Metastasis models: the green fluorescent revolution?

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Metastases are the leading cause of treatment failure and death of patients affected by malignant tumors, which makes them a major therapeutic target. During the last decade, efforts made to understand the mechanisms governing the passage of a localized tumor cell to a metastatic one has led to significant advances in this field. In vivo models using nude mice largely contributed to the understanding of this multi-step phenomenon, thus allowing the discovery of new targets and the development of therapeutic agents. These models were however hampered by the difficulties to detect micrometastases. The recent introduction of the green fluorescent protein (GFP) as a marker for tumor cells has radically changed the use of these models, in particular by allowing detection of single cell metastases in vivo. In this review, we discuss the major advantages of models using GFP-labeled cells and their limits.

Introduction

The metastatic process is a series of complex interactions between cancerous cells and host cells or tissues (1). A cell originating from a typical solid tumor must undergo several modifications to become metastatic. These include loss of adhesion with surrounding cells, migration towards vessels, destruction of the basement membrane, passage in the blood stream and escape from the immune system. The cells must then arrest and extravasate into the target tissue, and growth in this tissue where a neoangiogenesis leads to its blood supply. Each of these events requires that the cell acquire particular capacities and thus numerous molecular modifications, which must successively appear in the same clone. Such transformations are also dependent upon microenvironmental conditions. For example hypoxia leads to induction of the hypoxia inducible factor-1, which is over-expressed in numerous progressing cancers (2).

Many points of the metastatic process are still undefined and characterization of their molecular basis is crucial in the development of new cancer therapies. Accordingly, contemporary experiments are attempting to define molecular changes and then modify them. For example, several studies are directed towards the inhibition of angiogenesis by blocking the action of the vascular endothelial cell growth factor (reviewed in refs 3,4). Indeed, as soon as the center of a primary tumor reaches 1 mm3, it becomes hypoxic; in order to get nutrients and oxygen, the tumor has to develop new blood vessels. Thus, prevention of angiogenesis could concomitantly inhibit tumor growth and metastatic spread. Other approaches are directed towards the inhibition of matrix metalloproteinases (MMP) (reviewed in ref. 5). MMPs are involved in the modification of the extracellular matrix during tumor growth and dissemination; while cancerous cells are proliferating, they produce MMPs that degrade peritumoral tissues in order for the cells to migrate towards the blood stream and to metastasize. The use of specific inhibitors has promise because of this [e.g. TIMP (6)].

Whatever the pathway under investigation, it is essential to have at one’s disposal an in vivo model mimicking as far as possible the cascade of biological events leading to the formation of metastases. This review will focus on the use of the green fluorescent protein (GFP) in metastasis models by highlighting their main advantages and by describing some of the most representative current studies. Then, we will discuss their limits and try to underline their weaknesses.

The models of studies of the metastases

Nude mice are widely used to study the metastatic process in vivo. These immunodeficient animals (7,8) may be xenografted with human cancer cells (9,10). Historically, Rygaard and Povlsen realized the first successful transplantation of a human tumor in these mice in 1969 (11). Whatever the method of cell grafting [intravenous (i.v.), subcutaneous (s.c.), orthotopic, etc.], a major problem is difficulty visualizing micrometastases in host tissues. Current methods of metastases detection are generally restricted to histology or immunohistochemical analysis of target tissues. Direct macroscopic observation of the metastases is even less sensitive. As they do not address to micrometastases, such observations considerably underestimate the actual number of metastases; further, the number of tissues reasonably analyzable limits them. Such investigations are therefore restricted to a qualitative rather than a quantitative analysis of the effect of a molecule or a gene on the metastatic process.

