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HISTOLOGICAL EVALUATION OF THE ZONATION OF COLLOIDAL GOLD UPTAKE BY THE RAT LIVER

Keywords: Kupffer cell, liver acinus, light microscopy, ultrastructure, endocytosis, rat

ABSTRACT. The distribution and endocytotic function of Kupffer cells in the rat liver were studied after administration of fibrinogen stabilized colloidal gold suspensions either by injection directly into the circulatory system of anaesthetized rats or by application to the isolated perfused liver. After exposure to gold particles the livers were perfused with fixative and studied using several microscopic techniques. Gold was predominantly endocytosed by a highly active population of Kupffer cells surrounding the portal spaces resulting in distinct dark patterns around the terminal portal veins. In cross-sections of lobules the pattern appeared as incomplete networks composed of dark triangular areas with distinct borderlines towards light areas concentric with the terminal hepatic veins (central veins). The light areas contained few and relatively inactive small Kupffer cells. A wide variation of conditions gave essentially the same uptake pattern compatible with the concept of microcirculatory zones concentric with the terminal hepatic veins (Lamers *et al.*, 1989; Quistorff and Rømert, 1989), but contradicting the traditional view of microcirculatory zones advanced by Rappaport *et al.* (1954). Since the same pattern developed during conditions of anoxia, it seems that oxygen is not the stimulus for the developmental distribution of Kupffer cells with high endocytotic activity. *In vivo* and perfusion experiments gave identical patterns, but a higher endocytotic activity of endothelial cells was found in perfused isolated livers.

Introduction

In recent years it has been shown that hepatocytes are not a homogeneous population of cells, but differ both structurally and functionally along the length of the sinusoid (reviews by Klinger *et al.*, 1988; Quistorff, 1990). Using the digitonin-pulse perfusion method (Quistorff and Grønnet, 1987) in order to correlate metabolic zonation with morphology we found a digitonin discoloration pattern which we suggested was a reflection of the microcirculatory flow distribution (Quistorff and Rømert, 1989). The results obtained could not be adequately explained by the acinus (zone) definition of Rappaport (1973; *et al.*, 1954) and we pro-

posed that the microcirculatory zones of hemodynamic equipotential were concentric with the terminal hepatic veins and not berry-shaped shells concentric with the terminal portal veins. This interpretation is in agreement with other recent reports (Lamers *et al.*, 1989; Matsumoto *et al.*, 1979; Teutsch, 1984, 1988). While studying the uptake of well-defined fibrinogen-gold markers by different cell types, including Kupffer cells, we discovered that the Kupffer cells gave rise to an uptake pattern, visible on the liver surface, which was nearly identical to the discoloration pattern we had seen earlier applying digitonin-pulse perfusion (Quistorff and Rømert, 1989). The present study elaborates on this finding and evaluates the pattern of fibrinogen-gold uptake by the Kupffer cells (and also by the endothelial cells) both in perfusion and *in vivo* experiments. Previously it was shown that Kupffer cells are more numerous around portal vein branches and that these periportal cells are larger and more active than centrolobular macrophages

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(Kaneda and Wake, 1983; Sleyster and Knook, 1982). It was further suggested by Sleyster and Knook (1982) that Kupffer cells form two cell populations which are functionally heterogeneous. The present study also investigates this aspect by different microscopic techniques. Furthermore, the gold uptake was studied in the isolated perfused rat liver as a function of flow rate, flow direction, oxygen supply and the presence of corpuscular elements in the perfusion medium.

Materials and Methods

32 male Wistar rats weighing 180–200 g were used. The rats were on a 12 hr dark/light cycle, changing at 9 a.m. All animals had free access to water and food pellets. Experiments were carried out between 10 a.m. and 2 p.m. The rats were anaesthetized with sodium pentobarbital (400 μ l, 50 mg/ml) given intraperitoneally. Suspensions of 15 or 50 nm colloidal gold, stabilized with human fibrinogen (Sigma) following standard stabilization procedures (Behnke *et al.*, 1986) were used as macrophage tracers. Gold conjugates were stored concentrated at 4°C without additives and used within one week.

