A Scanning Electron Microscopic Study of Liver Microcirculation Disarrangement in Experimental Rat Cirrhosis

EUGENIO GAUDIO,[1] LUIGI PANNARALE,[2] PAOLO ONORI[2] AND OLIVIERO RIGGIO[3]

[1]Department of Experimental Medicine, State University of L'Aquila, 67100 Aquila; Departments of [2]Anatomy and [3]Gastroenterology, State University of Rome "La Sapienza," 00161 Rome, Italy

Hepatic microcirculation has been related to liver function in several studies. The principle of this relationship lies in the sequential distribution of blood from the feeding vessels of the hepatic acinus to the central vein. This study was undertaken to investigate the progressive changes at different sites of the liver microvascular bed in the developing cirrhosis, both by light microscopy and scanning electron microscopy of corrosion casts. Experimental cirrhosis was induced with intragastric carbon tetrachloride. The most important vascular changes progressively observed are the reduction of the distance between the pre- and postsinusoidal vessels, the presence of newly formed shunting vessels bypassing the sinusoids and, finally, the development of a perinodular vascular plexus composed of pre- and postsinusoidal vessels. Newly formed vessels grow through preformed tissue septa. These vascular modifications make any zonal gradient hardly possible. The loss of the zonal gradient of perfusion could highly modify liver function, along with the structural changes of hepatic laminae. Hepatocyte regeneration cannot recover the original vascular relationships: this makes the morphological and functional destructuralization of cirrhotic liver irreversible. (HEPATOLOGY 1993;17:477-485.)

Morphological studies have provided in-depth knowledge of hepatic microvasculature both on light microscopy (1-6) and scanning electron microscopy (SEM) of corrosion casts (7-13). Hepatic microcirculation has been related to liver function in several studies (3, 14-17). The principle of this relationship lies in the sequential distribution of blood from the feeding vessels of the hepatic acinus to the central vein.

The influence of vascular modifications during cirrhosis has been studied for many years (18-21). Rappaport et al. (22) underlined the fundamental role of microvascular changes in cirrhosis after studying cirrhotic human livers with serial sectioning and light microscopy. They hypothesized that the microvascular changes in the liver are not the mere consequence of fibrosis and nodule formation: they documented that vascular adaptation starts as soon as reparative processes start and influences the entire development of the pathological condition.

More recently, microvascular casts observed on SEM have proved themselves valuable tools for showing the three-dimensional changes of the microvasculature. Studies of human (23, 24) and experimental (25, 26) material have been performed.

Corrosion casts have also proved useful in demonstrating the microvascular changes in hepatic cirrhosis at the level of the perinodular plexus (22, 24, 27), the peribiliary microvascular bed and the sinusoids (25-27). But no study employing this technique provided valuable information concerning the development of the cirrhotic lesion in its different stages or the possible reversibility and physiological impact of the microvascular lesions. Corrosion casts of autopsy livers (22, 24, 27) were only able to show advanced terminal lesions of cirrhosis. Moreover, casting conditions were less than ideal because of postmortem alteration of organs.

Hence experimental and autopsy studies have only considered well-developed cirrhotic lesions. For this reason, progressive modification of the nodular circulation and its influence in the genesis of cirrhosis and metabolic impairment of the liver remain to be clarified.

This study was undertaken to investigate the progressive changes at different sites of the liver microvascular bed at different stages in developing cirrhosis. We employed an updated technique for obtaining SEM corrosion casts of rat livers treated with intragastric CCl_4 (28-29).

MATERIALS AND METHODS

Experimental Protocol. Thirty-six male Wistar rats aged 8 wk and weighing about 200 gm were used in this study. In performing this protocol we adhered to the National Research Council's criteria for the care and use of animals.

The animals were divided into six groups. Normal (N) rats received no treatment except barbital for 10 wk (n = 6). Thirty

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Address reprint requests to: Prof. E. Gaudio, Department of Experimental Medicine, State University of L'Aquila, Via S. Sisto, 67100 L'Aquila, Italy.

