Development of arterial blood supply in experimental liver metastases

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Abstract

Here we present a mechanism for the development of arterial blood supply in experimental liver metastases. Before analyzing the arterialization process of experimental liver metastases we had to elucidate a few key questions regarding the blood supply of hepatic lobules in mice. The microvasculature of the mouse liver is characterized by numerous arterioportal anastomoses and arterial terminations at the base of the lobules. These terminations supply one hepatic microcirculatory subunit (HMS) per lobule, which we call an arterial hepatic microcirculatory subunit (aHMS).

The process of arterialization can be divided into the following steps: 1/ distortion of the aHMS by metastasis, 2/ initial fusion of the sinusoids of the aHMS at the tumor parenchyma interface, 3/ fusion of the sinusoids located at the base of the aHMSs, leading to the disruption of the vascular sphincter (burst pipe), 4/ incorporation of the dilated artery and the fused sinusoids into the tumor. 5/ Further development of the tumor vasculature (arterial tree) by proliferation, remodeling and continuous incorporation of fused sinusoids at the tumor - parenchyma interface. This process leads to inevitable arterialization of liver metastases above the size of 2000-2500μm regardless of the origin and growth pattern of the tumor.

Introduction

It is widely accepted that hepatic metastases and tumors are predominantly supplied by arterial blood, a notion that serves as the basis for hepatic arterial chemotherapy and chemoembolization (1-7). The most cited article on this field dates back to the 1950s (1). Since then numerous papers have been published using human and experimental materials and different methods such as corrosion casting, confocal and electron microscopy, angiography, radiolabeled microspheres, and in vivo microscopy, have been used to study the blood supply of liver metastases (2-16). A large proportion of these articles have confirmed the original observation of Breedis and Young, (1) but no mechanism for the development of the arterial blood supply in metastases has ever been presented (2-7). On the other hand, numerous papers, including ours, have emphasized the contribution of the portal vein, either directly or through the sinusoids in the blood supply of hepatic metastases (8-14). This apparent contradiction might result from the observed continuity of the sinusoidal with the tumor vasculature and the presumption that blood flows in an "outside-in" direction from the sinusoids toward the tumor vasculature. Most of the studies dealing with the blood supply of metastases have neglected the importance of arterioportal anastomoses and other interspecies differences in the hepatic microcirculation, which could lead to seriously biased results. According to the observations of Yamamoto et al. (15) there are extensive arterioportal anastomoses throughout the vascular tree in rats, whereas a separate arterial and portal tree, without direct arterioportal communication, can be observed in hamster and human liver. Opinions about the presence of arterioportal anastomoses in mice are controversial (10, 16); therefore, we have addressed this question first.

The classic lobule can be divided into several conical hepatic microcirculatory subunits (HMS) supplied by a single inlet portal venule. Hepatic arterioles terminate either on the inlet

venules or directly on sinusoids. The number of these terminations within a lobule is species dependent. The blood flow through the inlet venules and terminal arterioles is regulated by sphincters (17). The most detailed studies on microcirculation of the liver and vessel architecture of liver metastases were performed by corrosion casting. However, in these studies the livers were completely filled with uncolored resin which made analyzing the 3D organization of the deep interlobular vessels difficult (10, 14, 15).

In the present study, we used a two color corrosion casting technique to analyze the blood supply in liver metastases of experimental tumors in mice. A special filling method was used to prevent the mixing of the "portal and arterial resin" upstream of the hepatic sinusoids. This technique enabled us to analyze separately the contribution of the two vascular systems to the blood supply of liver metastases and to establish the steps of the arterialization process.

Materials and Methods

Animals and tumor lines

The C38 colorectal carcinoma line was maintained by serial subcutaneous transplantations in C57Bl/6 mice, as described earlier (13). Liver metastases were produced by injecting $2x10^4$ tumor cells into the spleen of C57Bl/6 mice. Vascular casting was performed 15-18 days following tumor cell injection.

The highly metastatic Lewis lung carcinoma (3LL-HH) tumor line was maintained by serial intrasplenic transplantations of tumor cells obtained from liver metastases. Single cell suspensions were prepared from 14-day-old 3LL-HH liver metastases, as described earlier (12). 10^3 tumor cells were injected into the spleen of C57Bl/6 mice. Vascular casting was performed 12 days following tumor cell injection.

