Selective Implantation and Growth in Rats and Mice of Experimental Liver Metastasis in Acinar Zone One

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ABSTRACT

The functional zonation of liver tissue provides a framework for studying the implantation and growth of metastatic colonies within given zones of the hepatic acini. A very exact method for calibrating the position of metastatic foci in the hepatic acini was made possible by using the succinate-dehydrogenase reaction, which reveals the functional differences of the acinar zones. In mouse livers, metastasis was induced by intrasplenic injection of both high and low metastatic-capacity B16 melanoma and Lewis lung carcinoma cells. In rats, liver metastasis resulting from rhabdomyosarcoma was studied by injecting these cells into the s.c. tissue. All cases of metastasis occurred in hepatic acinar zone 1, and no significant differences were detected resulting from the type of tumor, its metastatic potential, or the procedure used for obtaining the metastasis. In subsequent experiments, metastasis was induced after first altering the zonal distribution of the hepatic extracellular matrix; distribution of the sinusoidal macrophages; and the sinusoidal diameter. However, even under these conditions, metastasis continues to occur exclusively in hepatic acinar zone 1. Thus, metastatic predilection for hepatic acinar zone 1 cannot be explained solely in terms of hemodynamic causes or the influence of extracellular matrix. Although it may still turn out that these elements play some secondary role in the phenomenon, our research points to the sinusoidal endothelial cells as a factor directly responsible for metastatic predilection for zone 1.

INTRODUCTION

To understand the metastatic process of malignant tumor cells, we assume that a complex series of sequential steps exists in which there is a constant interaction of tumor cells with both host defenses and peripheral colonized organs (1, 2). A selected metastatic phenotype of tumor cells has long been under investigation and many different properties have so far been shown to be preferentially expressed by highly metastatic cell variants (3–6). However, these metastatic properties cannot be the only factor accounting for colonization of peripheral organs since a suitable “soil” must exist for there to be successful implantation and focal growth of tumor cells as metastases. This fact was clearly stressed by Paget (7) in 1889 and, since then, many works have focused on the biological properties of target organs and the sites where the selective arrest, survival, and growth of circulating tumor cells occurs (8–14).

According to “soil” studies, two different factors could be of interest. The first factor is the anatomical makeup of the organ and, more specifically, both its position in the blood circulation and its tissue architecture. In this regard, some positive correlations between these anatomical properties and the metastatic patterns of malignant tumors have been found both in clinical and experimental systems (15–17), lending support to the functional sites within the liver tissue structure where metastatic colonization coincides with the colonization pattern in metastasis is a natural extension in cancer cells of the biological suitability of host defenses and peripheral colonized organs (1, 2). A selected populations of malignant cells with a definite preference for metastasizing certain organs (9). Although specific mechanisms of interaction between tumor cells and the normal microenvironment are not clearly understood, certain studies have shown that organ-specific selective mediators for metastasizing tumor cells could be the capillary endothelial cells (10, 11), extracellular matrix molecules (21), parenchymal cells with their secreted products (12, 22–24), and antitumor host-reaction systems (25, 26).

What must be determined is whether or not specific colonization in metastasis is a natural extension in cancer cells of the selective processes of homing, transvascular migration, and clonogenicity which have long been recognized as critical in processes such as embryonic colonization (27), lymphocyte recirculation and homing (28, 29), and the colonization and development of hemopoietic stem cells in specific environments (30, 31).

In order to analyze the functional interrelationship between hepatic tissue and metastatic cells, this study examines the functional sites within the liver tissue structure where metastatic tumor cells specifically implant as nascent micrometastatic foci. Assuming the possibility of a dual involvement of the biological properties of tumor cells and the particular conditions of host tissue for determining sites of metastatic implantation, the metastatic position in the liver was calibrated following a precise procedure which enabled us to relate the functional zones of liver parenchymal cells to the position of metastasis within the liver acinus.

Our results definitely show that the implantation of tumor cells in the liver tissue is not a random process, but one of highly specific, and therefore predictable, location in the periportal domain of liver acini (Rappaport’s zone 1). Thus, metastatic colonization coincides with the colonization pattern produced by hemopoietic stem cells when they develop in the adult liver tissue of phenylhydrazine-treated mice (31).

