Tumour metastasis to the liver, and the roles of proteinases and adhesion molecules: New concepts from in vivo videomicroscopy

Alan C Groom PhD[1], Ian C MacDonald PhD[1], Eric E Schmidt BSc MCS[1]*, Vincent L Morris PhD[1,2,3], Ann F Chambers PhD[1,2,3,4]

AC Groom, IC MacDonald, EE Schmidt, VL Morris, AF Chambers. Tumour metastasis to the liver, and the roles of proteinases and adhesion molecules: New concepts from in vivo videomicroscopy. Can J Gastroenterol 1999;13(9):733-743. Most preclinical studies of tumour metastasis and effects of molecular interventions have been based on end point assays, and little is known about the fate of cells at sequential steps in the metastatic process. In vivo videomicroscopy permits direct observations of sequential steps in hematogenous metastasis as they occur in living animals over time. These steps include initial arrest of cells in the microcirculation, extravasation, postextravasation migration and growth in the target organ. In the mouse liver model, cells are arrested in periportal sinusoids based on size restriction, survive in the circulation and extravasate into the tissue by 48 to 72 h regardless of metastatic potential. Thereafter, cells may migrate to preferred sites for growth. Critical steps responsible for cell losses and metastatic inefficiency occur at the level of postextravasation cell growth. Many extravasated cells may remain dormant, and growth to form micrometastases is initiated in only a small subset of cells. Most early micrometastases may disappear after a few days, and only a small subset continue growth into macroscopic tumours. Angiogenesis is a prerequisite for continued growth of metastases, as shown previously by others. Integrin-based interventions can modulate postextravasation cell migration and cell growth. Matrix metalloproteinase inhibitors can inhibit tumour angiogenesis and thus reduce growth. Key targets against which future therapeutic strategies should be directed include the initiation and maintenance of growth of micrometastases, and the activation of dormant solitary cells.

Key Words: *Integrins; In vivo assays; Liver; Matrix metalloproteinases; Metastasis; Videomicroscopy*

Métastases tumorales au foie et rôles des protéinases et des molécules d'adhésion : nouveaux concepts provenant de la vidéomicroscopie *in vivo*

RÉSUMÉ : La plupart des études précliniques des métastases tumorales et des effets des interventions au niveau moléculaire sont basées sur des analyses du résultat final, et l'on sait peu de choses au sujet du destin des cellules à des étapes séquentielles du processus métastatique. La vidéomicroscopie *in vivo* permet une observation directe des étapes séquentielles de la formation des métastases hématogènes survenant chez des animaux vivants au cours d'une période de temps. Ces étapes comprennent l'arrêt initial des cellules dans la microcirculation, l'extravasation, la migration postextravasation et la croissance dans l'organe cible. Dans le modèle de foie de souris, les cellules sont arrêtées dans les sinusoïdes périportes sur la base d'une restriction par la taille, survivent dans la circulation et s'extravasent dans les tissus dans les 48 à 72

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*Deceased. [1]Departments of Medical Biophysics, [2]Microbiology and Immunology, and [3]Oncology, University of Western Ontario, and [4]London Regional Cancer Centre, London, Ontario

Correspondence and reprints: Dr AC Groom, Department of Medical Biophysics, University of Western Ontario, London, Ontario N6A 5C1. Telephone 519-661-2111 ext 86548, fax 519-661-2123, e-mail acgroom@julian.uwo.ca

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heures indépendamment du potentiel métastatique. Ensuite, les cellules peuvent migrer vers des sites préférés pour la croissance. Les étapes critiques responsables des pertes de cellules et l'inefficacité métastatique surviennent au niveau de la croissance cellulaire postextravasation. De nombreuses cellules extravasées peuvent demeurer inactives, et la croissance permettant la formation des micrométastases est initiée seulement chez un petit sous-groupe de cellules. Les micrométastases les plus précoces peuvent disparaître après quelques jours, et seul un petit sous-groupe continue à croître pour devenir des tumeurs macroscopiques. Une angiogenèse préalable va permettre la croissance continue des métastases, tel que démontré précédemment par d'autres chercheurs. Les interventions basées sur l'intégrine peuvent moduler la migration cellulaire post extravasation et la croissance cellulaire. Les inhibiteurs des métalloprotéinases de la matrice peuvent inhiber l'angiogenèse tumorale et, ainsi, réduire la croissance. Les cibles clés contre lesquelles les futures stratégies thérapeutiques devraient être dirigées comprennent le début et le maintien de la croissance des micrométastases, et l'activation de cellules solitaires inactives.

Metastasis to the liver is an all too common feature of disease progression in many types of cancer, especially those that originate within the splanchnic organs. Cells detach from the primary tumour and invade the blood or lymphatic circulations, where they are carried along by the flow to distant sites. Evidence (1-3) suggests that fewer than one in 1000 of these cells ever gives rise to a secondary tumour (referred to as 'metastatic inefficiency' [4]), but it is uncertain at which steps in the metastatic process cells are lost. The general view has been that, on entering the circulation, most cancer cells are destroyed by hemodynamic forces (5) or the immune system (6,7) before they can extravasate into surrounding tissues and grow to form tumours.

Experimental studies have indicated that adhesion molecules and proteinases are important in metastasis development. However, because the methodology has consisted largely of in vitro assays and end point metastasis assays in mice, conclusions about the key steps affected by these molecules have been based on logical inference rather than direct observations of the metastatic process itself. It has been assumed that cancer cells in the bloodstream behave much like leukocytes, which, after flowing through the capillaries, may become arrested by adhesion to venular walls in readiness for extravasation. The secretion of proteinases, which degrade the basement membrane and stromal proteins, then permits cell migration across the vessel walls into the tissue. Thus, the roles of adhesion molecules and proteinases in metastasis in vivo have been viewed as strictly analogous to their roles in cell adhesion and invasion assays in vitro.

What has been lacking in experimental metastasis methodology until now is the means to watch directly the process in vivo, as it occurs over time. In vivo videomicroscopy (IVVM) meets this need. Its use has led to a new conceptual understanding of early steps in tumour metastasis to the liver and some of the molecular mechanisms involved. Some of the key insights obtained thus far are presented.

NEW TOOLS FOR STUDYING HEMATOGENOUS METASTASIS

IVVM: The basic approach underlying the use of IVVM in experimental metastasis is that cancer cells are fluorescently labelled in vitro, then injected into an animal and viewed later in vivo in thin tissues or superficial (less than 50 µm) regions of thick tissues by both fluorescence and oblique transillumination. In this way, individual cancer cells may be positively identified in vivo and viewed at high magnification in relation to their immediate environment. Detailed descriptions of the methodology have been presented (8,9); therefore, only a brief summary is given here.

