Metastasis: new perspectives using multiphoton microscopy

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Cancer is a disease of multiple systems and components that interact at both molecular and cellular levels leading to initiation, progression and spread of the disease. Lately there has been a tremendous increase in systems-level study of cancer and the use of integrative approaches to understand mechanisms of cancers and their metastases. Metastasis is one of the most enigmatic hallmarks of cancers characterized by complex molecular interactions. It is responsible for as much as 90% of cancer-associated mortality, yet remains the most poorly understood component of cancer pathogenesis. Many hypotheses have been postulated to explain the intricate nature of the metastatic process, but none of them completely accounted for the actual biological and clinical observations. Consequently, metastasis still remains an open issue with only few metastasis-inducing proteins experimentally validated so far. New advances in intravital microscopy (IVM) have recently been used to visualize the behavior of single metastasizing cells at subcellular resolution over several days, yielding new and unexpected insights into this process. In this chapter, we will provide a current overview of intavital microscopy, and discuss recent findings in this field obtained with IVM.

Keyword: cancer, metastasis, intravital microscopy.

1. Introduction

1.1 Cancer

Cancer is a disease where uncontrolled cell growth has taken place in tissues of the body. The mechanisms that normally prevent cells from excessive proliferation have somehow been rendered dysfunctional in cancer cells and this can lead to the formation of a primary tumor. The capacity to invade and metastasize is what distinguishes a malignant tumor from a benign one and it is the most dangerous aspect of cancer; metastasis is the cause of 90% of deaths from cancer [1].

1.2 Metastasis

Overt metastasis is the end result of a multistep process that involves dissemination of tumor cells to distant organs and subsequent adaptation to foreign tissue microenvironments [2]. Metastasis, however, displays a remarkable diversity in clinical features depending on the cancer type, the pattern of organ dissemination and the different courses of disease [3,4]. The essential steps in the metastatic cascade include invasion of the adjacent tissues, intravasation, survival in circulation when detached from the extracellular matrix, a condition called anoikis [5], transport in blood and lymphatic vessels, extravasation, colonization and growth at distant sites with unrelated microenvironments [6]. To successfully complete the 'metastatic journey' cancer cells acquire distinct mechanisms and activate several gene expression programs and hence several models of metastasis have been proposed [7]. One of the models suggests that metastatic ability is an acquired event wherein the primary tumor cells develop genetic alterations during the course of time and become more invasive and metastatic [8]. The other model proposes that the metastatic capacity of primary tumors is pre-determined at the onset of tumorigenesis and gene expression signatures exist which can distinguish localized tumors from tumors that metastasize to distant locations [9]. Despite opposing views, one invariable feature of the metastatic process is deregulated gene expression [2]. Genes that promote metastasis have been categorized into three distinct classes based on their level of participation [2]. They include *metastasis-initiation genes* that promote tumor invasion, cell motility and angiogenesis. The gain or loss of function of these genes allows tumor cells to invade the basement membrane and escape into the circulation. The second class of genes is referred to as *metastasis-progression genes* that confer a specific organ tropism. They are involved in functions such as vascular remodeling and provide the malignant cells the ability to infiltrate the distant organs. They could be enriched at the primary site but possibly have a unique role at the distant site. The third class is called *metastasis-virulence genes* that promote growth and colonization at distant sites. They do not affect the primary growth but provide selective advantage at the secondary site [2]. Metastasis suppressors such as caspase 8 have also been strongly implicated in tumor metastasis [10].