MATERIALS AND METHODS

Animals. Inbred C57Bl/6 mice (6 weeks old), and Wistar AG adult rats (10 weeks old) were purchased from Ifa Credo Laboratories (France). They were housed five animals to a cage and given food and water ad libitum.

Tumor Cell Lines and Culture Conditions. Mouse tumor cell lines: the B16F1 melanoma cell line was isolated by Dr. I. J. Fidler (32) and...
produces experimental hepatic metastasis following its intrasplenic injection in C57BL/6 mice (33). The B16F10 melanoma cell variant was also isolated by I. J. Fidler and has a higher efficiency than B16F1 melanoma cells in producing experimental hepatic metastasis following its intrasplenic injection (33, 34). The parental Lewis lung carcinoma (LLC-P) was made available by Dr. G. Vase (International Institute of Cellular and Molecular Pathology, Brussels, Belgium) and produces experimental hepatic metastasis following its intrasplenic injection in C57BL/6 mice (33). The high-metastatic Lewis lung carcinoma variant cell line (LLC-MH10) was derived from the selection of 10-times passaged hepatic metastasizing LLC cells (33). Rat tumor cell line: rhabdomyosarcoma cells (35) (54MH subline) were kindly supplied by Dr. Poupou (Villejuif, France) and they produce spontaneous hepatic metastases from the local primary tumor developed in the back following their s.c. injection. All these cell lines were grown in plastic tissue culture flasks, in the culture medium and atmosphere described earlier (33) and were routinely monitored for mycoplasma infection. For passage, cells were detached with 0.1% trypsin-EDTA 2 mm.

Experimental Procedure for Inducing Liver Metastasis. At least 20 animals were used for each experiment. Both B16 melanoma and LLC experimental hepatic metastases were produced according to the method of Kopper et al. (36). Briefly, a standard inoculum of 5 x 10^5 viable tumor cells in 0.1 ml of NaCl and MgCl2-free Hank's balanced salt solution was injected into the upper pole of the spleen of the anesthetized mouse (Nembutal, 50 mg/kg) (34). Spontaneous hepatic metastases from the RMs were obtained in the Wistar AG rat bearing a back s.c. primary tumor formed by the third week following s.c. injection of 2.5 x 10^5 viable tumor cells in 0.2 ml of medium without fetal calf serum.

Assessment of the Retention of Intrasplenically Injected B16F10 Melanoma Cells in the Liver Microcirculation. B16F10 melanoma cells were incubated for 18 h in DME medium in which 10 μM BrdUrd was added (Sigma Chemical Co., St. Louis, MO). These cells were then harvested as described above, and 5 x 10^3 viable cells in 0.2 ml of medium were intrasplenically injected, also as described above. At 5-min intervals in the first 0.5 h postinjection, mice were sacrificed (five mice at each time point) and the liver was collected from each mouse and frozen in liquid nitrogen. Ten cryostat sections were obtained from each liver and incubated in the SDH histochemical substrate as described below. Then, liver sections were washed in PBS, transferred to 70°C alcohol for 30 min, washed first in borate buffer (pH 8.5), and afterwards in NaOH 0.07 M for 2 min. They were then incubated in PBS-Tween 20 0.5% solution diluted 1:10 anti-BrdUrd purified antibody (fluorescein conjugated) (Becton Dickinson, Mountain View, CA) for 45-60 min; rinsed in PBS, and air dried. Finally, they were examined under fluorescent light microscopy and the number of tumor cells in high and low SDH activity hepatic tissue areas was recorded.

Assessment of the Transhepatic Passage of Intrasplenically Injected B16 Melanoma Cells. Mice were intrasplenically injected with 5 x 10^4 B16F10 melanoma cells and the presence of viable tumor cells was studied in the lungs. Injected mice were sacrificed on the first 1 h postinjection and lungs were removed and rinsed in cold Hanks' balanced salt solution and DME medium. Pulmonary tissue was cut into 0.5-1 mm^2 pieces which were placed in tissue culture plastic containing DME medium supplemented with 15% fetal calf serum, and incubated at 37°C in a humidified 5% CO2 atmosphere. The medium was changed each 2 days and tumor cells grown from organ explants were resuspended and intrasplenically injected in syngeneic mice as described above.

