Tumorigenesis and Neoplastic Progression

Organ-Specific Metastatic Tumor Cell Adhesion and Extravasation of Colon Carcinoma Cells with Different Metastatic Potential

Kerstin Schlüter, Peter Gassmann, Andreas Enns, Timo Korb, Andre Heping-Bovenkerk, Jens Hölzen, and Jörg Haier

From the Department of General Surgery, Molecular Biology Laboratory, University Hospital Münster, Münster, Germany

Adhesive and invasive characteristics appear to be crucial for organ-specific metastasis formation. Using intravital microscopy we investigated the relation between the metastatic potential of colon carcinoma cells and their adhesive and invasive behavior during early steps of metastasis within microvasculatures of rat liver, lung, intestine, skin, muscle, spleen, and kidney *in vivo*. Colon carcinoma cells with low (HT-29P), intermediate (KM-12C), and high (HT-29LMM, KM-12L4) metastatic potential were injected into nude or Sprague-Dawley rats. Initial interactions with host organ microvasculatures were semiquantitatively analyzed throughout 20 to 30 minutes. Circulating cells passed microvessels in all observed organs without size restriction. All cell lines showed high adhesion rates, independent from their metastatic potential, within liver and lung but very rarely in other organs. Diameters of involved microvessels were larger than diameters of adherent tumor cells. Cell extravasation of highly metastatic HT-29LMM and KM-12L4 cells into liver parenchyma was significantly higher compared to low metastatic cells ($P < 0.05$). Our results indicate that colon carcinoma cells can arrest in target organs without size restriction. Cell adhesion of circulating tumor cells occurred in metastatic target organs only, likely attributable to specific interactions. Migration into target organs correlated with their metastatic potential. (Am J Pathol 2006; 169:1064–1073; DOI: 10.2353/ajpath.2006.050566)

Colorectal carcinomas will affect ~6% of the population in the Western countries during their lifetime. These carcinomas are the third leading cause of cancer-related deaths among women and men and are the most impor-

tant malignancies of the gut.¹ Approximately 50% of the cancer patients die within 5 years because of cancer-related problems, mostly attributable to metastatic lesions caused by the spread of cancers to near and distant sites. Even after potentially curative surgery, more than 30% of the patients with colorectal carcinomas subsequently develop metastases demonstrating that the spread from primary tumors to distant organs is usually the life-limiting aspect in colorectal carcinoma patients.²

Similar to other cancer entities, colorectal carcinomas often show organ preference of metastasis formation. The liver is the most common organ in which colorectal carcinomas establish distant metastases, and the liver is involved in more than 70% of patients with colorectal metastases. However, in 40 to 50% of these patients with liver metastases, other organs are also involved in metastatic colonization. The second most important organ for colorectal cancer metastasis is the lung, and 20 to 30% of all distant colorectal carcinoma metastases are found primarily in the lung. Isolated lung metastases with no evidence of other organ involvement are found in 5 to 10% of colorectal cancer patients. Other organs, such as the central nervous system, adrenal glands, spleen, skeleton, or skin together account for less than 10% of all colorectal metastases.^{2,3}

The metastatic process consists of a number of sequential, interrelated steps that can all be rate limiting.^{4,5} An important and early step during formation of distant metastasis appears to be the arrest of circulating tumor cells within the host organ.^{6,7} Approximately a century ago, two major hypotheses on the mechanisms of tumor cell arrest in metastatic host organs were proposed. Ewing⁸ assumed that the random mechanical lodgment of

Supported by the Innovative Medizinische Forschung Fund (University Hospital Münster) (Ha 1 2 01 01 to J.H.).

K.S. and P.G. contributed equally to this study.

Accepted for publication May 18, 2006.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

Address reprint requests to Jörg Haier, Molecular Biology Laboratory, Department of General Surgery, University Hospital Münster, Waldeyerstr. 1, 48149 Münster, Germany. E-mail: haier@uni-muenster.de.

circulating tumor cells in the first capillary system encountered is the determining factor and that the hemodynamics of the vascular system based on specific anatomical structures predicts the locations of secondary cancers. In contrast, Paget⁹ postulated in his "seed and soil" hypothesis that successful interactions of tumor cells (seeds) with the microenvironment of a particular target organ (soil) leads to formation of distant metastases in specific organs. Both models as well as the requirement for tumor cell extravasation into the host organ parenchyma are still intensively debated.^{10–14}

Most experimental data were obtained using *in vitro* systems that likely simplify biological responses and reduce their complexity within the host organs or using *in vivo* endpoint assays with macroscopic tumors as target structures. These endpoint assays are unable to differentiate between different steps of the metastatic cascade, such as cell adhesion, invasion, survival, and proliferation. Furthermore, biochemical interactions between adhesion molecules and their ligands during tumor cell adhesion within circulatory systems appear to be influenced by biophysical factors, such as shear stress caused by fluid flow, and cellular and soluble components of the circulating fluid.^{15–17} Intravital microscopy technologies have been recently used to investigate metastatic tumor cell adhesion within host organ microcirculation, such as in liver and lung.^{10–14} In these studies contradictory results were reported regarding the type of entrapment (mechanical entrapment^{10–12} versus active cell adhesion^{14,18}) and the requirement of invasion into host organ parenchyma (invasion^{10,11,13,18} versus intravascular proliferation¹⁴). Using colon carcinoma cells, we recently reported a lack of mechanical entrapment in rat liver sinusoids and a rapid extravasation into the liver parenchyma. These specific adhesive interactions were independent from the route of cell application, and tumor cells showed similar behavior within the liver sinusoids, if they were injected intravenously, intra-arterially, or intraportally.¹⁸ Furthermore, using this model we previously showed that specific cell adhesions mediated by different integrins and selectin ligands at the tumor cell surfaces appear to be required for successful tumor cell arrest within the hepatic microcirculation,^{19,20} with tumor cell adhesion modulated by the integrity of cytoskeletal components.²¹