Pilot experiments were performed to assess the amount of gold suspension necessary to obtain an uptake which was readily visible on the surface of the liver. This amount which was similar in the different types of experimentation (see below) was measured spectrophotometrically ($E_{\text{max}} = 535 \text{ nm}$) and used in the experiments as the basic quantity unit, in the following called x. Gold suspensions were applied in the amounts: 0.5x, 1x, 2x, 4x, and 8x. (The indication of the relative amounts of gold administered is more relevant with regard to the different experiments described below than giving the exact numbers measured spectrophotometrically).

The uptake of gold particles by the liver was studied in three types of experiments:

1. *In vivo experiments* with intact, anaesthetized rats where the different amounts of gold suspension (0.5x–8x) were injected directly into the posterior vena cava or the portal vein using the blood of the intact circulatory system as a vehicle. After exposure periods of 30 and 60 min the liver was isolated and then connected to a perfusion system for a brief perfusion with buffer prior to fixation as described below.

2. *Perfusion experiments* on isolated perfused rat livers which were connected *via* the portal vein and the posterior vena cava to a perfusion system (perfusion technique described in detail by Quistorff, 1985; Quistorff and Grunnet, 1987). The isolated liver was perfused at 35°C for 10 min with a Krebs-Henseleit (K-H) buffer (Krebs and Henseleit, 1932) equilibrated with 95%/5% O_2/CO_2 at a rate of 25 ml/min. The different amounts of gold suspensions (0.5x–8x) were diluted with 75 ml buffer or buffer containing 20% washed bovine erythrocytes and perfused through the liver either in the direction portal vein–vena cava (antegrade perfusion) or vena cava–portal vein (retrograde perfusion). The rates of perfusion flow were: K-H buffer: 25 ml/min and 20% bovine erythrocytes in K-H buffer: 10 ml/min. The perfusion periods were: 2, 4, 8, 15, and 30 min. The uptake from the gold-K-H buffer medium was followed by measuring the concentration of gold in the medium spectrophotometrically every minute during the perfusion. In some experiments flow-through perfusion was employed, while in others recirculation was established during the gold perfusion in order to save on the expensive gold preparations.

3. *Installation experiments*. In order to test a possible effect of anoxia, so-called installation experiments were carried out with gold suspensions in KH buffer or in KH buffer

Fig. 1. Liver *in situ* after *in vivo* injection of a low amount of gold suspension (= x1) into the posterior vena cava, exposure for 60 min, and finally perfusion with buffer and fixative. A regular net-like pattern is seen on the surfaces of the liver lobes. The large median lobe is seen to the left, the left lobe to the right. FL: falciform ligament. K: right kidney. $\times 4$.

Fig. 2. Micrograph of a tissue block from a liver after gold exposure (amount = x1) *in vivo* for 30 min, taken through a stereo microscope. The mesothelial (intact) surface shows a regular net-like pattern of triangular areas with approaching points. $\times 14$.

with 20% washed bovine erythrocytes which had been exposed to 95%/5% N_2/CO_2 for 1 hr. Five ml diluted gold suspension were injected *via* the portal vein and the inlets and outlets closed for 4 min.

After exposure to gold the '*in vivo*', 'perfusion', and 'installation' livers were perfused for 3 min with K-H buffer and finally fixed by perfusion for 3 min with 2% glutaraldehyde in 67 mMol phosphate buffer (pH 7.2; 4°C, 10 ml/min) or 0.5% formaldehyde + 1% glutaraldehyde in the same buffer. Then the liver was transferred to the same fixative for 4–6 hr and left in buffer for the following procedures.

For visualization of the liver the following methods were applied: (1) The surface of the liver was studied and photographed during and after gold uptake. (2) Tissue blocks and slices were cut by hand or with a vibratome (100, 200, and 400 μ m sections) and studied in a stereo microscope. Vibratome sections were dehydrated with ethanol and *via* xylene transferred to anise oil to make sections transparent. (3) Smaller slices of tissue (1–2 mm thick) were cut from both superficial and deep parts of the liver lobes, some post-fixed in 1% OsO_4 for 1 hr, dehydrated and embedded in Epon 812. For light microscopy, 2 μ m and 8 μ m Epon sections were studied, some after staining with toluidine blue. For electron microscopy 400 nm sections were contrasted with uranyl acetate and lead citrate.