1.3 Steps of Metastasis

1.3.1 Tumor Migration and Invasion

Tumor invasion is the initial event in the metastatic cascade and is primarily mediated by regulated interaction of tumor cells with the surrounding extracellular matrix (ECM). This step of metastasis requires tumor cells to attach proteolyze and migrate through the basement membrane and enter into the circulation [11]. The extracellular matrix is composed of both interstitial component and the basement membrane. The extracellular-matrix components, such as fibronectin, collagen, and Laminin, act as a scaffold allowing cells to attach and move by making contacts with the cell-surface receptors called integrins [12]. As one of the most abundant constituents of the extracellular matrix, fibronectin binds multiple integrins [13], and this binding results in the activation of focal adhesion kinase (FAK) [14], Src [15], Akt [16], as well as modulation of the small GTPases of the Rho family [17]. In response to these signals, cells remodel their actin cytoskeleton [18] express matrix metalloproteinases [19], become migratory [20], invade basement membranes [21], and acquire the ability to resist apoptosis [22]. Increased fibronectin expression has been shown in various tumor cell types like melanomas [23], breast cancers [24], and thyroid carcinomas [25]. In addition, fibronectin has been shown more recently to play an important role in the colonization of primary tumor cells at the distant sites by forming a 'premetastatic niche' [26].

Integrins are heterodimeric transmembrane proteins containing 1 of 18 α- and 1of 8 β subunits. The different α- and β chain combinations dictate the specificity for different ECM components [27,28]. A number of studies have demonstrated that more migratory and invasive cells show dramatic alterations in the levels of integrin and ECM expression [29,30]. Integrins form a functional link between the ECM and the intracellular signaling pathways that influence cell shape, motility, proliferation and migration and invasion [31]. 'Outside–in signaling' mediated by binding of ECM [18] to integrins leads to the clustering of integrins that initiates several downstream signaling events. Since integrins lack intrinsic catalytic activity, signals initiated by ECM–integrin interactions are transduced inside the cells through activation of some non-receptor tyrosine kinases like focal adhesion kinase (FAK) and Src.

1.3.2 Focal Adhesion Kinase (FAK)

FAK is a 120 Kilodalton cytoplasmic protein tyrosine kinase that co-localizes with integrins to structures referred to as 'focal contacts' [32]. Structurally, FAK contains several distinct domains. It contains a central catalytic domain flanked by an amino-terminal Four-point-one, ezrin, radixin, moesin (FERM) domain and a carboxy terminal proline-rich and FAK targeting domain (FAT). The FERM domain is a key regulatory element as it interacts with several proteins including cytoplasmic tails of integrins [33], integrins associated proteins [34] and epidermal growth factor receptor (EGFR) [14]. The focal adhesion targeting (FAT) domain at the carboxy terminus is important in FAK localization by interacting with focal adhesion protein, paxillin [35], whereas the proline rich domains are involved in binding with proteins containing SH3 domains including Crk-associated substrate (Cas), p130 Cas [36], GRAF [37] and ASAP1 (ADP ribosylation factor [ARF]- GTPase-activating protein [GAP] containing SH3, ANK repeats, and PH domain) [38]. FAK is maintained in an inactive state by binding of its FERM domain to the kinase domain [39]. Integrin clustering leads to intracellular signals that relieve the autoinhibition, resulting in stimulation of FAK catalytic activity by autophosphorylation at tyrosine 397 (Y397). This auto-phosphorylation event creates a high-affinity binding site for the SH2 domain of Src family kinases and leading to the activation of Src [40,41]. The activated Src in turn phosphorylates additional tyrosine residues on the kinase domain (Y576 and Y577) and carboxy-terminal domain (Y861 and Y925) of FAK. These phosphorylated residues form docking sites for other cytoplasmic proteins including p130 Cas and paxillin [40], p21-activated kinase and mitogen-activated protein kinase (MAPK) pathways and the small GTPases, Rac and Rho leading to the activation of various signaling cascades involved in migration and invasion [42]. In normal cells, FAK activity is under tight regulation by several mechanisms including gene amplification, alternative gene splicing [43] and action of phosphatases [44]. On the contrary, FAK levels are dramatically altered in a wide range of malignancies. The levels are increased in tumors of prostate [45], ovary [46], colon and breast [47]. Increased FAK expression and activity is closely linked to metastasis and poor prognosis [47,48,49]. They are expressed at higher levels in invasive tumors as compared to benign preneoplastic lesions [48]. FAK has also been implicated in deregulation of E-cadherin expression, a protein involved in epithelial mesenchymal transition (EMT). This is another prominent mechanism involved in tumor cell invasion. During this process epithelial cells lose the cell-cell contacts and epithelial markers like E-cadherin and γ-catenin and acquire fibroblast like phenotype and mesenchymal markers like fibronectin, vimentin, N-cadherin. This switch from epithelial to mesenchymal phenotype is important for most tumors to become invasive and aggressive [50].