This observation of specific adhesive interactions within the hepatic microcirculation suggested an involvement of this step in the organ-specificity of metastasis formation. Therefore, the direct intravital observation of metastatic tumor cell adhesion and invasion of colon carcinoma cells with different metastatic properties into the host organ parenchyma was used to investigate their specificity in different potential target organs. Cells were able to pass all capillary beds of the investigated organs without size restriction. We found that circulating tumor cells can specifically adhere within the microvasculature of liver and lungs, leaving remaining perfused vessel lumen. In contrast, in other organs (kidney, mesenteric, muscle, skin) no or only single cells were able to arrest within their microvasculature. Within the spleen circulating tumor cells were trapped, but diffuse fluorescence

suggested their rapid destruction. The metastatic properties of various colon carcinoma cell lines correlated with the numbers of cells that rapidly migrated into the host organ parenchyma.

Materials and Methods

Materials and Cells

Media (RPMI 1640, DME/F12) and fetal bovine serum were purchased from Gibco-BRL (Karlsruhe, Germany). All other chemicals were purchased from Sigma (Deisenhofen, Germany). Colon carcinoma cells with low (HT-29P), intermediate (KM-12C), and high (HT-29LMM, KM-12L4) metastatic potential were cultured in RPMI 1640 or DME/F12 medium containing 10% fetal bovine serum without antibiotics in humidified 5% CO₂/95% air at 37°C. Confluent cell monolayers were used during the log phase of growth. For experiments cells were rinsed with calcium-magnesium-free phosphate-buffered solution (CMF-PBS), trypsinized, and kept in serum-free adhesion medium (RPMI 1640 or DME/F12, bovine serum albumin 1%) for 60 minutes. Trypsinized cells were resuspended as single cell solutions in CMF-PBS at a final concentration of 1×10^6 cells/ml. This preparation did not interfere with adhesive and migrative properties *in vitro*.²²

Intravital Fluorescence Video Microscopy

Intravital microscopy was performed as previously described.^{18–21} Briefly, CD rats (200 to 250 g) or nude rats (80 to 100 g; Charles River, Sulzfeld, Germany) were cared for in accordance with standards of the German Council on Animal Care, under an approved protocol of the local animal welfare committee. Rats were anesthetized using inhalation of isoflurane (Curamed, Karlsruhe, Germany). Permanent catheters were introduced into the left heart via the carotid artery and the right heart via the jugular vein. After a wide median laparotomy was performed, the left liver lobe was carefully mobilized without disturbing hepatic microcirculation. Using a heated operating table, animals were fixed under an upright microscope and positioned on their left side. This positioning allowed a partial luxation of the mobilized left liver lobe that was placed on a specific holder to investigate its lower surface. During the experiments the liver was continuously irrigated with isotonic saline solution.

Alternatively, the anterior chest wall was removed in mechanically ventilated animals without disturbance of lung surfaces (careful avoidance of atelectases). An artificial pleura was placed on the left upper lobe or the right lower lobe enabling a direct observation of the ventilated and perfused lung *in situ* with minimal moving artifacts. Moreover, the anterior surface of the left kidney, mesenteric microcirculation closed to the small intestine, the abdominal rectus muscle, abdominal skin (from subcutaneous side), or the ventral part of the spleen were prepared for the investigation similarly to the liver surface.

Figure 1. Semiquantitative determination of the numbers of adherent and migrated cells. Thirty microscopic fields were analyzed within a 5-minute observation period. The numbers of adherent and migrated cells were counted in each interval. This procedure was repeated every 5 minutes. The numbers of arrested cells and relative migration rates were calculated as described within the text.

An upright epifluorescence microscope (Zeiss, Oberkochen, Germany) was used with a 20-fold objective that was located over a glass slip covering the organ surfaces. The microscope was connected with a video enhancer-zoom lens system and a low-light charge-coupled device video camera (Peiper, Düsseldorf, Germany) allowing real-time imaging via a separate monitor. Fluorescence images were recorded using a timer-containing S-VHS video system for further analysis.²³ In some experiments Vybrad-Dil (Molecular Probes, Leiden, The Netherlands) was used for intravital staining of endothelial cells, resulting in nonhomogenous *in vivo* labeling within the liver sinusoids.

In Vivo Observation of Metastatic Tumor Cell Adhesion

For intravital observation of adhesive interactions between circulating tumor cells and the host organ microcirculation, single cell suspensions of CalceinAM (Molecular Probes) fluorescence-labeled tumor cells (1×10^6 cells in CD rats, 0.5×10^6 tumor cells in nude rats) were injected intra-arterially throughout 60 seconds. Previously, we have shown that the route of cell application (left heart, right heart, portal vein) did not influence the adhesive or migratory behavior within the liver sinusoids.¹⁸ This technique did not interfere with cardiocirculatory or pulmonary functions of the animals. In case of the lung microcirculation, injection time was 120 seconds.

An off-line analysis was used to determine tumor cell behavior within the target organs. Various parameters

were used for further investigation and semiquantitative analysis of these interactions. First, occurrence of cell rolling was monitored. In addition, the latency was measured until first stable tumor cell adhesions were observed within the host organ microcirculation. The localization of stable tumor cell adhesions within the vascular tree and in relation to the diameter of the involved vessels was evaluated. If tumor cells were able to arrest within the microvessels, the diameter of the involved vessel was determined compared to the diameter of the adherent tumor cell. Furthermore, remaining blood flow within this vessel or its occlusion was investigated using fluorescence-labeled dextran or fluorescein isothiocyanate. A semiquantitative analysis of tumor cell adhesion and extravasation was performed throughout a 30-minute observation period (lung, 20 minutes), and the numbers of adherent cells were counted for each of the 5-minute intervals. Using a standardized procedure, all fields were analyzed in each observation period and average numbers of adherent cells, migrated cells, and total cells observed were counted (Figure 1).^{18–21} The numbers provided represent the total numbers of cells within 30 microscopic fields for each 5-minute period. In addition, the latency of tumor cell invasion into host organ parenchyma was determined. Relative migration rates were calculated as percentage of cells within the host organ parenchyma in relation to the total number of observed cells.^{19,20} In case of the pulmonary microcirculation, we were unable to differentiate between adherent and migrated cells, and therefore the numbers of arrested cells were counted in this organ.