A2058 human melanoma cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO). To produce liver metastases, $2x10^4$ cells were injected into the spleen of anesthetized male SCID mice. Vascular casting was performed 28-33 days following tumor cell injection.

Vascular corrosion casting

A two color corrosion casting procedure was used to analyze the arterialization of liver metastases. Mice were anesthetized and a ligature was placed onto the vena cava just above the renal veins to prevent retrograde filling of the liver. The portal vein was cannulated (22G, Braun Melsungen AG, Melsungen, Germany) and secured with a tie, the chest was opened and the vascular system was flushed through the left ventricle with PBS containing heparin. When the effluent was clear, the thoracic aorta was cannulated (22G, Braun Melsungen AG, Melsungen, Germany). To remove air from this cannula a ligature was placed onto the aorta above the bifurcation into the iliac arteries. The aortic cannula was filled retrograde with PBS through a 30G needle connected to a syringe inserted into the abdominal aorta below the ligature. After filling, the ligature was tightened. The ligature on the vena cave was also tightened. Blue casting medium (Mercox 2-CL) was injected through the portal vein. The injection was monitored under a dissecting microscope and stopped when the resin reached the sinusoids (~0.2 ml resin) (Fig. 1. A, B). The blue resin was allowed to become thick and 1ml of red casting medium was injected through the thoracic aorta. The filling of the portal system with resin was necessary to prevent the flow of the red resin through arterioportal anastomoses (described in the results section) into the portal system and subsequently into the sinusoids and metastases resulting in false observations. However, all routes were left open where the arterial system was in direct connection with the sinusoids and metastases.

Altogether 53 animals were used for corrosion casting: 10 control, and 16, 15 and 12 mice bearing C38, 3LL-HH and A2058 metastases, respectively.

Determination of the percentage of arterial metastases and the size of the metastases

After the injection of the blue and red resins into the portal and arterial system, the livers were removed and cut in lobes; then every lobe was photographed from each side (Olympus SZ61 dissecting microscope, Olympus 7070 or DP 50 camera, Olympus Japan). Once the resin was cured completely the lobes were placed overnight in 35% KOH at 60^{o}C. The casts were washed in running tap water and placed in distilled water. Again, every lobe was photographed under water from each side. Metastases on the surface of uncorroded specimens were counted and their diameters were measured (Quick Photo Micro, Olympus, Japan). Most of the arterial metastases were completely filled with red resin, but some were only partially filled. In this case, the resin was not always visible on the surface of the uncorroded specimens. Therefore, the determination of the origin of the blood supply was performed by comparing the uncorroded and corroded specimens (Fig. 1. A, B). On the corroded specimens, metastases filled with any amount of the red resin through an artery directly connected to the metastasis were designated as "arterial". Metastases not having an arterial blood supply appeared on the corroded specimens as holes. In some cases the red color turned white either because the color particles were filtered out or the color was lost during the corrosion procedure. To determine the size of the metastases and the origin of the blood supply, 484, 907 and 485 metastases were analyzed from the C38, 3LL-HH, and A2058 tumor lines respectively.

Determination of the diameter of the arteries and the accompanying portal veins

Livers from four control animals were used to determine normal porto-arterial diameter ratio in 167 randomly chosen branch pairs originating from 7 different orders of the vascular tree of the mouse liver. Altogether 729 arterial metastases were isolated (252, 305, 172; C38, 3LL-HH, A2058), under the dissecting microscope using fine forceps. Each metastasis was photographed under water, and if metastases were supplied by one arterial branch, the diameter of the portal vein and the supplying artery was determined at the entry into the metastases. The diameter of the portal and arterial branches were measured ~1000 μm upstream from the metastasis wherever possible. The size of the completely filled metastases was easily determined by measuring the extension of the structure filled with the red resin. With incompletely filled metastases, the extension of the hollow space left by the metastases in the portal tree was measured.