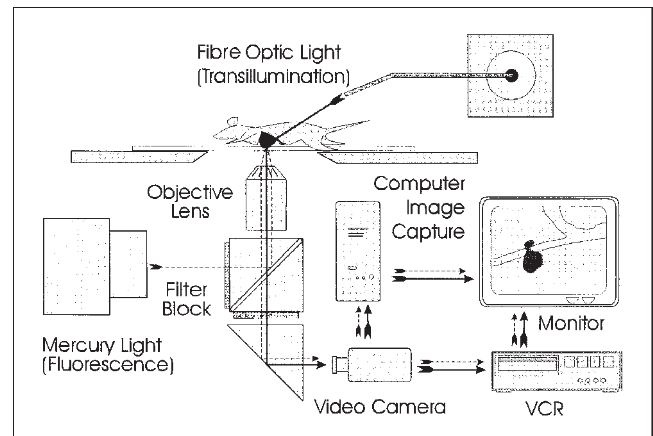

Figure 1) *In vivo videomicroscopy method. The animal is placed on the platform of an inverted microscope and an exposed lobe of the liver is visualized through a coverslip using ×10 to ×100 (dry) objectives. Oblique transillumination using a fibre optic light guide and/or episcopic fluorescence illumination with appropriate filter blocks is employed for observing fluorescently labelled cancer cells in liver tissue. Images are viewed with a video camera and monitor, and recorded on super VHS tapes and/or sent to a computer image capture system for further analysis. VCR Video cassette recorder*

Unambiguous identification by IVVM of individual cancer cells within intact organs and tissues requires that these cells emit a strong fluorescence when excited. Cytoplasmic labels such as Calcein-AM (Molecular Probes Inc, Eugene, Oregon) or fluorescent polystyrene 'nanospheres' 0.05 to 0.07 µm in diameter have proved to be very effective for this purpose, and leave both membrane integrity and growth potential of the cells undiminished (10). Recently, the transfection of cells to allow expression of green fluorescence protein (GFP) has also become an option (11-13). Because the gene encoding GFP is passed on to the progeny after cell replication and because metastases have been shown to be clonal in origin (14-16), this approach will permit longer term studies of metastatic growth and therapeutically induced growth inhibition.

Labelled cancer cells are injected into the circulation of an anesthetized mouse via a mesenteric vein to target the liver. At selected times later, animals are placed on a plexiglass platform resting on the stage of an inverted microscope (Figure 1), with one lobe of the liver partially exteriorized

and lying on a glass coverslip window above the objective lenses (magnification ×10 to magnification ×100, dry). In this way, the lower surface of the lobe remains stationary and flat within the plane of focus of the microscope, in spite of the respiratory motion to which the rest of the tissue is subjected. Epifluorescence illumination makes it easy to locate cancer cells within the tissue and to assess their overall shape (round, elongated, pseudopodial extensions). However, to obtain high resolution images of cells, and to see cells clearly in relation to their immediate surroundings, transillumination of the tissue is essential.

Visualization of cancer cells in the microcirculation by transillumination is difficult because the optical density of the cells differs so little from that of plasma. However, oblique illumination of the tissue (via a fibre optic light guide) results in more light being refracted from one side of the cells than the other. This gives a shadowing effect that enhances the contrast greatly and imparts a three-dimensional quality to the image (8,9). Cells are then seen by virtue of their shape rather than their optical density. Nevertheless, because of the large volume fraction of blood in the path of the incident light, a monochromatic red image is produced that is extremely difficult to view. The use of a Newvicon tube camera (eg, Panasonic WV1550, Panasonic Canada, Mississauga, Ontario) with extended red sensitivity attached to the microscope yields black and white images that may be viewed on the monitor and recorded on super VHS tapes for subsequent analysis. When desired, fluorescence and transillumination can be used simultaneously to visualize the cells in the context of the surrounding tissue. By focusing up and down, 'optical slicing' of the tissue may be carried out at high magnifications.

Cancer cell 'accounting' in tissues: To quantify cancer cell survival at successive key steps in the metastatic process, it is necessary to express the number of cells actually observed in a sampled volume of tissue relative to the number of cells that originally entered it. This is done by including in the cell suspension injected, inert plastic 10.2 μm microspheres at a known ratio, eg, five cells per one microsphere. After injection, the microspheres are trapped by size restriction within periportal sinusoids and remain in the tissue indefinitely, providing a reference marker for the number of cancer cells that originally entered the particular region under study. In this way, cell accounting may be based on the total number of injected cells rather than the percentage of observed cells (17).

To quantify the percentage of injected cells surviving in the liver at various times after injection, the cell to microsphere ratio in the tissue is compared with the ratio in the syringe before injection (Figure 2). The percentage cell survival is calculated as follows:

$$\frac{\text{cell:microsphere ratio in the liver after injection}}{\text{cell:microsphere ratio in the syringe before injection}} \times 100\%$$

This technique may be used in conjunction with IVVM to sample superficial regions of the liver, or after fixation the organ may be sampled throughout its entire thickness by counting cells and microspheres in 30 μm sections of tissue (18). Because metastases are clonal in origin, this technique can be used to quantify them as well.

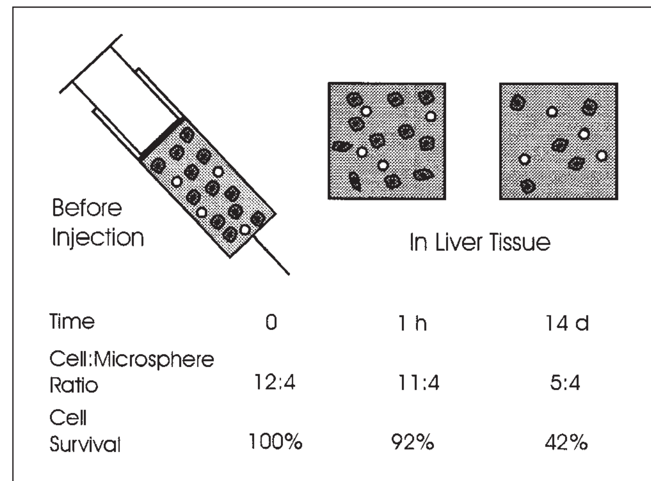

Figure 2) *Cell accounting procedure for measuring cell survival in vivo. Inert plastic 10.2 μm microspheres, which are included in the cell suspension injected, are arrested by size restriction and remain indefinitely in periportal sinusoids, providing a reference marker for the number of cells that reached the tissue originally. The ratio of cells to microspheres is determined in the syringe before injection and in liver tissue at various times after injection. The percentage of cells that survived can be determined from these ratios. An example is provided. d days*