For the differentiation of free moving and rolling tumor cells, we tried to use the formula that was introduced by Ley and Gaehtgens^{24,25} for the description of leukocyte behavior. This formula calculates a critical moving velocity based on the velocity of cells at the center of the involved microvessel and the ratio between cell diameter and vessel lumen. Glinsky and colleagues²⁶ adapted this formula for investigations on tumor cells within 80- to 100- μm arterioles. Applying this method in our methodological setup, however, did not result in reliable discrimination caused by the anatomical conditions within the rat microcirculation (sinusoids $\sim 10\ \mu\text{m}$). Because circulating tumor cells used in this study had a diameter that was approximately only 1 to 2 μm smaller than the diameter of the microcapillaries in liver and lung, central velocities cannot be determined. In addition, the ratio of diameters that is used in this formula is close to 1 and therefore also not applicable in our *in vivo* system.

Localization of Tumor Cells within Target Organs

To validate the intraparenchymatous localization of the observed tumor cells after rapid migration, in some experiments cells were preincubated with BrdU 24 hours before injection into the rats. Intravital microscopy of the hepatic microcirculation was performed as described above. After the 30-minute observation period the portal vein was cannulated and the liver was perfused with formalin solution using physiological hydrostatic pressure (8 to 10 cm H_2O). Finally, prefixed organs with maintained microvascular structures were removed, completely fixed, and paraffin embedded. Serial sections of the liver were used for anti-BrdU fluorescence staining. Alternatively, tumor cells were labeled using CellTracker Green CMFDA fluorescence staining (Molecular Probes). In the latter experiments, Vybrad-Dil acLDL was injected into the blood circulation of rats. Both dyes can be used in paraffin-embedded tissues. In the rat liver, however, this staining procedure resulted in a discontinuous staining pattern of endothelial cells and a slight uptake of the dye by parenchymatous cells. Similarly, anti-rat CD31 monoclonal antibody (BD Pharmingen, Heidelberg, Germany) also discontinuously stained sinusoidal endothelial cells. Therefore, BrdU- and fluorescence-based labeling techniques were used to confirm the localization of tumor cells within the liver tissue. Three-dimensional reconstruction of fluorescence-labeled slices was done using a laser-scanning confocal microscope and the Lucia5-software package (Nikon, Düsseldorf, Germany).

Because of the limitations of histological techniques, we also used raster electron microscopy to detect the localization of tumor cells within the liver sinusoids. At the end of the 30-minute observation period, the liver was perfusion-fixed using glutaraldehyde-based buffer solution and remained in this buffer for at least 48 hours. After stepwise dehydration using various concentrations of ethanol, small slices were cut for further processing. Slices were mounted on silicium wafers and dehydrated using increasing ethanol concentrations. Immediately be-

Figure 2. *In vivo* cell adhesion within liver sinusoids. Autofluorescence of liver parenchyma enabled differentiation between sinusoids and parenchyma. Circulating colon carcinoma cells adhered within the liver sinusoids. Remaining perfused vessel lumen suggested specific adhesive interactions without mechanical entrapment. Scale bar = 20 μm .

fore electron microscopy, samples were sputtered with ultra thin layers of carbon and platinum using an argon plasma sputter coater (Polaron). Dry and coated slices were used for raster electron microscopy (LEO 1530 VP; Zeiss SMT, Oberkochen, Germany). Figures were obtained using 5 kV and secondary electrons.

Results

In Vivo Metastatic Tumor Cell Adhesion

All circulating colon carcinoma cell lines were usually able to easily pass the microvascular vessels of potential target organs without mechanical entrapment. Even at the end of the observation period circulating cells were observed passing the capillary system of liver or other investigated organs. In liver sinusoids and pulmonary microvessels, first cells were observed that arrested within 2 minutes after injection of the cell suspensions (Figure 2). However, in contrast to adhering leukocytes, rolling of colon carcinoma cells has not been observed in all investigated organs and all cell lines used. Although the movement velocity of tumor cells within the capillaries differed with their length in few cells, this was probably not caused by rolling-like interactions with the underlying endothelial cells but by the small differences between tumor cell and microvessel diameters. In addition, this behavior was also previously observed independent from the inhibition of selectin ligands.¹⁹ In some cases, adherent cells appeared to lack adhesion stabilization and lost their adhesive bonds after several seconds resulting in recirculation of these cells (see Supplemental Videos S1 and S2 at <http://ajp.amjpathol.org>).