Determination of the rate of cell proliferation in the arteries supplying the metastases

This procedure was performed only with the C38 tumor, because this tumor line produced a large proportion of metastases with centrally localized (described below), easily discernible arteries. BrdU labeling and tissue processing, with the exception of vascular casting were performed as described above. Large metastases (>2 mm, 8 pieces) located at the periphery of the liver were chosen and cut perpendicular to the flat surface of the lobe. The upper half of the metastasis was cut away. Subsequently, serial sections were cut until the artery was discernible on toluidine blue stained cryosections. Further 6-10 serial sections were cut and double labeling was performed for BrdU (Becton Dickinson) and NG2 proteoglycan (Chemicon). Nuclei were counterstained by DAPI. BrdU labeled and the total number of arterial wall cells (endothelial and smooth muscle cells, >2000) was determined, using a 40x or 60x objective (Nikon TE 300 fluorescent microscope).

Determination of the proliferation rate of tumor cells in the arterial and mixed blood metastases

BrdU labeling of proliferating tumor cells was performed as follows. One hour before the above described vascular casting was performed, 200 mg/kg BrdU was injected intraperitoneally. Following the casting procedure livers were removed and frozen. Cryostat sections (10 μm) were fixed in methanol (-20^{o}C) treated with 2 N HCl (15 min, 20^{0}C), anti-BrdU antibody (dilution 1:50, Cat. No: 347580, Becton-Dickinson) and fluorescent secondary antibody (Jackson Immunoresearch Inc., West Grove, PA). Nuclei were counterstained by TOTO3 or DAPI. BrdU labeled and total number of tumor cells were determined using micrographs captured by the Bio-Rad MRC-1024 (Bio-Rad, Richmond, CA) confocal microscope (four animals for each tumor, 3-5 metastases from each animal). Counting was performed using the morphometry system described above. Blood supply of the metastases was identified according to the autofluorescence of the dye in the resin. Arterial metastases had a strong red florescence (Ex568/Em580±32). In contrast, the mixed blood metastases were dark, or when filled with blue resin, had weak green fluorescence (Ex488/Em522±32).

Scanning electron microscopy (SEM)

Isolated vascular trees and metastases (over 240 specimens) were glued wet on metal stubs. After drying, the samples were coated with gold by a HBA 1 high-vacuum metal evaporator (Carl Zeiss, Jena, Germany). Observations were made using a Hitachi S-2360 N scanning electron microscope (Hitachi, Tokio, Japan) at 15-25 KV accelerating voltage.

Results

Microvascular architecture of the normal mouse liver

Arterioportal anastomoses were observed throughout the hepatic vasculature. The arterial blood entered the portal veins either directly or through the peribiliary plexus (Fig. 2. A, B). Arteries run in the vicinity of the peribiliary plexus. From the peribiliary plexus blood was also shed directly into sinusoids, which inlets were regularly spaced between terminal portal venules at the base of the lobules (Fig. 2. C, D, E). Short arterio-sinusoidal twigs were also observed in this region. Since the blood was shed from the peribiliary plexus into the closest lobules, asymmetry could be observed in the distribution of the arterial blood around larger portal tracts (Fig. 2. C, D). Other lobules, especially those situated on the opposite side (according to the artery) of the portal tract, were supplied by arterioles (marginal branches) running around the large portal tract ending either at the base of the terminal portal venules forming arteriopotral anastomoses or running up on the portal venules. These latter arterioles terminated on sinusoids at the base of lobules (Fig. 2. E, F). Similar terminations were observed at the peripheral areas of the vascular tree. Usually one or two terminations were detectable per lobule. Since the sinusoids were intentionally not filled through the portal system, only that part of the lobule was visible which was also fed directly by the arterial system. The resin entering the lobules through the arterioles formed conical structures which corresponded to the hepatic microcirculatory subunits (HMS) (17). To distinguish these HMSs from the others supplied exclusively by portal inlet venules, we called this subunit arterial microcirculatory subunit (aHMS). However it should be kept in mind that this subunit also drained mixed blood. No aHMSs were observed at the surface of the liver.