NEW CONCEPTS ABOUT METASTASIS FROM IVVM AND CANCER CELL ACCOUNTING IN TISSUE

Cancer cell arrest in the microcirculation: By cannulating a mesenteric vein and injecting cells after the animal is positioned on the microscope stage, the way in which blood-borne cancer cells become arrested in the liver has been observed directly (19). It has become clear that the 'leukocyte model', whereby cells are arrested in venules larger than the cell diameter and roll along the endothelium before the formation of shear-resistant bonds, is not appropriate in the case of cells from solid tumours. Such cancer cells, which are much larger than leukocytes, become arrested by size restriction in periportal sinusoids (Figure 3a,b). Similar results have been obtained for several different types of cells, including melanoma, mammary carcinoma, rhabdomyosarcoma, and normal and oncogene-transformed fibroblasts (10,18-21). In other tissues (mouse muscle, chick embryo chorioallantoic membrane [CAM]) the cells are arrested in a similar manner – in capillaries at the input side of the microcirculation (10,19,22,23). It was previously believed, based on extrapolation from in vitro studies, that whether blood-borne cancer cells become arrested in an organ or tissue is determined solely by specific adhesive interactions between the cells and the endothelium. An attractive feature of this concept was that it also offered a rational explanation for organ specificity of metastasis. However, direct observations by IVVM show that, for the systems we have studied, the

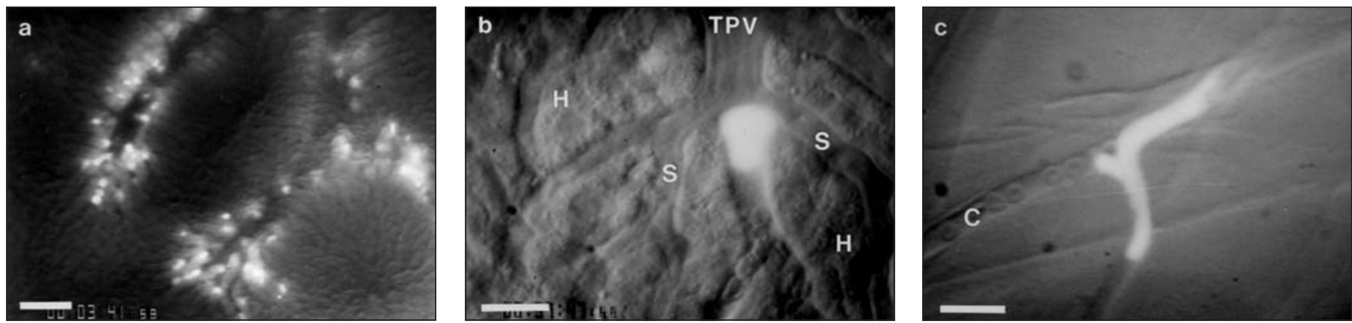

Figure 3) *Arrest of cancer cells in the microcirculation of mouse liver and muscle, seen by in vivo videomicroscopy using epifluorescence plus transillumination. Reproduced with permission from reference 19.* **a** *In the liver, B16F10 melanoma cells were arrested where sinusoids branch from terminal portal venules (acinar zone 1). Note the absence of cells in the vicinity of the terminal hepatic venules (zone 3, eg, lower right). Three minutes forty-one seconds after injection. Bar = 100 μm.* **b** *In the liver, a D2A1 mammary carcinoma cell was arrested on entering a sinusoid from a terminal portal venule (TPV) and was only slightly deformed by the portal pressure. Blood flow continued via anastomosing sinusoids (S) but was blocked downstream from the arrested cell. Four minutes after injection. Bar = 20 μm. H Hepatocytes.* **c** *In the cremaster muscle, two B16F10 melanoma cells were arrested at a capillary bifurcation. The degree of deformation is much greater than that in liver (shown in* **b***). Erythrocytes are seen within the capillary (C). Four minutes after injection. Bar = 20 μm*

above view is no longer tenable. These results suggest that cancer cells in the bloodstream are arrested on the basis of size restriction in the microcirculation of whatever organ or tissue they enter (8,9,19,22). This is in agreement with findings in mesentery by Thorlacius et al (24). Scherbarth and Orr (25) confirmed these results in mouse liver but also found that, when mice are pretreated with interleukin-1alpha, the cancer cells can become arrested by adhesion to the walls of portal venules twice the cell diameter. It is thus possible that the release of cytokines may extend hepatic arrest of tumour cells to presinusoidal vessels.

It has been generally believed that the majority of cancer cells entering the bloodstream are rapidly destroyed by the immune system and/or hemodynamic forces. Studies with ^{125}I-IUdR-labelled cells injected intravenously to target the lung showed that the radioactivity in this organ fell by a factor of more than 100 over the first 24 h. Based on the premise that loss of radioactivity corresponds to loss of cells, more than 99% of the cells may have been destroyed (1). A potential explanation for such destruction was later put forward, based exclusively on in vitro studies (26). It was proposed that cell deformation and membrane stretching to the point of rupture could occur, due to hemodynamic pressures, if a cancer cell were unable, because of size restriction, to pass through a blood capillary. An in vivo experiment carried out later, using cells labelled with the nuclear fluorescent marker acridine orange, appeared to support this proposal (27).

Direct observations by IVVM do not support the concept that cancer cells arrested in the microcirculation of an organ or tissue are rapidly destroyed. Cells arrested in periportal sinusoids of the liver (a low pressure circulation) showed little deformation, whereas in cremaster muscle (a high pressure circulation) the cells became extremely deformed (Figure 3b,c). However, under both conditions the cells maintained their membrane integrity (based on exclusion of ethidium bromide) throughout the 120 min period of observation (19).

An important proviso for studies such as these is that fluorescent labelling of cells must be carried out using cytoplasmic as opposed to nuclear markers. When the above experiments were repeated using cells labelled with the nuclear stain acridine orange, most of the arrested cells in both liver and muscle ceased to exclude ethidium bromide within the first 15 mins, indicating loss of membrane integrity and leading to rapid lysis (19). (These results were identical to those obtained by Weiss and colleagues [27].) In contrast, studies by IVVM and cell accounting in liver demonstrated that when a cytoplasmic label was used, more than 80% of the injected cancer cells survived arrest and deformation within the microcirculation and then went on to extravasate (18).

If these results from IVVM can be translated to other cell types and the clinical situation, most cells that escape from a solid tumour into the bloodstream may not undergo rapid destruction, but survive.