Adherent tumor cells were located within sinusoidal or pulmonary microvessels with larger diameters compared to the arrested tumor cells. This resulted in a remaining lumen and persistent blood circulation of these microves-

Figure 3. HT-29LMM cell with specific arrest to lung capillaries. A circulating cell adhered within the lung microcirculation without rolling. Remaining perfused lumen is visualized using fluorescence-labeled dextran, suggesting specific interactions between the tumor cell and vessel wall structures. Scale bar = 20 μ m.

sels with red blood cells passing the arrested tumor cell. In addition, the intravascular space has been contrasted using fluorescence-labeled dextran. Using this technique we visualized the remaining perfused vessel lumen in hepatic sinusoids and pulmonary microvessels with adherent cells (Figures 2 and 3; see Supplemental Video S3 at <http://ajp.amjpathol.org>). In contrast, circulating HT-29LMM cells did not arrest in subcapsular microcapillaries of the kidneys, muscles, skin, or mesentery; some of which have an even smaller diameter than hepatic sinusoids or pulmonary microvessels (Figure 4; see Supplemental Videos S4 and S5 at <http://ajp.amjpathol.org>). Although circulating tumor cells were observed even at the end of the observation period, the numbers of adherent

Figure 4. HT-29LMM cell arrest in different target organs of distant metastasis. Intravital microscopy was performed for 30 minutes (lung 20 minutes) in liver, lung, intestine, muscle, and skin ($n = 5$ rats in each organ). Numbers of arrested cells at the end of the observation periods adjusted to 30 microscopic fields for comparison between different organs are shown. In liver and lung, as major metastatic targets for colon carcinomas HT-29LMM cells became adherent, whereas in other organs that are rarely or not involved in metastatic progression of this tumor entity few or no cells adhered within their microvessels. Differences between liver/lung and the other organs were highly significant ($P < 0.0001$).

Figure 5. Rapid migration of adherent cells into the liver parenchyma. All cell lines were able to rapidly extravasate and migrate into the liver parenchyma, but the numbers of cells and relative migration rates differed between the various cell lines. An intravital microphotograph of a HT-29LMM cell is shown where the fluorescence-labeled cell is located outside the sinusoidal lumen. Scale bar = 20 μ m.

tumor cells mostly increased within the first 15 minutes, whereas afterward only a slight increase in the total numbers of arrested cells was observed resulting in plateau-like patterns of the time curves. In the spleen, few fluorescence-labeled cells were found, but fluorescence mostly occurred in small but diffuse areas suggesting rapid destruction of circulating tumor cells.

In the liver small numbers of adherent cells were observed that rapidly started to migrate from the intravasal compartment into the hepatic parenchyma within the 30-minute observation period. First, migrated cells were observed within 5 minutes after injection of the cell suspension. However, the ability to invade into the liver parenchyma differed between the different cell lines used (Figure 5).

To evaluate potential interference of rat's immune system, we compared adhesive tumor cell behavior within the hepatic microcirculation of immunocompromised nude rats and immunocompetent CD rats. Using weight-adapted numbers of injected HT-29LMM cells (0.5×10^6 tumor cells in nude rats versus 1×10^6 tumor cells in CD rats), all characteristics of tumor cell adhesion and extravasation were very similar in both strains. Although the diameter of nude rat liver sinusoids was slightly smaller than that of CD rats, significant differences were not found for the numbers of adherent cells, totally arrested cells, or relative migration rates (Table 1). Similarly, passing of circulating cells and cell arrest in the lung were comparable in both rat strains.

Metastatic Properties Correlate with Invasive Behavior

For investigation of the potential role of organ-specific cell adhesion and migration into the host organs as rate-limiting steps during metastasis formation we compared

Table 1. Tumor Cell Arrest Within Hepatic Microcirculation of Nude and CD Rats

Interval (minutes)		5	10	15	20	25	30
Adherent cells	Nude	23.1 ± 12.5	23.7 ± 7.8	25.5 ± 6.3	22.6 ± 6.3	22.5 ± 6.8	21.8 ± 7.6
	CD	22.3 ± 7.5	22.7 ± 11.7	23.2 ± 8.4	20.0 ± 7.6	20.1 ± 11.5	19.4 ± 9.9
Total arrested cells	Nude	23.3 ± 12.5	28.8 ± 10.4	30.7 ± 7.8	27.8 ± 7.6	26.8 ± 8.2	26.2 ± 9.4
	CD	22.9 ± 7.4	25.8 ± 10.8	29.1 ± 7.7	25.8 ± 7.1	25.4 ± 11.5	25.9 ± 8.8
Relative migration rates (%)	Nude	1 ± 2%	17 ± 5%	17 ± 3%	19 ± 4%	16 ± 6%	16 ± 3%
	CD	0 ± 0%	9 ± 6%	21 ± 13%	23 ± 10%	22 ± 6%	23 ± 10%

Numbers of adherent HT-29LMM cells, arrested (adherent and migrated) cells, and relative migration rates were compared between both rat strains using Student's *t*-test (CD, *n* = 8; nude, *n* = 7). Significant differences were not found.

various colon carcinoma cell lines with different metastatic potential. All cell lines showed comparable adhesive behavior with high numbers of adherent cells in liver and lungs. Although the numbers of adherent cells differed between the various cell lines, we did not find a correlation between their metastatic potential and their adhesive properties in liver and lungs. In contrast, migration of adherent cells into the liver parenchyma was limited in low metastatic HT-29P and nonmetastatic Caco-2 cells (relative migration rates up to 11 to 18%), whereas intermediate metastatic KM-12C (relative migration rate up to 38%) and highly metastatic HT-29LMM and KM-12L4 cells (relative migration rates up to 23 to 45%) showed significantly higher percentages of migrated cells (*P* < 0.005 to 0.05). All other parameters, such as absence of rolling, localization of cell adhesion, and lack of size restriction with remaining perfused vessel lumen were similar (Table 2).

Furthermore, we directly compared the HT-29P versus HT-29LMM and KM-12C versus KM-12L4 cells in which the latter clones were derived as highly metastatic subclones from the parental lines. In HT-29 cells the highly metastatic subclone HT-29LMM showed comparable cell adhesion within the liver sinusoids but significantly higher numbers of migrated cells after 15 minutes (*P* < 0.05). Similarly, in KM-12 cells the migration rates were significantly higher in the highly metastatic KM-12L4 cells (*P* < 0.05), although using the intermediate metastatic KM-12C subclone significantly higher numbers of adherent cells were observed (*P* < 0.05) (Table 2).