Assessment of the Size and Frequency of Metastatic Foci at Onset of Liver Colonization. On the 7th day of tumor evolution (for B16 melanoma and LLC) and on the 17th day (for RMs cells), animals were sacrificed under anesthesia and their livers fixed in formaldehyde 10%, dehydrated, and embedded in paraffin. Next, mice were serially sectioned (4-μm thick, 100-μm apart) and stained with hematoxylin &

The abbreviations used are: LLC, Lewis lung carcinoma; DME, Dulbecco's modified Eagle's medium; BrdUrd, bromodeoxyuridine; PBS, phosphate buffered saline; RMs, rhabdomyosarcoma; 17a:EE, 17α-ethynylestradiol; SDH, succinate-dehydrogenase; PV, portal vein; CV, central vein; DFVP, distance from the portal vein.

eosin. Quantitative information on the size of micrometastatic foci present in liver tissue in at least five sections per liver was obtained by using an integrated automatic image analysis system (Southern Microcomp. Instruments, Inc., Atlanta, GA). The number of foci per standard unit volume of liver tissue was calculated according to a stereological procedure described previously (33).

Pretreatment of Mice Injected with Tumor Cells. Partial hepatectomy was performed according to the experimental protocol described by Higgins and Anderson (37), 24 h before the intrasplenic injection of B16F10 melanoma cells in 20 mice. 17α:EE (Sigma) was prepared by dissolving the powder in ethanol and by subsequent dilution with paraffin oil prior to being administered to mice five days before the intrasplenic injection of B16F10 melanoma cells, as daily s.c. injections of 2.5 mg/kg in 0.1 ml of oil (38). Before tumor injection, however, several livers from treated mice were studied to ascertain whether the desired effect of the drug (dilatation of sinusoids of acinar zone 1 and constriction of sinusoids of zone 3) (38) had occurred. Carbon tetra-

Functional Labeling of Differentiated Domains in Liver Tissue and Quantitative Measurement of the SDH Activity of Hepatic Parenchymal Cells. For the selective delineation of hepatic acini and their functional zones as described in Rappaport (40), an in situ histochemical reaction for the SDH activity of liver parenchymal cells was performed on 10-μm cryostat sections from untreated and tumor-colonized livers and from treated-tumor-free and tumor-colonized livers, as described previously (41). Assessment of the hepatocellular heterogeneity at the acinar level was made by measuring the cytoplasmic SDH activity at a significant number of points selected at random in the tissue sections. Hepatocyte SDH-activity values were obtained by means of a Zeiss microspectrophotometer (Zeiss, Oberkochen, WR) (31, 41) and expressed as optical density units.

Obtention of the Hepatocyte SDH-activity Acinar Gradient for Calibrating Positions within Liver Tissue. For the quantitative determination of the position of micrometastatic foci within the liver acini, a reference scale was first applied to the portal-to-central vein pathway by step-by-step recording of the cytoplastic SDH activity of hepatocytes
at 10 consecutive equidistant points on an imaginary line drawn on the portal-to-central vein distance (Fig. 2). This procedure was repeated in 20 different acini per liver, with at least 10 animals per experimental group. All the spectrophotometric values were expressed in optical density units normalized with respect to the hepatocyte SDH-activity average value obtained at the point nearest to the PV. These values were plotted and the following third-degree equation (correlation coefficient, 0.995) was calculated from relative values of the gradient:

\[ y = 30.21 - 83.03x + 98.48x^2 - 44.62x^3 \] (A)

where \( y \) is the distance from the portal vein to the metastatic foci (DFPV) on a scale going from 0 at the PV to 10 at the CV, and \( x \) is the normalized optical density value determined for a light absorption wave length of 570 nm on SDH-stained hepatocytes occupying the area selected for positioning. New DFPV values can be interpolated from this equation (Figs. 2 and 3). A similar equation was obtained from rat liver sections.