The arterialization process of metastases

The rate of arterialization

Three different tumor lines, that frequently metastasize into the liver, and exhibit different growth patterns were used for the experiments. The highly invasive 3LL-HH tumor line is characterized by replacement type growth, whereas the C38 colon carcinoma shows pushing type of growth and the A2058 human melanoma line has an intermediate growth pattern (12, 13, 18). The earliest arterialization of the metastases was observed in the case of the highly invasive Lewis lung tumor line (Fig. 3.). 40 percent of the metastases had well developed arterial blood supply below the diameter of 800μm. The other two lines, especially the differentiated colon carcinoma, acquired their arterial blood supply at a considerably slower rate. Only half of the metastases of the colon carcinoma were arterialized at the size of ~1500μm. However, the arterialization process accelerated later and almost all C38 metastases had arterial blood supply at the diameter of 2000μm, similar to the fast growing Lewis lung carcinoma. For the slow growing human melanoma, this value was about 2500μm.

Sequential events of the arterialization

Small avascular metastases and metastases supplied with mixed blood through the sinusoids generally appeared on the corrosion specimens as holes, since the liver vasculature through the portal system was filled only to the sinusoids (Fig. 1. A, B). However, micrometastases which early invaded the area of terminal portal venules were readily filled through the portal system and stained blue (Fig. 4. A, B). The majority of these metastases were not in contact with aHMSs. The process of arterialization was deduced mostly by the examination of the well organized C38 metastases allowing a clear view of the intra- and

peritumoral vessels. The first step of the arterialization process was the distortion of the aHMSs by the metastases (Fig. 4. C), i.e. the spherical metastasis impressed into the aHMS while the sinusoidal structure of the aHMS remained intact. This was followed by initial fusion of the sinusoids of the aHMS owing to the compression of the tumor (Fig. 4. D, E). The process of sinusoidal fusion was described earlier in detail by our group for the C38 tumor line (13). The present corrosion casting studies confirmed these data and demonstrated sinusoidal fusion at the periphery of the two other studied tumor types as well. However, the extent of the fusion differed among the tumor lines. It was most pronounced around the differentiated colon tumor followed by the highly invasive 3LL-HH tumor line (Fig. 4. F). The sinusoidal fusion was least advanced in the neighborhood of the metastases of the human melanoma cell line, which corresponded to the delicate vessel structure of its metastases (Fig. 4. G, supplemental Figure S1 A, B at http://ajp.amjpathol.org). Vascularization of the metastases was initiated by the incorporation of these fused sinusoids. Extensive sinusoidal fusion in the case of the C38 colon carcinoma led to the development of vascular lakes on the surface of the metastases that were directly connected to the arterial system (Fig. 5. A, B). When the fusion reached the base of the aHMS, the arterialization of the metastasis was just complete (Fig. 5. C, D). The fused sinusoids, together with the supplying artery, were incorporated into the tumor (Fig. 5. E). As the aHMSs were located at the base of the lobules, the artery entered the majority of the metastases from the hilar region.

Architecture of the supplying arteries

A large proportion of the metastases were supplied by one arterial branch (Table 1.). However, there was a tendency, especially in the case of the A2058 tumor line, for larger tumors to acquire more supplying arterial branches (supplementary Table S1, supplementary Figure S1 C at http://ajp.amjpathol.org). The arteries supplying the metastases became

strongly dilated while the neighboring arterial branches originating from this supplying artery were generally collapsed (Fig. 5F). A high proportion of the arterial "trees" was centrally positioned within the metastases of the C38 colon carcinoma but this phenomenon was also observed in the metastases of the other tumor lines (Table 1, Fig. 6. A-C, supplemental Figure S1 D at http://ajp.amjpathol.org).