Localization of Cells within Liver Parenchyma

During the intravital observation, we were able to differentiate between adherent cells that were located within the microvessels and extravasated cells that had left the blood circulation. However, the endothelial cell lining was not clearly visible *in vivo* and three-dimensional visualization was not available in the intravital system. Therefore, arrested tumor cells were fixed within the target organ. Using BrdU and fluorescence dye staining techniques, HT-29LMM tumor cells were traced within the liver after fixation of the organs maintaining its microvascular structure. Adherent tumor cells were identified within the liver sinusoids, and remaining vessel lumens were confirmed using serial sections and three-dimensional reconstruction. In addition, some HT-29LMM cells were found that had left the microcirculation within 30 minutes after cell injection and were clearly located between the hepatocytes (Figure 6, left). Despite the discontinuous staining of hepatic sinusoidal endothelium extravasated tumor cells were detected. This was confirmed by raster electron microscopy (Figure 6, right). Using three-dimensional reconstruction of fluorescence-labeled tissue samples, adherent cells were found that were still intravascularly located (Figure 6, left; see Supplemental Video S6a at <http://ajp.amjpathol.org>). Some cells were partially covered by red-labeled structures suggesting that they started to extravasate (Figure 7B; see Supplemental Video S6b at <http://ajp.amjpathol.org>). Other green-labeled tumor cells were completely surrounded by Dil-

Table 2. Statistical Comparison of Low and Highly Metastatic Cells

Interval (minutes)		5	10	15	20	25	30
HT-29P/HT-29LMM							
Total arrested cells	HT-29P	20.8 ± 6.4	22.0 ± 8.4	22.8 ± 6.9	21.2 ± 7.9	20.6 ± 5.7	19.8 ± 6.7
	HT-29LMM	19.1 ± 9.5	20.6 ± 10.5	24.7 ± 11.4	21.4 ± 9.3	21.0 ± 12.8	21.8 ± 11.4
	p	ns	ns	ns	ns	ns	ns
Relative migration rate (%)	HT-29P	4 ± 6%	9 ± 6%	14 ± 11%	15 ± 7%	18 ± 13%	16 ± 12%
	HT-29LMM	0 ± 0%	12 ± 9%	23 ± 12%	25 ± 9%	29 ± 17%	29 ± 15%
	p	ns	ns	0.042	0.019	ns	0.019
KM-12C/KM-12L4							
Total arrested cells	KM-12C	28.5 ± 10.8	45.9 ± 16.7	51.6 ± 13.8	51.1 ± 15.1	51.6 ± 23.7	52.2 ± 14.3
	KM-12L4	25.0 ± 6.7	33.4 ± 7.5	39.0 ± 10.2	36.6 ± 10.2	37.4 ± 8.7	44.2 ± 11.7
	p	ns	ns	0.043	0.043	ns	ns
Relative migration rate (%)	KM-12C	0 ± 0	18 ± 5	26 ± 11	35 ± 6	38 ± 7	38 ± 7
	KM-12L4	14 ± 5	31 ± 5	40 ± 6	39 ± 5	44 ± 7	45 ± 5
	p	<0.0001	<0.0001	0.032	0.049	0.045	0.012

Numbers of arrested cells (adherent and migrated) and relative migration rates were pair-wise compared between HT-29P/HT-29LMM and KM-12C/KM-12L4 cells using Student's *t*-test (*n* = 10 in each group). ns, not significant; p, level of significance.

Figure 6. Early extravasation into liver parenchyma. HT-29LMM cells were labeled and injected intracardially as described. After 30 minutes of intravital microscopy the liver was fixed using infusion (formalin or glutaraldehyde) with physiological hydrostatic pressure via the portal vein. Subsequently, different detection methods were used to determine the localization of the tumor cells. Left: BrdU was visualized using immunofluorescence (green) and the resulting picture was fused with conventional H&E staining; the tumor cell is clearly located within the liver parenchyma. Right: A migrating tumor cell leaving the liver sinusoid is shown using raster electron microscopy.

labeled tissue showing a complete extravasation of these cells (Figure 7C; see Supplemental Video S6c at <http://ajp.amjpathol.org>).

Discussion

It has long been accepted that most malignant tumors show an organ-specific pattern of metastasis. For example, colon carcinomas metastasize usually to liver and lung but rarely to bone, skin, and brain and almost never to kidneys, intestine, or muscle. In contrast, other tumor entities, such as breast carcinomas, frequently form metastases in most of these organs. This specific formation of secondary tumors at distant organs appears to require a number of steps that have to be successfully completed by metastasizing tumor cells.²⁷ After these cells enter the blood circulation directly or indirectly via the lymphatic system, they have to arrest in secondary host organs. Mechanical entrapment^{11,12} and receptor-specific seed- and-soil adhesions^{14,18} are currently discussed as determining factors for this cell arrest. However, experimental data using different types of assays revealed contradictory results regarding the specificity of initial interactions between host organs and circulating tumor cells.