Calibrating the Position of Micrometastatic Foci within the Liver Acini of Treated Animals. In order to quantitatively determine the position of metastasis within the liver acinus, in each experiment we examined every metastatic focus found in sections taken from the entire liver of each animal (at least 100 foci), and measured the SDH-activity of the hepatocytes surrounding each focus. The resulting average value was used as value \( x \) to interpolate the value \( y \), or expression of the DFPV, in the function (Equation A).

Statistical Evaluation. The level of significance in the differences between mean values was computed with Student's t test.

RESULTS

Size Distribution of Metastatic Foci in the Early Period of Liver Colonization. Under light microscopy, liver micrometastases were clearly identifiable on the 7th day after tumor cell injection (Fig. 1). By this time, they appeared as small rounded foci of around 20–30 nuclei per section. Individual foci occupied an average surface area of 4834 ± 457 \( \mu m^2 \) for B16 melanoma, 1981 ± 259 \( \mu m^2 \) for LLC, and 3180 ± 579 \( \mu m^2 \) for RMS (diameter range, 25–50 \( \mu m \)), which represented between one-tenth and one-fortieth of the average surface of individual rat and mouse liver acini. In addition, for all the tumor lines used, the volume occupied by metastatic tissue never exceeded 5% of the hepatic volume, and the average density calculated for all the tumor lines used was two to six micrometastatic foci per \( mm^3 \) of liver tissue, a distribution that did not significantly disturb liver tissue structure (42).

Identification of Liver Acini and Definition of Their Functional Zones in B16F10 Melanoma-Colonized Livers. Following a SDH histochemical reaction, the liver acini and their zones were clearly delineated on tissue sections from control and colonized livers (Fig. 1). High SDH-activity hepatocytes were revealed as a dark-stained parenchymal cell population around distributing and terminal portal vessels which defined acinar zones 1. By contrast, low SDH-activity hepatocytes appeared as a pale cell group around central veins which, therefore, marked acinar zones 3. The boundary area connecting these two separate regions was considered as zone 2. These segregated hepatocyte SDH-activity tissue domains marked, however, the functional extremes of a metabolic gradient at the level of the hepatic acinus which was easy to reveal by “stepwise” recording (10 steps) of this enzyme activity on an imaginary line drawn from the PV to the CV (Fig. 2). Repetition of this procedure in a large number of hepatic acini gave us a hepatocyte SDH-activity correlation on the PV-CV scale, where 0 would represent the initial point (at the PV) and 10 would represent the terminal extreme (at the CV) (Figs. 2 and 3).

Monitoring Passage through the Liver of Highly Metastatic B16F10 Melanoma Cells. The lung fragments extracted 20 min after intrasplenic injection of B16F10 melanoma tumor cells caused the growth of cells clearly identifiable as B16 melanoma 48 h after their explantation as primary culture. When intrasplenically injected once again into other animals, these cells produced liver metastases with the same efficiency as the original B16F10 melanoma cells.

Assessment of the Retention of B16F10 Melanoma Cells throughout the Liver Sinusoidal Network. The injection of BrdUrd-labeled B16F10 melanoma cells made it possible to observe that 15 min later, approximately \( 1.5 \times 10^6 \) had been retained inside the hepatic sinusoidal network. Of these cells, 65% were found in hepatic acinar zone 1, with the remaining 35% found in zones 2 and 3.

Distribution of Extracellular Matrix Molecules along the Sinusoidal Pathway within Liver Acini from Normal and Treated Mice. In normal animals, fibronectin, laminin, and collagen I and IV molecules were found homogeneously distributed...
Caliber of Hepatic Sinusoids in Normal and Treated Mice. Caliber of the hepatic sinusoids run from the terminal portal venules (PV) to the central vein (CV) and define a functional pathway that we have scaled 0 (at the PV end) to 10 (at the CV end). Dotted area, specific domain of metastatic foci implantation and growth of tumor cells. The average position of metastatic foci on the sinusoidal pathway was 3.19 (range, 2.31–4.49).

Throughout all the hepatic acinar zones (Figs. 2 and 4). This homogeneous distribution pattern of the extracellular matrix molecules did not vary significantly in the animals studied 24 h after partial hepatectomy, nor in those treated with 17αEE for 5 days. In animals treated for 5 days with CCl₄, there did occur a high concentration of laminin, fibronectin, and collagen IV in the central zone of the hepatic lobule—that is, in hepatic acinar zone 3 (Figs. 2 and 4; Table 1).

