus cessation of liver regeneration induced by oval cells is achieved by apoptotic cell death. Administration of 2-AAF induces apoptosis of oval cells in the portal areas, a process that contributed to the overall retention of liver morphology after liver regeneration, as well as mitogenic response of oval cells (2).

During normal hepatic regeneration as well as during renewal from the stem cell compartment (oval cell), several growth factors appear to affect the proliferation and differentiation of hepatic cells (3-5). Furthermore cessation of cell growth, after liver mass has been restored, appears to be caused by growth inhibitors produced in the liver. It has been proposed that transforming growth factor-β1(TGF-β1) is a negative growth signal controlling liver size after the compensatory hyperplasia that occurs after loss of liver mass (6). TGF-β1 has varied and often conflicting effects. It is a growth inhibitor to most epithelial cell types in cultures. But, its effects on fibroblasts and smooth muscle cell proliferation favor fibrogenesis, and indeed there is increasing evidence that TGF-β1 is involved in the development of fibrosis in a variety of chronic inflammatory conditions (7, 8). Also, it has been reported that TGF-β1 plays an important role in inducing apoptosis in hepatoma cell line, hepatocytes and oval cells (9-11). But its cellular and temporal expression in oval cell proliferation and apoptosis is uncertain. We studied oval cell behaviour by histologic and ultrastructural method, expression of TGF-β1 and proliferation of oval cells by immunohistochemical method, and apoptosis of oval cells by in situ by specific labeling of nuclear DNA fragmentation (TUNEL method) after PHx in 2-AAF treated liver regeneration.

MATERIALS AND METHODS

Chemicals

2-acetylaminofluorene (2-AAF), carboxymethylcellu-

lose and dimethlysulfoxide were all obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2-AAF was dissolved in a small volume of dimethylsulfoxide and suspended in 1% aqueous solution of carboxymethylcellulose to obtain a final concentration of 1.5 mg/mL.

Experimental design

2-AAF/PHx model

Male Sprague-Dawley rats, weighing 200 g, were used and maintained on standard pelleted chow and had access to water ad libitum. To inhibit hepatocyte cell proliferation, all rats received a daily oral gavage of 10 mg/kg of 2-AAF for a period of up to 14 days. After seven days of the regimen, all rats underwent a two-thirds PHx under diethyl ether anesthesia; five animals were killed as indicated 1, 2, 3, 6, 8, 10, 14, 21 day after initiation of the PHx. For routine light microscopy, immunohistochemcial stain, and TUNEL method, sections of liver were fixed in 10% neutral buffered formalin. Control rats received only carboxymethylcellulose-dimethylsulfoxide without PHx.

PHx model

Without a daily oral gavage of 10 mg/kg of 2-AAF, rats underwent a two-thirds PHx under diethyl ether anesthesia; one animal was also killed as indicated 1, 2, 3, 6, 8, 10, 14, 21 day after initiation of the PHx.

Routine morphological examinations and immunohistochemical stain

Tissue from each specimen was fixed in 10% neutral buffered formalin and embedded in paraffin. Routine histological examinations were made for all liver tissue samples on paraffin sections stained with hematoxylin and eosin. For electron microscopic observation, tissue samples not exceeding 1 mm^3 in volume were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, postfixed with 1% osmium tetroxide, dehydrated, and embedded in Epon. Serial semithin sections were cut from three to four pieces of each liver. A series of ultrathin sections were obtained from a selected area of semithin sections. The sections were stained with uranyl acetate and lead citrate, and studied by JEOL electron microscope.

For immunohistochemcial stain, sections were dewaxed and immunostained by the avidin-biotin peroxidase complex (ABC) method with aminoethylcarbazole (AEC) as chromogen using LSAB kit (Dako Corp., Carpinteria, CA, U.S.A.). For the detection of TGF-β1, activated hepatic stellate cells(HSCs), and proliferating cell, primary antibodies against TGF-β1 (Santa-Cruz, CA, U.S.A.), α-smooth muscle actin (SMA) (Dako Corp., Carpinteria, CA, U.S.A.), and proliferating cell nuclear antigen (PCNA) (Dako Corp., Carpinteria, CA, U.S.A.) were used at dilution 1:50, respectively. For each, antibody-negative controls were performed by either blocking with the appropriate nonimmune serum or by omitting the primary antibody from the protocol.

