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Imaging of cancer invasion and metastasis using green fluorescent protein

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Abstract

The use of green fluorescent protein to fluorescently tag tumour cells has allowed investigators to open the ''black box'' of metastasis in order to visualise the behaviour of tumour cells in living tissues. Analysis of cells leaving the primary tumour indicates that highly metastatic cells are able to polarise more effectively towards blood vessels while poorly metastatic cells fragment more often when interacting with blood. In addition, there appear to be greater numbers of host immune system cells interacting with metastatic tumours. After arresting in target organs such as the lungs or liver, most tumour cells become dormant or apoptose. A small fraction of the arrested cells form metastases. In some target organs, migration of tumour cells may enhance the ability to form metastases.

Keywords: GFP; Green fluorescent protein; Cancer; Metastasis; Motility; Intravasation; Extravasation; *In vivo* videomicroscopy; Confocal microscopy

1. Outline of the metastatic process

This review will be focused on metastasis of mammary adenocarcinoma cells. Metastasis can be crudely broken down into four steps: growth of the primary tumour, entry of tumour cells into the blood from the primary tumour, arrival of tumour cells in a target organ and cell division to form the metastases (Fig. 1) [1–4]. These four steps can be further dissected into finer steps and processes that are required for each step. This review will not focus on growth of the primary tumour, but on the direct visualisation of the subsequent steps of the metastatic process. For example, vascularisation of the primary tumour is important for providing blood vessels or lymphatics as entry points for metastasising tumour cells, as well as allowing rapid growth of the primary tumour [5,6].

Entry into the circulation requires that cells detach from the primary cell mass and move into a vessel. The detachment process can require a reduction in cell–cell adhesion mediated by molecules such as E-cadherin [7,8]. Movement into a vessel requires degradation of the extracellular matrix which is present as basement membranes surrounding the tumour and vessel, as well as present in the connective tissue separating the tumour from the vessel [9–11]. Depending on the vascularity of the tumour and the size of the blood vessel, the distance travelled from the tumour to the blood vessel can range from less than a cell diameter to many cell diameters. Extension of cell processes into spaces generated by degradation may be aided by chemotaxis to external factors including matrix degradation products [12]. This is followed by the generation of new cell–substratum contact sites which stimulate focal contact formation and contraction of the cell posterior [13,14]. Net flow of cytoplasmic contents into the extension shifts the centre of mass of the cell forward. Older cell–substratum contacts are dissolved and the cell pulls up its trailing edges. This coupled process involving proteolysis, cell movement and adhesion has been termed the three-step model for metastasis [15] and can be repeated until the cell contacts the blood vessel.

If tumour cells enter a lymphatic rather than a blood vessel, additional motility may occur to facilitate reaching the general circulation. Although it is possible that metastases are seeded in new target organs traversed by

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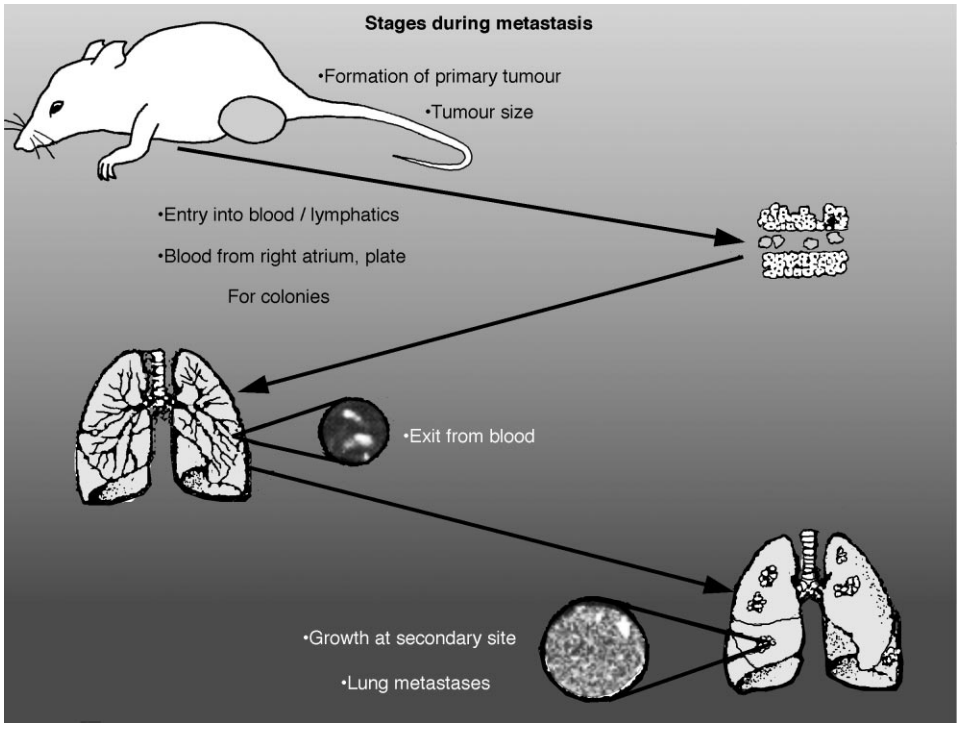