Quantitative Distribution of Kupffer Cells in the Sinusoidal Network of Liver Acini from Normal and Treated Mice. As shown in Table 2, in normal animals, intrasinusoidal macrophagic cells were found especially in acinar zone 1. In sinusoidal segments of zone 3, they account for 35% of the total number excluding the zone 2 subpopulation. Following hepatectomy, there is a global increase of macrophages in the animals, with a greater concentration in zone 1 (71%). The animals subjected to 5 days of 17αEE treatment showed a decrease in the total density per unit of surface area, but no significant variation was noted in the acinar distribution of macrophages. Finally, in animals treated with CCl₄ for 5 days, there was a clear inversion of the macrophage distribution pattern, with a decrease in zone 1 accompanied by a clear increase in zone 3 (90.14%) (Table 2).

Caliber of Hepatic Sinusoids in Normal and Treated Mice. There was no significant difference in the caliber of the hepatic sinusoids in normal animals and in those that had been partially hepatectomized or treated with CCl₄. In those that had been treated for 5 days with 17αEE, the previously described (38) dilatation of sinusoidal diameter occurred in zone 1, while the diameter of the sinusoidal pathway in zone 3 remained unaltered. These modifications caused an inversion of the normal shape of the sinusoids, which now tapered down from their widest point at the portal end to their narrowest point at the distal end (Fig. 5).

Calibrating the Position of Micrometastatic Foci from B16 Melanoma, LLC and RMS within the Liver Acinus of Normal Mice and Rats. The metastatic focus of B16F1, B16F10, 3LLP, and 3LLMH10 cells injected intrasplenic in mice, as well as the spontaneous metastases produced in rats by s.c. RMS tumor cells, all were distinctly located in the hepatic areas clearly identifiable as hepatic acinar zone 1 (Figs. 1 and 2). In order to calibrate their exact position, and after verifying that the SDH-activity gradient curves of all experimental groups coincided with that of the normal liver (once their optical density values had been relativized), the average value of the SDH activity of the hepatocytes surrounding each type of metastatic foci was interpolated on the standard curve. It was then possible to determine the position of these values on the 0 to 10 scale that goes from the PV to the CV of any hepatic acinus (Fig. 2 and 3). The result of this interpolation (Table 3) shows that the metastases of all the lines studied grow in the sinusoidal stretch running through hepatic acinar zone 1 (Fig. 2). In terms of distance, the position of all the metastases of all the tumors studied were found 2.31 to 4.49 units away from the portal end of the sinusoid (Table 3). The sinusoidal segment that runs from point 4.49 to the end emptying into a CV was never found to be the site of metastatic foci (Fig. 2).

Calibrating the Position of B16F10 Melanoma Micrometastatic Foci in Livers Altered by Various Treatments. Once animals had been sacrificed, the SDH-activity gradients derived from the livers of each experimental group were found to coincide with that of the standard curve (Fig. 3). As in the case of the experiments above, the position of the metastasis was calibrated. In animals injected intrasplenic with B16F10 melanoma cells, 24 h after hepatectomy the metastatic frequency increased (4.16 ± 1.46 foci/mm²) with respect to control mice (1.28 ± 0.6 foci/mm²) and yet, metastasis developed at mean point 3.00 on the sinusoidal pathway; that is, the same place as under normal conditions (Table 4). In animals injected with B16F10 melanoma cells 5 days after treatment with 17αEE, the number of foci was also higher (3.64 ± 0.44 foci/mm²) than in control mice and the average site where metastasis developed was at 3.97 (on a range between 3.48 and 4.49); that is, somewhat further from the portal end of the sinusoid, but always within the stretch that corresponds to hepatic acinar zone 1 (Fig. 2). In no case was metastasis observed to occur in the second half of the sinusoids (zones 2 and 3). In animals treated for 5 days with CCl₄ the number of foci was, once again, higher (7.11 ± 1.55 foci/mm²) than in control mice and yet metastatic foci occurred at average point 3.86, within a range of between 3.37 and 4.38. Metastasis was never observed to occur in the distal stretch of the sinusoid, that is, between points 5 and 10 (Table 4).

