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THE STELLATE CELLS PHENOTYPIC TRANSFORMATION IN THE CCl_4- INJURED LIVER FIBROSIS OF ICR MICE : Their desmin immunoreactivity and vitamin A storage

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SYNOPSIS

To elucidate the process of phenotypic transformation of stellate cells which were responsible in the production of collagen fibers, ICR mice were injected subcutaneously with CCl_4 twice weekly for 5 weeks and the livers were examined by light and electron microscopy every week. Stellate cells were detected by desmin immunoreaction and vitamin A-autofluorescence. In the first week, following cell necrosis in the pericentral areas, granulomas were formed. Two distinct stellate cell phenotypes were developed i.e. desmin-negative cells in the granuloma and a row of intense desmin-positive cells in its boundaries. Both phenotypes seemed to be derived mostly from perisinusoidal stellate cells and involved in collagen synthesis as evidenced by the presence of reticulin meshwork within the granulomas. The meshwork formation was the initial stage of a new septum formation since in the second week the intense desmin-positive cell rows were closer to the central vein concomitant with the growth of several protrusions to form thin layer septa towards neighboring central or portal veins. The myofibroblast-like cells in the septa with features interposed between stellate cells and myofibroblasts were likely derived from the stellate cells phenotypes. During the transformation of stellate cells, the decline of vitamin A storage was accompanied by the formation of connective tissue septa. The desmin immunoreactivity and vitamin A storage in the stellate cell phenotypes during liver fibrogenesis described in this study may reveal their phenotypic transformation in association with the collagen synthesis.

INTRODUCTION

In an injured liver, perisinusoidal fibrogenesis contributes a major part to the development of liver cirrhosis (cit. Gressner 1991). It has been known that a liver comprises of various cell types i.e. the parenchymal cells which occupy 80-85 % of the total volume and the remainder non-parenchymal cells which include endothelial cells,[36,38] Kupffer cells,[6,37] pit cells or liver-associated large granular lymphocytes [20] and perisinusoidal stellate cells.[33,25] Although the number of stellate cells is much smaller than hepatocytes,[33] they are postulated as the most important extracellular matrix-producing cell types.[10,11,14,26,31] Stellate cells are characterized by vitamin A-containing lipid droplets[2,19] and 10 nm desmin filaments in their cytoplasm.[39] In the carbon tetrachloride-induced liver fibrosis, stellate cells transform into the transitional cells and produce collagen type III,[21]

collagen type I, type IV, laminin and fibronectin, [23] entactin [25] and tenascin. [32]

A number of cells in the stellate cell lineage have been demonstrated to interpose morphologically and functionally between the endothelial and smooth muscle cell axis. [34] This view is supported by the presence of desmin cytoplasmic filaments [39] and their vitamin A-storage in the stellate cells. From the above view, the detail process of stellate cell phenotypic transformation with their desmin and vitamin A content correlated to the enhancement of collagen production needs a profound answer. Therefore in the present study, we have concomitantly examined the desmin immunoreactivity, vitamin A-storage and the collagen formation of stellate cell phenotypes during the hepatic fibrosis induced by CCl_4. We have also compared the histochemical and immunohistochemical data with the morphology obtained by electron microscopy.

MATERIALS AND METHODS

Twenty four male mice of ICR strain weighing 30 to 35 gram were fed on standard mice cake diet (Clea Japan, Tokyo, Japan) and water *ad libitum*. The animals received subcutaneous injections of CCl_4 0.3 ml/kilogram of body weight (10% v/v in liquid paraffin) twice weekly. Each group of 4 animals was sacrificed in weeks 1, 2, 3, 4 and 5 after treatment. The livers were perfused through the portal vein with saline at room temperature by a pump system. One or two lobes of each liver were removed and excised into several blocks and processed for light microscopy. While for electron microscopy, the remaining lobes were perfused-fixed with 1.5% glutaraldehyde in 0.062 M cacodylate buffer (pH. 7.4) containing 1% sucrose.

Light microscopy

Gold chloride method : Tissue blocks were stained by the Kupffer's gold chloride method for detecting retinol-containing lipid droplets in the stellate cells. [35] Blocks were immersed in 0.05% chromic acid for about 2 hours and cut with a freezing microtome at 50 μm in thickness. The sections were rinsed in the same solution for 10 minutes and quickly unfolded with a glass stick. These sections were incubated in the gold staining solution containing 1 ml of 1% gold chloride, 1 ml of 0. 1N HCl and 98 ml of distilled water for 12-16 hours at 18-20 °C in total darkness. The sections were dehydrated with graded alcohol, cleared with xylol and mounted in

balsam.