Fig. 1. Stages of metastasis that are easily quantitated. Quantitation of tumour growth, viable cells in the blood, single cells in the lungs and metastases in the lungs is performed as shown in the diagram.

the lymphatic system, in most cases metastases are found mainly in organs that contain downstream capillary beds (i.e. the lungs), suggesting that tumour cells do not exit the lymphatic system except through entry into the blood via the thoracic duct. This may reflect the general increase in the lymphatic walls (and presumed increased barrier to tumour cell exit) that occurs as the lymph moves towards the thoracic duct. In addition, traversal of the lymphatic system requires movement through lymph nodes. The rate of movement through nodes may be slow enough that tumour cells grow and divide during this process, allowing the formation of lymph node metastases.

During and after entry into the blood, whether directly or via the lymphatics, tumour cells must survive the physical stresses generated by the rapid flow of blood [16]. The cells may be single or in clumps, possibly coated by platelets. The immune system may also have the most direct exposure to the tumour cells when in lymph nodes or blood vessels.

In the target organ, tumour cells must stop and (at least in some cases) extravasate. The arrest may be due to physical plugging up of a capillary that is too small to allow flow of the tumour cell through the capillary, or due to adhesion of the tumour cell to the walls of the blood vessel. Although expression of specific integrins has been shown to increase the metastasis of tumour cells injected directly into the blood [17,18], it is unclear whether the contributions of integrins to metastatic ability is due to the enhancement of growth of cells arrested in blood vessels, or increased adhesion during extravasation and growth. The extravasation process may require the three steps of proteolysis, motility and adhesion similar to the invasion and intravasation events. Finally, whether in the blood vessel or in the parenchyma of the target organ, tumour cells must grow and divide in order to form metastases that affect patient survival.

2. Evaluating specific steps in metastasis

It is important to determine which steps are inefficient or rate-limiting during tumour cell metastasis. By understanding the molecular events underlying the rate-limiting steps, one can hope to design prognostic indicators or therapeutic interventions that will more effectively aid in patient treatment. Thus, the efficiency of the steps described in the previous section should be measured and compared in order to identify the stage at which tumour cell metastasis is most vulnerable.

For evaluation of these steps in patients, studies have been limited to situations in which the patient's health is not endangered. For entry into the circulation, the levels of circulating tumour cells have been evaluated under several different conditions. Patients with ascite tumours and high levels of circulating tumour cells have been studied for the correlation of tumour cells in the blood with metastases [19,20]. In these studies, little increase in metastasis was observed when there was an

increased tumour cell level in the blood due to surgical intervention. However, it is possible that this subset of patients may have tumours which are blocked at later stages in the metastatic process. More recently, antibody and polymerase chain reaction (PCR) based assays with increased sensitivity have allowed initial studies of patients with far lower circulating cell levels. The results are not consistent — in some cases circulating cell levels correlated with poor prognosis [21,22] and in other cases there was not a direct correlation [23,24]. These studies were limited in that they did not distinguish dead or dying cells from viable cells. In addition, in many cases blood is taken after filtration by the lungs, and cells not trapped by the lungs might be atypical. Better studies would evaluate cell viability and examine blood prior to filtration by the lungs. Such studies are possible using animal models as described below.

Animal studies of metastasis typically make use of either of two experimental paradigms. In the 'experimental metastasis' case, tumour cells are directly injected intravenously (i.v.) and then cell arrest and/or growth in the lungs or other organs is measured at various times after injection. In the "spontaneous metastasis" case, tumour cells are injected into a tissue (preferably the source tissue from which the tumour cells are derived, such as the mammary gland for mammary adenocarcinoma), and the injected cells grow to form a primary tumour from which cells then metastasise. The experimental metastasis assay is useful in examining extravasation with defined numbers of cells injected at a specific time. However, it utilises cells cultured *in vitro* rather than cells metastasising from a primary tumour, and the injection of a bolus of cells at one time may also introduce artifacts. The spontaneous metastasis assay ameliorates some of these problems, although the structure of the primary tumour formed by injected cells is unlikely to exactly match tumours that arise from a normal tissue.