1.3.3 Src family of Kinases (SFKs)

The other family of non-receptor tyrosine kinases is the Src family of kinases. They are associated with FAK and integrins at focal contacts and play an important role in the integrin signaling. This family includes c-Src, Fyn, Yes, Blk, Lyn, Hck and Lck [51]. c-Src is the most extensively studied member of this family and has been implicated in

tumor progression [52]. Discovered by Michael Bishop and Harold Varmus, c-Src is the first cellular homologue of the retroviral v-Src protein identified in the late 1970s by Francis Peyton Rous [53]. c-Src and v-Src share similar structures except for a truncation in the regulatory carboxy terminus which features loss of the auto-inhibitory tyrosine 527 residue [54], making v-Src constitutively active. Unlike v-Src, c-Src is nontransforming in nature but once activated, can be converted to a transforming protein by various modifications including dephosphorylation of tyrosine-527 which is consistent with its role as a proto-oncogene [55]. Src protein is characterized by several distinct domains. It contains C-terminal domain with a negative regulatory tyrosine residue (Tyr-527), four SH (Src homology) domains and a N-terminal domain which may undergo myristoylation [56]. Src is present in an inactive state and inhibited state under normal conditions [57]. Binding of the Src-SH2 domain to FAK brings about a conformational change in Src relieving the auto inhibition of Src kinase and phosphorylation of the activating tyrosine residue (Tyr-416) on the first SH1 kinase domain of Src [42]. The activated Src and FAK form a functional bipartite kinase complex leading to the activation of several downstream signaling cascades important in cell motility, adhesion and invasion [58]. FAK-Src signaling has been shown to be involved in activation of Rac1 activity [59,60], a member of the Rho family of GTPases involved in cytoskeletal remodeling [61]. Overexpression of c-Src and an increase in its catalytic activity has been implicated in various types of cancers [52] and increased c-Src kinase activity has been implicated with enhanced metastatic potential [62,63] through effects on motility and invasion [64].

1.3.4 Survival in circulation-Anoikis Resistance

Once tumor cells detach from the primary tumor mass, they enter into the circulatory or lymphatic system. This is called intravasastion. Once in the circulation, they no longer get the survival signals they normally receive from cell-cell and cell-ECM interactions. In normal epithelial cells the lack of cell-ECM interactions activates a special form of cell death called 'anoikis'(Greek word for homelessness) [65]. This is a safeguarding mechanism for normal cells to maintain tissue homeostasis and development and has significant physiological relevance. Various studies have focused on the importance of anoikis in the process of neoplastic transformation as seen in malignant mammary [66] and colon cancers [67]. Tumor cells with metastatic potential acquire several mechanisms to evade this process and acquire resistance to anoikis which not only provides them with increased survival time but also facilitates reattachment and colonization at secondary sites. The molecular mechanisms involved in anoikis resistance are still not completely understood. Some of the prominent mechanisms include activation of survival pathways (Phosphoinositide 3-kinase -Akt), upregulation of matrix metalloproteinases, inactivation of p53, overexpression of focal adhesion kinase (FAK) and anti-apoptotic proteins (BCL-2, BCL-XL, XIAP) [68,69,70].

1.3.5 Colonization and Growth at Distant Sites

The last and final stage of the metastatic cascade is the colonization of the secondary site. Stephen Paget proposed a 'seed and soil' hypothesis according to which the disseminated tumor cells (seed) colonize to specific organs whose microenvironment (soil) is compatible for their growth and proliferation [71]. For example, breast cancers frequently metastasize to lungs, liver, bone and brain whereas prostate cancers colonize to bone. This hypothesis was refuted by James Ewing who proposed that distant metastasis at specific sites is primarily determined by the vasculature at the primary site [72]. Recent advances and emerging data strongly support Paget's hypothesis and stresses on the concept of viable tumor microenvironment or 'premetastatic niche' in grafting primary tumor cells to distant sites [26].