Fluorescence microscopy for vitamin A : The liver tissue was fixed in 10% formol for 12-24 hours at 4∞C in total darkness then cut on 10 μm thickness with a freezing microtome. The sections were mounted with water and photomicrographed immediately under a fluorescence microscope using a U (UGL) filter for excitation and a U or V filter for emission. The film was developed with Konidol Super (Sakura Co. Ltd.).

Immunohistochemistry for desmin : Frozen tissue blocks were cut at 20 μm thickness with a cryostat (Reichert-Jung 2800 Frigocut). After being fixed with cold acetone at 4° C for 20 minutes, the sections were incubated with rabbit anti-desmin polyclonal antibody (diluted 1:200, Dako, Copenhagen, Denmark) for 1 hour at 37 °C. After being washed thoroughly with PBS, they were incubated with goat anti-rabbit immunoglobulin (diluted 1:50, Dako, Copenhagen, Denmark) for 1 hour at room temperature and then incubated with peroxidase labelled Avidin-Biotin complex (Vectastain ABC, Vector laboratories CA) for 30 minutes. The immuno-reaction was visualized by adding 0.025% 3,3-diaminobenzidine tetrachloride (Sigma) and 0.015% hydrogen peroxide in 0.1 M Tris-HCl buffer (pH 7.3) for 10 minutes. The sections were counterstained with methyl green. Normal rabbit serum (diluted 1:500) was substituted for primary antibody to serve as a non-immune control.

Hematoxylin-eosin and the silver staining : The tissues were fixed with 10 % formol and embedded in paraffin wax. Five μm sections were stained either with hematoxylin-eosin or the Suzuki's silver impregnation method[7] for observing general histology and reticulin fibers, respectively.

Electron microscopy

The perfused-fixed tissues were cut into small pieces approximately 1 mm^3 and postfixed in phosphate-buffered (pH. 7.4) containing 1% osmium tetroxide for 2 hours at room temperature. They were dehydrated and embedded in Poly/Bed 812. Semithin sections of 1 μm thickness were stained with toluidine blue for ultrathin preparation, The ultrathin sections were double-contrasted with uranyl acetate and lead citrate and then examined with a JEOL-100CX electron microscope at an acceleration voltage of 100 kV.

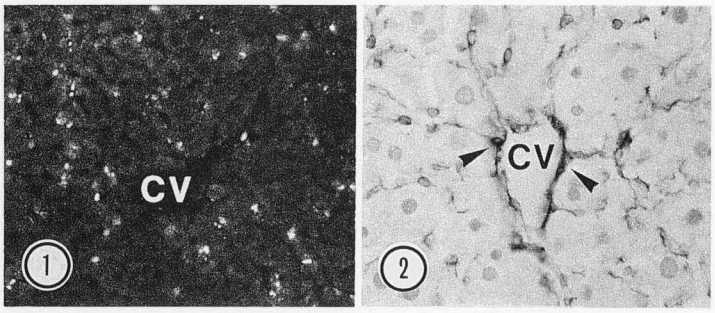


Fig. 1. Liver of the normal mice, demonstrated the distribution of vitamin A-storing cells within the lobulus. **CV**, central vein. Fluorescence micrograph ×100.

Fig. 2. Intense desmin immunoreaction (***arrowhead***) of smooth muscle cells surrounding central vein (**CV**) and desmin-positive stellate cells with elongated cytoplamic extension within the lobules. Normal liver mice. ABC method ×200.

RESULTS

Light microscopy

Livers of control animals

Under a fluorescence microscope, a quick fading vitamin A-fluorescence scattered within the hepatic lobules (**Fig. 1**). The sources of the fluorescence coincided with the lipid droplets of stellate cells reacted to gold chloride. Reduced gold was precipitated primarily on the surface of lipid droplets and dispersed in the cytoplasm of cell body and main processes of the cells, hence the star-shaped configuration of the cells appeared among a red background of parenchymal cells. Intensity of vitamin A fluorescence as well as the gold reaction per one individual cell was stronger in the periportal area than that in the centrilobular area. Intense desmin immunoreaction was observed in smooth muscle cells of the large blood vessels and central veins while a less intense reactivity was encountered in the cell body and processes of perisinusoidal stellate cells (**Fig. 2**).