More extensive studies have been performed using the experimental metastasis paradigm. From such studies, it is clear that only a small fraction of the injected tumour cells are able to generate metastases in the lungs. Radioactive labelling of cells indicated that most of the cells injected i.v. arrest in the lungs but then most of the radioactivity is released, presumably reflecting cell death over the next 24–48 h [25–27]. However, using non-radioactive labelling methods, Chambers and colleagues have reported higher initial survival of several cell types [28,29]. This raises the question of what precisely is occurring at the early stages after arrest in target organs. The examination of the arrest, extravasation and growth of metastases in the lungs using green fluorescent protein (GFP) labelling is an active area of research and will be discussed below.

Relatively few studies have been performed that examine the efficiency of the early stages of metastasis. Liotta and colleagues studied the release of cells during perfusion of primary tumours formed in muscle [30]. The cells tended to be released in clumps, which might aid in the formation of metastases. We have recently completed an analysis of the various steps of metastases for two cell lines derived from the 13762 NF rat mammary adenocarcinoma [31]. The two cell lines were moderately metastatic (MTLn3) and poorly metastatic (MTC) [32–34]. We compared primary tumour size, number of circulating viable cells, single cell numbers in the lungs and micrometastases in the lungs six weeks after injection of cells into the mammary fat pad (Table 1). Tumours generated by the poorly metastatic MTC cells were significantly worse at seeding the blood with circulating tumour cells compared with tumours generated by MTLn3 cells. This indicated that intravasation efficiency could correlate with metastatic efficiency.

In addition, we evaluated the importance of the different steps of the metastatic cascade for the MTLn3 tumours by comparing these parameters within the same animal. Using rough estimates of survival times, a consistent picture regarding the efficiency of the various steps of the metastatic cascade could be constructed for these two lines (Table 2). From this analysis, we identified two steps as being inefficient and, very likely, rate limiting. Intravasation was quite inefficient for the MTLn3 cells with less than 1 in 10 000 cells successfully entering the blood. Consistent with many previous studies [4,36], we found that cells in the blood arrested with high efficiency in the lungs. In addition, a second inefficient step was the growth of metastases from the arrested cells. Thus, the two inefficient steps during the metastatic cascade for the moderately metastatic MTLn3 cell line are the intravasation and growth in the lung stages. To gain further insight into the factors

Table 1
In vivo tumour cell distributions[a]

	Tumour size (cm^3)	Cells in blood (per 4 ml)	Cells in lungs (per 40 HPF)	Lung mets (per section)
MTLn3	31.0±5.5	22.8±13.6	35.7±10.1	17.9±13.6
MTC	44.5±9.9	0.25±0.16	0.75±0.75	0
P value	< 0.4	< 0.002	< 0.003	< 0.003

[a] For MTLn3, 15 rats were injected and for MTC, 8 rats were injected. Tumour cell distributions were measured, means and standard errors of the mean calculated, and statistical significance evaluated using the non-parametric Wilcoxon test. From [31].

Table 2
Metastatic efficiency

	Primary tumour[a]	Blood[b]	Single cells[c]	Metastases[d]
Cell Number	2.2×10^{10}	3.9×10^{5}	2.1×10^{5}	3.3×10^{2}
Efficiency[e]		1.8×10^{-5}	Approximately 0.5	1.6×10^{-3}

[a] Given the average volume of an MTLn3 cell of 1.4 pl (unpublished results) and an average volume of the MTLn3 tumours of 0.031 l, the number of cells in the tumour is of the order of 2.2×10^{10}.

[b] Using the average cell density of 5.7 tumour cells/ml and a cardiac output for female Fischer rats of 47 ml/min [35], we estimate that the average MTLn3 tumour is releasing approximately 390 000 cells into the blood each day.

[c] Using our measurement of 36 cells/40 high power fields, a field diameter of 0.373 mm, and a depth of field of about 0.04 mm, a lung volume of 1 cc (35) would contain about 210 000 cells.

[d] With a surface area per section of roughly 110 mm^2, an average diameter of metastases of about 0.05 mm, and 17.9 metastases per section, we estimate that a lung volume of 1 cc would contain about 3255 metastases.