Our results provide evidence that the successful colonization of distant organs by circulating colon carcinoma cells is, at least in part, mediated by specific adhesive interactions between these cells and the microvasculature of the host organs. The observed cell arrest in microvessels with diameters larger than the diameters of the tumor cells and the remaining perfused lumen suggest that mechanical arrest caused by size restriction is less important for the cell arrest in host organs in our rat model. The requirement of specific cell interactions within the microvasculature of metastatic host organs is further supported by our observation that the cell arrest occurred in organs that are usually targets for colorectal metastases. In renal and other capillary systems (mesenteric, skin, muscle) with even smaller

diameters, cell arrest was not or very rarely found, whereas in the relatively large pulmonary microvessels circulating tumor cells were able to successfully adhere to the vessel wall. Similar results of specific cell adhesions within the microvasculature of target organs were reported by others.^{14,28} For example, using different experimental approaches and breast and prostate cancer cells, Glinskii and colleagues^{29,30} demonstrated that mechanical entrapment is not sufficient for metastatic cell arrest in distant organs, whereas intercellular adhesion appears to be essential for this process. Using isolated, ventilated, and perfused rat and mouse lungs Al-Mehdi and colleagues¹⁴ reported that metastatic HT1080 fibrosarcoma cells attached to the endothelia of pulmonary precapillary arterioles and capillaries that had diameters considerably larger than the tumor cells. In addition, tumor cell deformability can contribute to their passage through narrow capillaries, which has been shown in our previous study and by others.^{21,31}

Although circulating tumor cells were observed throughout the 20- to 30-minute observation period, the numbers of arrested cells increased mainly during the initial 10 to 15 minutes, followed by a plateau-like shape of the curve. Both in liver and lung observed microvessels were not overloaded with arrested cells and, therefore, the question was raised whether specific characteristics of target organs might be responsible for this time dependence. In our view various hypotheses can explain this pattern. 1) In metastasis-prone organs only certain adhesion sites are able to sufficiently provide anchorage areas, eg, neighborhood of Kupfer cells or intraorgan variance of endothelial cells. 2) Although clonal cell lines were used only few cells are able to interact with target organs (eg, cell cycle dependence). 3) Finally, interactions with other circulating cells, such as platelets, increase throughout time and can inhibit successful cell arrest within the target organs. Future studies will investigate underlying mechanisms.

Extravasation of tumor cells was rare, and nontransmigrated cells have been reported to be rapidly cleared by

Figure 7. Double-fluorescence staining of tumor cells within liver parenchyma. Methods were as for Figure 6. HT-29LMM cells were labeled with CellTracker Green CMFDA (green) and Vybrad-Dil acLDL (red) was injected intravascularly for fluorescence staining of endothelial cells (other cellular components of liver parenchyma were also stained using this method). Three-dimensional reconstruction was performed after confocal microscopy. **I:** Three-side view rectangles represent area for volume reconstruction. **II:** Three-dimensional surface volume reconstruction with intensity histograms for green and red staining along the marked lines. **A:** A tumor cell that started to migrate but is mostly localized within the sinusoidal lumen (see Supplemental Video S6a at <http://ajp.amjpathol.org>). **B:** Two HT-29LMM cells showing one that is partially covered by red-stained cells whereas the other cell almost completes extravasation (see Supplemental Video S6b at <http://ajp.amjpathol.org>); intensity histogram confirms a red-labeled cell layer covering the green tumor cell. **C:** The tumor cell is completely surrounded by parenchymal cells; volume reconstruction and intensity histogram show faint green color behind red-labeled cells (see Supplemental Video S6c at <http://ajp.amjpathol.org>).

the lung. Similarly, in our study various colon carcinoma cell lines specifically adhered to the vessel wall of pulmonary capillaries without size restriction. However, we were unable to differentiate between adherent and migrated cells in our *in situ* lung model.

Currently, it remains controversial if and where tumor cell apoptosis within the host organ microvessels occurs. For example, using HT-1080 fibrosarcoma cells and the above-mentioned isolated lung model only vascular endothelial cell-bound tumor cells developed into metastases, while extravasated cells underwent apoptosis within 24 hours.¹⁴ In contrast, a recent publication by Tsuji and colleagues³² demonstrated different apoptotic fate of two colon carcinoma cell lines within the hepatic circulation. Invasive cells were able to survive whereas intravascular cells became apoptotic after 6 to 12 hours. Carcinoma cells might be more susceptible to external forces, such as under shear exposure, whereas sarcoma cells originate from tissues that have to resist external forces physiologically and might be more stable within the blood flow.

In our experimental system some modifications were used compared to other investigators. Steinbauer and colleagues³³ reported size restriction in a mouse system, but they used fluorescent beads ($>10\ \mu\text{m}$) for comparison that appear to be more rigid than circulating tumor cells. In contrast to Chambers' group,^{11,12} who also found mechanical cell arrest of circulating tumor cells in mice as the main mechanism, we used an upright microscope that enabled us to investigate the surface of the organs. This approach can avoid additional tissue pressure at the lower side of the liver lobe caused by the weight of the tissues above. This additional tissue pressure might be responsible for increased intravascular pressure and/or reduced size of the microvessels subsequently interfering with tumor cell adhesion. Mook and colleagues³⁴ injected very high numbers of cells (5×10^6 in 0.5 ml) directly into the portal vein. In our previous study¹⁸ using a similar technique, we found that this procedure can result in severe disturbances of the hepatic microcirculation with subsequent occlusion of sinusoids with cell clusters. In our opinion, these findings do not reflect the physiological conditions during metastasis formation. Therefore, we used injection of smaller numbers of tumor cells into the left heart. We had previously compared this approach with other routes of cell application and found that cellular adhesive interactions were independent from whether the cells were injected intracardially (left or right heart) or into the portal vein, but intra-arterial application resulted in better reproducibility, less disturbance of the microcirculation, and less complications.

In addition, a comparison of the adhesive patterns of highly metastatic HT-29LMM cells in immunocompetent and immunocompromised animals did not show any observable difference, suggesting that T-cell-associated reactions do not interfere with these properties during the early steps of metastasis formation. Furthermore, although immunocompetent rats were used in our previous experiments, human and rat carcinoma cells with comparable malignant properties (HT-29P and CC531 cells)

showed similar patterns of adhesive interactions within the liver sinusoids.¹⁸ Moreover, Steinhauer and colleagues³³ demonstrated that metastatic tumor cell arrest and extravasation were similar in immunocompetent and immunodeficient mice, whereas further progression was influenced by immunological phenomena.