Detection of apoptotic cells

Cells undergoing programmed cell death (apoptosis) were detected in situ by specific labeling of nuclear DNA fragmentation (TUNEL method). For TUNEL method, we used ApopTag In situ apoptosis detection kit/peroxidase (Oncor, Gaithersburg, MD, U.S.A.). Paraffin-embedded thin sections (5 μm) were deparaffinized and digested with 20 μg/mL proteinase K (Dako Corp., Carpenteria, CA, U.S.A.). Endogenous peroxidase activity was blocked with a treatment of 2.0% hydrogen peroxide in phosphate buffered saline (PBS) for 5 min. After application in equilibration buffer, the sections were incubated with working strength TdT enzyme in a humidified chamber at 37℃ for 1 hr. Sections were rinsed with working strength stop/wash buffer and incubated with anti-digoxigenin-peroxidase for 30 min. Diaminobenzidine (Dako Corp., Carpenteria, CA, U.S.A.) was applied for color development.

Measurement of cellular proliferation and apoptosis

The selected portal/periportal and centrilobular areas were examined for detection of PCNA positive proliferating cells and apoptotic cells under light microscope at magnification of 400×. Proliferating and apoptotic cells were measured in 10 randomly selected separate portal/periportal and centrilobular areas per animal. Five animals were used for each experimental point. Mean and standard deviation of the total number of proliferating cells and apoptotic cells per animal at each experimental point was used to generate the data shown in Table 1 and Fig. 4. Statistical analysis included Kruskal-Wallis test using SPSS program. Results were considered statistically significant, if $p<0.05$.

RESULTS

Morphological changes in 2-AAF/PHx model

At day 1 after PHx, small basophilic cells with high nuclear: cytoplasmic ratio (oval cells) were evident in and around the portal tracts. Oval cells were close to hepatocytes at limiting plates (Fig. 1A). Oval cells became more

Table 1. Distribution of PCNA-positive cells and apoptotic cells after PH in AAF-treated rat

Cell type	Area	Days after PH							
		1	2	4	6	8	10	14	21
PCNA(+) Cell*	Portal/periportal	44.4±8.9	258.2±27.9	438.4±25.2	628.6±61.1	874.4±91.5	567.0±35.0	70.8±12.8	15.0±5.3
	Centrilobular	3.2±2.4	41.6±14.4	132.8±18.1	320.0±34.0	422.6±18.5	232.4±25.8	29.8±10.9	17.0±5.0
Apoptotic Cell*	Portal/periportal	5.2±3.9	20.4±9.4	113.4±11.0	220.6±27.9	296.2±19.3	407.4±22.7	58.2±16.3	20.4±9.8
	Centrilobular	1.2±1.3	8.0±3.5	48.6±12.1	80.0±14.0	120.8±15.5	214.0±21.9	45.0±16.6	12.0±3.7

PH, partial hepatectomy
Values are mean±SD.
*Significance of value difference among each experimental point ($p<0.05$)