In the *in vivo* experiments, spleen and bone marrow were studied to estimate the uptake of gold by the macrophages of these tissues.

Results

In vivo experiments

Injection of a colloidal gold suspension into the posterior vena cava or the portal vein resulted in a golden tinge of the liver surface

gradually turning into reddish-brown. The staining was observed almost simultaneously on all lobes after a few minutes and in experiments with large dosages of gold particles reached its maximum after approximately 15 min. Thus the experimentation periods of 30 and 60 min probably allowed a complete uptake of the injected amount of gold suspension. After isolation and removal of blood by buffer perfusion, the liver displayed the deposition of gold as a regular reddish-brown or black pattern of confluent areas with well-defined borders towards pale brown areas (Fig. 1). Seen with the naked eye and through the stereo microscope the dark pattern was composed of triangular areas with approaching points isolating more or less circular areas (Fig. 2). Sections of the liver showed identical patterns on cut surfaces, but also variations of this pattern depending on the direction of cutting (Fig. 3). The dark areas were situated around small portal vein branches including terminal portal veins, while the light areas were concentric to small and terminal hepatic veins (central veins) (Fig. 4). Blocks cut to show the transition between the mesothelial surface and the cut surface showed continuity of the surface pattern and the cross-section pattern (Fig. 3). Increasing the amounts of gold resulted in an accentuation and blackening of the periportal pattern, while the shape of the pattern remained unchanged with constant borders. When equal amounts (measured spectrophotometrically) were applied, 50 nm gold particles gave a slightly darker pattern than 15 nm particles. 30 and 60 min experiments gave identical patterns.

The stereomicroscopic examinations of cleared slices (100, 200, 400 μ m thick) showed the same above-described pattern of dark areas in a regular pattern with rather well-defined borders towards light areas (Figs 5, 6, 7, 8). The network appeared incomplete since the points of the periportal areas

Fig. 3. Micrograph of a block from a liver after gold exposure (amount = $\times 4$) *in vivo* for 30 min. The dark pattern is seen both on the mesothelial and the cut surfaces. Note the continuation of the dark periportal areas at the cut edge (arrows). $\times 12$.

Fig. 4. Micrograph of a cut surface after 60 min exposure *in vivo* to a high gold dose (8x). The dark triangular areas show well-defined borders towards pale areas surrounding terminal hepatic veins (central veins: c). $\times 50$.

approached each other but did not fuse. The dark areas around larger ($100\text{ }\mu\text{m}$) portal branches exhibited wedges of light tissue reaching the portal veins. The shape of dark areas around terminal portal veins varied depending on the plane of section from oblong thorn-like to circular.

Light microscopy showed that the dark pattern was due to an uptake of gold by the Kupffer cells situated in the sinusoids around the portal vein branches (Fig. 9). The Kupffer cells were found along the walls of the sinusoids, often traversing these (Fig. 10). Accumulations of gold particles appeared as smaller and larger spheres in the cytoplasm, in increasing number with increasing amount of injected gold suspension. Smaller spheres were also found in endothelial cells, often forming a regular chain of spheres (Fig. 10), being less numerous in centrilobular areas. Around the terminal hepatic veins (central veins) a few Kupffer cells containing small amounts of gold were found and the transition to the dark pattern of the gold loaded Kupffer cells in the periportal areas was strikingly abrupt.

Electron microscopy showed the periportal Kupffer cells with various endocytotic structures (see below) containing distinct 15 and 50 nm gold particles. The Kupffer cells were rounded or oblong with elongated cytoplasmic processes (Fig. 11A). They were found upon the endothelium or partly between the endothelial cells forming part of the endothelial wall. They often traversed the endothelial lumen. Kupffer cell microvilli were found in close contact with microvilli of hepatocytes. The gold particles were found in: coated vesicles, tubular (worm-like) structures, fuzzy coat (pinocytotic) vacuoles, and secondary lysosomes. After exposure to high doses of gold the number increased and some of the fuzzy coat vacuoles were very large. The few centrilobular Kupffer cells contained few and small endocytotic structures.

In the endothelial cells gold particles were found in coated vesicles and larger uncoated vesicles (Fig. 11B).