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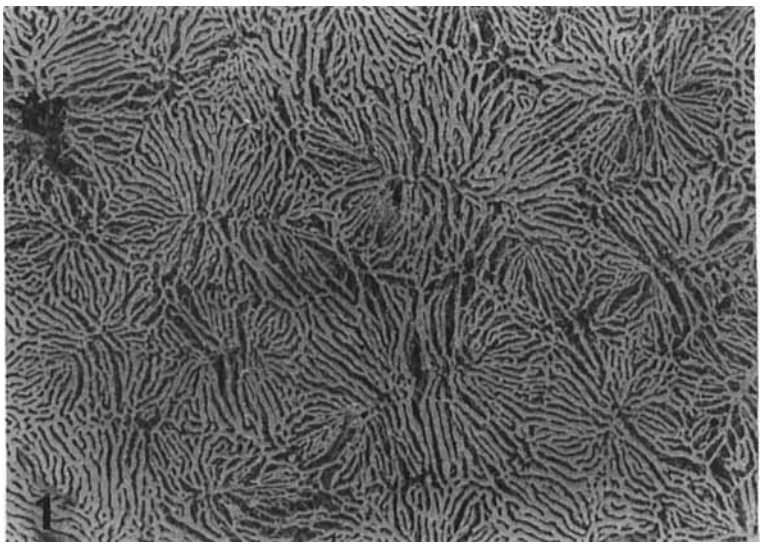

FIG. 1. SEM of a microvascular corrosion cast of the superior surface of a control liver. A continuous network of sinusoids is visible (original magnification ×65).

rats were treated with weekly intragastric doses of CCl_4. These animals were subdivided into the following groups: C2 (six rats treated for 2 wk); C4 (six rats treated for 4 wk), C6 (six rats treated for 6 wk), C8 (six rats treated for 8 wk) and C10 (six rats treated for 10 wk).

One rat from group C8 and two rats from group C10 died before the end of treatment. Four rats each from groups N, C2, C4, C6 and C8 and three rats from group C10 were processed for corrosion casting. Two rats each from groups N, C2, C4 and C6 and one rat each from groups C8 and C10 were processed for light microscopy.

Cirrhosis-induction Procedure. Weekly intragastric doses of CCl_4 were administered to rats under light ether anesthesia. Dosages were regulated on the basis of individual body weight. The initial dosage was 0.04 ml, and the maximum final dosage was 0.3 ml. Standard pellet diet and water containing barbital (100 mg/dl) were supplied *ad libitum*. Once ascites was detected, CCl_4 dose was reduced to 0.04 ml (28). Treatment was withdrawn when stable ascites appeared. This technique has proved useful in several of our studies (26, 29-31) because it reproduces structural and biochemical features of micronodular cirrhosis.

Corrosion Cast Preparation. We modified our casting procedure (26, 32) by using a standard injection pressure and more fluid casting compound.

Rats were anesthetized with an ether-oxygen blend. The thorax was cut open, and the ascending aorta was cannulated with a Venflon cannula (1.7 mm; Viggo-Spectramed, Helsingborg, Sweden). The right atrium was cut open, and the vascular bed was washed out with heparinized 0.9% saline solution. Mercox CL2R resin (Okenshoji, Tokyo, Japan), diluted with methyl methacrylate (methyl methacrylate/resin ratio = 1:4) (33-34) and mixed with an amount of its catalyzer adjusted to obtain polymerization in 10 min, was injected at room temperature until polymerization was visible. Injection pressure at the level of the cannula was 250 mm Hg.

After 24 hr, livers were dissected and macerated in 15% NaOH and 5% trichloroacetic acid (CCl_3COOH) (35-36) solutions. Then the livers were rinsed in distilled water. When the casts were completely free of tissue remnants, they were frozen in distilled water and freeze-dried (33-34). The casts were glued onto stubs, coated with gold in an S150 sputter coater (Edwards, London, UK) and observed with a model S4000 field emission scanning electron microscope (Hitachi Ltd., Tokyo, Japan).

Light Microscopy. Samples of the median lobe of the liver parenchyma, measuring 125 mm^3, were immediately immersed in 10% buffered formalin. Normal procedures for paraffin embedding were followed. Five-micrometer-thick sections were stained with hematoxilin and eosin, by the Gomori method for reticular fibers and by the Fast green–Sirius Red and Azan-Mallory trichromic method for collagen fibers. They were observed with a Zeiss FO-MI 2 photomicroscope (Carl Zeiss, Oberkochen, Germany) at magnifications of 10, 25 and 40.

RESULTS

In control animals, on SEM observation of corrosion casts a normal microvascular tree was evident (Fig. 1).

Sinusoids always had spaces between them, with even distribution of their diameters. Casts of sinusoids showed no extravasations.

Casts of portal venules showed regular circular sections. The surfaces of the casts were characterized by round or oval endothelial imprints. Terminal branches gave off short inlet venules, which gave rise to sinusoids. Around portal branches a space free of sinusoids was always present.