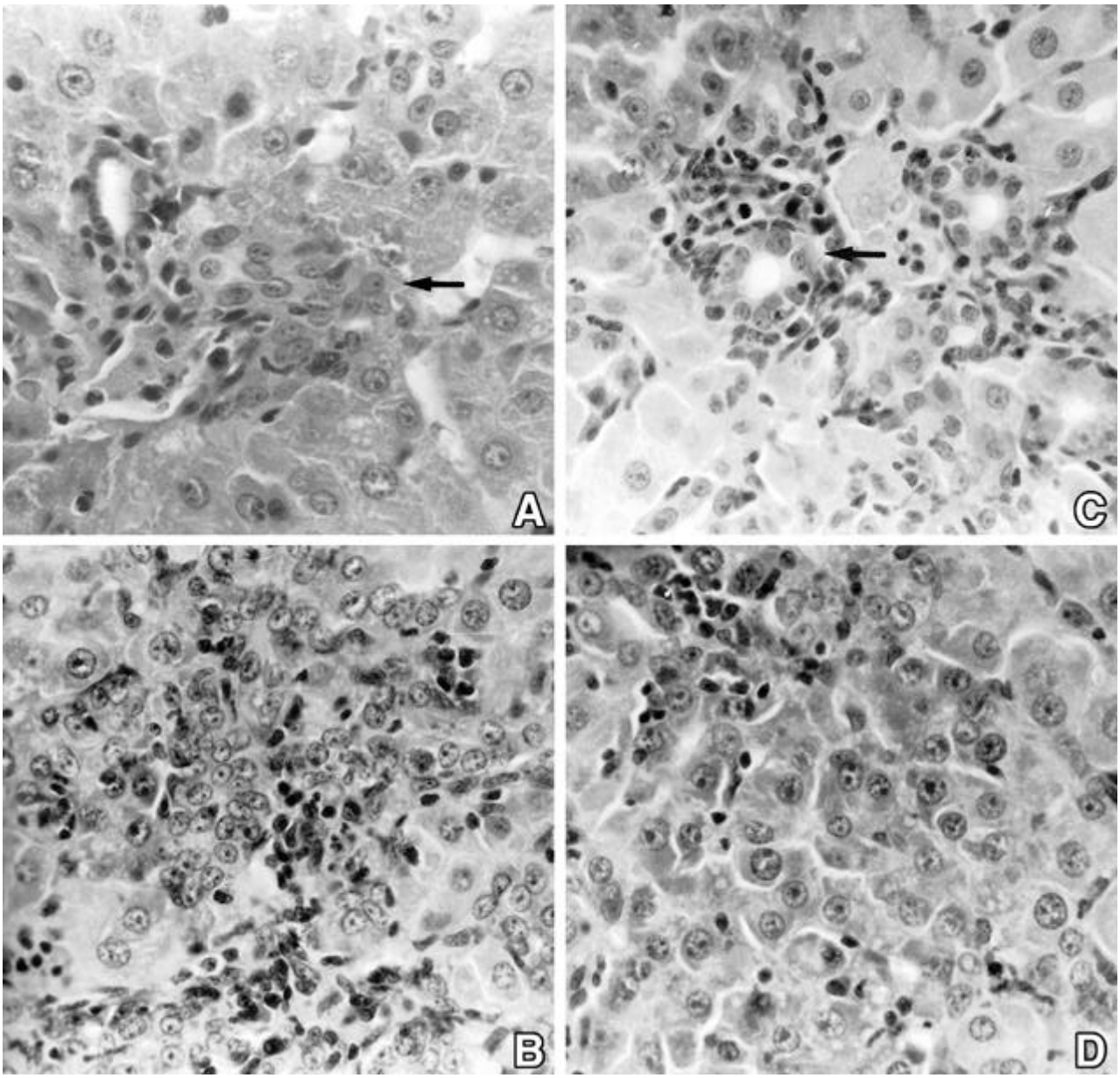

Fig. 1. Oval cells (arrow) close to hepatocytes at limiting plates at 1 day after PHx (A), long strips of oval cells fan out into the periportal and midzonal parenchyma (B), small lumina (arrow) surrounded by clustered ductular cells (C), and small strings of oval cells at the periphery of the small basophilic hepatocytes at 8 days after PHx (D) (H&E, ×400).

numerous with time and by day 8 long strings of these cells could be seen fanning out into the periportal and midzonal parenchyma (Fig. 1B). They formed small lumina surrounded by clustered ductular cells (Fig. 1C). Simultaneously, periportal areas were colonized by small basophilic hepatocytes, though small strings of oval cells could still be discerned at the periphery of the basophilic hepatocytes (Fig. 1D). Spindle cells adjacent to proliferated oval cells formed a meshwork around the oval cells and hepatocytes. These features were more apparent on immunostain for smooth muscle actin for activated HSCs (Fig. 5B). And with time, inflammatory cells, mainly polymorphonuclear leukocytes, infiltrated periportal areas. Control rats, which received only carboxymethylcellulose-dimethylsulfoxide without PHx, showed unremarkable findings.