Extravasation of cancer cells into surrounding tissues: Two conflicting views about the way cancer cells extravasate can be found in the literature. Both views are based on histological and ultrastructural examination of tissues after cancer cell injection. However, uncertainties in interpretation of static two-dimensional images have made it difficult to reconcile these views. One view is that intravascular replication of arrested cells occurs, followed by proteolytic destruction of adjacent vascular basement membrane and cell extravasation en masse (28-30). The second view is that before replication ever occurs arrested cancer cells extravasate singly in the manner of leukocytes (31). Our direct observations in vivo of the process of cancer cell extravasation in both chick CAM and mouse liver support this second view (8,9).

For several hours after their initial arrest in periportal sinusoids, cancer cells continue to block blood flow completely in the individual vessels involved. They then gradually move away from one side of the vessel and stretch out along the opposite wall (Figure 4a), allowing flow to resume progressively. Clearly, adhesion molecules (identity

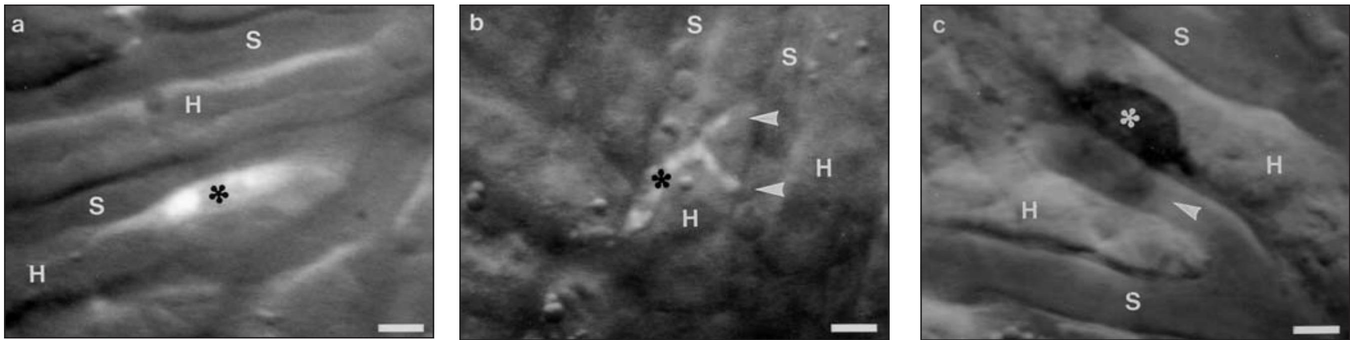

Figure 4) *Cancer cells in the process of extravasation in mouse liver two days after injection. Bars = 10 µm.* **a** *B16F1 melanoma cell (*) that has begun to extravasate adheres to and stretches out along one side of the sinusoid (S), allowing blood flow to resume (blurred image). Fluorescence and transillumination. H Hepatocyte. Reproduced with permission from reference 20.* **b** *D2.OR mammary carcinoma cell (*) in the process of extravasation one day after injection. Part of the cell body is within the S lumen, with pseudopodial projections (arrowhead) between H. Fluorescence and transillumination. Reproduced with permission from reference 10.* **c** *B16F1 melanoma cell (*) that has completed extravasation and displaced H one day after injection. At a deeper plane of focus, the cell was seen wrapping around S (shown blurred in this image [arrowhead]). Rapid flow in the sinusoid has resumed. The cell can be seen by transillumination alone because of its melanin content. Reproduced with permission from reference 20*

unknown) must be involved at this stage, forming shear-resistant bonds between the cells and the vessel wall. Each cell then forms pseudopodial projections that extend through the vessel wall and between the surrounding hepatocytes (Figure 4b). This stage is followed by migration of the cell body into extravascular tissue, displacing hepatocytes and allowing the complete restoration of blood flow within the sinusoid (Figure 4c). Often, the extravasated cell wraps around the abluminal surface of the vessel. The mechanism of extravasation appeared to be the same for all the cell types examined, and no signs of disruption of vessel walls due to the extravasation of cancer cells were observed (19).

The time course of extravasation for a population of cancer cells is longer than that for individual cells because not all cells begin the process at the same time. Two to three hours after intraportal injection, the first extravasated cells are seen, and two to four days later virtually all cells have extravasated. In each animal, IVVM observations may be carried out for approximately 3 h, permitting the locations of large numbers of individual cancer cells to be assessed at that particular time point. Cells are classified as intra- or extravascular, or in the process of extravasation, and the percentages in each category are determined. The population time course of extravasation may be obtained by studying a number of animals at successive time points after injection and combining the data (10,20,22).

The ability of cancer cells to extravasate into surrounding tissue, by degrading basement membrane and extracellular matrix, has been regarded as an important rate-limiting step in metastasis. However, comparisons of the population time course of cell extravasation in mouse liver (incomplete basement membrane) with that in chick CAM (complete basement membrane by day 11, when cells were injected) showed that, contrary to expectation, extravasation took longer in the liver than in CAM. For instance, 40% of injected cells had extravasated by aproximately 18 h in liver versus 7 h in the CAM (10,22). The lack of correlation between the extent of the physical barriers in these two different models and the times required for the cell population to extravasate show that the in vivo situation is more complex than that expected on the basis of in vitro invasion assays. Clearly, other factors such as differences in cell signalling molecules present in CAM versus liver must also play a major part in cancer cell extravasation.

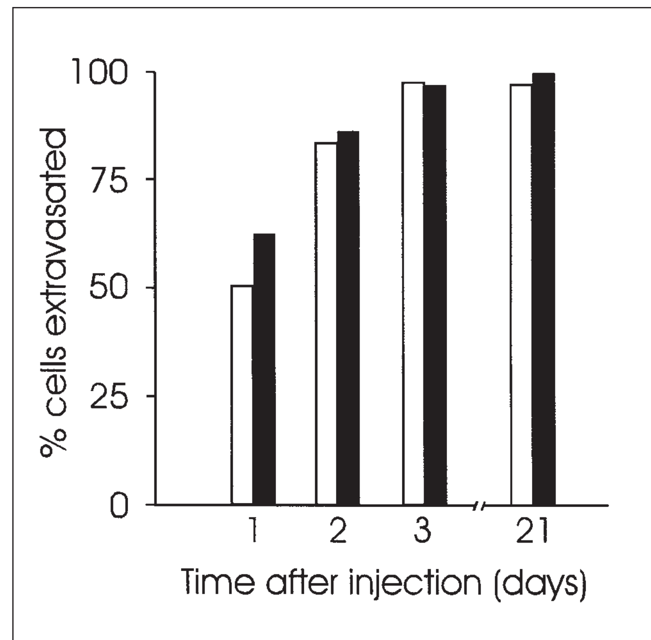

Figure 5) *Time course of extravasation of D2A1 (highly metastatic: open bars) and D2.OR (poorly metastatic: filled bars) mammary carcinoma cells in mouse liver. Bars show the percentages of observed cells that had completed extravasation at various times after injection. Extravasation was essentially completed for both cell lines by day 3. Data from reference 10*