Casts of arterial vessels were much less numerous than were venous casts. Terminal branches of the hepatic artery had smaller diameters than did the portal vessels running next to them. The surfaces of arterial casts were characterized by spindle-shaped endothelial imprints.

Terminal hepatic vein branches (central veins) took rise directly from sinusoids that joined the vessels, mostly at right angles. Sinusoids also reached preterminal branches of the hepatic vein.

On the superior surface of the liver, we observed frequent terminal hepatic vein branches taking rise from the outermost layer of sinusoids (Fig. 2). At the

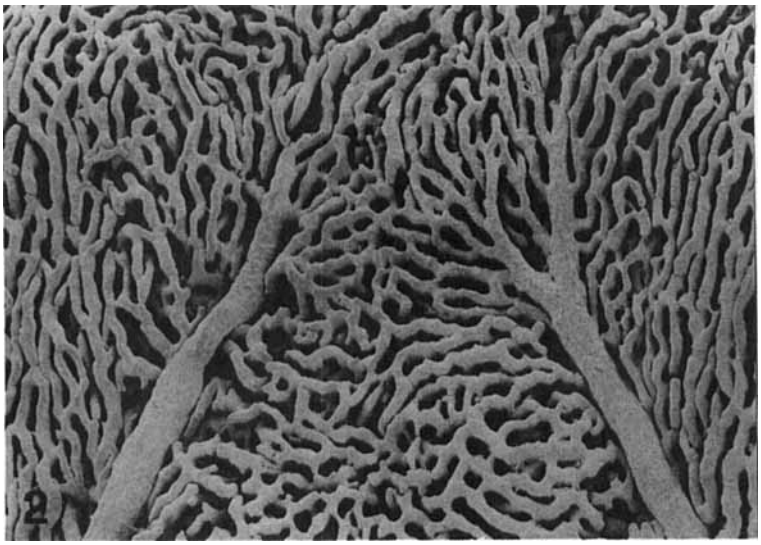

FIG. 2. SEM of a microvascular corrosion cast of the superior surface of a control liver. Two central veins take rise directly from sinusoids that join to the vessels, mostly at right angles. Almost no space between central veins and sinusoid network is present (original magnification ×175).

same level, portal and arterial branches were much less frequently encountered.

Intrahepatic peribiliary vessels were observed more often in the part of the parenchyma nearest the hepatic hilum. Light microscopy showed normal parenchymal arrangement.

During the second week of treatment, SEM corrosion cast observation showed that the most diffusely noticeable feature of this stage of treatment is the appearance of sinusoid-free spaces around central veins (Fig. 3). On the surface of the liver, it was much easier to observe portal branches (Fig. 4). These vessels approached hepatic vein branches closely. Sinusoids running directly from pre- to postsinusoidal vessels were observed. These sinusoids had larger-than-average diameters.

Light microscopy showed an increase of connective tissue around the central veins. Sparse necrotic hepatocytes, accompanied by rare monocyte infiltration, were encountered.

During wk 4, SEM corrosion cast observation showed microvessels very similar to those of livers treated for 2 wk. One new feature in this phase was the presence of spaces, free of vessels, radiating from central veins (Fig. 5).

Light microscopy showed an increase of connective tissue septa extending from the central veins into the neighboring parenchyma. Almost no vascular lumen was observed in these septa.

At wk 6, SEM corrosion cast observation showed that most of the liver is subdivided into nodules of different sizes (between 100 and 500 μm) that are separated by spaces free of sinusoids and crossed by some vessels, mainly of portal origin (Fig. 6). Presinusoidal vessels give off sinusoids at very short distance from central veins. These sinusoids run almost directly from the afferent to the efferent vessels and have diameters larger than those of neighboring sinusoids.

On light microscopy, a net of connective septa subdivided the liver parenchyma into nodules of different sizes (Fig. 7). Vessels with different diameters were evident in most of the connective tissue.

At wk 8, SEM corrosion cast observations showed that the spaces separating nodules of sinusoids had become crowded by vessels of portal (the most numerous), arterial and hepatic venous origin. Larger branches often appeared flattened. Central veins appeared sometimes inside nodules and showed an almost normal pattern, but their afferent sinusoids took rise at an extremely short distance. Many small (10 to 20 μm in diameter) portal and arterial branches were present at this stage. These vessels gave rise to sinusoids and were involved in shunting between presinusoidal vessels and between pre- and postsinusoidal vessels (Fig. 8).

Peribiliary plexus showed increased vascularization (Fig. 9).

Thicker connective septa and smaller nodules were observed on light microscopy (Fig. 10).