1.4 Studying cancer metastasis

It is important to study cancer and especially metastasis, not only to find out how tumor cells behave *in vivo*, but also the underlying molecular mechanisms of this process are important. The knowledge gained about metastasis can be used in the clinics to develop drugs and the right treatment program for patients. For this reason, intravital microscopy become very important as tool for studies about the underlying mechanisms of cancer and metastasis [73].

2. Intravital microscopy Technique

2.1 Intravital microscopy

Intravital microscopy consists of microscopically imaging intact living tissue. Tissue preparations for intravital microscopy can be either chronic transparent windows, acute preparations (where the animal in research is prepared surgically to gain direct access to inner organs), or *in situ* preparations [74]. Transparent window chambers implanted in the dorsal skinfold of mice are commonly used models for the study of tumor vasculature with intravital microscopy. In this case, a tumor grows inside of one of the skin layers, under a glass window. This glass window makes it possible to image the vascular network of the tumor. In order to achieve good visualization of the vasculature, fluorescent contrast agents can be injected intravenously which, after a short time, distribute throughout the vascular network of the tumor.

2.2 Interactions between light and matter

The transit of light through a material leads to exchange processes between the light and the molecules in the material [74]. These interactions can result in changes of the direction, polarization and energy of light through different absorption and energy distribution processes. If the energy of the incoming photons is equal to or greater than the energy difference between two energy levels in a molecule, it can absorb the energy and excite to a higher energy level. Electronic, vibrational and rotational energy levels can be excited. Since the energy of the incoming photons is dependent on the wavelength of light, it is possible to use light with different wavelengths to selectively excite different molecules or different energy levels in the same molecule. After an excitation takes place, the excited molecule will expel the absorbed energy and return to the basal state. This de-excitation can occur through different processes. The most important ones are illustrated in the Jablonski diagram (Figure 2) [75]. From these processes, fluorescence and phosphoresce involve the emission of light. Phosphorescence comprises forbidden transitions between electronic energy levels with different spin quantum number *J*. Fluorescence consists of electronic relaxation without changing the spin quantum numbers for the electrons ($\Delta J = 0$), with further emission of a photon with an energy equal to the excited energy gap. Fluorescence only occurs from the lowest excited condition to the basal state [76]. In the absence of other relaxation processes fluorescence has a life-time of about 10−9 to 10−8 seconds. Other non-radiative processes compete with fluorescence, among them, internal conversion, dissociation, chemical reactions and ionization. The emitted fluorescent light has less energy than the excitation light. The molecule releases the remaining excitation energy through non light emitting transitions between vibrational levels within the same electronic energy level. The result is that the fluorescent light gets a shift towards longer wavelengths compared to the excitation light. This is called *Stokes shift* and makes it possible to separate excitation light from fluorescence light with the help of optical filters. Fluorescence has a life-time of about 10−9 to 10−8 seconds. Other non-radiative processes compete with fluorescence, among them, internal conversion, dissociation, chemical reactions and ionization. The emitted fluorescent light has less energy than the excitation light. The molecule releases the remaining excitation energy through non light emitting transitions between vibrational levels within the same electronic energy level. The result is that the fluorescent light gets a shift towards longer wavelengths compared to the excitation light. This is called *Stokes shift* and makes it possible to separate excitation light from fluorescence light with the help of optical filters.