Based on in vitro invasion assays and the view that extravasation is a major rate-limiting step in metastasis, it has been believed that the ability of cancer cells to extravasate is

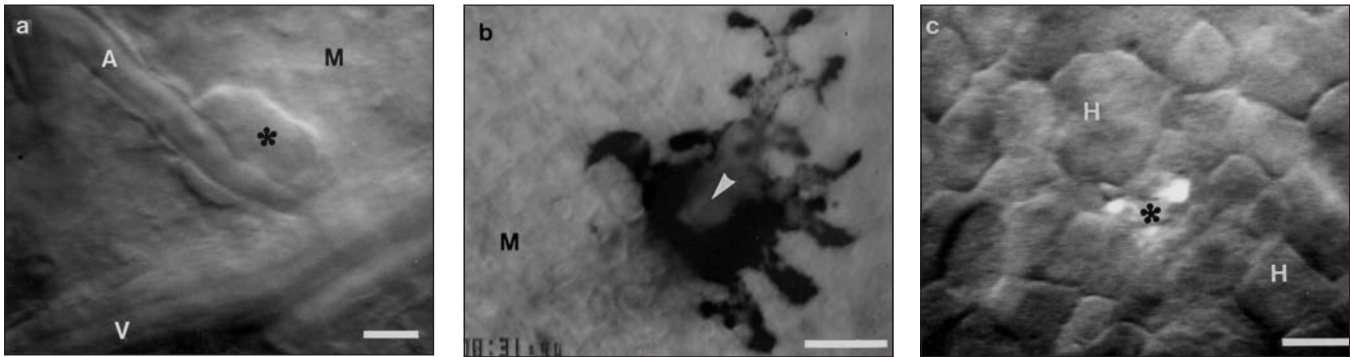

Figure 6) *Postextravasation cancer cell migration and growth. Bars = 20 µm.* **a** *In chick embryo chorioallantoic membrane (CAM), 40 h after injection, an extravasated B16F10 melanoma cell (*) wraps around a terminal arteriole (A). An adjacent venule (V) lies slightly below the plane of focus. Mesenchyme tissue (M); transillumination only. Reproduced with permission from reference 22.* **b** *In chick embryo CAM, three days after injection of B16F1 melanoma cells, a highly melanotic micrometastasis grows within M, surrounding an arteriolar orifice (arrowhead). Blood flowed from A (bottom left, below plane of focus) through the tumour up into the capillary plexus (above the plane of focus). The cancer cells extended pseudopodia laterally under the plexus. Transillumination only. Reproduced with permission from reference 47.* **c** *In mouse liver, two days after injection, an extravasated B16F1 melanoma cell (*) has migrated up to the hepatocyte (H) layer in the subcapsular region. Note the outlines of H. Transillumination plus epifluorescence. Reproduced with permission from reference 20*

dependent on their metastatic potential. However, our IVVM findings do not support this view. We employed two mammary carcinoma cell lines, one (D2A1) highly invasive in Matrigel (Collaborative Research Inc, Bedford, Massachusetts) invasion assays in vitro and highly metastatic, and the other (D2.OR) essentially noninvasive in vitro and poorly metastatic (10). The results (Figure 5) showed that both cell lines extravasated with exactly the same population time course in mouse liver (10). Similar findings were obtained when the extravasation ability of highly metastatic *ras*-transformed NIH3T3 cells (PAP2) was compared with that of control nontumourigenic nontransformed NIH3T3 cells and primary mouse embryo fibroblasts in chick CAM (23). All three cell types extravasated with the same kinetics, and within 24 h after injection more than 89% of observed cells had completed the process. Thus, a key new concept from IVVM studies is that highly and poorly metastatic cells, or even nonmetastatic cells, may extravasate equally well. This suggests that, contrary to previous belief, most blood-borne cancer cells can extravasate successfully into the tissues; thus, extravasation does not appear to be a rate-limiting step in metastasis.

Postextravasation migration of cancer cells to preferred sites in tissues: Cells that have successfully extravasated need to be able to grow at their new locations, and IVVM has revealed that, before replication, the extravasated cells may migrate short distances to preferred sites within host tissue. This was first observed in chick CAM (22), where cancer cells that had extravasated from the capillary plexus exhibited directed migration through the mesenchyme toward arterioles, and encircled or spread over their abluminal surfaces (Figure 6a). This behaviour was seen for every cell type studied (melanoma and mammary carcinoma cell lines, *ras*-transformed fibroblasts) and was independent of metastatic potential or transformed status of the cells. Primary mouse fibroblasts also showed the same behaviour (23), indicating that directed migration may be of widespread significance.

In the CAM, extravasated cells migrated preferentially toward arterioles rather than venules or lymphatics (22,23). However, the reasons for this behaviour and the molecular basis of these events remain to be determined. Directed migration cannot be explained by the need for oxygen because the CAM is a respiratory organ and the arterioles contain deoxygenated blood. Cells might be expected to migrate to regions rich in fibronectin, laminin and collagen type IV, but the distribution of these proteins appears to be similar in arterioles and venules of the CAM (32). Attachment of cells to various extracellular matrix components can protect them from undergoing apoptosis (33,34); thus, the attachment of cells to arterioles may result in improved cell survival. The cells remain wrapped around arterioles following cell division, and micrometastases form as perivascular 'collars' from which cell pseudopodia extend along the vessel's abluminal surface (Figure 6b). Thus, the directed migration of extravasated cancer cells to preferred sites within the CAM may be a necessary, although hitherto unrecognized, component of the metastatic process in this tissue.

The question arises whether postextravasation cell migration is peculiar to the CAM, possibly related to the unique arrangement of its microcirculation, or whether it is a more general process that occurs in other organs and tissues as well. Experiments by IVVM have also demonstrated migration of extravasated cancer cells in mouse liver for all cell lines analyzed (melanoma, mammary carcinoma, rhabdomyosarcoma, erythroleukemia [10,20,21,35]). After extravasation from liver sinusoids, the cells wrap around the abluminal surface of these vessels, in a manner similar to that seen in the CAM. However, cells that have extravasated from the most superficial sinusoids may send up long pseudopodia (up to 30 µm) to the subcapsular region, and this is followed by migration of the cell body to this location. Cells

from either poorly or highly metastatic cell lines may be seen squeezed between hepatocytes at the liver surface (Figure 6c); in the mouse, this is the primary location where hepatic metastases from many cancer cell lines develop.