The hypothesis of specific cell adhesions instead of size-restricted arrest is also supported by our previous findings that inhibition of different integrins and selectins can completely and selectively diminish cell adhesion and/or migration within the liver sinusoids.^{19,20} Similarly, Kikkawa and colleagues³⁵ described an increased arrest of CHO cells within sinusoids if they injected cells transfected *de novo* with $\alpha v \beta 3$ -integrins. Wang and colleagues³⁶ reported that inhibition of $\alpha 3 \beta 1$ -integrins can reduce metastatic tumor cell arrest of HT-1080 fibrosarcoma cells within the lung microvessels. If size-restricted cell arrest would be responsible for organ targeting, this modulation of cell adhesion molecules could not affect the numbers of arrested cells.

Similar to others^{35,37} we found an early extravasation of carcinoma cells, whereas Al-Mehdi and colleagues¹⁴ and Mook and colleagues³⁴ reported intravascular proliferation using sarcoma cells. The velocity of this process might be different depending from the cell lines and/or tumor entities. Using time-lapse microscopy and single cell tracking, we also found that the motility of various carcinoma cell lines (mean velocity on extracellular matrix components up to $4\ \mu\text{m}/\text{minute}$; unpublished results) is sufficient for an early extravasation that requires approximately a distance of 10 to $15\ \mu\text{m}$ corresponding to one cell diameter. Because endothelial cell gaps occur within the liver sinusoids (up to $4\ \mu\text{m}$ in diameter) that also lack a basement membrane, this tumor cell migration appears to be sufficient for their successful extravasation. Additionally, this early migration of adherent tumor cells into the hepatic parenchyma correlated with the metastatic potential of different colon carcinoma cell lines, suggesting that this step is rate-limiting for the formation of distant metastases.

In summary, our study shows for the first time that organ-specific formation of colorectal metastases appears to be mainly mediated by specific interactions between circulating carcinoma cells and the vessel wall of potential target organs. The low importance of mechanically size-restricted entrapment is also supported by remaining perfused microvessel lumen after successful tumor cell arrest. Moreover, we found a correlation between the metastatic potential of colon carcinoma cells and their ability for cell adhesion within potential target organs. For the first time we directly observed circulating tumor cells within the pulmonary microcirculation *in situ* and found specific cell adhesions without size restriction comparable to the liver sinusoids, whereas cells were unable to arrest within the renal and other capillaries *in situ*. In addition, the metastatic potential of various colon carcinoma cell lines correlated with their ability to rapidly extravasate into the liver parenchyma, suggesting the rate-limiting character of this step for the organ-specific formation of distant metastases. Further studies are required to investigate the underlying molecular mecha-

nisms of these specific adhesive interactions in metastatic target organs.

Acknowledgments

We thank Ms. K. Hagen and Mrs. I. Baumeister for their technical assistance and M. Bühner for his support during electron microscopy.