Histopathology of the CCl_4 treated liver

In the first week of the experiment, after twice injections of CCl_4, necrotic hepatocytes occurred in the pericentral areas. Within the necrotic zone, inflammatory mononuclear cells and mesenchymal fibroblastic cells accumulated to form

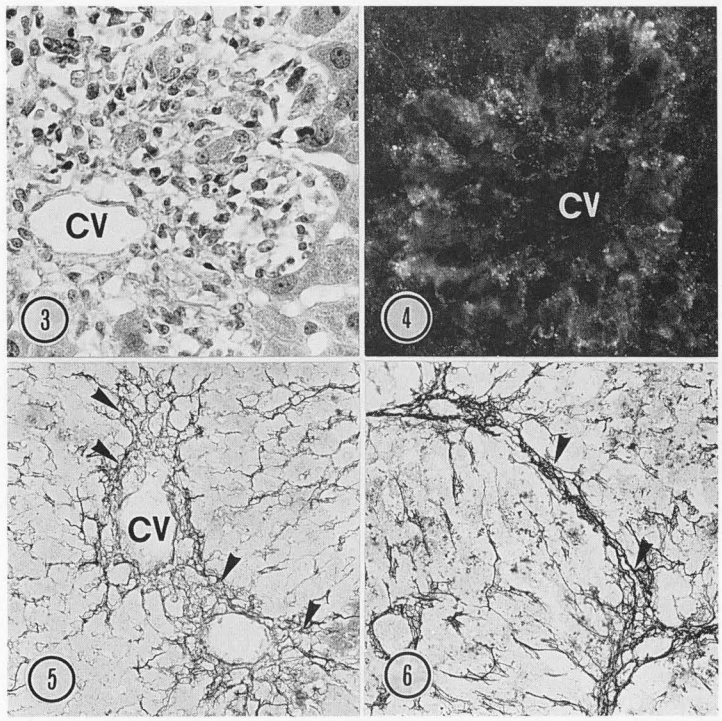
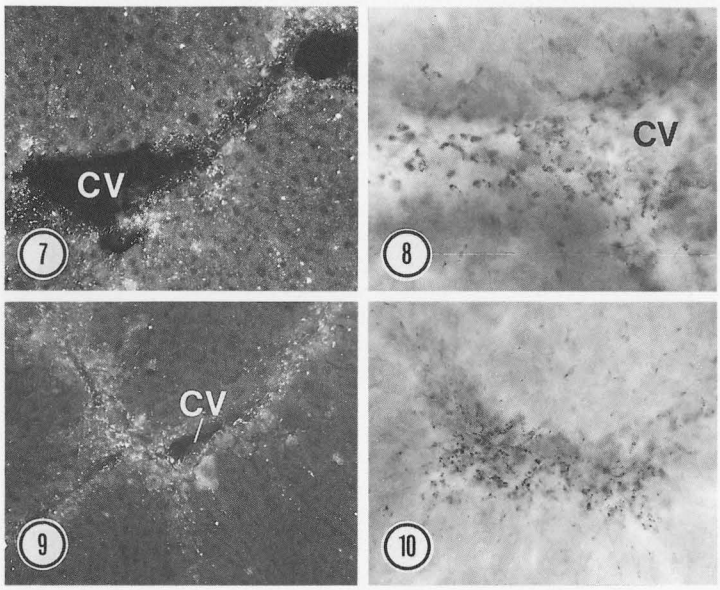


Fig. 3. On week 1, inflammatory cells with round denser nuclei and mesenchymal cells with oval nuclei accumulated in the necrotic pericentral area. **CV**, central vein. Hematoxylin-eosin × 150.

Fig. 4. In the granuloma, small vitamin A-containing lipid droplets resided closely to the central vein (**CV**) were surrounded by a row of stronger vitamin A-fluorescence. After 2 times injections of CCl_4. Fluorescence micrograph. × 100

Fig. 5. On week 1, a course meshwork of reticulin fibers (***arrowhead***) was prominent within the granuloma. **CV**, central vein. Suzuki silver impregnation method. × 300

Fig. 6. On week 4, condense arrangement of reticulin fibers (***arrowhead***) connected two blood vessels. Suzuki silver impregnation method. × 300.