Electron microscopic examination showed that there are at least two "oval" cell types. Oval cells with or without lumina, which exist as individual cells or clusters. They may be classified into two types; oval cells showing features of ductular cell differentiation or oval cells showing features of hepatoid cell differentiation. Both of these types had characteristics of proliferating cells with a cytoplasm rich in polysomes. Ductular cell-differentiating oval cells had ductule characteristics; microvilli, tight junctions, basement membranes (Fig. 2A). Hepatoid cell-differentiating oval cells showed characteristics of hepatocytes; abundant cytoplasmic organelles with prominent nucleoli and canalicular microvilli (Fig. 2B). Ultrastructural studies also showed that HSCs containing cytoplas-

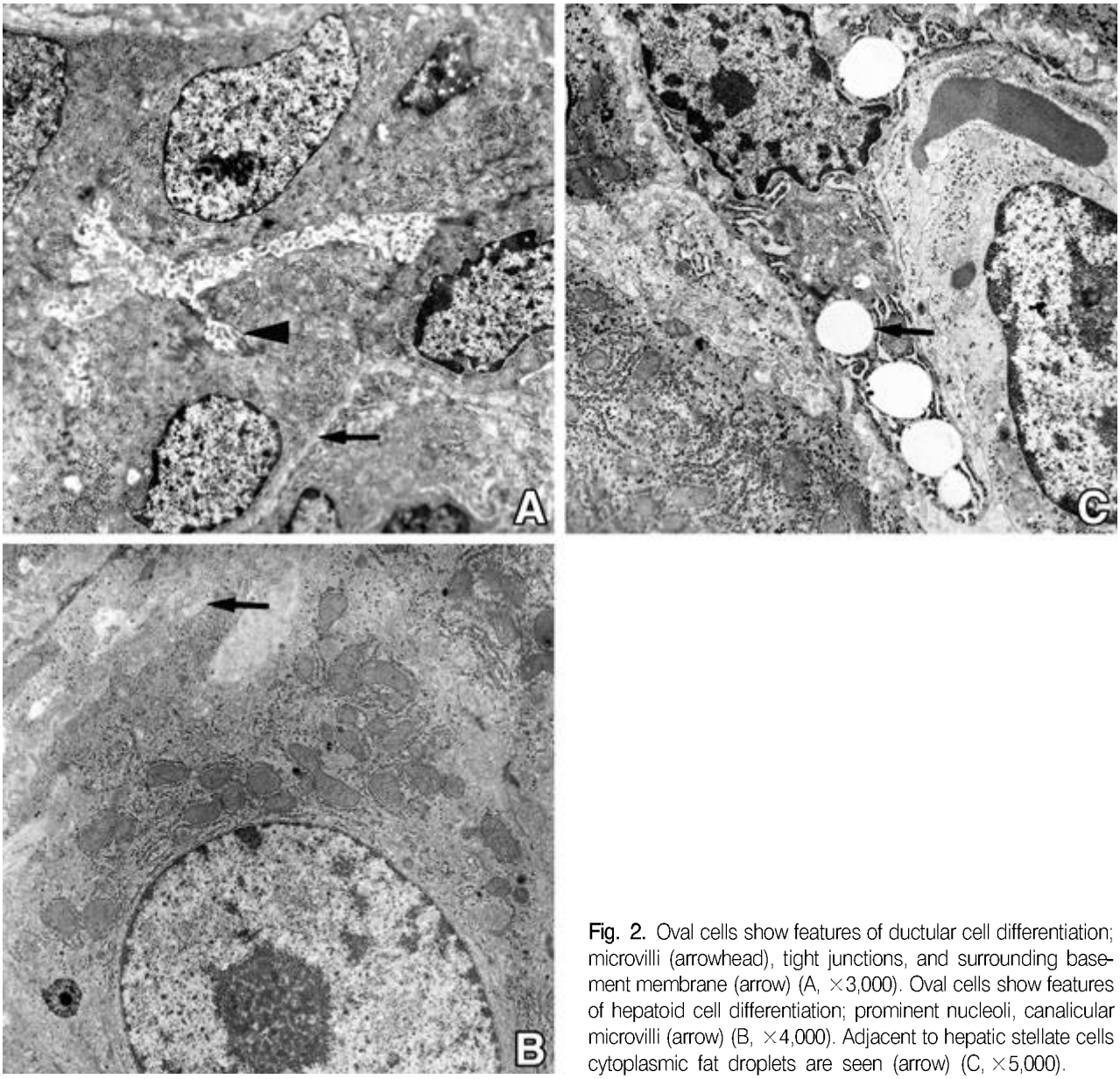

Fig. 2. Oval cells show features of ductular cell differentiation; microvilli (arrowhead), tight junctions, and surrounding basement membrane (arrow) (A, ×3,000). Oval cells show features of hepatoid cell differentiation; prominent nucleoli, canalicular microvilli (arrow) (B, ×4,000). Adjacent to hepatic stellate cells cytoplasmic fat droplets are seen (arrow) (C, ×5,000).

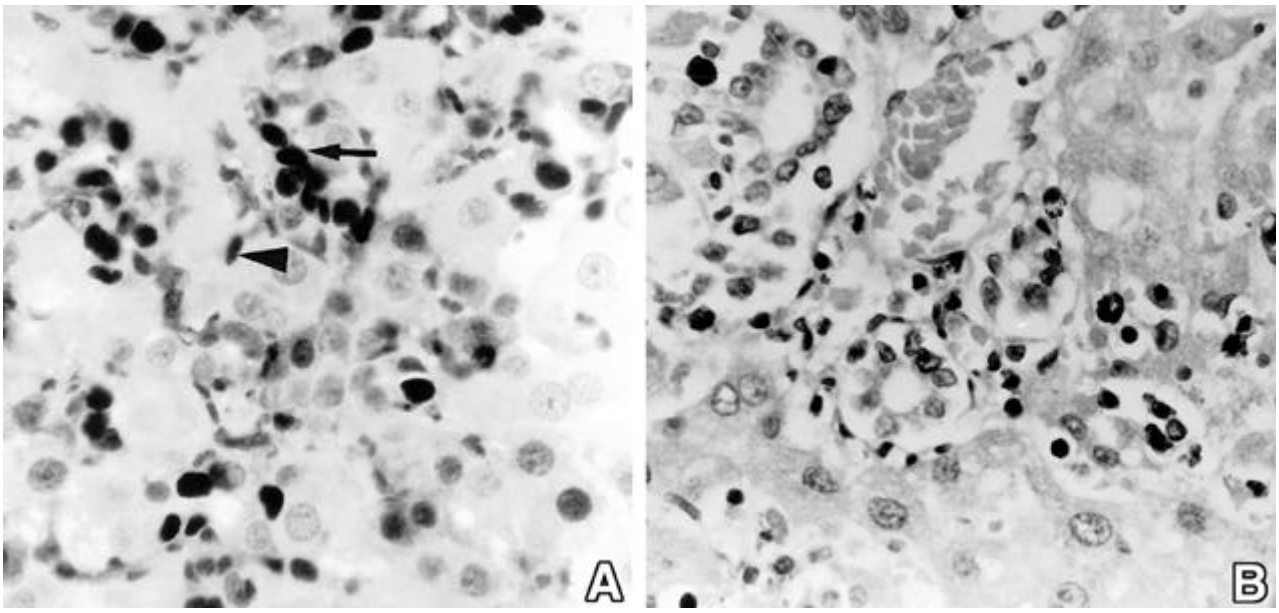

Fig. 3. PCNA positive proliferating oval cells (arrow) and spindle cells (arrowhead) (A) (immunostain for PCNA, ×400), and apoptotic cells (B) at 8 days after PHx (TUNEL method, ×400).

mic fat droplets were seen adjacent to oval cells and hepatocytes (Fig. 2C).