In summary, in two very different organs the use of IVVM has revealed a new step in the metastatic process, the migration of extravasated cancer cells through the extracellular matrix to selective sites for tumour growth. The reasons for such preferences in location are still unknown, but it is possible that these sites contain substances that may attract cells and presumably enhance tumour growth. Therefore, the possibility exists that hepatic tumour metastasis could be suppressed by blocking the postextravasation migration of cancer cells.

Growth of extravasated cells to form metastases – the major rate-limiting step in tumour metastasis to the liver: The traditional concept of metastasis formation by blood-borne cancer cells is that very few cells can survive in the circulation and successfully extravasate into surrounding tissue, but those that are able to do so grow into tumours. Our IVVM results do not support this view but suggest that, in syngeneic or immune-deficient models, most cancer cells that enter the circulation survive and extravasate successfully. Therefore, that the number of tumours produced is so small must mean that very few extravasated cells ever succeed in growing into tumours. This led us to propose a new and different concept – the major rate-limiting step in metastasis is the survival and growth of extravasated cells in tissue.

To test the validity of this concept, it was necessary to quantify the percentage of injected cells surviving in the host tissue at a given time by using the cell accounting procedure described above. In the mouse liver model, the proportions of injected melanoma cells (B16F1) remaining at progressive stages of the metastatic process were quantified: cell arrest in the microcirculation, extravasation, and growth into early micrometastases and macroscopic tumours (18). The key findings are summarized in Figure 7. At 90 mins after injection, 87% of the injected cells remained, all of them still within periportal sinusoids. Three days later, more than 80% of the injected cells remained and had extravasated into the surrounding tissue; 2% of these had developed into micrometastases consisting of four to 16 cells. Interestingly, after two weeks, only 0.07% of injected cells remained as micrometastases and 0.02% had formed macroscopic tumours, whereas 36% remained within hepatic tissue as solitary cells. Immunohistochemical staining for apoptosis (TdT dUTP nick end labelling) and proliferation (Ki-67) revealed that 95% of these solitary cells were neither proliferating nor undergoing apoptosis, signifying that they were dormant. In contrast, only 3% of cells within tumours were dormant (18).

These studies demonstrate quantitatively that for B16F1 cells in the mouse model, early destruction in the microcirculation and an inability of cells to extravasate are not rate-limiting steps in tumour metastasis to the liver. However, only one in 40 extravasated cells had formed tumours by day 3, and the rest remained as solitary cells. This indicates that failure of the extravasated cells to initiate growth in the target organ constitutes one major rate-limiting step in metastasis. In addition, only one in 100 of the micrometastases produced by day 3 went on to form macroscopic tumours, and the rest disappeared. This indicates that failure of micrometastases to continue growth into tumours constitutes a second major rate-limiting step in metastasis.

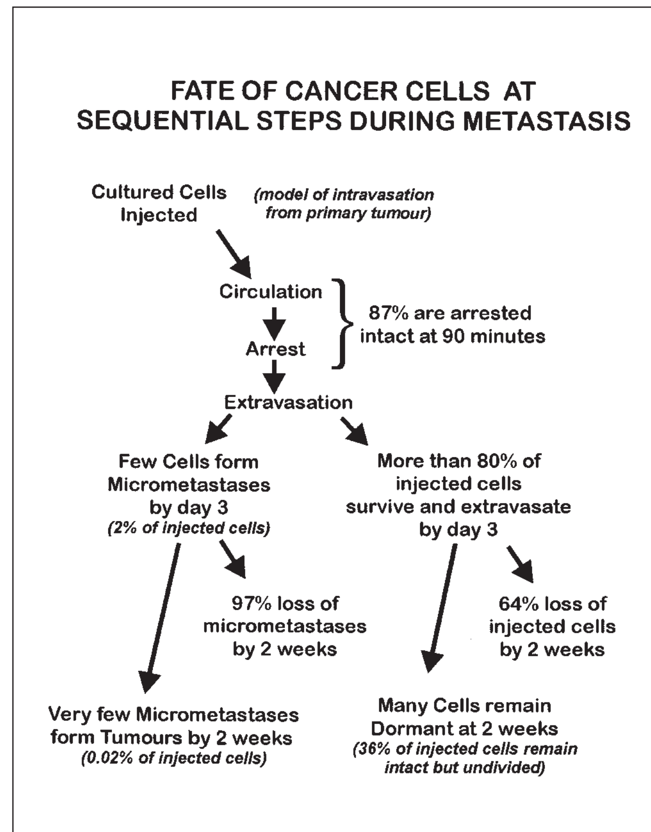

Figure 7) *Flow chart of the fate of cancer cells at sequential steps during hematogenous metastasis. Conclusions regarding cell arrest in the microcirculation and extravasation into the surrounding tissue were based on several different cell types and organs, whereas data on the fate of cells after extravasation were obtained from B16F1 melanoma cells in mouse liver (18)*

The discovery that more than one-third of the total number of intraportally injected melanoma cells remained two weeks later in liver tissue as dormant cells is not encouraging. It is conceivable that dormant cells may have the potential to be activated at some later time and commence growth, analogous to time bombs buried in the tissue. This would be consistent with clinical evidence that human malignancies can recur years after apparently successful treatment of a primary tumour (36,37). Furthermore, these dormant cells would be spared by standard cytotoxic chemotherapies directed against actively dividing cells. Our results also show that the survival rate of early micrometastases (four to 16 cells) is only one-10th that of solitary cells, suggesting that cancer cells that start to replicate in vivo are much more vulnerable to destruction than dormant cells. Our findings suggest that the activation of dormant cells, together with the initiation and growth of micrometastases,

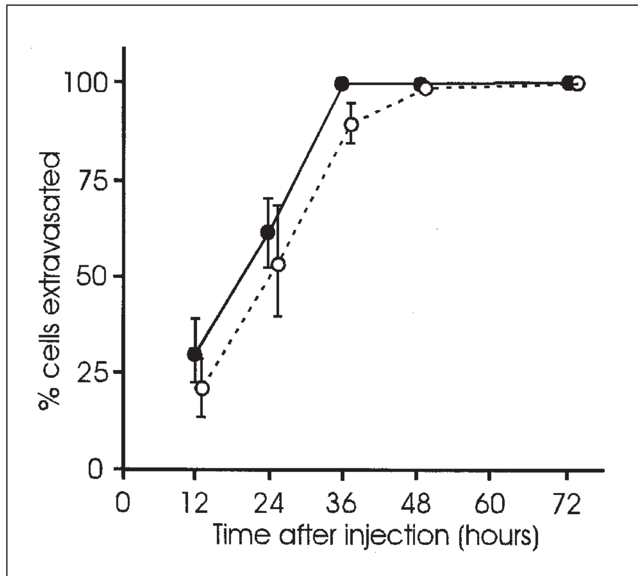

Figure 8) *Time course of extravasation of parental B16F10 (●) and transfectant tissue inhibitor of metalloproteinases-1 overexpressor (○) melanoma cells in chick chorioallantoic membrane. Mean percentage of observed cells that had extravasated was plotted as a function of time after injection. A total of 36 embryos was used for each cell line. Bars = SE. Reproduced with permission from reference 22*

are key targets against which therapeutic strategies should be developed.