[e] Efficiency was determined by dividing the number in each column by the number in the previous column.

regulating the intravasation efficiency, we have made use of GFP-tagging of cells as described below.

3. *In vivo* imaging methodology using GFP

Although the quantitation of individual steps of metastasis as described in the previous section is important for providing an initial rough identification of the critical steps for metastasis, these steps are of necessity treated as black boxes in such analyses. Imaging technology utilising GFP has provided the mechanism for looking inside the black box. GFP is a protein originally derived from the jellyfish *Aequorea victoria* [37]. It spontaneously folds to generate a chromophore composed of amino acids 65–67 which is fluorescent independent of additional cofactors. The most commonly used form (eGFP) has an excitation peak of 488 nm and emission peak of 508 nm. Modification of the amino acid sequence and codons has enhanced the fluorescence efficiency, provided for proteins with different spectra, and increased expression in mammalian cells. Most *in vivo* studies have used either the original GFP or the enhanced (e)GFP proteins, but we anticipate that future work will make use of various forms of GFP and other fluorescent proteins for labelling of multiple cell types to examine cell to cell relationships in more detail.

Imaging of cells in living tissue using GFP fluorescence is subject to several constraints. First, expression levels in the cells of interest must be high enough to make the cells clearly distinguishable from background. Expression using promoters such as the cytomegalovirus (CMV) promoter or retroviral long terminal repeats (LTR) are sufficient. Early problems with efficiency of protein expression and rate of formation of the chromophore are resolved by using the humanised eGFP protein with the S65T mutation. A second constraint can be preparation of the tissue in a state which is sustainable for the length of the observation. If a tissue is removed from the animal, a substitute source of oxygen and energy should be used to perfuse the blood vessels and maintain the tissue. With time after removal of the tissue, cells lacking oxygen and/or nutrients will die and become autofluorescent. Dead and dying cells will fluoresce in both the rhodamine and GFP channels, and can thus be distinguished from healthy, GFP-labelled cells (which will fluoresce only in the GFP channel). The third, and perhaps most challenging problem is imaging of cells inside the tissue. The cell/matrix layers between the GFP cells and the microscope objective can severely limit resolution due to multiple refractile boundaries between the layers. The optimum imaging method will vary with the particular tissue preparation that is imaged. We have found that for imaging of cells in the primary tumour in the mammary fat pad, confocal imaging is essential. Using single photon confocal microscopy, imaging of the primary tumour tends to work best with a 20× objective. Even under these conditions, the depth of imaging is limited and bleaching can occur. The adipose tissue of the mammary fat pad is a significant cause of the imaging problems. Preliminary studies indicate that image quality, depth of imaging and bleaching rates are better using a two-photon confocal microscope.

The use of GFP as a tool for visualising various aspects of tumour formation and metastasis is growing rapidly. Whole body imaging of primary tumours and metastases in severe combined immunodeficient (SCID) mice has the potential to allow screening of antimetastatic drugs [38,39]. A wide variety of tumour cell types have been labelled with GFP, including brain [40–42], breast [31,43,44], skin [45] and ovary [29,46]. Although GFP can induce an immune response in immunocompetent animals [47], such effects have not been generally observed. The following sections will focus on the use of GFP to follow single cell movement in the primary tumour and in target organs.

4. *In vivo* imaging of the primary tumour

Given the results presented earlier indicating that intravasation is an inefficient step for the MTLn3 and MTC cell lines, we have made use of GFP imaging of these cell lines in the primary tumour in order to examine more directly cell behaviour in the primary tumour [31,44]. Cells are injected into the mammary fat pad of a Fisher 344 rat and a primary tumour forms within 2–3 weeks. In order to stabilise the imaging conditions and

to reduce the motions due to breathing, the animal is anaesthetised and a skin flap containing the tumour is opened [44]. Care is taken to ensure that the blood supply is maintained intact and that the tumour is not disturbed. Excess adipose tissue is cleared in order to improve image quality. The animal remains anaesthetised and is laid on an imaging stage such that the exposed primary tumour can be imaged using an inverted microscope. The skin flap is attached to the stage in order to minimise motions occurring during imaging. Under these conditions, imaging of multiple fields can occur over several hours.