References

1. American Cancer Society, Surveillance Research. Vital Statistics of the United States, 1997. 1998
2. Hermanek Jr P, Wiebelt H, Riedl S, Staimmer D, Hermanek P: Long-term results of surgical therapy of colon cancer. Results of the Colorectal Cancer Study Group (German). *Chirurg* 1994, 65:287–297
3. Haier J: Indication and strategy of surgical treatment in metastatic colorectal cancer. *Operationskurs: Kolorektales Karzinom*. Edited by Buhr HJ, Runkel N. Leipzig, Ambrosius Barth Verlag, 1997, pp 138–145
4. Fidler IJ: The pathogenesis of cancer metastasis: the 'seed' and 'soil' hypothesis revisited. *Nat Rev Cancer* 2003, 3:1–6
5. Weiss L: Metastatic inefficiency. *Adv Cancer Res* 1990, 54:159–211
6. Nicolson GL: Cancer metastasis: tumor cell and host properties important in colonization of specific secondary sites. *Biochim Biophys Acta* 1988, 948:175–224
7. Feldman M, Eisenbach L: What makes a tumor cell metastatic? *Sci Am* 1988, 259:40–47
8. Ewing J: *A Treatise on Tumors, Neoplastic Diseases*, ed 3. Philadelphia, Saunders, 1928
9. Paget S: The distribution of secondary growth in cancer of the breast. *Lancet* 1889, 1:571–573
10. Chambers AF, Schmidt EE, MacDonald IC: Early steps in hematogenous metastasis of B16F1 melanoma cells in chick embryos studied by high-resolution intravital microscopy. *J Natl Cancer Inst* 1992, 84:797–803
11. Naumov GN, Wilson SM, MacDonald IC, Schmidt EE, Morris VL, Groom AC, Hoffman RM, Chambers AF: Cellular expression of green fluorescence protein, coupled with high-resolution in-vivo microscopy, to monitor steps in tumor metastasis. *J Cell Sci* 1999, 112:1835–1842
12. Koop S, MacDonald IC, Luzzi K, Schmidt EE, Morris VL, Grattan M, Khokha R, Chambers AF, Groom AC: Fate of melanoma cells entering the microcirculation: over 80% survive and extravasate. *Cancer Res* 1995, 55:2520–2523
13. Ito S, Nakanishi H, Ikehara Y: Real-time observation of micrometastasis formation in the living mouse liver using green fluorescent protein-tagged rat tongue carcinoma cell line. *Int J Cancer* 2001, 93:212–217
14. Al-Mehdi AB, Tozawa K, Fisher AB, Shientag L, Lee A, Muschel RJ: Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells—a new model for metastasis. *Nat Med* 2000, 6:100–102
15. Takahashi M, Ishida T, Traub O, Corson MA, Berk BC: Mechanotransduction in endothelial cells: temporal signaling events in response to shear stress. *J Vasc Res* 1997, 34:212–219
16. Bastida E, Almira L, Bertomeu MC, Ordinas A: Influence of shear stress on tumor-cell adhesion to endothelial-cell extracellular matrix and its modulation by fibronectin. *Int J Cancer* 1989, 43:1174–1178
17. Kitayama J, Nagawa H, Tsuno N: Laminin mediates tethering and spreading of colon cancer cells in physiological shear flow. *Br J Cancer* 1999, 80:1927–1934
18. Haier J, Korb T, Hotz B, Spiegel HU, Senninger N: An intravital model to monitor steps of metastatic tumor cell adhesion within the hepatic microcirculation. *J Gastrointest Surg* 2003, 7:507–515
19. Enns A, Gassmann P, Korb T, Schlüter K, Spiegel HU, Senninger N, Haier J: Integrins can directly mediate metastatic tumor cell adhesion within the liver sinusoids. *J Gastrointest Surg* 2004, 8:1049–1060
20. Enns A, Korb T, Schlüter K, Spiegel HU, Senninger N, Mitjans F, Haier J: $\alpha v \beta 5$ -integrins mediate early steps of metastasis formation. *Eur J Cancer* 2005, 41:1065–1072
21. Korb T, Schlüter K, Enns A, Spiegel HU, Senninger N, Nicolson GL, Haier J: Integrity of actin fibres and microtubules influences metastatic tumor cell adhesion. *Exp Cell Res* 2004, 299:236–247
22. Haier J, Nicolson GL: PTEN regulates tumor cell adhesion of colon carcinoma cells under dynamic conditions of fluid flow. *Oncogene* 2002, 21:1450–1460
23. Uhlmann S, Uhlmann D, Spiegel HU: Evaluation of hepatic microcirculation by in vivo microscopy. *J Invest Surg* 1999, 12:179–193
24. Ley K, Gaehdgens P: Endothelial, not hemodynamic differences are responsible for preferential leukocyte rolling in rat mesenteric venules. *Circ Res* 1991, 69:1034–1041
25. Gaehdgens P, Ley K, Pries AR, Muller R: Mutual interactions between leukocytes and microvascular blood flow. *Prog Appl Microcirc* 1985, 7:15–28
26. Glinsky OV, Turk JR, Pienta KJ, Huxley VH, Glinsky VV: Evidence of porcine and human endothelium activation by cancer-associated carbohydrates expressed on glycoproteins and tumor cells. *J Physiol* 2004, 554:89–99
27. Chambers AF, Groom AC, MacDonald IC: Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer* 2002, 2:563–572
28. Glinsky VV, Glinsky GV, Glinsky OV, Huxley VH, Turk JR, Mossine VV, Deutscher SL, Pienta KJ, Quinn TP: Intravascular metastatic cancer cell homotypic aggregation at the sites of primary attachment to the endothelium. *Cancer Res* 2003, 63:3805–3811
29. Glinskii OV, Huxley VH, Turk JR, Deutscher SL, Quinn TP, Pienta KJ, Glinsky VV: Continuous real time ex vivo epifluorescent video microscopy for the study of metastatic cancer cell interactions with microvascular endothelium. *Clin Exp Metastasis* 2003, 20:451–458
30. Glinskii OV, Huxley VH, Glinsky GV, Pienta KJ, Glinsky VV: Mechanical entrapment is insufficient and intercellular adhesion is essential for metastatic cell arrest in distant organs. *Neoplasia* 2005, 5:522–527
31. Yamauchi K, Yang M, Jiang P, Yamamoto N, Xu M, Amoh Y, Tsuji K, Bouvet M, Tsuchiya H, Tomita K, Moossa AR, Hoffman RM: Real-time in vivo dual-color imaging of intracapillary cancer cell and nucleus deformation and migration. *Cancer Res* 2005, 65:4246–4252
32. Tsuji K, Yamauchi K, Yang M, Jiang P, Jiang P, Bouvet M, Endo H, Kanai Y, Yamashita K, Moossa AR, Hoffman RM: Dual-color imaging of nuclear-cytoplasmic dynamics, viability, and proliferation of cancer cells in the portal vein area. *Cancer Res* 2006, 66:303–306
33. Steinbauer M, Guba M, Cernaianu G, Köhl G, Cetto M, Kunz-Schughart LA, Geissler EK, Falk W, Jauch KW: GFP-transfected cells are useful in examining early metastasis in vivo, but immune reaction precludes long-term tumor development studies in immunocompetent mice. *Clin Exp Metastasis* 2003, 20:135–141
34. Mook ORF, van Marle J, Vreeling-Sinderlarova H, Jonges R, Frederiks WM, van Noorden CFJ: Visualization of early events in tumor formation of eGFP-transfected rat colon carcinoma cells in liver. *Hepatol* 2003, 38:295–304
35. Kikkawa H, Kaihou M, Horaguchi N, Uchida T, Imafuku H, Takiguchi A, Yamazaki Y, Koike C, Kuruto R, Kakiuchi T, Tsukada H, Takada Y, Matsuura N, Oku N: Role of integrin $\alpha v \beta 3$ in the early phase of liver metastasis: PET and IVM analyses. *Clin Exp Metastasis* 2002, 19:717–725
36. Wang H, Fu W, Im JH, Zhou Z, Santoro SA, Iyer V, DiPersio CM, Yu QC, Quaranta V, Al-Mehdi A, Muschel RJ: Tumor cell $\alpha 3 \beta 1$ integrin and vascular laminin-5 mediate pulmonary arrest and metastasis. *J Cell Biol* 2004, 164:935–941
37. Wong CW, Song C, Grimes MM, Fu W, Dewhirst MW, Muschel RJ, Al-Mehdi AB: Intravascular location of breast cancer cells after spontaneous metastasis to the lung. *Am J Pathol* 2002, 161:749–753