Figs. 7 and **8**. On week 3, vitamin A-containing lipid droplets accumulated in the necrotic and septum area. **CV**, central vein. **Fig. 7**. Fluorescence micrograph × 100. **Fig. 8**. Gold chloride method × 100.

Figs. 9 and **10**. On week 5, a weak vitamin A fluorescence were emitted from lipid droplets distributed in the connective tissue of the septum. **Fig. 9**. Fluorescence micrograph. × 100. **Fig. 10**. Gold chloride method × 100.

granulomas. In the hematoxylin eosin preparations, fibroblastic cells were identified by the oval nuclei while the inflammatory cells by the round and denser appearance of nuclei (**Fig. 3**). Under a fluorescence microscope, cells containing small granular sources of vitamin A-fluorescence were scattered in the granulomas (**Fig. 4**). The silver impregnation method demonstrated a loose coarse meshworks of reticular fibers in the granulomas (**Fig. 5**). Those reticular meshworks became more compact and extended a newly-formed septa to the neighbouring central or portal veins on week 2 to 3. These septa became thicker on week 4 to 5 (**Fig. 6**).

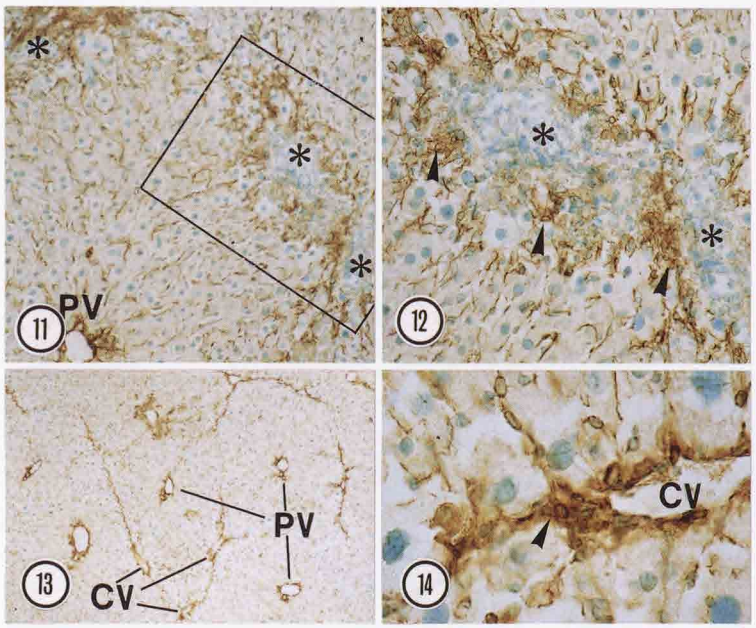


Fig. 11. On week 1, numerous green cell nuclei which showed negative desmin reactivity in the centre part of granuloma (***asterisk***) were circumscribed by intense desmin-positive cells. ABC method × 200.

Fig. 12. Higher magnification of the area surrounded by lines, demonstrated intense desmin-positive cells with long cytoplasmic processes (***arrowhead***). ABC method. ×400.

Fig. 13. On week 5, thin layer of intense desmin-positive cells divided the liver tissue into pseudolobules. ABC method × 100.

Fig. 14. Higher magnification of those intense desmin-positive cells (***arrowhead***) in the pericentral and extended septum **CV**, central vein. ABC method. × 400.

Cells with vitamin A-containing lipid droplets which emitted their fluorescence reacted also with gold chloride during the hepatic fibrosis. In week 3, a row of cells which released strong vitamin A-fluorescence in the boundaries of granulomas was