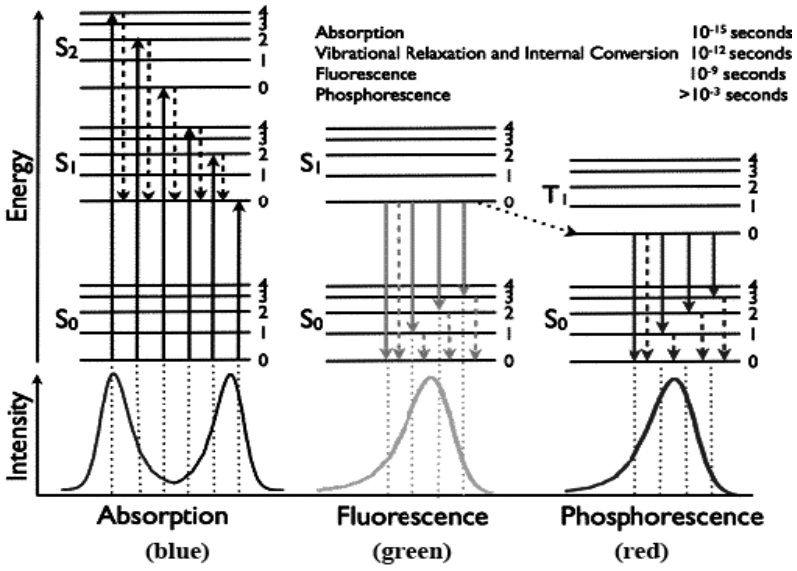

Fig. 1 The Jablonski diagram and spectra of absorption, fluorescence and phosphorescence.

Solid arrows point to radiative transitions through absorption (shown in violet and blue) or emission of a photon by fluorescence (displayed in green) or phosphorescence (marked in red). Non-radiative transitions are pointed with dashed arrows. Transition S1,0 !T1,0 (subscripts correspond to the electronic state and vibrational sublevel, respectively) is termed *intersystem crossing* since it occurs between two states of distinct spin multiplicity and is non-radiative. Under the Jablonski diagram, representations of the absorption, fluorescence and phosphorescence spectra are shown [75].

2.3 Fluorescent markers and contrast agents

The ability of molecules to absorb and emit light with certain frequencies can be exploited to detect special molecules in a test volume based on the emission spectrum. Molecules can be naturally fluorescent (a phenomenon called

autofluorescence) or can fluoresce due to the presence of other fluorescent molecules. *Endogenous fluorophores* are autofluorescent contrast agents that occur naturally in a sample. Several molecules that occur naturally in cells are auto-fluorescent, such as NADH, flavins, elastin and collagen [77]. This property can be utilized to image special structures inside the cell in addition to different cellular processes. *Exogenous fluorophores* are fluorescent molecules that can be attached specifically to biological structures. There exist a variety of these fluorophores which vary in specificity, sensitivity and emission spectra. Some basic requirements for an exogenous flurophore are solubility, specificity for association with targets, high quantum emission efficiency, stability and low probability of photobleaching. When using different fluorophores simultaneously, it is important that the emission spectra are sufficiently separated in order to be detected individually.

2.4 Green and Red fluorescent protein

Green fluorescent protein (GFP) is a fluorescent protein that occurs naturally in certain cells of the jellyfish *Aequoria Victoria* [77]. This protein can be introduced in cells grown *in vitro*, or in specific cells in a living organism by means of recombinant DNA methods. A gene that codes for GFP is introduced in the DNA of the cell, and results in synthesis of GFP in the cell. These cells are called *GFP transfected cells*, and can be detected with fluorescence microscopy by using filter packages that detect the fluorescence in the green region of the light spectrum. The unit in GFP that absorbs and emits light is well protected within the compact folded structure, which reduces the probability of de-excitation through non-light-emitting processes as a result of interactions with the surroundings. This makes GFP a robust and stable protein. Several GFP mutant proteins have been produced. These mutants have higher emission than the original GFP and some have also different emission spectra [77]. The other discovered red fluorescent protein (RFP) shares many properties of GFP and can be applied in a similar manner. One form of red fluorescent protein (DsRed), isolated from *Discosoma striata* has an optimal absorption at 558 nm and emits light at 583 nm. Another form of RFP (cob A) has been isolated from *Propionibacterium freudenreichii* [78]. One advantage of RFP is that it produces less background interference than GFP. Although the greatest advantage of RFP is that it can be used in conjunction with GFP to colabel cells.

3. Microscopic imaging techniques

In microscopy the ability of convex lenses to concentrate light is utilized to form real magnified images of objects that are currently outside the focal plane of the lens. This simple principle forms the basis of a number of advanced imaging systems.