DISCUSSION

In an earlier study (31), we showed that hemopoietic cells induced to form colonies do not do so at random in adult organs like the liver capable of recuperating their hemopoietic function. Indeed, in the liver there exist certain well-defined areas where the induction and proliferation of these colonies takes place.
This study confirms the fact that individual tumor cells do not randomly implant and proliferate in the liver, but do so only in certain specific locations, and shows, moreover, that the process is apparently independent of physical factors such as mechanical or hemodynamically induced arrest.

In order to carry out this study, we have used a method for pinpointing the location of metastases by measuring where they occur along the hepatic sinusoidal path. This method is based on the functional identification of different zones in the hepatic acinus, and thus avoids the morphometric errors inherent in measurements based solely on anatomical criteria. Also used for the study were several lineages of B16 melanoma and Lewis lung carcinoma with different metastatic potentials, following intrasplenic inoculation. The results show that all the colonies, regardless of their nature and metastatic potential, implant in the periporal zone of the hepatic lobule (or perilobular area, to use a more anatomical term). Moreover, this same zonal selectivity occurs in spontaneous metastases produced by rat rhabdomyosarcoma following their s.c. implantation. On the whole, the mean position for the location of metastases is 3.19 (range, 2.31–4.49) starting from the portal vein, that is, within the first third of the hepatic sinusoidal path (Fig. 2). In the maximum probability range (where 98% of the metastases would be found), we find that for this frequency, metastases occur in the proximal half of the hepatic sinusoidal path, as shown in Fig. 2. This finding coincides with observations concerning the implantation and proliferation of hemopoietic stem cells in adult rat and mouse livers (31).

The arrest of circulating tumor cells and the consequent development of metastatic foci have traditionally been associated with factors such as hemodynamic characteristics (flow velocity, capillary pressure, and diameter) (2, 18), the presence of certain subendothelial matrix molecules (laminin, fibronectin, collagen IV, etc.) (43), the presence of macrophages partially blocking the sinusoidal lumen (44), and the presence of adhesion molecules on the endothelial cell surface (10, 11, 45).

We have developed experiments in which metastases were implanted into livers whose normal characteristics had previously been altered in the four aspects mentioned above. Partial hepatectomy of two-thirds of the liver mass produces a hemodynamic alteration of the residual stump involving a rise in pressure and flow velocity (46). Under these conditions, the distribution of the extracellular matrix is homogeneous throughout the entire length of the sinusoids, and the macrophages concentrate in the immediate periportal zone. Under

**Table 1** Quantitative evaluation of the laminin, fibronectin, and types I and IV collagens in the liver acinus of normal and carbon tetrachloride-treated (CCL4) mice

<table>
<thead>
<tr>
<th>Extracellular matrix molecules</th>
<th>Normal mice</th>
<th>CCL4-treated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin</td>
<td>0.88 ± 1.0</td>
<td>1.30 ± 1.3</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>4.08 ± 1.4</td>
<td>6.12 ± 5.2</td>
</tr>
<tr>
<td>Type IV collagen</td>
<td>9.58 ± 4.0</td>
<td>6.58 ± 4.9</td>
</tr>
<tr>
<td>Type I collagen</td>
<td>11.87 ± 5.0</td>
<td>7.01 ± 3.1</td>
</tr>
</tbody>
</table>

* Data are expressed as mean values ± SD from measurements of the fluorescent light intensity per unit area separately taken in periportal (zone 1) and perivenous (zone 3) areas of liver acini. Acinar zones 2 were excluded in the quantification.

**Table 2** Quantitative recording of the Kupffer cell distribution in high (acinar zone 1) and low (acinar zone 3) SDH-activity tissue domains of normal and treated mice

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Cells/field</th>
<th>Zone 1 (%)</th>
<th>Zone 3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mice</td>
<td>72.5 ± 2.0</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>Partially hepatectomized mice</td>
<td>136.6 ± 12.4</td>
<td>71</td>
<td>29</td>
</tr>
<tr>
<td>17β-Ethynylestradiol-treated</td>
<td>11.9 ± 0.6</td>
<td>82</td>
<td>18</td>
</tr>
<tr>
<td>Carbon tetrachloride-treated</td>
<td>102.8 ± 7.3</td>
<td>10</td>
<td>90</td>
</tr>
</tbody>
</table>

* Data are expressed as mean number of dianiminobenzidine positive cells (Kupffer cells) ± SD in 30 microscopic fields from at least ten mice per group. Microscopic field area = 0.84 mm².