The structure of the supplying artery inside the tumor, especially in the case of the C38 colon carcinoma, was unique. The diameter increased toward the center of the metastasis. No ramification could be observed along the trunk; all branches originated from a small area of this artery, which was approximately located in the center of the metastasis (Fig. 6. A-D). The ratio of the diameters of the portal vein running parallel to the metastasis supplying artery dropped significantly compared to that in the control liver (supplemental Table 2. at http://ajp.amjpathol.org). This ratio decreased with increasing tumor size (Fig. 7. A). The diameter of the supplying artery increased linearly with increasing tumor size (Fig. 7. B). The extent of dilatation dropped rapidly upstream, but persisted to some extent up to 1mm form the metastasis (supplemental Table 2 at http://ajp.amjpathol.org). The BrdU labeling index of the arterial wall cells at the base of the metastasis was 6.9±2.3%, suggesting that cell proliferation contributed significantly to the dilatation of the arteries. 10-15% of the metastases were supplied with arterial blood through the peribiliary plexus (Table 1). The artery accompanying the peribiliary plexus was not directly involved in the supply of these metastases. The portal vessels in the majority of the metastases were displaced, although a portion remained central but severely compressed (Fig. 6. D). Rarely (<5%), the metastases were supplied by arterial blood through arterioportal anastomoses inside the tumor.

Consequences of the arterial blood supply

We questioned whether the metastases acquired an arterial blood supply because they had grown bigger or whether the arterial blood supply provided a growth advantage to the metastases. There was no difference in the rate of tumor cell proliferation between metastases supplied arterially or portally (mixed blood) in two of the mouse cell lines (C38 and 3LL-HH). However, the proliferation rate was slightly but significantly increased in the arterially supplied metastases of the A2058 human melanoma cell line (Table 2, supplemental Figure S1 E, F at http://ajp.amjpathol.org).

Discussion

Using three different tumor lines, we have shown that metastases more than 2000-2500μm in diameter in the mouse liver inevitably become arterialized. Although the importance of arteries in nourishing metastases has long been recognized, mechanisms for the evolution of arterial blood supply have never been presented (1-7). Here we describe a mechanism for the arterialization of metastases in the mouse liver. This process can be divided into the following steps (Fig. 8): 1/ distortion of the aHMS by the metastasis, 2/ initial fusion of the sinusoids of the aHMS at the tumor parenchyma interface, 3/ fusion of the sinusoids located at the base of the HMS, leading to the disruption of the sphincter (burst pipe), 4/ incorporation of the dilated artery and the fused sinusoids, 5/ further development of the tumor vasculature (arterial tree) by proliferation, remodeling and continuous incorporation of fused sinusoids at the surface of the tumor.

The key element in the arterialization of the metastases is the so called arterial hepatic microcirculatory subunit (aHMS) observed in the mouse liver. The situation is more complicated in the human liver, where no direct arterioportal anastomoses are present but

arteries run in the interlobular vascular septa terminating on sinusoids along the whole circumference of the lobules (15, 17). Thereby, all HMSs are connected to arterioles, (in that sense all HMSs in the human liver are aHMSs) increasing the probability that an arteriole will be hit by a metastasis. This strongly suggests that metastases in the human liver become arterialized even earlier than in the mouse liver. Thus, arterialization takes place at the level of liver lobules. The size of a surface lobule in the mouse liver is approximately 500x500x600μm (unpublished observation). However, the actual size of the arterialized metastases is considerably larger, which can be explained by the expansive growth of the tumors resulting in certain displacement of bases of the surrounding aHMSs. This displacement is probably the lowest in the highly invasive rapidly growing tumor (3LL-HH), which becomes arterialized at a size closest to that of a lobulus.

Arteries enter the metastases from the hilar direction, which can explain the failure to detect direct connection between arteries and metastases in an *in vivo* microscopic study (16). The authors of the above study suggested that arterial blood entered the metastases through portal branches. Our observation contradicts this hypothesis as in the vast majority of metastases a separate arterial "tree" is responsible for nourishment of the metastases. The unique structure (no ramification along the trunk) of the central arterial tree suggests that no sprouting type angiogenesis takes place from this vessel and the moderate proliferation rate of cells constructing the arterial wall contributes only to the dilatation of the artery. The area of extensive ramification probably represents the original inlet of the artery into the sinusoids, modified by fusion, cell division, and incorporation.