Imaging of the primary tumour yielded several surprising results that have important implications for mechanisms limiting cell metastasis. We had hypothesised that the metastatic MTLn3 tumours might show more highly active and motile cells, and that such motility would contribute to the enhanced intravasation. However, comparisons of MTC and MTLn3 tumours indicated minor (1.3-fold) differences in protrusiveness and cell translocation, not on a level to account for the >50-fold difference in tumour cell blood burden or metastasis (Table 1). These results suggest that random motility *per se* was not a critical difference between metastatic and non-metastatic cells. Three unexpected differences were observed which suggest potential explanations for the difference in metastatic efficiency between MTLn3 and MTC cells. First, metastatic cells showed polarisation mainly around blood vessels, while the poorly metastatic MTC cells were randomly polarised throughout the tumour (Fig. 2). A second difference was that increased rates of fragmentation of MTC cells in association with blood vessels occurred. The third difference was increased numbers of host cells associated with the metastatic MTLn3 tumours. Each of these three differences will be briefly discussed below.

In vitro, MTLn3 cells are strongly chemotactic towards epidermal growth factor (EGF) (Fig. 3). The polarisation of MTLn3 cells around blood vessels could indicate chemotactic orientation. During early tumour formation and angiogenesis, chemotaxis-like movement of rodent mammary tumour cells towards host blood vessels is observed using a dorsal skin window model [49]. MTLn3 cells not next to blood vessels are more rounded and not polarised. MTC cells tend to be extended with cell projections independent of their relationship to blood vessels [50]. Although both MTLn3 and MTC cells might be equally motile, the orientation of MTLn3 cells towards blood vessels could increase the efficiency with which the MTLn3 cells contact blood vessels and intravasate compared with MTC cells. If the orientation of MTLn3 cells is indeed due to chemotactic orientation, the components of the blood or the blood vessels could be the source of the chemoattractants. In the blood, serum proteins or secretory products of platelets are chemoattractants. If the blood vessels are

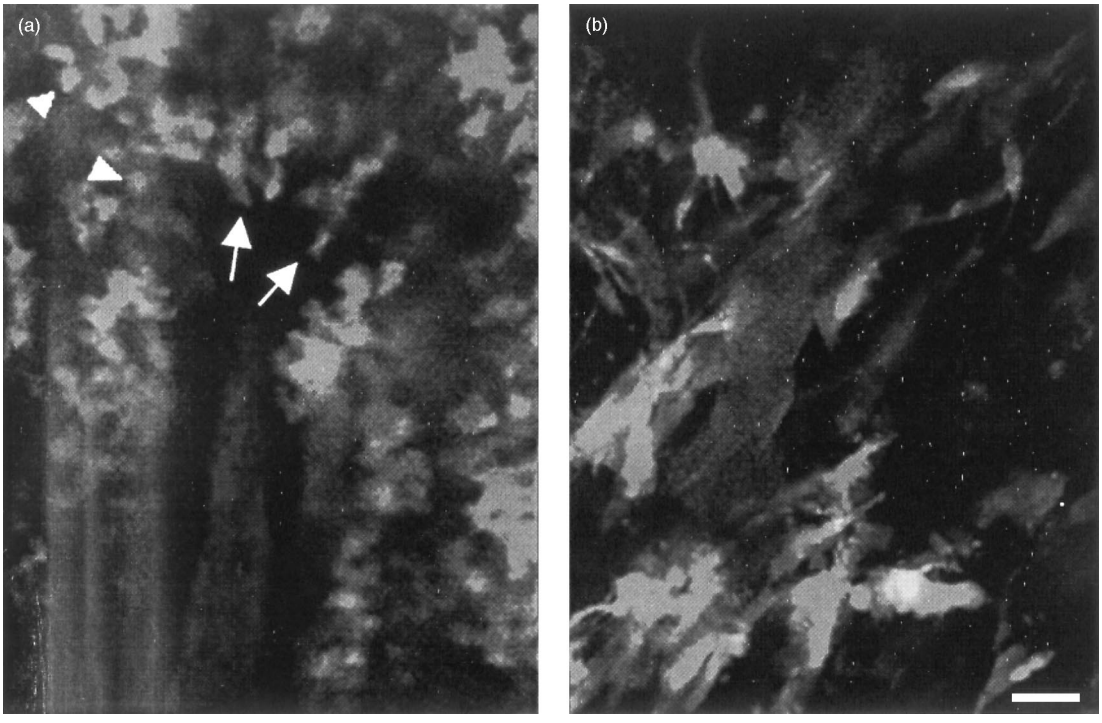

Fig. 2. MTLn3-GFP cells orient towards blood vessels, while MTC-GFP cells do not. Orientation of MTLn3-GFP cells (a) and MTC-GFP cells (b) to blood vessels in the primary tumour. (a) MTLn3-GFP cells (green) near the vessel (red) are seen to orient themselves toward the vessel in an elongated fashion (arrows) as opposed to those away from the vessel (arrowheads). (b) MTC-GFP cells (green) randomly associate with the vessel (red), and remain elongated away from the vessel. Scale bar = 25 μm. GFP, green fluorescent protein. From [31].