**Fig. 4.** Immunofluorescent micrographs of liver tissue sections from normal (a) and CCL4-treated C57Bl/6 adult mice (b), stained by fluorescein-labeled antifibronectin antibody. An enhanced immunofluorescence was evident in the perivenous domain (zone 3) of CCL4-treated livers. PV, portal vein; CV, central vein; zone 1, periportal area (× 200).
these conditions, metastasis continues to occur at position 3.00, which we interpret as a proof of the lack of relation between regularity of arrest position and sinusoidal hemodynamics (pressure and flow).

However, this experiment does not exclude the possibility that arrest in this place may be related to the diameter of the sinusoid, which in general is narrower in the first half of its path from the portal end (42). We therefore carried out an experiment in which we took advantage of the known effect of estrogens on hepatic architecture, to obtain a significant dilatation of the proximal sinusoidal stretch near the portal venule, without modifying the distal stretch close to the center of the lobule (37). Under these conditions, the narrowest stretch of the sinusoids corresponds to the terminal zone 3. The metastases, however, continued to appear in the proximal zone, despite its having been greatly dilated. It does not seem likely, therefore, that such a restrictive, repetitive implantation site for metastases can be explained simply in terms of an area favored by conditions of blood circulation or the mechanical features of the vessels. In addition, the lower hydrostatic pressure and the higher hematocrit concentration in zone 3 sinusoids (40), make the possible involvement of hepatic circulation appear even less likely.

Macrophages, however, could play a role by creating obstacles in the path of cells traveling through the sinusoids (47). In fact, under normal conditions, macrophages tend to concentrate in greatest numbers in hepatic acinar zone 1, the proximal half of the hepatic sinusoid (48). This distribution pattern does not mean that there are no macrophages in zone 3 (distal third of the sinusoid), for they do exist there in great numbers (49), in contrast to the total absence of metastatic foci in this zone. What is more, treatment with CCl4 causes macrophages to concentrate preferentially in zone 3 (39). Even under these experimentally induced conditions, however, our results show the persistence of metastases in zone 1, together with the total absence of metastasis in zone 3. In our opinion, it would therefore be difficult to attribute to the macrophages the repetitive and selective location of metastases in the proximal stretch of the hepatic sinusoids.

At one point, we suspected that the metastatic cells were retained and destroyed in the liver itself upon their first reaching this organ (44, 50), and that they never actually reached the distal half of the sinusoid. But it is evident that tumor cells do travel through the distal half of the sinusoid: first, because we have detected, in the lung, cells with metastatic potential following their intrasplenic injection; and second, because we have also observed them stopped in the hepatic acinar zone 3 sinusoids (in the terminal zone of the sinusoid), 15 min after having been injected.

Table 3 Hepatocyte SDH activity around different micrometastatic foci and relative position of metastases within the liver acini (DFPV) of normal mice and rats

<table>
<thead>
<tr>
<th>Tumor cell type</th>
<th>Average SDH activity (RODU)</th>
<th>DFPV*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average value</td>
<td>Range</td>
</tr>
<tr>
<td>B16 melanoma (F1)</td>
<td>0.80 ± 0.18</td>
<td>3.97 (3.48-4.49)</td>
</tr>
<tr>
<td>B16 melanoma (F10)</td>
<td>0.89 ± 0.23</td>
<td>2.86 (2.44-3.39)</td>
</tr>
<tr>
<td>LLC (parent)</td>
<td>0.88 ± 0.19</td>
<td>3.00 (2.56-3.53)</td>
</tr>
<tr>
<td>LLC (MH10)</td>
<td>0.85 ± 0.21</td>
<td>3.39 (2.91-3.92)</td>
</tr>
<tr>
<td>RMS (S4MH)</td>
<td>0.90 ± 0.19</td>
<td>2.73 (2.31-3.23)</td>
</tr>
</tbody>
</table>

* SDH activity was expressed as relative optical density units (RODU) ± SD. The number of samples per experimental group was higher than 100.