Cellular proliferation and apoptosis in 2-AAF/PHx and PHx models

PCNA immunostain revealed that cell proliferation was at first most noticeable in the small interlobular bile ducts, and as time elapsed in the strings of oval cells and intervening meshwork of spindle cells, which were probably SMA-positive HSCs (Fig. 3A). Table 1 showed that PCNA positive cells became more numerous with time and by day 8 reached its peak and fanned out into centrilobular areas. Thereafter, PCNA positive cells were decreased. But most of the PCNA positive cells were hepatocytes in the PHx group. Apoptotic cells were evident mainly in periportal areas. With time apoptotic cells were far more numerous and by day 10 reached its peak, and were also seen in centrilobular areas (Fig. 3B) (Table 1). Fig. 4. showed that PCNA-positive proliferating oval cells and spindle cells reached its peak of proliferation by day 8 after PHx while apoptotic cell death reached its peak by day 10 after PHx. This result explained why the peak of apoptotic cell death appeared after the peak of oval cell proliferation measured by PCNA immunostaining. Control rats, which received only carboxymethylcellulose-dimethylsulfoxide without PHx, showed few PCNA-positive cells.

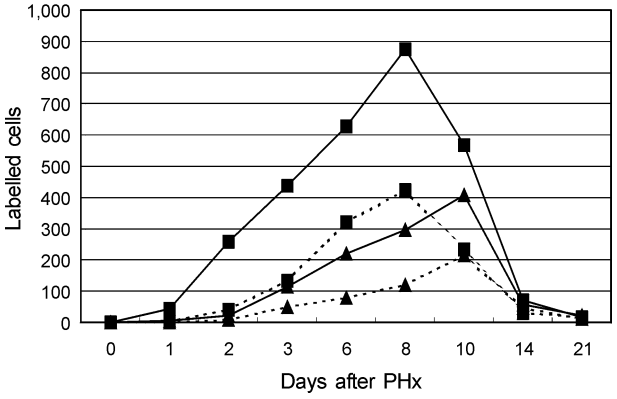

Fig. 4. PCNA-positive proliferating cells (■) and TUNEL-positive apoptotic cells (▲) in 2-AAF/PHx model. Data represent the mean and SD from measurements from 10 separate periportal/portal areas (solid line) and centrilobular areas (dotted line) per animal.

Cellular distribution of TGF-β1 in 2-AAF/PHx and PHx models

At day 1 after PHx, some TGF-β1-positive non-parenchymal cells and some inflammatory cells were seen in the periportal areas. By day 8, TGF-β1 expression reached its peak and TGF-β1 positive cells were observed in the meshwork of non-parenchymal cells around the oval cells and hepatocytes (Fig. 5A). Both SMA and TGF-β1 immunostain revealed similar linear positivity around the oval cells and hepatocytes. We speculated that TGF-β1 positive cells were SMA-positive HSCs (Fig. 5B). TGF-β1 positivity was also apparent in the fibrous stroma of widened portal areas and some inflammatory cells (Fig. 5C). In PHx model, TGF-β1 positivity was