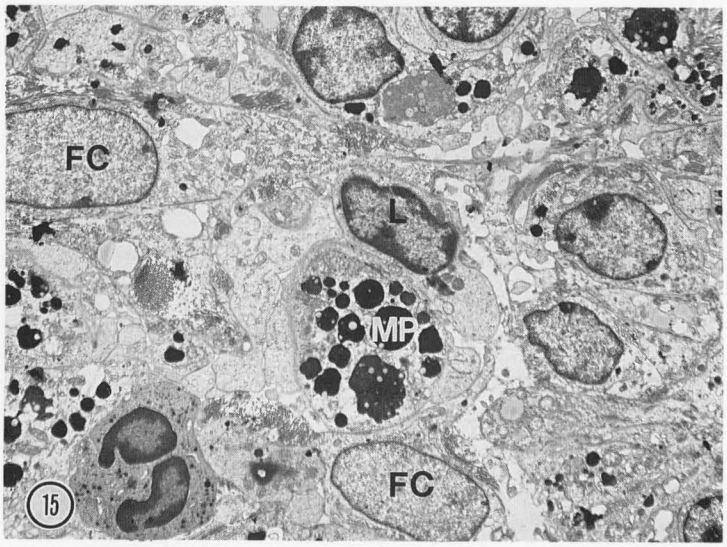


Fig. 15. One week after injection of CCl_4, within the necrotic zone, macrophages (**M P**) containing cell debris and large lysosome contacted with lymphocytes (**L**) and fibroblastic cells (**FC**) characterized by their electron-lucid oval nucleus. Electron micrograph. ×5400.

closer to central veins and expanded to form new septa (**Fig.7**and **8**). Eventually on week 5, a weaker vitamin A-fluorescence emitted from the cells was located along the septa (**Figs. 9** and **10**).

Desmin immunoreaction was expressed intensely in the cells bordering the negative desmin reactive granulomas while a weaker desmin reaction was demonstrated from stellate cells scattering in the surrounding parenchymal zones after twice injections of CCl_4 (**Fig. 11**). Those intense desmin-positive cells with long cytoplasmic processes appeared larger than the weak desmin positive-cells in the parenchymal area (**Fig. 12**). On week 2 to 3, the size of desmin-negative granuloma gradually became smaller and the intense desmin-positive cell rows continued as a thin septum. Those septa developed and divided the liver tissue into pseudolobules (**Fig. 13** and **14**).

Electron microscopy

Ultrastructure of perisinusoidal stellate cells in normal mouse liver demonstrated lipid droplets, granular endoplasmic reticulum, Golgi complex, free ribosomes, and small mitochondria in their cytoplasm. Basal lamina was not studded on their cytoplasmic membranes. However, interrupted basal lamina was recognized in the narrow space between the stellate and endothelial cells. Collagen fibrils were frequently seen close to the abluminal cell surfaces of the stellate cells.

In the first week after twice injections of CCl_4, the stellate cell phenotypes within the granuloma were packed in close contact with lymphocytes and large lysosomes containing macrophages (**Fig. 15**). The features of those phenotypes were slightly different according to their location. In the centre part of granulomas, they were characterized by elongated cell bodies, oval electron-lucid nuclei, well developed endoplasmic reticulum and small lipid droplets (**Fig. 16**), whereas in the periphery, they were irregular in shape and contained larger lipid droplets in their long cytoplasmic processes (**Fig. 17**). Indeed, bundles of collagen fibers appeared adjacent to both phenotypes.

In week 4, when new septa developed, septal cells encountered among abundant collagen bundles increased in number. They demonstrated dilated cisterns of rough surfaced endoplasmic reticulum which contained flocculus materials. Abundant intracytoplasmic filaments converged to subplasmalemmal dense plaques. The cell membrane was covered partially by the basal lamina-like structure (**Fig. 18**). Elongated membrane-bounded vacuoles containing characteristic striped structures recognized as zebra bodies were observed in the vicinity of the well developed Golgi complex (**Fig. 19** and **inset**).

DISCUSSION

After twice injections of CCl_4, in the granulomas bordered by a row of strong desmin-positive cells, desmin-negative cells were scattered. Two possible cells are proposed to develope into those desmin-negative cells i.e. perisinusoidal stellate cells distributed in the pericentral area or cells residing around central veins between subendothelial myofibroblasts / smooth muscle cells and parenchymal cells which Bunchet and Wake (1992) have designated as "second-layer cells". Ultrastructurally, the desmin-negative cells resemble the second-layer cells which show moderate desmin immunoreaction. On the other hand, Takase *et al.* (1988) has