Light microscopic studies of spleen and bone marrow indicated that compared to liver little gold was taken up by these organs.

Liver perfusion experiments

Perfusion of the liver with the Krebs-Henseleit buffer resulted in an evenly pale yellow liver (Fig. 12). After antegrade perfusion with gold-containing, oxygenated K-H buffer a periportal red stained pattern developed which turned still darker until the perfused gold had been taken up (almost) completely (Fig. 13). Independent of the dosages of gold used, the pattern developed already after about 30 sec and (almost) all gold had been taken up after about 15 min. The pattern as such was independent of the amounts of gold applied and the exposure periods, and similar to that described above in the *in vivo* experiments. In contrast to the *in vivo* experiments, the liver also developed a pale red staining of the areas found in the meshes of the web-like periportal pattern. The periportal uptake of gold was uniform as judged from studying the surfaces, but seemed to develop a little faster in the large median lobe. The stereo- and lightmicroscopic studies showed gold uptake by many periportal and a few centrilobular Kupffer cells (Figs 14, 16, 17) as described for the *in vivo* experiments. These studies revealed that the pale red staining was due to a much more efficient uptake of gold by the endothelial cells, especially in the centrilobular areas (except for the inner 30–40 μm), as opposed to the *in vivo* experiments. Electron microscopy confirmed the light microscopic observations. The retrograde gold perfusions of the liver gave a periportal pattern like that of the antegrade perfusions. It was a little less distinct, probably because of an even larger uptake by centrilobular endothelial cells. But still the

Figs 5–6. Micrographs of cleared slices ($200\text{ }\mu\text{m}$ thick) after 30 min *in vivo* exposure to two different amounts of gold suspension (Fig. 5: 1x; Fig. 6: 4x). The regular dark patterns are similar, but there is an accentuation after the higher dosage. $\times 6$.

Figs 7–8. Higher magnifications of cleared slices show dark portal areas (p) and terminal portal veins (t). Note that the points of the dark areas approach, but do not fuse. Central vein: c. $\times 40$.

uptake by the few centrolobular Kupffer cells was moderate, like that in antegrade perfusions.

Antegrade perfusion with the gold Krebs-Henseleit buffer containing 20% oxygenated erythrocytes gave results which were similar to those obtained with the gold K-H buffer alone (Fig. 15), but the uptake by the periportal Kupffer cells was more efficient resulting in a slightly more distinct periportal pattern. The uptake by the centrolobular endothelial cells was less prominent. Thus the addition of oxygenated erythrocytes to the K-H buffer seemed to give an antegrade uptake pattern which was intermediate in sharpness of borders between the *in vivo* and the buffer perfusion patterns. When the retrograde perfusion was used, these features were less evident.

Installation experiments

In order to test whether the uptake pattern of gold particles reflects the oxygen gradient along the sinusoid, experiments were performed with anoxic media installed in the liver and left without circulation for 4 min before fixation. The results showed the same distinct periportal uptake pattern as seen in the *in vivo* and perfusion experiments, thus indicating that neither the oxygen tension gradient nor a possible concentration gradient is decisive for the uptake pattern.

Gold particles were not found in hepatocytes in any of the above-described types of experiments.

Discussion

The present investigation shows that the distinct periportal pattern which is seen on the intact and cut surfaces of the liver after injection

of a gold-fibrinogen suspension into the bloodstream is due to a particularly high uptake of gold by periportal situated Kupffer cells. This pattern is remarkably similar to the pattern which was described previously in a study on the effect of digitonin-pulse perfusion of the rat liver (Quistorff and Rømer, 1989) (Fig. 18). An antegrade digitonin-pulse perfusion caused a permeabilization of hepatocytes in the afferent part of the sinusoid. This was followed by an elution of cytoplasmic matrix (cytosol), resulting in a light periportal pattern (Rømer *et al.*, 1990). We suggested that the observed pattern reflected the flow front of the hepatic microperfusion. As discussed in detail previously (Quistorff and Rømer, 1989) this hypothesis does not harmonize with the Rappaport acinar model (Rappaport, 1973; Rappaport *et al.*, 1954), since the demarcation lines obtained by the digitonin method form contours concentric with the terminal hepatic vein (central vein) rather than with the axis of the terminal portal vein. Recent angio-architectural studies by Matsumoto *et al.* (1979) and McCuskey (1988), histochemical studies by Teutsch (1984; 1988), and an immunohistochemical study by Lamers *et al.* (1989) reach the same conclusions. The Kupffer cell pattern of the present study appears identical to the patterns obtained with the above-mentioned different techniques. This fact suggests that the localization of the highly active Kupffer cells is defined by the natural flow distribution of the liver microcirculation. Figure 19 shows the two interpretations of the microcirculation and their relation to the Kupffer cell pattern described in this paper.