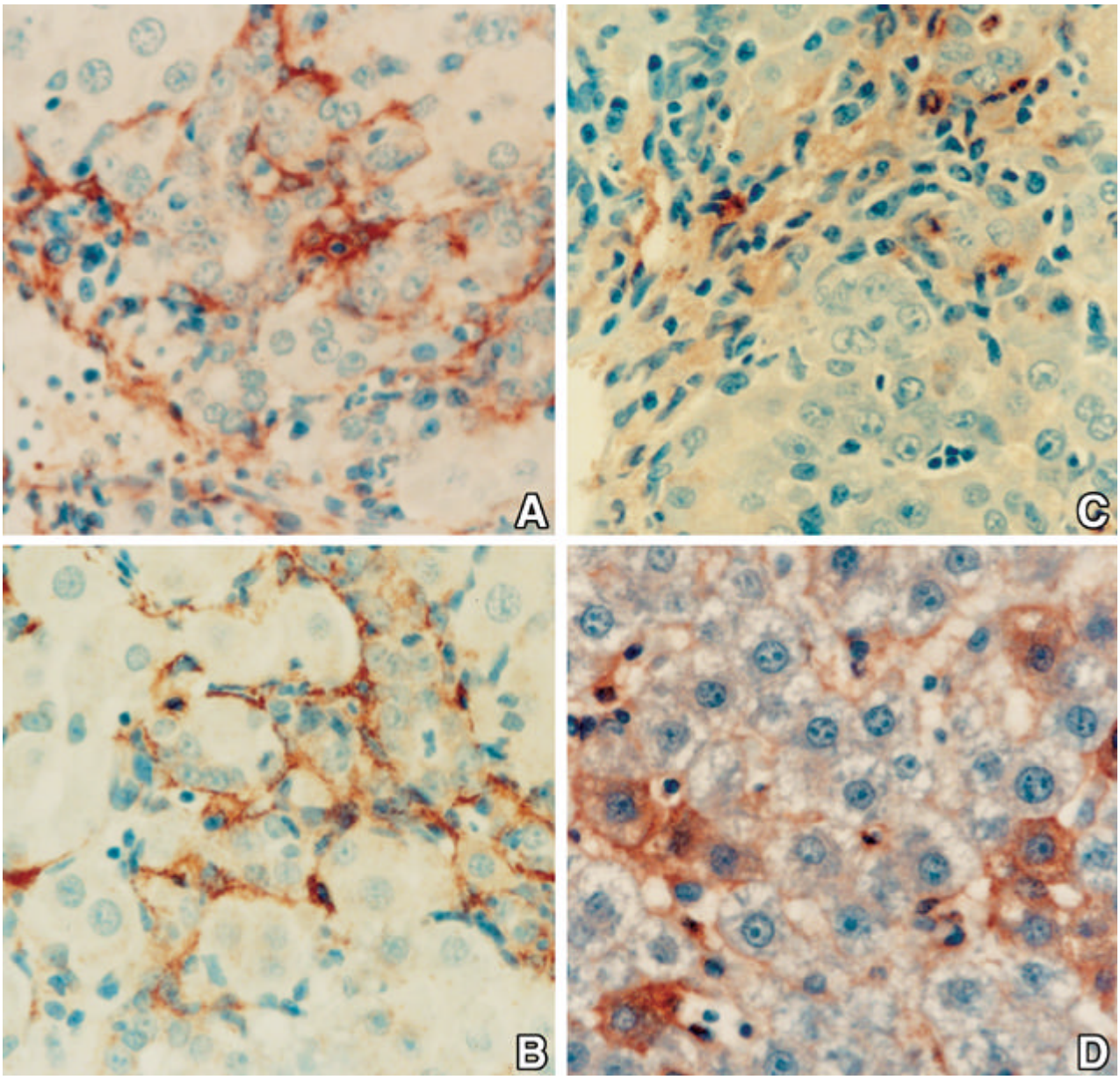

Fig. 5. Similar linear positivity around hepatocytes and oval cells in TGF-β1-immunostain (A) and smooth muscle actin-immunostain for hepatic stellate cells (B), and TGF-β1 positivity also in fibrous portal stroma (C) in 2-AAF/PHx model at 8 days after PHx. TGF-β1-positive hepatocytes in hepatic parenchyme in PHx model (D) at 8 days after PHx (immunostain for TGF-β1, ×400).

seen only in the cytoplasm of hepatocytes (Fig. 5D). Control rats, which received only carboxymethylcellulose-dimethylsulfoxide without PHx, showed minimal TGF-β1 expression in fibrous stroma of portal areas

DISCUSSION

The two new major cell populations that emerged after partial hepatectomy in the AAF-treated rats were oval cells and SMA-positive activated HSCs. Our studies showed that oval cells acquired features of both hepatoid and ductular cell differentiation and had an intimate relationship with HSCs. Several studies clearly showed that oval cells are epithelial cells, which are capable of differentiating, in addition to bile duct epithelium, into at least two lineages in vivo, including hepatocytes and intestinal type epithelium (12-14). Also, it had been established that proliferation of HSCs is closely associated with the early stages of oval cell proliferation in AAF-treated rats after PHx (3).