ROLES OF PROTEINASES AND ADHESION MOLECULES IN METASTASIS

It is known that proteinases and integrins are important in metastasis, based on end point analyses such as number and size of metastases (21,38-40). However, it is not clear at which steps of the process these molecules have their effect. It is important to identify the specific steps at which these molecules act so that therapeutic interventions can be better designed and targeted.

Members of the matrix metalloproteinase (MMP) family are believed to contribute significantly in cancer metastasis, primarily by degrading the components of vascular basement membranes and thus promoting intravasation and extravasation of cells. However, IVVM and cell accounting studies indicate a more complex role for MMPs in metastasis. The arrest, extravasation and growth of B16F10 melanoma cells transfected to overexpress tissue inhibitor of metalloproteinases-1 were first studied in chick CAM (TIMP-1) (22). These cells produce fewer metastases than do control cells in vivo (41,42) and exhibit reduced invasive ability through Matrigel in vitro (43). However, the experiments demonstrated that TIMP-1 overexpressor and control cells extravasate with the same time course in chick CAM (Figure 8), although, after extravasating the TIMP-1 overexpressor cells show reduced homotypic adhesion and reduced metastatic potential compared with the control cells. These results indicate that the decreased metastatic ability of these TIMP-1 overexpressor cells is due to effects on tumour growth, after cancer cell extravasation has occurred, as opposed to a decreased ability of cells to extravasate.

A second study on the role of MMPs in metastasis used an exogenous instead of endogenous inhibitor of MMPs, and extended the investigation to the mouse liver (44). Daily administration of the synthetic MMP inhibitor BB-94 (batimastat [45]) reduced the size but not the number of liver tumours produced by intraportally injected B16F1 melanoma cells. Observations by IVVM showed that the kinetics of cell extravasation were not altered by the drug treatment, nor was there any inhibition of cell survival or initial growth of cells to form metastases. However, histological analysis revealed that BB-94 treatment significantly decreased tumour vascularity, indicating an inhibition of the angiogenesis that is needed if tumours are to grow beyond a limited size. The IVVM investigations described above, together with results from other laboratories, have altered significantly the understanding of the role of MMPs in the metastatic process (reviewed in 46). These studies demonstrated that inhibitors of MMPs can interfere with the metastatic process by means of effects on postextravasation growth of tumours rather than at the level of cancer cell extravasation.

Adhesion molecules, including integrins, are believed to be important for the attachment of cancer cells to endothelial surfaces and the extracellular matrix, providing traction during extravasation and postextravasation cell migration. However, IVVM studies indicate that integrins may also affect postextravasation cell replication. First, B16F1 melanoma cells were treated with eristostatin (20), a ‘disintegrin’ or naturally occurring peptide that binds to selected integrins and blocks their action. After intraportal injection these cells gave rise to markedly fewer liver tumours than were produced by control cells. However, IVVM studies showed that the initial arrest and kinetics of extravasation of cells were unchanged by treatment with eristostatin. Furthermore, eristostatin had no effect on the ability of extravasated cells to migrate to the subcapsular region of mouse liver where tumours form. It must be concluded, therefore, that the reduction of metastasis by eristostatin was caused primarily by inhibition of postextravasation cell growth.

In a second study on the contributions of integrin-mediated adhesion to liver metastasis, human rhabdomyosarcoma cells transfected (in Dr B Chan’s laboratory) to express either intact alpha$_2$beta$_1$ ($\alpha_2\beta_1$) integrin or a nonfunctional variant (as a control) were injected intraportally in mice (21). IVVM experiments showed that the cells that expressed functional $\alpha_2\beta_1$ on their surface extravasated with the same time course as the control cells. However, the expression of functional $\alpha_2\beta_1$ markedly reduced the ability of extravasated cells to migrate to the subcapsular region of the liver where tumours form, and reducing $\alpha_2\beta_1$function with a blocking monocolonal antibody (mAb) restored the ability of cells to migrate (Figure 9) and increased their ability to form tumour foci in an end point study. Interestingly, the expression of functional $\alpha_2\beta_1$ integrin in weakly adherent erythroleukemia cells enhanced their postextravasation migratory ability, whereas treatment with a blocking

$\alpha_2\beta_1$-specific mAb markedly reduced cell migration through the extracellular matrix (35). It has been suggested that cell migratory function increases with increased adhesive ability up to an optimal level of cell-matrix interactions. However, if the adhesive forces become too strong the cells simply wrap around the basement membrane of blood vessels and cease to migrate (35). These results demonstrate that integrin interactions with matrix proteins may modify the migratory abilities of cancer cells in vivo and cause cells to reside in sites that may differ in their ability to support tumour growth.

SUMMARY AND SIGNIFICANCE

IVVM has made it possible to observe sequential steps directly in experimental tumour metastasis to the liver as they occur over time and, when used in conjunction with a novel cell accounting strategy, to quantify the contributions of these individual steps to metastatic inefficiency. These procedures may be used to determine which steps in metastasis are affected by a particular treatment or genetic manipulation, to test molecular mechanisms of metastasis and to identify potential therapeutic targets.

It has been held traditionally that most cancer cells that enter the circulation and escape the immune system are quickly destroyed by hemodynamic forces. Cell arrest in a target organ was thought to depend on specific adhesive interactions with vascular walls. Few arrested cells were thought to be able to extravasate; extravasation was considered the major rate-limiting step in metastasis. However, examination by IVVM of steps in metastasis by using the mouse liver model has provided new conceptual understanding of tumour metastasis to the liver and some of the molecular mechanisms involved. Our findings challenge several previously held beliefs about early steps in the metastatic process.