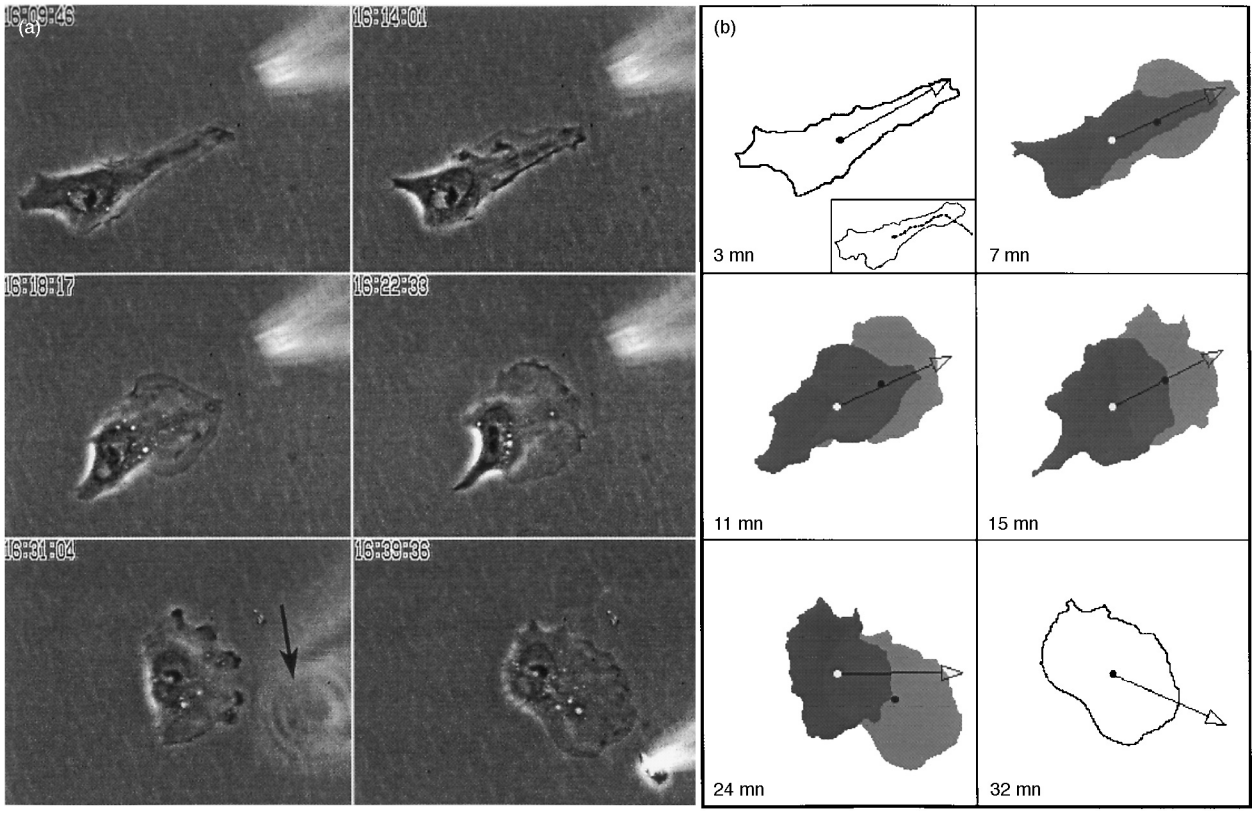

Fig. 3. MTLn3 cells display amoeboid chemotaxis. (a) An MTLn3 cell oriented toward a pipette filled with a solution of 50 μM epidermal growth factor (EGF) (time in the top left corner). The cell crawled toward the pipette and changed direction when the pipette was moved (arrow marks the moving pipette on image 16:31:04). (b) Difference images showing lamellipod extension at the front of the cell (green) and retraction at the rear (red). Time is indicated as minutes after initial positioning of the pipette. The cell path over the course of the experiment is shown as the position of the centroid of the cell once every minute on the top left image. From [48].