PCNA-positive proliferating oval cells first emerged in the small bile ductules and more numerous with time. In addition to oval cells, PCNA-positive spindle cells,

which were thought to be SMA-positive HSCs, appeared and were more numerous with time. By day 8 after PHx in AAF-treated rats, PCNA-positive proliferating oval cells reached its peak of proliferation and thereafter sharply decreased. Our data clearly showed that major sources of oval cells were lining cells of the biliary ductules and proliferation of oval cells was intimately associated with activation and proliferation of HSCs. Other detailed time course study of activation of HSCs after PHx in the AAF-treated rats demonstrated that the earliest population of proliferating OV-6 positive cells is located in the small bile ductules, utilizing a combination of immunohistochemistry with oval cell specific OV-6 and desmin antibodies and autoradiography after [^{3}H] thymidine administration (15). Our study also revealed that proliferated oval cells seemed to move from periportal areas to centrilobular areas. These features reinforce the morphologic evidence of oval cell behavior, oval cells could be seen fanning out into the periportal and midzonal parenchyma and acquired features of ductular cell differentiation or hepatoid cell differentiation.

Apoptosis has major roles in cell deletion in proliferating cell population, such as involution of liver hyperplasia, deletion of hyperplastic biliary epithelial cells following removal of the ligation of bile ducts, and removal of proliferated hepatic non-parenchymal cells (Kupffer's cells, endothelial cells, pit cell, and HSCs) (16-18). In our study, apoptotic cells were also seen in the small bile ductules and periportal areas, and became numerous with time and seemed to move from periportal areas to centrilobular areas. Apoptotic cell death reached its peak by day 10 after PHx in AAF-treated rats. A detailed time course examination suggested that the peak of apoptotic cell death appeared after cellular (oval cells, HSCs) proliferation peaked. Thus, we thought that cessation of cellular proliferation coincided with apoptotic cell death and proliferated oval cells, and HSCs were removed by apoptotic cell death with cessation of cellular (oval cells, HSCs) hyperplasia.

Soluble growth factors which are concerned with controlling apoptotic cell death, are important in remodeling hepatic parenchyma that had been damaged by toxins, radiation injury, or other stimuli (19). TGF-β1 is a multifunctional polypeptide, promoting embryogenesis (20) and collagenesis (21), while inhibiting hematopoeisis (22) and growth of epithelial cells (23, 24), including hepatocytes (25, 26) and most malignant cells (27-29). Furthermore, its expression at the later stage of liver regeneration has been reported. It proposed that TGF-β1 is a negative growth signal controlling liver size by induction of apoptosis in the compensatory hyperplasia that occurs after loss of liver mass (30). Expression of TGF-β1 is cell-specific. After injury TGF-β1 increases selectively in the cells which are the target of the factor, i.e., in hepatocytes after partial hepatectomy and in HSCs in inflammation and fibrosis (31). In our study, expression of TGF-β1 was evident in the meshwork of SMA-positive activated HSCs around the oval cells in 2-AAF/PHx model, but in hepatocytes in PHx model. Proliferation of TGF-β1-producing, SMA-positive activated HSCs were intimately associated with proliferation of multipotent oval cells. Furthermore coinciding with the proliferation of oval cells, an increased expression of TGF-β1 produced by SMA-positive HSCs was observed, then apoptosis of oval cells reached its peak. Thus, TGF-β1, which is produced by activated HSCs, might play an important role in controlling cessation of oval cell proliferation by inducing apoptosis after PHx in AAF-treated rats. Other studies suggest that TGF-β1 might play major roles in inducing apoptosis in hepatoma cell line, hepatocytes and oval cells (9-11). Therefore, we think that TGF-β1 produced by HSCs might have important roles in inducing apoptosis of oval cells, resulting in cessation of oval cell activation and remodeling of liver parenchyma in 2-AAF induced liver regeneration. Therefore, future investigation should focus on exact molecular mechanisms of TGF-β1 action on proliferation and apoptosis of oval cells.

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