If our results from IVVM can be translated to other models and to the clinical situation, the findings suggest several new concepts. First, most cells that escape from a solid tumour into the bloodstream and evade the immune system may not undergo rapid destruction, but survive and extravasate into the surrounding tissue, where they may migrate within the stroma to preferred sites for growth. Second, after extravasation, many cells can persist as individual dormant cells that are neither dividing nor undergoing apoptosis, whereas very few cells may begin to replicate and form small micrometastases of four to 16 cells within a few days. However, most of these micrometastases subsequently disappear, and very few progress to form macroscopic tumours. Third, dormant cancer cells disappear from tissue at a much slower rate than is the case for micrometastases, and this raises concerns that they may have the potential to be activated at a later time and commence growth. Regardless of this last possibility, our IVVM findings suggest that the rate-limiting steps in metastasis occur during the postextravasation growth of cancer cells in tissue, and are not the survival of cells in the circulation or their ability to extravasate.

End point assays from several laboratories have shown

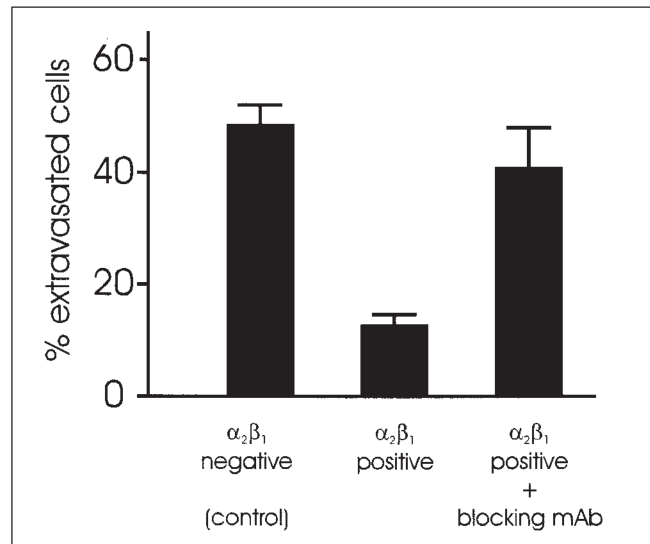

Figure 9) *Ability of extravasated rhabdomyosarcoma transfectant cells to reach the subcapsular region of the liver by day 4 after cell injection, analyzed by in vivo videomicroscopy. Integrin $\alpha_2\beta_1$-negative cells used as a control expressed a nonfunctional variant of $\alpha_2\beta_1$. Expression of functional $\alpha_2\beta_1$ markedly reduced ($P<0.006$) the ability of cells to migrate to the subcapsular region. However, injection of a blocking monoclonal antibody (mAb) on day 1 after cell injection restored to $\alpha_2\beta_1$-positive cells the ability to migrate ($P<0.0001$). Data from reference 21*

that when animals are treated with inhibitors of metalloproteinases the metastatic ability of injected cancer cells is decreased, and this reduction has been attributed to a reduced ability of cells to penetrate the vascular basement membrane and extravasate. In contrast, IVVM studies have demonstrated that MMP inhibitors can have a direct effect on the growth of metastases, due to altered cell behaviour and a reduction of tumour vascularization. The role of MMPs in metastasis is undoubtedly complex, but our findings provide preclinical data in support of MMP inhibitors reducing angiogenesis within macroscopic tumours.

Interactions between cancer cells and host tissues depend on several classes of cell surface molecules, including integrins and the ligands that bind to them. These cell surface molecules do more than simply bring about attachment because the adhesive interactions in turn initiate signal transduction and lead to functional consequences. It was not surprising, therefore, to discover that integrin inhibition via disintegrins or blocking antibodies can interfere with the growth of metastases. The disintegrin eristostatin produced a direct reduction of growth, without affecting the ability of melanoma cells to migrate from sinusoids to the subcapsular region of the liver (the preferred site for growth). In contrast, $\alpha_2\beta_1$ function modulated both the ability of cells to migrate to the subcapsular region and the numbers of metastatic tumours formed. Our findings suggest, therefore, that integrins can exert a direct effect on the postextravasation growth of cancer cells in the liver, as well as an indirect effect that is mediated by modifying the cell's ability to migrate through the stroma to preferred sites for growth.

CONCLUSIONS

A goal of our IVVM research on early steps in hematogenous metastasis is to identify steps that would be most appropriate for therapeutic intervention. We have shown that, in the mouse liver model, blood-borne cancer cells are arrested in periportal sinusoids based on size restriction, survive in the circulation and extravasate into surrounding tissue. Virtually all arrested cells extravasate by 48 to 72 h, regardless of their metastatic potential. We have been unable to prevent cells from extravasating, which suggests that this step may be a poor therapeutic target. In contrast, postextravasation cell migration to preferred sites for growth may offer a suitable target for integrin-based interventions, modulating cell migration and leading indirectly to suppression of cell growth.

Our findings indicate that critical or rate-limiting steps in metastasis, which are responsible for the cell losses that lead to metastatic inefficiency, occur at the stage of post-extravasation cell growth, as follows:

- Many extravasated cells may remain in a state of dormancy, and initiation of growth to form micrometastases occurs in only a small subset of cells.
- Most early micrometastases disappear after a few days, and only a small subset continue growth into macroscopic tumours.
- Angiogenesis is a prerequisite for continued growth of metastases, as shown previously by others.

These three stages may represent good targets for the design of new antimetastasis strategies. Drugs that may inhibit activation and growth of dormant cancer cells, and drugs that inhibit angiogenesis, would complement the use of conventional cytotoxic therapies. IVVM and cell accounting procedures offer the means to evaluate, in the mouse model, how drugs of potential therapeutic value can diminish individual steps in tumour metastasis to the liver.

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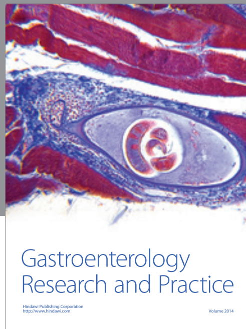

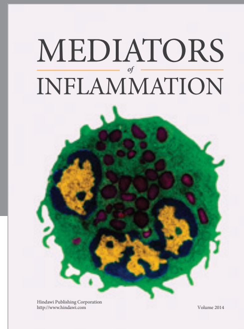

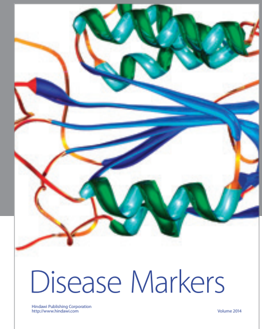

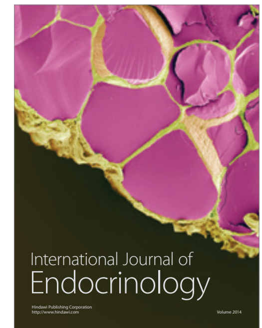

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