The preferred growth of the tumors around the arteries may be related to the pressure difference between the arterial and portal systems. The low pressure portal and central veins are pushed aside by the metastasis, while the tumor grows around the firm standing, dilated, high pressure artery. The high percentage of metastases supplied by only one artery can be

explained if we view a metastasis as a burst pipe (reduced resistance owing to the dilated artery and the fused sinusoids) in the arterial system, which results in subsequent drop in the pressure and collapse of the neighboring arteries. These arteries are then pushed away by the growing tumor preventing the development of further supplying artery branches. The observation that the A2058 human melanoma has a larger percentage of metastases supplied by more arteries may be related to the small caliber intratumoral vessels, which may cause higher resistance to blood flow through the tumor, leaving the neighboring arteries uncollapsed. From these arteries new supplying branches can develop. Larger metastases could acquire further arterial blood supply from more distant, large arterial branches where the effect of the pressure drop caused by the metastasis is not so pronounced.

The finding that metastases with an arterial blood supply had no or only slight growth advantage over metastases supplied by mixed blood suggests that metastases become arterialized as a result of their increased size. This supports the notion, that the process of arterialization is purely mechanical in nature, governed by the pressure relationships in the liver vasculature.

The significant differences in the microvascular architecture between the mouse and human livers can have other consequences besides the possible earlier arterialization of the metastases in the human liver. Since arterioles in the human liver terminate on the whole surface of the lobules, a growing metastasis can hit more arterioles simultaneously resulting in a higher portion of metastases supplied by more arteries. This phenomenon can also reduce the number of human liver metastases having their arterial entry from the hilar direction. It is also important to note, however, that arterial connections on the surface of the metastasis might not all be functional (they will not feed the metastasis from an outside-in direction) if a dilated artery within the metastasis forces these arteries into collapse.

The observation that metastases, during their growth, develop an arterial blood supply contradicts the suggested role of sinusoids in nourishing liver metastases. The sinusoidal system is continuous with the vasculature of the tumor, but according to the frequently observed central localization of the arteries and considering that central veins are always located outside the metastases, blood should flow in an inside-out direction. In fact, in vivo microscopic studies have demonstrated that fluorescent dye or microspheres could not enter the metastases when injected into the portal system, whereas following arterial delivery the fluorescence appeared first within the metastasis, and the blood drained into the surrounding sinusoids (6, 16). Sinusoids surrounding the metastasis play no role in supplying the inner part of the metastases. They serve only as building blocks during the development of the tumor vasculature by sinusoid fusion and incorporation. However, portal vessels and sinusoids could have a role in the nourishment of the periphery of the metastases, especially when the arterial flow is blocked.

The rationale for using hepatic arterial infusion (HAI) is that it can maximize the exposure of metastatic colorectal cancer cells in the liver to high target concentrations of chemotherapeutic agents by their localized infusion (19). Although our experimental results provide further theoretical background for this therapeutical approach and HAI has demonstrated superior response rates compared to systemic chemotherapeutic treatments (19), its impact on the overall survival of colorectal cancer patients with hepatic metastasis is still unclear (20, 21, 22). Nevertheless, our results may also serve as a theoretical basis for further research into the effects of other anticancer drugs (such as novel targeted agents) administered intraarterially.

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Table 1.

Percent of metastases supplied by the different number of arterial branches

	Percent of metastases supplied directly by **one** arterial branch	Percent of metastases supplied directly by **two** arterial branches	Percent of metastases supplied directly by **3-or more** arterial branches	Percent of metastases supplied through the peribiliary plexus	Total number of metastases analyzed
3LL-HH	**68 (n.d.)**	**16,5**	**1**	**14,5**	**294**
C38	**82 (50)**	**9**	**-**	**9**	**252**
A2058	**63 (9)**	**22**	**6**	**9**	**172**

( ), percent of metastases with centrally positioned arterial branch. n.d.; not determined

Table 2.

Labeling indexes of metastases supplied preferentially by arterial or portal blood

	Arterial metastases	**Portal (mixed blood) metastases**
3LL-HH	**50,1±3,7**	**50,7±5,4 n.s.**
C38	**55,2±1,1**	**55,0 ±4,2 n.s.**
A2058	**38,3±2,5**	**34,1±1,9 #**

Mean ±SD; n.s.: not significant, #: $p<0.05$

Figure legends

Figure 1.

A. Liver lobes after the casting procedure containing arterial (red) and mixed blood (white) metastases of the C38 tumor line. The blue resin fills only the terminal portal venules.