damaged or poorly formed (common in tumour vasculature), exposure of the parenchymal extracellular matrix to the blood is likely to result in platelet binding and activation, leading to the release of the platelet contents and generation of a chemoattractant gradient that could be detected by tumour cells. Alternatively, the endothelial cells or smooth muscle cells forming the walls of the blood vessel might secrete chemoattractants that lead to tumour cell orientation towards the vessel [51]. A candidate for a chemoattractant is EGF or transforming growth factor (TGF)alpha. MTLn3 cells express the EGF receptor, while expression of the EGF receptor in MTC cells is not detectable [52]. Correspondingly, MTLn3 cells respond to EGF or TGFalpha as a chemoattractant while MTC cells do not [34]. Transfection of the EGF receptor into MTC cells results in chemotactic responses of MTC cells to TGFalpha and increased metastatic ability [34,53]. However, the metastatic ability of MTC cells expressing the EGF receptor is still significantly less than the metastatic ability of MTLn3 cells, indicating that there are other differences between the two cell lines that are important for metastasis.

We believe that the fragmentation observed in MTC tumours is due to cell disruption upon contact with the blood flow. The fragments that form move away from the cell rapidly and are often associated with visible blood vessels, consistent with the flow in the blood. The fragments we observe must be enclosed by membranes, because the GFP fluorescence is not dramatically reduced. It is possible that additional fragments are formed that are not intact (and which we would not detect), in which case the fragmentation rate is even greater. The increased fragmentation observed in MTC tumours compared with MTLn3 tumours could be related to the lack of polarisation of the MTC cells towards blood vessels. If MTC cells randomly extend a process into the bloodstream while maintaining tight adhesion of the rest of the cell to the extracellular matrix, shear forces exerted on the protrusion in the blood may cause the fragmentation. In contrast, for MTLn3 cells, if the cell is polarised to move into the blood rapidly and is in the process of deadhering its tail during motility, then the shear forces encountered in the blood would drag the entire cell intact into the blood. These observations have implications for biochemically based assays of the blood. The presence of non-viable cell fragments could be an indication of a poorly metastatic tumour, while the presence of intact cells would indicate a more metastatic tumour. Highly sensitive

PCR or antibody-based assays which do not distinguish between cell fragments and whole cells may not provide prognostic value. This might explain the lack of correlation between blood burden and metastasis reported in some studies.

We believe that we can detect host cells as dark, non-fluorescent but motile objects viewed against the fluorescent background of the tumour cells. These objects are approximately the right size for neutrophils or macrophages and move at appropriate speeds for such cells. Macrophages could assist tumour cell invasion in several ways. First, macrophages secrete metalloproteases that could aid in degrading extracellular matrix, creating pathways that tumour cells might use in moving away from the tumour. Second, macrophages could secrete chemoattractants that would orient cells toward the macrophages. If the macrophages enter the tumour from the blood, and remain concentrated around the blood vessels, such orientation could result in polarization of the tumour cells towards the blood vessels. Alternatively, macrophages may be recognising the metastatic tumour cells but not effectively mounting an immune response.

5. Imaging of arrest, extravasation and growth in the target organ

Analysis of the arrest and growth of cells in target organs such as the lung and liver has been perhaps the most extensively analysed aspect of metastasis, using the experimental metastasis paradigm of injecting tumour cells into the circulation and measuring the number of metastases that form. Overall, this phase is also quite inefficient, with typically less than 1% of the injected cells forming a metastasis [4]. Radiolabelling or fluorescent labelling studies have indicated that the injected cells arrest at the first capillary bed that they encounter with high efficiency, suggesting that there is relatively little lysis during the initial stages [26,27,54]. However, over the next 24 h, there is extensive loss of label suggesting massive cell death, with eventually only a small number of metastases forming. Visualisation of this process using fluorescent cells allows a more detailed mechanistic evaluation of the key steps involved, but has also generated some controversy.

Ann Chambers and colleagues have combined detailed cell accounting methods together with *in vivo* videomicroscopy for tracking the fates of cells injected into the hepatic portal vein and CAM [29,55]. By co-injecting microspheres with fluorescently labelled cells, they provide an internal normalisation standard to which they can compare their cell counts and localisation. After injection into the hepatic portal vein, they find that over 80% of the injected cells are intact after 3 days. At that time, most have extravasated. Both tumour cells as well as non-tumour cells such as fibroblasts extravasate with high efficiency. However, only a small fraction of the injected cells start to form metastases and only a small fraction of the micrometastases form macrometastases.