3.1 Light microscopy

The main components in a light microscope are the light source, the condenser lens, the objective lens and the eye piece [77]. The light source in a conventional light microscope is usually a halogen bulb that emits white light. The light from this lamp is focused on a small area of the sample by the objective lens. This results in a magnified real image of the object in a image plane as shown in Figure 3. Typical magnifications in the objective lens are in the range of 4× to 100×. The image is subsequently magnified by the eye piece, which creates the resulting image on the retina. The total magnification is given by the product of the magnification in the objective lens and the magnification in the eye piece. A modern light microscope usually combines various lenses to improve the magnification and the image quality by correcting lens irregularities and chromatic aberrations. Optimal illumination of the sample is obtained when the sample is evenly illuminated in the field of view, and the sample is illuminated with a cone of light that is as wide as possible. These criteria are fulfilled using Köhlers illumination principle [77]. The lateral resolution d of a microscope is defined as the shortest distance between two points in the object which can be separated to be observed as two separate points in the image. This will be limited by diffraction, and is given by the Rayleigh criteria [77]:

$$d = \frac{0.61\lambda}{NA},$$

where NA is the numerical aperture and λ is the wavelength of light. The numerical aperture is determined by the refraction index n of the media between the sample and the objective, and the maximal angle θ the light rays can have relatively axis normal on the objective lens and still reach the objective as,

$$NA = n\,sin(\theta)$$

The thickness of the slice of the sample that is in focus is given by the axial resolution Z in the objective plane, as

$$Z = \frac{n\lambda}{NA^2},$$

and can be improved with increased magnification in the objective and eye piece.

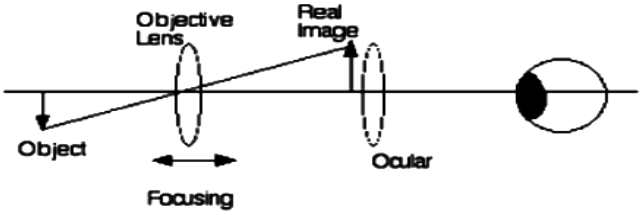

Fig. 2 Focusing of light by an objective lens results in a magnified real image of the object which can be imaged by the ocular.

3.2 Inverted microscope

One of the problems in conventional microscopy is the limited working distance between the object table and the objective lens. Objective lenses with magnification in the range of 25× to 40× yield working distances of 3 to 5 mm [79]. Inverted microscopes get an increased workspace since in an inverted microscope the light source and the condenser are located above the object table, and the objective lens is placed under the object table. In addition, gravity will facilitate the imaging, for example in the case of imaging cells in the bottom of growth bottles or Petri dishes. A disadvantage with inverted microscopes is that they have a more complex structure and lower maximum magnification compared to the conventional microscopes.

3.3 Fluorescence microscopy

In fluorescence microscopy, a mercury or halogen lamp excites fluorophores in the sample. By using an excitation filter, the wavelength of the light can be adjusted to the excitation wavelength of the used fluorophore. Then the emitted light is detected with a system of filters and dicromatic mirrors that separate the fluorescent light from the excitation light. This separation is possible because, as mentioned before, the fluorescent light have longer wavelength than the excitation light. An important advantage of fluorescence microscopy is that it gives a high signal-to-noise ratio which permits the detection of low concentrations of the molecule under investigation [77]. In fluorescence microscopy epi-illimination can be used since the fluorescent light has longer wavelengths than the excitation light. The sample is then illuminated with excitation light through the same lens that detects the fluorescent signal. The excitation light and the fluorescent light are separated by a dichromatic mirror that only allows light with wavelengths above a specific value to pass through and the rest of the light is reflected

4. Important new insights into tumor cell biology and Future perspectives

The combination of better microscopes, other fluorescent probes window makes it easier to study metastasis of tumors in real time. Intravital imaging can give insights in the action of primary tumors in living animals, can provide us with observations about migration and invasion of tumor cells into the microenvironment and in addition demonstrate the role of the microenvironment in the process of metastasis. In the future, this will provide more insight into the multi-stage process of metastasis, whereby especially invasion and intravasation can be studied. Different combinations of microscopic techniques with probes have unraveled already some of the questions of the microenvironment in metastasis.

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