Previously it has been shown by morphologic and endocytotic estimates that there

Fig. 9. Light micrograph of an 8 μ m thick unstained plastic section. 60 min exposure *in vivo* to a high dose of gold (8x). Most gold is seen in Kupffer cells surrounding a portal vein branch (p), while there is little gold around a central vein (c). $\times 230$.

Fig. 10. Light micrograph of a 2 μ m thick stained plastic section. 30 min exposure *in vivo* to a 4x amount of gold suspension. Periportal gold containing Kupffer cells are seen in the sinusoids along the walls and traversing. Small gold containing vesicles are seen in the endothelial cells (arrows). $\times 1500$.

Fig. 11. A. Electron micrograph of a periportal Kupffer cell after exposure *in vivo* to a 4x amount for 30 min. Gold particles (50 nm) are found in different endocytotic structures. $\times 5600$. B. Gold containing coated vesicles in an endothelial cell. $\times 15,000$.

is a higher number of larger and more active Kupffer cells around terminal portal veins (defined as zone 1 of the Rappaport acinus) compared to the number, size and activity of Kupffer cells around terminal hepatic veins (Sleyster and Knook, 1982; Bouwens *et al.*, 1986). It has also been suggested by the same authors that the Kupffer cells do in fact form two subpopulations which differ in morphology and activity. That the Kupffer cell population is morphologically and functionally heterogeneous is strongly supported by the present study, since we found little uptake of gold by the few small Kupffer cells identified by light and electron microscopy in the centrilobular (perivenous) area, regardless of the route in which gold is presented. The accentuation of the periportal pattern with unaltered shape when dosages of gold are increased agrees with the findings of Sleyster and Knook (1982) and Bouwens *et al.* (1986) which showed a relatively higher uptake of latex particles by periportal Kupffer cells even at overloading doses.

The liver perfusions with an oxygenated K-H buffer containing fibrinogen-gold particles resulted in periportal Kupffer cell patterns like those obtained in the *in vivo* experiments, with antegrade and retrograde perfusions, and with and without recirculation. This confirms that the major uptake is by a periportal population of very active Kupffer cells. However, a certain amount of gold is taken up by endothelial cells and found in vesicles which are localized in the sparse flattened cytoplasm. This rather diffuse uptake does not disturb the dominant Kupffer cell pattern, but gives a weak background staining. The higher endothelial uptake by perfusion compared to that *in vivo* might at least partly be explained by the higher flow rate (25 ml/min versus 10 ml/min), but also the absence of corpuscular elements could

be of importance (see below). The higher uptake found by retrograde perfusions (compared to antegrade perfusions) may be explained by higher concentrations of gold at the inlet. Also high doses seem to increase the endothelial uptake. These observations probably indicate that the kinetics of endocytosis are different not only for the subpopulations of Kupffer cells, but also for Kupffer cells and endothelial cells. Recently te Koppele *et al.* (1990) using a light guide technique on the intact liver surface found that the rate of carbon uptake was reversed from perivenous (centrilobular) regions to periportal when an antegrade perfusion was shifted to a retrograde perfusion. Assuming that gold and carbon are treated alike, the apparent disagreement with the present study could be explained by the possibility that they first measure a perivenous endothelial uptake in the antegrade perfusions. Their published photos of antegrade perfusions (Figs 6 a, b. te Koppele *et al.*, 1990) show a considerable uptake of carbon by endothelial cells, as well as Kupffer cells.