A technique devoted to the detection of micrometastatic foci, even to that of a single cancerous cell in healthy tissue, is to express in these cells an indicator gene such as the Escherichia coli lacZ gene (12–21). Although this system allows for the visualization of small groups of cancerous cells (15), it shows several restrictions: it is very difficult to detect a single malignant cell, it is time consuming and requires a complex preparation of the samples; it is impossible to enumerate all the metastatic foci in a target organ, and the endogenous β-galactosidase activity of some cells prevents a good interpretation of the results (22). Optical imaging via the

Abbreviations: GFP, green fluorescent protein; ITI, inter-alpha trypsin inhibitor; IVM, intra-vital microscopy; IVVM, intra-vital video microscopy; o.g., orthotopic graft; RFP, red fluorescent protein; s.c., subcutaneous; TIMP, tissue inhibitor of metalloproteinase.
luciferase reporter system is a good alternative (23,24), but it requires substrate delivery to the tissue under study and a special acquisition device; besides its low image resolution, this technique does detect single cells (25). These drawbacks have led to the use of the GFP as a marker for cancer cells. Initially isolated from the jellyfish _Aequoria victoria_ (26), the GFP gene was cloned in 1992 by Prasher _et al._ (27). It is a compact, acid and globular 27-kDa protein, composed of 238 amino acids (28), with an excitation peak of 488 nm and an emission peak of 508 nm. Its fluorescent properties are acquired by an autocatalytic mechanism giving rise to the fluorophore, that does not require any biochemical transformation, contrast agent or the use of ionizing radiation in order to be visualized (29,30). Zolotukhin _et al._ obtained a humanized mutant, hGFP-S65T, that displayed a high expression level in mammalian cell lines (31): in comparison with wild GFP, it is 35-fold more fluorescent, and much more easily expressed in mammalian cell lines. The GFP has been expressed in a variety of cells and organisms: bacteria (32), yeast (33), eukaryotic cell lines (34–36) and transgenic mice (37).

**The GFP as a cell marker to track metastases**

GFP has since become a reporter gene of choice. The fluorescence expressed by transfected cells allows one to select very high-level expression GFP transfectants _in vitro_ (38). It is then very easy to follow the stability of the fluorescence during time, in non-selective medium. This can be accomplished either by direct observation using a fluorescence microscope or by cytofluorimetry. Further, fluorescence-activated cell sorting (FACS) allows for purification of a polyclonal population of GFP-labeled cells. _In vivo_, one of the main advantages of GFP lies in the ability to visualize single cancer cells. Hoffman’s group first used this property to develop GFP-based cancer models. For example, Chishima _et al._ (39) stably transfected Chinese hamster ovary (CHO-K1) cells by a vector containing the cDNA of GFP. After surgical orthotopic implantation (SOI), they demonstrated for the first time that the expression of GFP in these cells allowed the detection in fresh tissue of single metastatic cells. Later on, they successfully used this system in several malignant cell lines: lung [ANIP 973 (40,41) NCI-H460 (42)] and prostate [PC-3 (43)], confirming the ability of GFP to mark cancer cells. Fluorescence is therefore the tool of choice for studying the genetic basis of metastatic processes in real time. This property has also permitted the study of metastatic effusion in the lymph nodes of rats with melanoma (44). Zolotukhin _et al._ obtained a humanized mutant, hGFP-S65T, that displayed a high expression level in mammalian cell lines (31): in comparison with wild GFP, it is 35-fold more fluorescent, and much more easily expressed in mammalian cell lines. The GFP has been expressed in a variety of cells and organisms: bacteria (32), yeast (33), eukaryotic cell lines (34–36) and transgenic mice (37).

**Analysis of gene involvement in the metastatic process**

Experiments aimed at analyzing the effect of gene expression or inhibition on the metastatic process can take advantage of GFP tagging. Using the highly metastatic lung cancer cell line (GFP-labeled H460 M cells), we have demonstrated the involvement of genes of the inter-alpha trypsin inhibitor family (ITI) in tumor growth and metastatic spreading. Transfection of fluorescent cells with the cDNAs corresponding to the various chains of the ITI family allowed us to study the influence of their expression on the metastatic process and the tumor growth after s.c. injection and analysis of the number of fluorescent lung metastases (58). We showed that ITI-H1 and ITI-H3 chains significantly decreased the metastatic scattering. The ITI-L chain was able to decrease the tumor growth and the number of lung metastases, possibly through the protease inhibitor characteristics of its bikunin moiety. Other experiments also demonstrated the feasibility of such an approach for the study of gene expression: Zhang _et al._ showed that overexpression of the bone sialoprotein (BSP) (for review, see ref. 59) in the human breast cell line MDA-MB-231 (60) increased the number of fluorescent lung metastases while transfection with an antisense BSP sequence reduced the number of metastases. In an elegant study taking advantage of GFP-tagged lymphomas and whole-body imaging in mice, Schmitt _et al._ (61) examined the p53 tumor suppressor pathway and tested the contribution of apoptosis to tumor suppression.