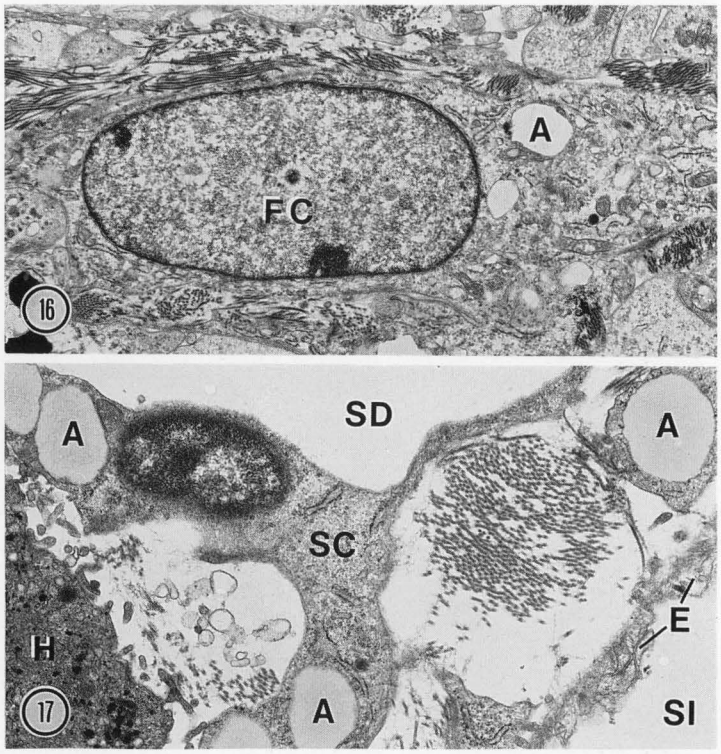
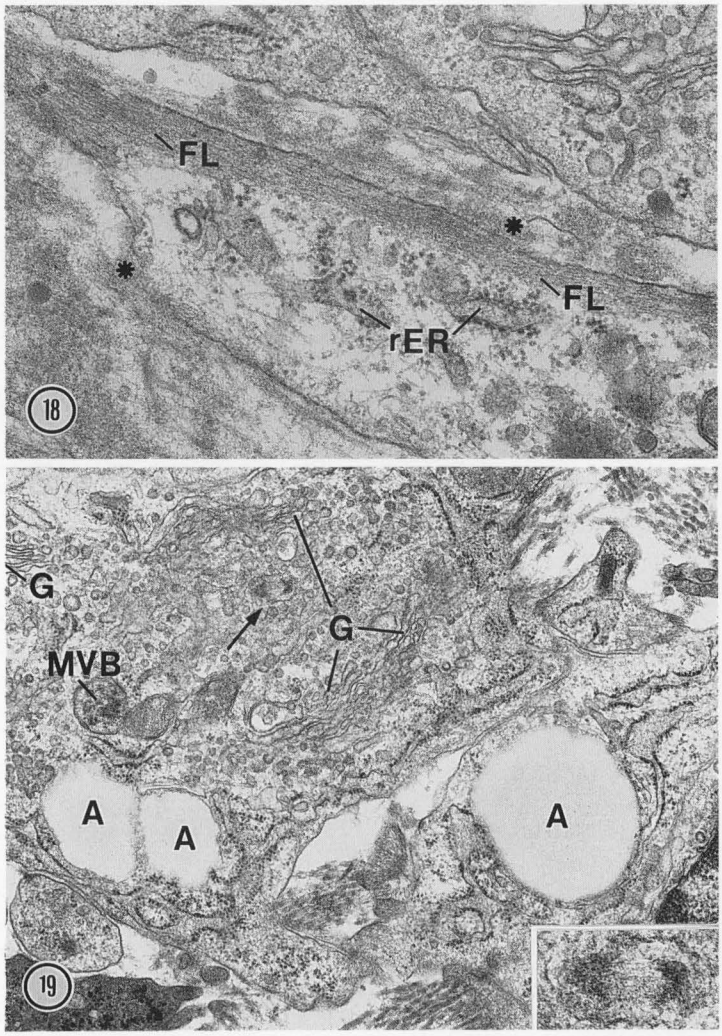


Fig. 16. Fibroblastic cell (**FC**) in the centre part of granuloma which contained granular endoplasmic reticulum and a small lipid droplets (**A**) was surrounded by bundle of collagen fibrils. One week after CCl_4 injection. Electron micrograph. × 11000.