Casts of livers at wk 10 were much less compact, on the whole. Larger sublobular vessels were constantly demonstrated. Sparse nodules were attached to these vessels (Fig. 11). These nodules comprised small cores of sinusoids wrapped to different extents with arterial and venous vessels. Many of these perinodular branches appeared dead-ended.

Light microscopy showed a pattern similar to that of livers treated for 8 wk (Fig. 12).

DISCUSSION

The technique of corrosion casting we employed has proved itself useful in the detailed observation of the courses of different vessels and these vessels' relation-

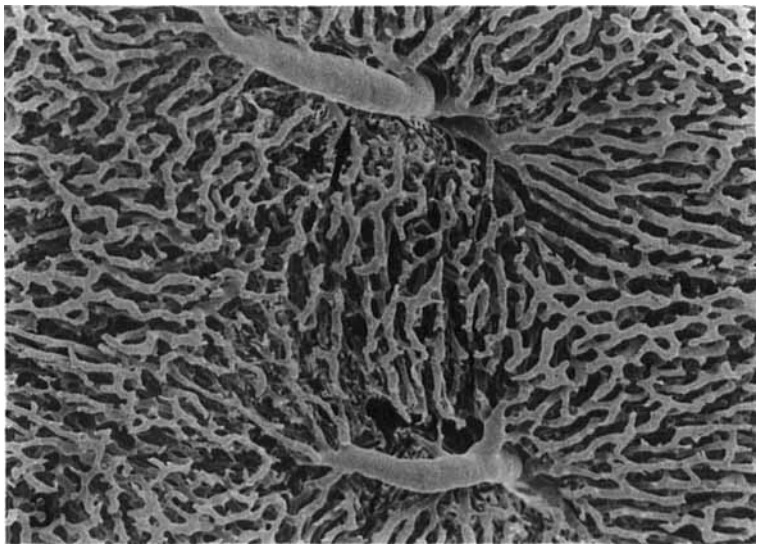

FIG. 3. SEM of a microvascular corrosion cast of the superior surface of the liver after 2 wk of CCl_4 treatment. Sinusoid-free space around two central veins is evident (original magnification ×175).

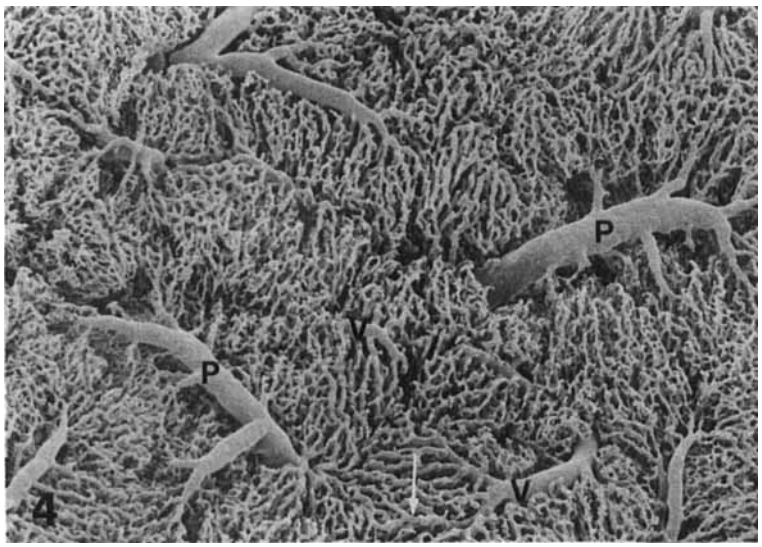

FIG. 4. SEM of a microvascular corrosion cast of the superior surface of the liver after 2 wk of CCl_4 treatment. Portal branches *(P)* and hepatic vein branches *(V)* are close. Sinusoids run directly between them *(arrow)*. These sinusoids have larger-than-average diameters (original magnification ×95).

ships. Moreover, the use of a resin with a lower-than-normal density and use of an electronically controlled injection pressure have made it possible to obtain microvascular casts even in advanced stages of cirrhosis (a situation in which high interstitial pressure opposes flow through the blood vessels subject to injection).

The use of experimental animals has made possible serial observations in consecutive stages of cirrhosis. This study has not only provided general information on the cirrhosis; it has also made it possible to recognize reversible and irreversible stages of this experimental model, thus providing a basis for the testing of treatments of the syndrome associated with cirrhosis.