The addition of bovine erythrocytes (20%) to the Krebs-Henseleit buffer gave an uptake more like that of the *in vivo* experiments. Since this addition claims a reduction of flow rate (25 ml/min to 10 ml/min) it is difficult to estimate if the uptake is influenced by rate of flow or presence of erythrocytes or both. The installation experiments with anoxia seem to indicate that neither the oxygen tension nor a possible concentration gradient is decisive for the difference in uptake by periportal and perivenous Kupffer cells. This is also supported by the observations that retrograde perfusion with buffer + 20% erythrocytes gives a distinct periportal pattern.

Considering the fact that the gold particle uptake pattern resembles the digitouin-cell

Figs 12-13. Isolated liver after the initial perfusion with buffer (Fig. 12) and after exposure to a high dose (4x) of gold suspension for 15 min (Fig. 13). The light yellow blood-empty liver develops a periportal pattern with a lighter background staining. $\times 3$.

Figs 14-15. Micrographs of cleared slices after perfusions with gold suspensions (2x) in Krebs-Henseleit buffer (Fig. 14) and in Krebs-Henseleit buffer containing 20% erythrocytes (Fig. 15). There is a higher uptake by periportal Kupffer cells and a smaller uptake by centrilobular endothelial cells with 20% erythrocytes, indicated by a more pronounced difference in staining. $\times 40$.

Fig. 19. Schematic drawings of liver lobules cut in the plane of the terminal portal vein and perpendicular to the terminal hepatic vein (central vein). 19A shows the zone-1 pattern according to the Rappaport model of the liver microcirculation. 19B shows the corresponding pattern in accordance with our interpretation of the microcirculation (as expressed in Fig. 18) assuming hemodynamic zones concentric with the terminal hepatic veins (central veins). Note that the distribution pattern of the highly active periportal Kupffer cells (Figs 1-8, 13, 14) is similar to that indicated in Figure 19B.

permeabilization pattern, it is important to realize the basic difference between the two processes: assuming even distribution of cholesterol in hepatocyte membranes along the sinusoids, the digitonin pattern arises as a consequence of a certain amount of digitonin (equivalent to the same molar amount of cholesterol) being distributed by the microcirculation. The gold uptake pattern, on the other hand, reflects one or both of the following factors: (1) Actual physical location of a certain population of Kupffer cells restricted to the area of the pattern and (2) uptake dependent on the dynamic distribution of gold by the microcirculation. The fact that the Kupffer cell labelling pattern remains the same with antegrade and retrograde perfusions; the fact that it is largely independent of flow rate, amount of gold and oxygen tension strongly suggests that the

macrophages taking up (endocytosing) gold are indeed selectively located in the area in which the pattern develops. This is quite unlike the digitonin distribution, which was found to correspond very closely to flow direction and amount of digitonin. Thus, the digitonin pattern created in pulse perfusion experiments reflects the distribution of the perfusion front, while the gold uptake pattern simply maps the location of Kupffer cells with a high capacity for gold uptake. The gold uptake by endothelial cells on the other hand, seems to be more dependent on rate and direction of flow and (to some degree) on the amount of gold. Consequently the endothelial cells could perhaps be used as an indicator of the flow distribution, as indeed is the case for digitonin. However, it would appear that the uptake speed of gold is much too slow for such an indicator function, and therefore the distribution pattern of gold uptake in endothelial cells must be reflecting concentration gradients rather than flow front distribution.

In conclusion, it can be stated that the periportal Kupffer cells form a functionally distinct population situated in a periportal zone concentric with the terminal hepatic veins (central veins). In our interpretation the zone does not correspond to that of the Rappaport acinus (zone 1), but rather to that found after antegrade digitonin-pulse perfusions. *In vivo* and perfusion experiments seem mostly to differ as far as the uptake by endothelial cells is concerned.

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Figs 16-17. Light micrographs of 8 μ m thick unstained plastic sections after 10 min exposure to a gold-buffer medium (4x). There is a high uptake in endothelial cells of all parts of the lobule. portal space: p. central vein: c. Fig. 16 $\times$ 120; Fig. 17 $\times$ 380.

Fig. 18. Micrograph of a liver block after a porta-cava digitonin perfusion. Note the regular net-like pattern of light, affected tissue on the mesothelial and cut surfaces with continuation at the cut edge (arrows). Compare with Figs 2 and 3. $\times$ 12.

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