B. The same lobes after corrosion. Arrows point to the hollow spaces of metastases not having arterial blood supply. Arrowhead points to a metastasis of which only the base was filled by the red resin; therefore its arterial connection was undetectable on the uncorroded specimen (arrowhead on figure 1. A).

Scale bar for A,B: 3mm

Figure 2.

Normal mouse liver

A. Direct arterioportal anastomosis (arrow) on a large trunk of the portal tree. A small amount of the red resin is spread on the previously hardened blue resin. Scale bar: 1mm

B. Detail of Fig.2A (arrowhead in Fig. 2A) viewed under the scanning electron microscope. Anastomosis between the peribiliary plexus and the portal vessel. Numerous capillaries of the peribiliary plexus (arrowhead) joined into one vessel which enters the large trunk of the portal vein (arrow). Scale bar: 300μm

C,D. Detail of a vascular tree of the liver viewed from opposite directions. The portal vein is filled with blue resin. Scale bar for C,D: 500μm

C. Shows the side where the artery (arrowhead) is running. Note the numerous aHMSs (arrows) spaced regularly between terminal portal venules (marked by arrows on D) along the portal vein. Each space between the terminal portal venules corresponds to one lobulus.

D. No aHMSs are visible on the other side of the portal vein.

E. Light micrograph of a single aHMS located above the peribiliary plexus and between terminal portal venules (arrows) at the base of the lobule. The space between the terminal portal venules determines the extension of the lobule. The red resin, which fills the sinusoids of the aHMS is in connection (arrowheads) with blue resin of the terminal portal venules, showing that the aHMS is part of the lobule. Scale bar: 200μm

F,G. An arterial HMS at the periphery of the vascular tree of the liver, SEM images.

F. The arteriole (large arrowhead) runs up on the portal venule and terminates in an aHMS at the base of the lobule (small arrow). The lobule is defined by the tree terminal portal venules (small arrowheads). Note that the red resin (inset) is present in the central venule (large arrow). The inset shows light micrograph of the same area. Scale bar: 200μm.

G. High power micrograph of the aHMS shown on Fig 2F. The main branch of the arteriole terminates in the aHMS (arrow). Smaller branches form direct anastomoses (arrowheads) with the portal venule. Scale bar: 90μm

Figure 3.

Percent of arterial metastases in relation to metastasis size.

Figure 4.

A. C38 micrometastasis on the surface of the liver grows close to a terminal portal venule (small arrowheads). The vascular lake (arrows) at the surface of the metastasis is filled with blue resin (inset) injected through the portal vein. An arteriole (large arrowhead) is visible close to the ramification of the portal venule which later represents the base of the lobule. The metastasis has not yet reached this region. Scale bar: 500 μm, Scale bar inset: 500μm

B. Detail of figure 4A. The arteriole branched off (arrows) is close the fork of the terminal portal venule. The resin hardly entered the aHMS (arrowheads). Scale bar: 90μm

C. Impression of a C 38 metastasis in an aHMS (small arrowheads). The sinusoids of the aHMS are nearly normal in structure. Inset shows the same aHMSs filled with red resin (arrow) at the base of the metastasis and the supplying arteriole (large arrowhead). Broken line marks the border of the metastasis. Small arrowheads point at the terminal portal venules entrapped within the metastasis. Scale bar: 100 μm. Scale bar for inset: 200μm

D. Compressed aHMS at the base (inset) of a C38 metastasis. Initial fusion of the sinusoids of the HMS is discernible (arrows). Other sinusoids of the aHMS are normal in structure (small arrows). On the inset the arrow points to an arteriole supplying the HMS. Drops of the resin (red dot on the light micrograph marked by arrowhead) are present in the central venule (arrowheads). Scale bar: 90 μm. Scale bar for inset: 300μm

E. The "nest" of a metastasis viewed from the top (from the surface of the liver). The sinusoids of the aHMS at the base of the metastasis are partially fused (arrowheads). Arrow points at a portal vessel within the metastasis projecting toward the surface of the liver. Scale bar: 100μm

F. Fusion of sinusoids in the metastasis of the 3LL-HH tumor. Large vascular lakes are not formed at the surface; instead tortuous vessels appear within the metastasis (large arrowheads). Note the impressions left by small tissue pillars (arrows) within the tumor vessels representing the last step of the fusion (reverse intussusceptive angiogenesis). The low density tortuous tumor vessels are continuous with the high density sinusoids of the surrounding liver tissue. Scale bar: 60μm

G. Small A2058 metastasis. The centrally located non-dilated artery (arrow) ramifies into delicate intratumoral vessels. Scale bar: 200 μm, Scale bar for inset: 500μm.