Observation of control livers has shown that the superior surface of the organ gives rise to numerous hepatic vein branches but that it is reached only by a few arterial and portal branches. This situation changes after nodule formation, when the three-dimensional relationship of afferent and efferent vessels is completely changed. Arterial portal and hepatic vein branches are mixed and densely represented on the outer surface of the organ.

Two main points must be considered among the vascular changes: the reduction of the distance between the pre- and postsinusoidal vessels and the new formation of shunting vessels.

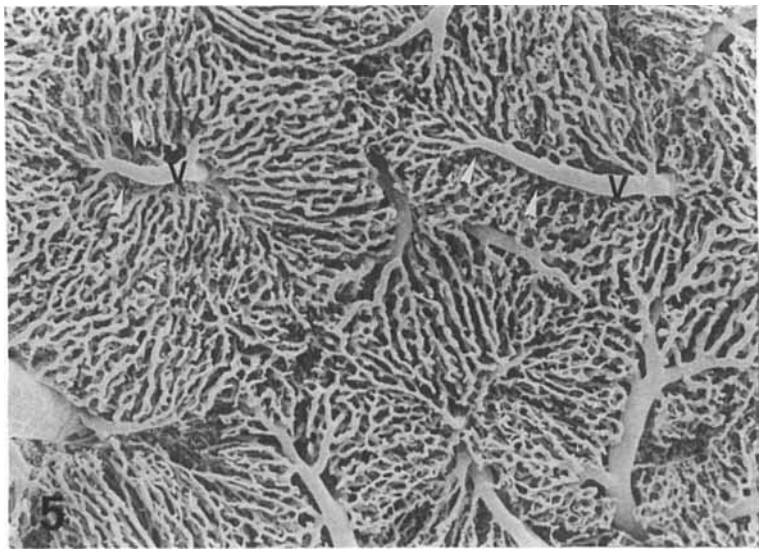

FIG. 5. SEM of a microvascular corrosion cast of the superior surface of the liver after 4 wk of CCl_4 treatment. Spaces free of vessels radiating from central veins *(V)* are present *(arrowheads)* (original magnification ×90).

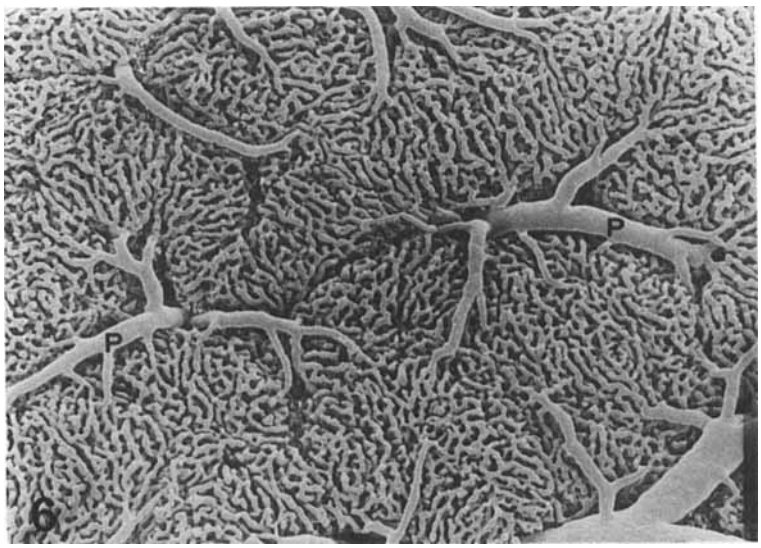

FIG. 6. SEM of a microvascular corrosion cast of the superior surface of the liver after 6 wk of CCl_4 treatment. Vessels of portal origin *(P)* run between nodules (original magnification ×90).

Reduction of the distance between pre- and postsinusoidal vessels occurs very early in the course of CCl_4 treatment (wk 2 to 4) and coincides with the observation of sinusoids between afferent and efferent vessels. These sinusoids show slightly larger-than-normal diameters. They may correspond to the "fast sinusoids" described by Sherman et al. (37). In fact, in *in vivo* studies on the experimental cirrhosis in the rat these researchers demonstrated shunting sinusoids with extremely rapid blood flow. These vessels were present when no signs of anomalous neovascularization were yet apparent.

Up to the sixth week of the CCl_4 toxicity protocol, changes seemed to have been caused more by fibrosis and tissue collapse than by vasoproliferative changes. In fact, the general pattern of the microvascular tree mirrored that of a normal microvascular bed except for the mentioned aspects. After wk 8, true shunting vessels appeared as a consequence of vasoproliferation.