**Screening of molecules that might have an effect on metastasis**

Besides several _in vitro_ cytotoxic assays built up with GFP-transduced cells for high-throughput screening of novel anti-neoplastic agents (62–64), various _in vivo_ experiments have been designed to study the therapeutic potential of drugs on tumor growth and metastasis. Among several promising molecules, Manni _et al._ (65) demonstrated that after grafting breast cancer cells (GFP-tagged MDA-MB-435 cells), the administration of alpha-difluoromethylornithine (DFMO) significantly decreased the number of mice presenting lung metastases; further, DFMO administration also reduced local recurrence of primary tumor growth after its removal. These cells were also used to demonstrate the inhibitory properties of a truncated form of a mammalian lectin involved in metastasis, galectin-3, on tumorigenicity, tumor invasion and metastasis _in vivo_ (66). Lawson _et al._ (44) showed that the use of a novel vitamin E
## Table I. Summary of stable GFP-expressing cell lines and localization of metastasis foci (*), according the species, the cell line origin and the inoculation type (i.p., intraperitoneal; i.c., intracardiac)

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue origin or cancer type</th>
<th>Cell line</th>
<th>Inoculation type</th>
<th>Target tissues of metastases</th>
<th>References</th>
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<td>Adrenal gland</td>
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<td>HCT-15</td>
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<td>Fibrosarcoma</td>
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<td>Lung</td>
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<td>ANIP 973</td>
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<td>H460 M</td>
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<td>NCI-H460</td>
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<td>MIA-PaCa-2</td>
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<td>CL1</td>
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<td>PC3</td>
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<td>Stomach</td>
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<td>o.g.</td>
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<td>Mouse</td>
<td>Colon</td>
<td>CT-26</td>
<td>i.c.</td>
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<td>Mammary</td>
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<td>Plasmacytoma</td>
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<td>MOPC315</td>
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<td>Rat</td>
<td>Lung</td>
<td>Lewis Lung</td>
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<td></td>
<td>Mammary</td>
<td>CC531s</td>
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<td>Tongue</td>
<td>RSC3</td>
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analog, the RRR-alpha-tocopherol ether-linked acetic acid analog (alpha TEA) had an inhibitory effect on lung metastases and tumor growth in a syngeneic BALB/c mouse mammary tumor model. The drug was delivered via an aerosolized liposomal formulation and its effect was correlated with an increase in apoptosis of cells. Sun et al. demonstrated the efficacy of the camptothecin analog DX-8951f (Exatecan mesylate) against two human pancreatic tumor cell line models (67): this new molecule was found to be more effective than gemcitabine, a standard treatment for pancreatic cancer, on metastases of MIA-PaCa-2 and BxPC-3 GFP-labeled cells. The antimetastatic activity of a 2'-deoxyctydine analog, CS-862, could also be demonstrated in another pancreatic model using the red fluorescent protein (RFP) (68). Mori et al. have studied the effect of S-1, a novel oral derivative of 5-fluorouracil (5-FU), on the development of peritoneal metastases induced by GFP-tagged gastric cancer cells (MKN-45) (69); they demonstrated that the therapeutic effect of S-1 was significantly greater than that of 5-FU. Finally, a murine GFP-tagged lymphoma model has been used to study the antitumor activity of cyclophosphamide and found that it depends on its ability to induce apoptosis and senescence (70).

**Activated cells**

The ability to retrieve live cells by FACS after passage in vivo opens the way to new studies; the genome, the transcriptome or the proteome of these 'activated' cells can be compared with that of parental in vitro cultivated cells and give molecular information on the metastatic process. Using this strategy, Dellacasagrande et al. (71) demonstrated that freshly sorted mouse plasmacytoma (MOPC315) GFP-labeled cells overexpressed the functional chemokine receptor CCR6. They suggested that chemotaxis via CCR6 might be a common mechanism by which malignant cells metastasize to liver that constitutively expresses CCL20, the natural CCR6 ligand.