Figure 5.

A,B. Formation of a vascular lake from an aHMS at surface of the metastasis of the C38 tumor line.

A. The arrow points to the non dilated arteriole feeding the severely distorted and fused aHMS (arrowheads). Broken line is drawn along two terminal portal venules which are pushed aside by the growing tumor mass and thereby outlines the border of the metastasis. Scale bar: 500μm

B. The area marked by arrowheads on A is viewed from above by the scanning electron microscope. Extensive fusion of the sinusoids leads to vascular lake formation (arrowheads). The arrow points to the supplying arteriole. Scale bar: 200μm

C. Part of an arterialized C38 micrometastasis. The supplying arteriole is severely dilated (arrow) and ramifying into vessels which delineate the surface (arrowheads) of the metastasis. The arteriole has not yet been incorporated into the metastasis. Scale bar: 100μm

D. Scanning electron micrograph of the same structure shown on C viewed from above. Fused sinusoids (arrows) and vessels (arrowheads) are organized into basket-like form. Scale bar: 200μm

E. Arterialized C38 micrometastasis. Dilated arteriole (arrow) feeds the metastasis. The area where most of the branches arise (arrowheads) is just incorporated into the tumor but the vessels run on or close to the surface of the metastasis. The centre of the metastasis is avascular. Scale bar: 200μm

F. 3LL-HH metastasis supplied by a strongly dilated artery (arrow). There is an extreme size difference between the artery running toward the tumor and the artery accompanying the neighboring portal vein (arrowhead). Note that the two portal veins are about the same size. Scale bar: 1mm

Figure 6.

A. C38 metastasis with a well developed central arterial tree (black arrowheads on the inset). The portal vein was pushed aside (arrows). No ramifications can be observed on the SEM micorgraph along the trunk of the arterial tree (white arrowheads). Scale bar: 600μm, Scale bar for inset: 1mm

B. Detail of figure 6A. Vascular branches originate from one small area of the arterial tree (arrow) located in the center of the metastasis. Scale bar: 300μm

C. 3LL-HH metastasis. The centrally located artery dilates gradually toward the center of the metastasis where it ends in tortuous vascular lakes. The metastasis is not completely filled by the resin its borders are defined by the portal branches (arrowheads). Scale bar: 600μm. Scale bar for inset: 500μm.

D. C38 metastasis with a strongly dilated funnel like artery (arrow). Both the artery and the portal vein (arrowhead) are located centrally. The portal vein is extremely compressed obstructing the resin flow. Note that there are no ramifications along trunk of the artery. The branching point of the artery is located in the center of the metastasis. Scale bar: 200μm. Scale bar for inset: 500μm

Figure 7.

A. Portal vein-artery ratio in relation to metastasis size.

B. Diameter of the supplying artery at the entry to the metastasis in relation to metastasis size.

Figure 8.

Schematic representation of the arterialization process in liver metastases.

Step 0/ Micrometastasis growing within the liver lobule. The arterial HMS has normal architecture. Step 1/ distortion of the aHMS by the metastasis, Step 2/ initial fusion of the sinusoids of the aHMSs at the tumor parenchyma interface. Step 3/ Fusion of the sinusoids located at the base of the HMSs, leading to the disruption of the sphincter (burst pipe). Step 4/ Incorporation of the dilated artery and the fused sinusoids. Step 5/ Further development of the tumor vasculature (arterial tree) by proliferation, remodeling and continuous incorporation of fused sinusoids at the surface of the tumor.

Blue - mixed portal and arterial blood, Purple - mixed blood in the arterial HMSs, Red - arterial blood, Green - hepatocytes, Grey - tumor tissue, Black boxes - arterial sphincters