Shunts between portal and arterial vessels (A-P shunts) have been observed rarely in this research. Other authors (23) have observed in SEM corrosion casts of human cirrhotic livers increases in numbers of A-P shunts. These researchers believed the shunts to have special value in the development of portal hypertension. This difference could be explained by the different types of livers observed (human and rat) or by the fact that the mentioned study was performed on

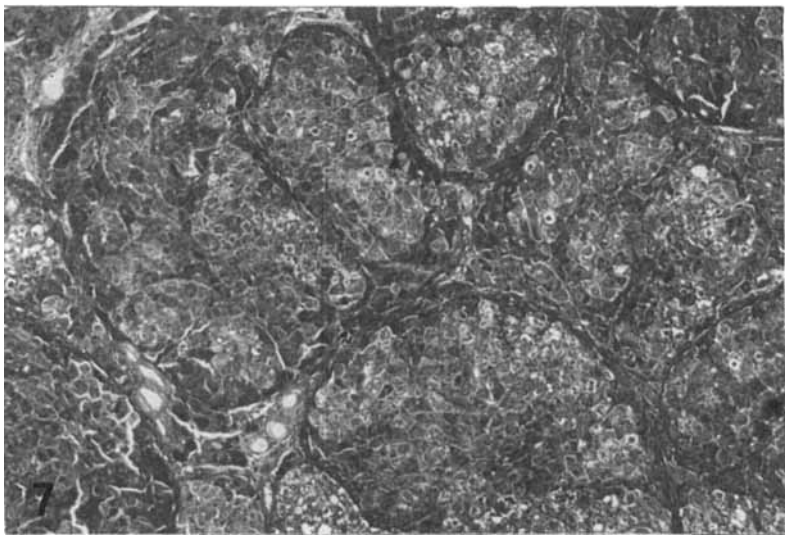

FIG. 7. Light microscopy of a histological section of a liver after 6 wk of CCl_4 treatment. Nodules of different size are delimited by thin connective septa (Azan-Mallory trichrome; original magnification $\times 100$).

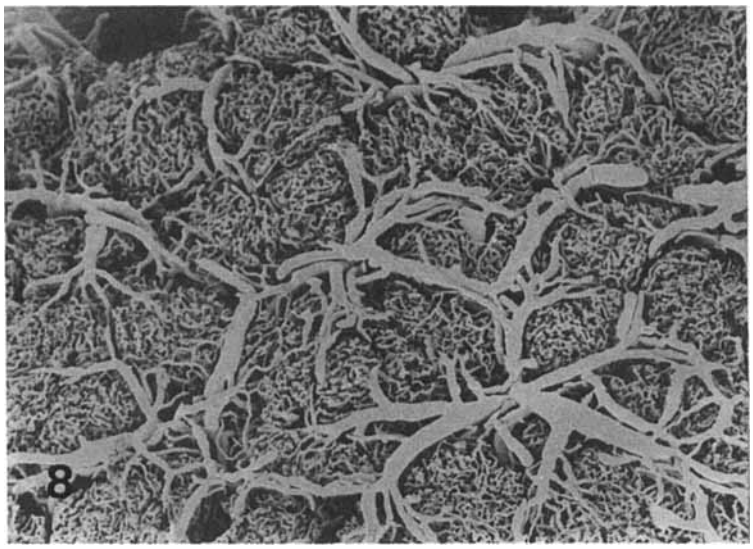

FIG. 8. SEM of a microvascular corrosion cast of the liver after 8 wk of CCl_4 treatment. Vessels of portal, arterial and hepatic venous origin run between nodules of different sizes. Shunting between presinusoidal and between pre- and postsinusoidal vessels is present (original magnification $\times 50$).

cirrhotic autopsy livers only, without appropriate controls. A-P shunts may be difficult to demonstrate on microvascular casting because they are frequently not perfused even under normal conditions (16).

Direct shunts between afferent and efferent vessels are present in casts as straight vessels with a peculiar shape. No measurement of vascular diameters has an absolute value with this technique, but we can argue that these shunting vessels could not be detected with microspheres in a previous study of *in vivo* blood flow because their diameters seem to be less than 15 μm (38). These vessels correspond to newly formed, poorly permeable vessels in fibrotic septa (38-40). Most do not seem to vascularize anoxic areas but seem to follow the course of spaces free of sinusoids corresponding *in vivo* to fibrous septa.

Intrahepatic peribiliary vessels represent an extremely limited part of the liver microvascular bed. There is no reason why vasoproliferative stimuli should not influence peribiliary circulation, but one could hardly imagine its importance in maintaining blood flow through the liver once cirrhosis is established.