**Metastatic potential**

The use of GFP and its by-products allows for dual-color studies to compare the metastatic potential of various cell lines. Glinskii et al. (72) have collected evidence demonstrating that there is a difference between cells ‘activated’ after passage in vivo (GFP-tagged) and the parental cells cultivated in vitro (RFP-tagged). Having isolated and cultured circulating GFP-tagged cells obtained after orthotopic implantation, they co-injected an equivalent number of ‘activated’ and parental cells and demonstrated that GFP-tagged cells have an increased metastatic propensity compared with the parental RFP-tagged cells. Dual-color studies were also performed to analyze the clonality of metastasis (73) and to follow RFP-tagged tumor cell dissemination in a GFP transgenic mouse host (74).

**Dormancy**

Although numerous cancerous cells have the capacity to migrate towards blood vessels (75) and to arrest in a target tissue, only a low percentage of them are able to form metastases. Studies recently realized by IVVM using GFP-labeled cells demonstrated that although the extravasation of cells is not limited, the metastatic cells do not all self renew as there are populations of dormant cells only with a potential for subsequent proliferation observed (76,77).

**Kinetics**

A key parameter in the study of the effect of a gene or a molecule on the metastatic process is the observation of development of metastases over time. Before the use of GFP, these studies could not be done. Using H460 MGFP cells (38), we have demonstrated that it is possible to quantify the metastatic foci present in lungs and thus to study their appearance with time. Fluorescent lung metastases are detected as soon as 2 weeks after s.c. injection, making it possible to study accurately the effect of genes, peptides or small molecules on the early steps of metastasis. A mouse bone marrow model was used to study the graft-versus-host-disease by IVM and follow cell homing and expansion according to time (78).

**Limits of the GFP models**

**Biological reality**

Generally speaking it is necessary to keep in mind that these in vivo models, based or not based on GFP, are far from the pathophysiological reality of carcinogenesis and only partially reflect the various mechanisms involved in the metastatic process. Indeed, models using ‘experimental’ metastases obtained by i.v. injection of cancerous cells do not study the early stages of metastasis prior to passage into the blood stream, i.e. they are restricted to the study of late events, such as the evaluation of the dormancy or growth of malignant cells in target organs (79). On the other hand, even if most human cell lines may form primary tumors after s.c. injection, they are rarely able to produce metastases. In fact cell lines selected for their strong potential metastatic capacity have been found to be inefficient in an attempt to form spontaneous metastases after intramuscular injection (80). Among models using cancerous cells and nude mice, those using orthotopic grafts (o.g.) get closer to physiological conditions because cancerous cells are introduced in the tissue of origin. Nevertheless, certain o.g. turned out to be technically difficult; for example, the SOI of lung cancer cells requires a thoracotomy to transplant to the mouse lung a fragment of histologically intact tumor tissue (81-83). Furthermore, the quality of transplanted cells can be very diverse since primary tumors are prone to necrosis and the tumor fragment cannot be identical in each animal thus producing a highly heterogeneous number of implanted cells.

**Problem of detection**

Although these models have allowed remarkable progress in the detection of the micrometastases, their enumeration may still be difficult. The GFP-expression level in stably transduced cells can be very diverse since primary tumors are prone to necrosis and the tumor fragment cannot be distinguished in host tissues. Moreover in liver, the tissue density hampers the fluorescence detection, thus a correct enumeration of the metastases; to get a correct evaluation of the fluorescence, the organ must be cut into slices with a thickness under 0.5 mm. This may distort the numbering of metastases with large metastasis being cut in two parts, and fluorescent cells spreading on non-malignant tissue. On the other hand, the autofluorescence of some tissues, such as lung, may hamper the analysis. Such difficulties are likely to be overcome by recent improvements in image acquisition and instrumentation (84,85).

**Enhanced sensitivity**

The cytotoxic activity of various anticancer drugs can be enhanced by reactive oxygen species (86,87) that can be generated by GFP (88). Goto et al. therefore hypothesized that GFP transfection could enhance the anticancer drug sensitivity
of human neuroblastoma cell lines (CHLA-20 and CHLA-90). They demonstrated that GFP transfection results in an oxidative stress that enhanced the sensitivity of neuroblastoma cell lines to various anticancer drugs such as carboplatin, melphalan, doxorubicin and etoposide (89); indeed, these drugs turned out to be much less effective than expected in pre-clinical tests. Although this enhanced sensitivity has been only observed once, care should be taken when assaying new drugs with these models.