Fig. 17. On week 1, a stellate cell (**SC**) located in the periphery of granuloma characterized with large lipid droplets (**A**) and long cytoplasmic processes adjacent to collagen fibers. **E**, endothelial cell ; **H**, parenchymal cell ; **SD**, space of Disse ; **SI**, sinusoidal lumen. Electron micrograph ×36000.


FL
FL
rER
18
G
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MVB
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A
19

reported that stellate cells developed into myofibroblasts in the 1st and 5th passage of a primary culture are stained negative to anti-desmin antibody. It leads to a suggestion that desmin-negative cells residing closely to central vein are derived from second-layer cells while the rest are from stellate cells. To support this view, an investigation is now being undertaken. Since the row of cells bordering the granuloma showing strong desmin reactivity has the cytological characteristics of stellate cells, we believe that those phenotypes are the activated stellate cells. The activated stellate cells show intense desmin reactivity and appear larger with long processes as identified in the perivenular zone of rats after single injection of CCl_4 by Burt *et al.* (1986) and Yokoi *et al.* (1988).

In advanced stages, although the septal cells reacted to anti-desmin antibody as strong as the activated stellate cells in the boundaries of granulomas did, they showed different character ultrastructurally. The septal cells demonstrated smaller lipid droplets with more abundant cytoplasmic filaments and were studded with basal lamina-like substances. Such features were also observed in the transitional cells or myofibroblasts found in the CCl_4-induced rat liver cirrhosis,[21] the liver of alcohol-fed baboons [9, 22] and during the primary culture of stellate cells.[5, 14] Moreover, Ballardini *et al.* (1988) has demonstrated the strong desmin-positive reactivity in the septal cells of rats after peritoneal injections of swine serum. Based on the features and supported by other investigations, we consider that the septal cells are myofibroblast-like cells and derived from the stellate cells phenotypes.

Fig. 18. A part of myofibroblast-like cells showing well developed granular endoplasmic reticulum (**rER**), intermediate filaments (**FL**) in the subplasmalemmal cytoplasm, and basal lamina-like substance (***asterisk***) in the intercellular space. Four week after CCl_4 injections. Electron micrograph. × 40000.

Fig. 19. On week 4, cytoplasm of myofibroblast-like cells demonstrated well developed Golgi complex (**G**) consisted of elongated and multilayered lamellae. A zebra body (***arrow***, **inset**) appeared in the vicinity of Golgi complex. **A**, vitamin A-lipid droplets ; **MVB**, multivesicular body. Electron micrograph × 24000. **Inset**, × 72000.

Although the quantification of desmin-positive cells accumulated in the granuloma was not conducted in the present work, they expressed an increasing in number. Similar conclusion was reported [4,15,40] by counting the positive cells with desmin as a marker. They suggested that the increased number of stellate cells which reached peak on days 3 to 5 were due to cell proliferation. The proliferation of stellate cells in rat acute liver injury induced by CCl_4 had been proven by ^3H-thymidine autoradiography.[8] Since we have observed an intimate contact between stellate cells phenotypes and the mononuclear cells in the granuloma, it is suggested that this phenomenon has an important role in the expansion of stellate cells and their active collagen synthesis. The collagen synthesized by stellate cell phenotypes is evidenced by the occurrence of reticulin meshworks within the granuloma. Our suggestion is supported indirectly by the *in vitro* studies which demonstrated the influences of mediators released from Kupffer cells to the proliferation of stellate cells [12,29] and to the activation of extracellular matrix protein synthesis in the stellate cells. [16,17,32] Vitamin A-storage in the stellate cells within the granuloma increase on week 1, and then decrease during the development of septa. This fact suggests that the level of vitamin A-storage in the stellate cell phenotypes may have a correlation with their activation in collagen synthesis. During the primary culture, stellate cells show a spontaneous loss of vitamin A lipid droplets [5] and a change of their phenotypes into myofibroblasts with increase of collagen production. [14] The correlation is also shown by the inhibition of stellate cell differentiation into myofibroblasts [13] and the suppression of connective tissue septa development in CCl_4-induced rat cirrhosis [27,28] after the administration of vitamin A.

In conclusion, following the liver injury by CCl_4, desmin-negative and desmin-positive cell phenotypes occur within the granulomas. Both phenotypes transform into septal myofibroblasts with the decline of their vitamin A-storage. Further investigation is needed to reveal the critical point of stellate cell transformation where they are still reversible.

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