Perhaps our technique is not the most appropriate for detecting vasoproliferative processes; it can be difficult to cast sprouting, dead-ended new vessels (33, 34). But the technique is certainly the most useful in detecting

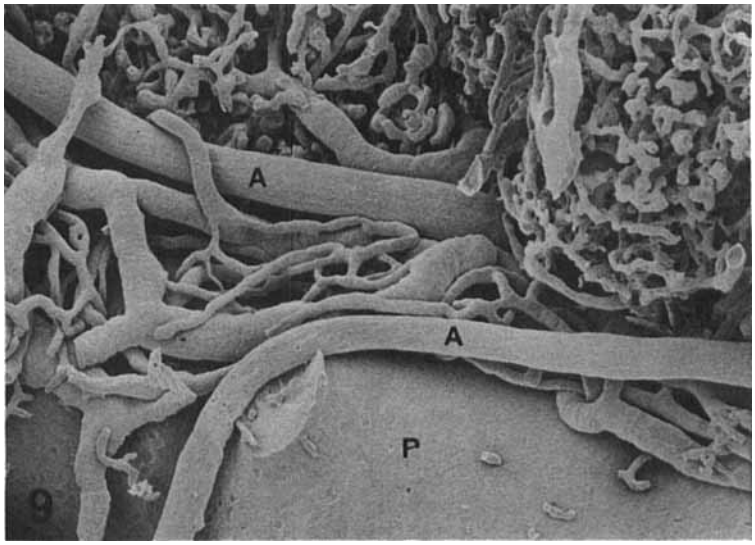

FIG. 9. SEM of a dissected microvascular corrosion cast of the liver after 8 wk of CCl_4 treatment. Peribiliary plexus *(PBP)* is evident next to portal *(P)* and arterial *(A)* branches (original magnification ×240).

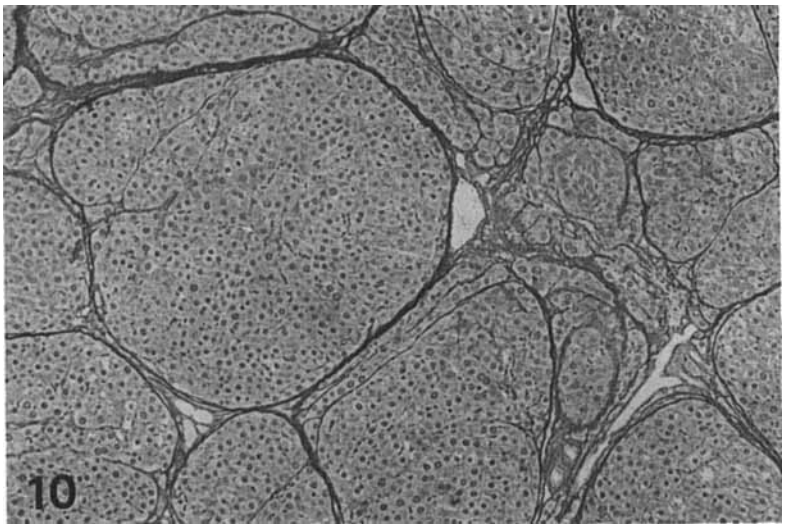

FIG. 10. Light microscopy of a histological section of the liver after 8 wk of CCl_4 treatment. Small nodules delimited by thick connective septa are present (Gomori reticulin stain; original magnification ×100).

new vessels once lumens develop and blood flow has been established. The use of lower-viscosity resins certainly helps (40).

Portal vessels seem more likely to form new vessels. In fact, perinodular plexus is mainly formed by portal vessels. The characteristics of surface, diameter and section are those of portal vessels. The easiest explanation of this phenomenon is that in normal livers portal vessels may be more numerous than are arterial vessels or hepatic veins. Thus when vasoproliferative processes are established portal vessels remain the most numerous.

Newly formed vessels bypass the sinusoids and run directly toward central veins; they follow the already formed septa that bridge from the central veins toward the tracts. (Spaces free of sinusoids are present before the newly formed vessels.) These vessels shunt blood with little or no diffusion of metabolites (38-41), thus reducing Po_2 in sinusoids next to the triad and increasing it near the hepatic vein branches.