Table 4 Hepatocyte SDH activity around B16F10 micrometastatic foci and their relative position within the liver acini of treated mice

<table>
<thead>
<tr>
<th>Tumor cell type</th>
<th>Average SDH activity (RODU)</th>
<th>DFPV*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average value</td>
<td>Range</td>
</tr>
<tr>
<td>Control mice</td>
<td>0.89 ± 0.23</td>
<td>2.87 (2.44-3.39)</td>
</tr>
<tr>
<td>Partially hepatectomized</td>
<td>0.88 ± 0.24</td>
<td>3.00 (2.56-3.53)</td>
</tr>
<tr>
<td>17α-EE-treated</td>
<td>0.80 ± 0.19</td>
<td>3.97 (3.48-4.49)</td>
</tr>
<tr>
<td>CC14-treated</td>
<td>0.81 ± 0.20</td>
<td>3.86 (3.37-4.38)</td>
</tr>
</tbody>
</table>

* SDH activity was expressed as relative optical density units (RODU) ± SD. The number of samples per experimental group was higher than 100.

17α-EE, 17α-ethyleneestradiol.
It might be thought that the selectivity is associated with extracellular matrix molecules, but there are two facts to contradict this hypothesis: (a) Under normal conditions, extracellular matrix molecules are distributed equally throughout the entire length of the sinusoid (51) and are also more exposed in zone 3 due to the porous nature of the endothelial cells in this zone (52). Metastases, however, concentrate in a zone 1 stretch. (b) In animals treated with CCl4, a high concentration of these molecules occurs in zone 3 (39), or the distal third of the sinusoid. Even under these conditions, however, the metastases occur only in the proximal half, with none appearing in the distal third. We would find it implausible therefore, to attribute the zonal selectivity of hepatic metastases solely to the distribution of matrix molecules.

Finally, there remains the question of what role the endothelial cells might play in defining the selective zone for the arrest and focal growth of neoplastic cells. In earlier studies, we showed that the hepatic sinusoidal endothelium is different in zones 1 and 3 (52), and that the differences develop prior to the functional definition of hepatocytes during the perinatal period (53, 54). We also showed that even though the traits of the endothelium may be altered by metastatic tumor cells, the endothelial response is different in the two zones (42). These permanent differences led us to think that the preference for zone 1 might somehow be related to the endothelial cells of this zone, bearing in mind the analogy with other transendothelial migration phenomena in certain areas of the lymph system (29), or of the bone marrow and other hemopoietic tissues (30).

Other studies have described the arrest and adhesion of migrating metastatic cells to certain specific capillary endothelial cells (11). The organ-specific seed and soil theory of metastasis (7, 8, 10) used in explaining this phenomenon is based on a set of microenvironmental factors present in the organ which influence the tumor cells (14). According to our data, in the zone of the sinusoid where metastases tend to locate, the following coincide: a specific, stable type of scarcely-fenestrated endothelial cell (52), a certain number of extracellular matrix molecules (51), a greater concentration of macrophages (48, 49), and of a special type of hepatocytes (40, 53), and a greater presence of pit cells (55). In addition, it is widely recognized that it is in the metastatic zone that the highest content of oxygen, nutrients, hormones, and growth factors, as well as certain specific metabolic characteristics coincide (40). We feel it possible that the endothelial cells play a major role in the homing of the metastatic cells, although we do not discard the possibility that the remaining local components, including metabolic and hormonal factors, also each play a role in the metastatic process. Whatever the case, the role played by the elements involved will be independent of the tumor line (malignoma, carcinoma, rhabdomyosarcoma) and its metastatic potential.

In conclusion, these experiments show that the specific implantation and proliferation of metastatic tumor cells, like that of hemopoietic stem cells, occurs in zone 1 of the hepatic acini, regardless of how the cells reach the liver, the caliber of the sinusoids, the concentration of Kupffer cells in each zone, and the distribution of extracellular matrix molecules in the subendothelial space.

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