Similar results were observed using the CAM and injection of melanoma cells into the inferior *vena cava* for arrest in the lungs [36]. The conclusions from these results are that tumour (and non-tumour) cells are able to extravasate efficiently. Cell migration to specific sites may be critical for initiation of cell division as well as survival during growth for the later stages of metastasis. However, how are the radiolabelling studies (which indicate a 24-h half-life) to be reconciled with the 30-day or longer survival times observed with GFP? Are cells damaged during the process or are radioactively labelled cells more susceptible to damage? Is protein turnover more rapid during arrest in the lungs? Do cells die because of an inability to extravasate? Or is progression to form lung metastases regulated by other factors?

Recently, studies by Muschel and colleagues have provided a different view of the formation of metastases in the lung [56]. They used a perfusion/ventilation procedure to allow visualisation of GFP-labelled cells in the lung that were injected into the circulation. For both metastatic fibroblasts and fibrosarcoma cells, they found that most of the cells arrested in precapillary arterioles as well as in capillaries. Surprisingly, few of the cells were found to extravasate and most of the growing colonies found 3 days after injection were also found to be intravascular.

6. Issues for further study

6.1. Determination of rate-limiting steps

As genomic/proteomic data accumulate, specific proteins will be identified that are correlated with metastatic capability. The precise molecular contributions that such molecules make will require methods of analysis that open up the black box of metastasis. Intravasation, extravasation and growth of metastases are three extremely different phenomena that are required for successful metastasis. Evaluating the stage at which particular molecules contribute to metastasis will rely upon being able to compare the efficiencies of these steps. Such information will be important for deciding which molecules should be designated as targets for drug design.

6.2. Blood vessels versus lymphatics as entry points at the primary tumour

An open question in the field involves the relative importance of lymphatic versus haematogenous entry to

the circulation. A great deal of emphasis has been placed upon angiogenesis, evaluating microvessel density and developing treatments that inhibit angiogenesis. Given that the presence of tumour cells in lymph nodes is an important prognostic indicator while tumour cell density in the blood is not generally recognised as being an important prognostic indicator, is entry of tumour cells into lymph nodes the most important route for metastasis? Is angiogenesis mainly important in growth of the primary tumour, and only indirectly important for metastasis because it is correlated with the formation of lymphatics in the tumour? Alternatively, is the major route of tumour-cell dissemination haematogenous? Does the presence of metastases in lymph nodes have prognostic value because it indicates that the tumour cells have the ability to survive in other tissues?

6.3. Role of blood-based assays

Simple, blood-based assays for the evaluation of metastatic capability of tumours are attractive because of the ease of sample collection. However, results from such assays have not been consistently correlated with overall patient prognosis. A reason for this lack of correlation may be that fragments of poorly metastatic cells present in the blood yield false positives. We recommend that additional animal studies utilise the collection of samples from blood prior to filtration by the lungs or other capillary beds in order to evaluate the value of measuring the viable cells present in the blood as an indicator of metastatic ability.

6.4. Growth of metastases in blood vessels or parenchyma

It is important to resolve the apparently contradictory results regarding the site of initiation of metastatic growth in lung and liver. One possibility is that these are organ-specific differences — the liver capillaries are discontinuous sinusoids that might easily allow egress while the continuous endothelial lining of the lung vasculature might inhibit extravasation. Alternatively, the efficiency and importance of extravasation after arrest in the target organ may vary with cell type. Depending on whether tumour cells remain in the vasculature for several days or not may have important implications for the design and delivery of blood-borne treatments.

6.5. Variation between tumours

A general issue that will become more important is the heterogeneity of properties of specific tumour types. It is likely that the efficiencies of individual steps in metastasis will vary between tumours. For some tumours, intravasation may be relatively efficient and growth in target organs relatively inefficient, while for other tumours the opposite may be the case. This variability may occur from one patient to the next — although both may have invasive ductal mammary adenocarcinoma, the specific molecular differences as eventually identified using genomic/proteomic technology may allow us to predict which steps are most critical and to design treatments accordingly.

7. Conclusions

The ability to image living tumours *in situ* in the process of metastasis is enabling researchers to perform a more mechanistic analysis of the process of metastasis. The combination of traditional, hypothesis-based studies with genomic/proteomic studies will provide candidate molecules that could be critical for metastatic efficiency. Intravital imaging along with cell collection from live animals will provide an analysis of cell lines and transgenic animals specifically altered in the function of these proteins. Such an analysis will further our understanding of the contributions of these molecules to the rate-limiting steps in tumour cell invasion and metastasis.

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