As we have seen, from the very beginning of the process leading to cirrhosis vascular modifications change the orderly diffusion gradient of metabolites through the liver acinus. In normal liver, this gradient is related to the different activities of hepatocytes in the three zones of the acinus (17). The presence of "high-flow sinusoids" in the earliest stages means that hepatocytes along them (not those lining portal tract) are immersed in the highest concentration of metabolites (37). Formation of direct shunts constituted by

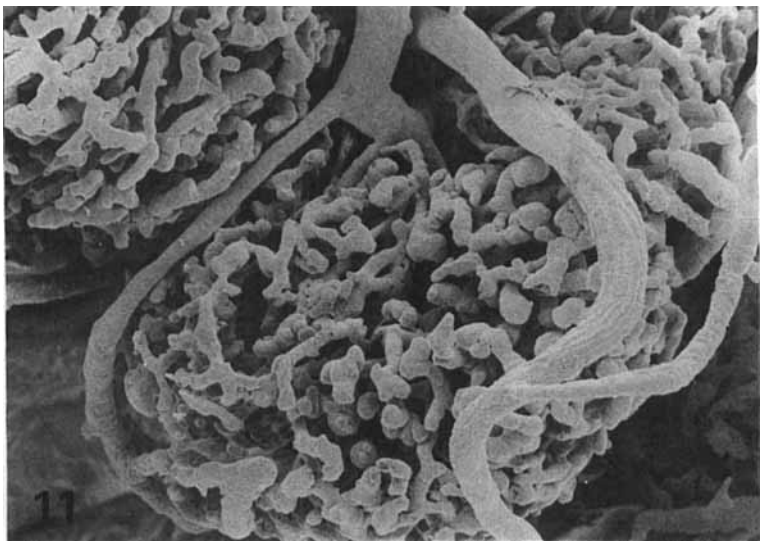

FIG. 11. SEM of a microvascular corrosion cast of the liver after 10 wk of CCl_4 treatment. An extremely small, isolated nodule is partially wrapped by arterial and venous vessels (original magnification ×300).

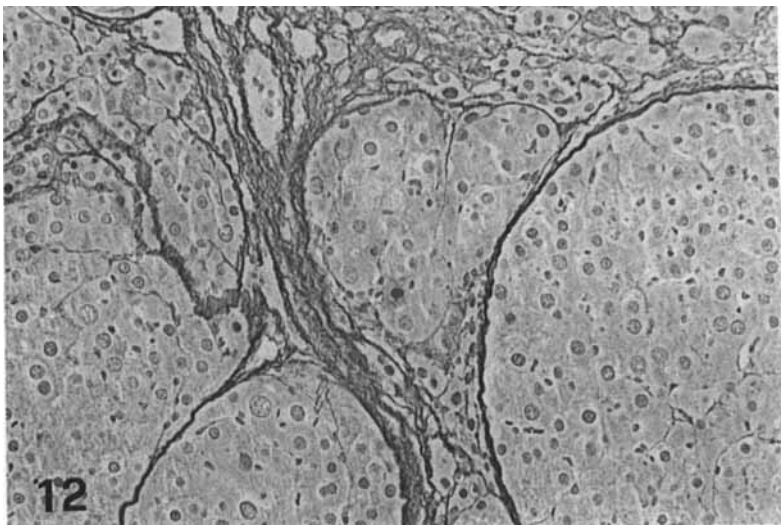

FIG. 12. Light microscopy of a histological section of the liver after 10 wk of CCl_4 treatment. Small nodules delimited by thick connective septa are present (original magnification ×200).

"nonsinusoids" and the formation of a perinodular plexus make any zonal gradient nearly impossible. These changes contribute dramatically to impairment of hepatocyte function.

According to other authors, changes at the level of the peribiliary plexus can definitely influence the blood flow parameters in the portal and arterial branches, thus possibly playing a role in portal hypertension (25). In our opinion, their influence on the function of the hepatic parenchyma is certainly much more indirect than that of the microvascular modifications that lead to perinodular plexus formation. In fact, these modifications involve the acinar vessel patterns that play a more direct role in the metabolism of the hepatocytes (17) and that maintain a zonal gradient of metabolites in the liver acinus. The loss of the zonal gradient of metabolites could impair liver function more than could the structural changes of hepatocytic laminae.(17, 42).

In conclusion, this study provided new information on the development of microvascular changes in the different phases of cirrhosis. This information may help determine which cirrhotic lesions are somewhat reversible and which are not. In the very early stages, the tissue collapse that approaches preexisting afferent and efferent vessels may be reversible. In later stages, the presence of newly formed tissue septa makes possible the onset of directed vessel proliferation. At that point, hepatocyte regeneration cannot recover the original

vascular relationships: this makes the morphological and functional destructuralization of cirrhotic liver irreversible.

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