Cytotoxicity

Although GFP has been used in many cell lines with no evidence of cytotoxicity (90,91), and transgenic animals were obtained (37,92), the establishment of stable GFP cell lines has a low efficiency. Several studies showed a GFP-induced toxicity; for instance, Liu et al. demonstrated that GFP transfection in various cell lines (NIH/3T3, BHK-21, Huh7 and HepG2) by different vectors led to programmed cell death or apoptosis (93) by promoting the activation of caspase 3. The observed toxicity may also be related to the vector used as a study showed (94) that the peptide generated by the multiple cloning site of the pEGFP (enhanced green fluorescent protein)-C2 vector (Clontech) induced apoptosis and that partial or total removal of this multiple cloning site abrogated the toxicity.

Immune response

In studies requiring the use of immunocompetent mice, for instance in pre-clinical evaluation of gene therapy, the use of GFP-tagged cells raises a problem. The difficulty is that this protein can induce immune response, mediated by a high cytotoxic T lymphocyte response against GFP-expressing cells (95). Although this has no direct influence on the presented models, it must be kept in mind when imagining new applications. It is worth noting that this phenomenon was not observed in other studies (96,97), possibly because of the high metastatic potential of the cell models used.

Conclusion and perspectives

Thanks to the introduction of GFP as a marker for cancerous cells, the detection of the micrometastases down to the unicellular level has become possible. Numerous cell lines of human or mammalian origin have already been tested with this system successfully. From now on it is therefore possible to track micrometastases in a simple and fast way within various tissues such as lung, liver, brain, bone, etc. Besides its increased resolution with respect to pre-existent models, the GFP-based technology, thanks to its non-invasive character, allowed us to implement new research strategies on the metastatic process among which IVVM and whole-body imaging must be highlighted because they allow us to study in a kinetic way the evolution of the metastases in living animals (53). The GFP tag also allowed us to obtain metastatic cells ‘activated’ after a passage in vivo in order to study various proteins and genes expressed in a differential way by comparison with the less aggressive parental cell line (71). The studies of genes and small anticancerous or antimetastatic molecules also took advantage of the GFP technology, because the study of their impact on these processes is made easy with a so far unequalled accuracy. They unfortunately suffer from some limitations such as, for example, the increased oxidative stress (89) induced by GFP. Results must be interpreted with caution and studies should come along with experiments determining the impact of GFP expression on the sensitivity of transfected cells for molecules or genes under investigation. Nevertheless, the interest raised by GFP models to track cancerous cells is clearly warranted and may undoubtedly lead to important developments in the next few years. Among future applications, there will be a greater use of these models for the in vivo screening of novel drugs and genes of interest. With the discovery of new targets and the progress in anticancer research, these models will certainly find a place of choice for testing new pre-clinical therapeutic strategies. Another possible purpose is the study of the homing of metastatic cells tracked by virtue of their fluorescence. Investigations on the modulation of the expression of genes supposedly involved in the homing of metastatic cells will allow us to know if there is actually a preferential change of target tissue for these cells. An interesting example to study would be that of the chemokine receptors CXCR4 and CCR7, recently involved in the homing of breast cancer metastasis (98). Efforts should also be made to study the influence of the GFP-expression on the sensitivity of cancerous cells for drugs under investigation in order to extend their possible use (89). It would also be interesting to study the reaction of dormant metastatic cells after excision of the primary tumor. Besides understanding the relationship between primary tumor cells and metastatic quiescent cells, such an investigation may allow us to estimate primary tumor recurrence and to test adjuvant therapies directed against these cells. Finally, different fluorescent labels may be combined to study the expression of a gene tagged by variants of the GFP (e.g. red fluorescent protein) under the control of a specific promoter in cells constitutively expressing GFP; the use of IVM or IVVM may then allow one to determine if and when the corresponding gene is activated during the metastatic process.

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