Metastasis movies, macrophages, molecules and more

Conference on Mechanisms of Invasion and Metastasis

Denise Montell

Department of Biological Chemistry, School of Medicine, Johns Hopkins University, Baltimore, Maryland, USA

Between 18 and 20 November 2002, the conference entitled 'Mechanisms of Invasion and Metastasis' took place, organized by Richard Hynes and Joan Massagué. Twenty-three speakers and a similar number of participants gathered in the auditorium of the gleaming Centro Nacional de Investigaciones Oncológicas (CNIO) in Madrid, Spain, under the gracious hospitality of its director, Mariano Barbacid.

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Metastasis begins with the local invasion of tumour cells into the surrounding stroma, and continues with their passage through the endothelial lining and into the vasculature. This is followed by their escape from blood vessels, and their colonization and growth in distant organs. One of the themes of this meeting was that there is more than one way for tumour cells to move around, and they are often unwittingly assisted by other cell types in their quest to survive, travel and proliferate.

Tumour cells do not do it alone

Tumour-macrophage interactions. Several speakers pointed out that, in contrast to textbook models of metastasis that focus primarily on tumour cells alone, accomplices such as tumour-associated macrophages (TAMs) are important in promoting metastasis (Fig. 1). Take, for example, the 'come hither' signals that macrophages send to tumour cells, beckoning them towards blood vessels. This property was beautifully illustrated by J. Condeelis (New York, NY, USA), who described a method that he and his collaborators have developed for observing individual tumour cells as they orient themselves towards and then enter blood vessels, in real time and in a living animal (Wang *et al.*, 2002). They found that tumour cells seem to be attracted to macrophages, which line the outside of the vessels. Condeelis described a paracrine signalling loop between tumour cells and macrophages, in which tumour cells secrete macrophage colony-stimulating factor (M-CSF, also known as Csf1). This, in turn, causes macrophages to secrete epidermal growth factor (EGF), a chemoattractant for the tumour cells. Interrupting either of these signals results in decreased tumour-cell motility.

Another indication of the fatal attraction between tumour cells and TAMs was described by J. Pollard (New York, NY, USA), who studied the effects of M-CSF on tumour metastasis by using a mouse mammary tumour model. This involves the transforming polyoma middle T antigen, which is expressed under the control of the mouse mammary tumour virus (MMTV) promoter (Lin *et al.*, 2002). In otherwise wild-type mice, mammary tumours form and invariably metastasize to the lung. However, when mammary tumours are induced in a mouse that lacks the gene that codes for M-CSF, there is no change in the growth or incidence of primary tumours, but metastasis is completely eliminated. Furthermore, in this cancer model, wild-type mice had numerous TAMs, whereas the M-CSF-knockout mice had few. Pollard speculated that the myriad of growth and angiogenic

Introduction

This timely and illuminating conference revealed that the study of metastatic disease is advancing at a rapid pace. No longer simply descriptive or phenomenological, the investigation of malignancy is now a modern, molecular endeavour that takes advantage of complex genetic manipulations, sophisticated mouse models and real-time *in vivo* imaging techniques, as well as global analyses of gene expression. Such approaches are yielding mechanistic insights into this complex and crucial aspect of cancer aetiology and treatment. In turn, these are leading to the development of new therapeutic compounds, some of which are now in promising clinical trials. In this report, I highlight some of the main findings that were described and trends that emerged at the conference.

Metastasis refers to the dissemination of cancer cells from an initial tumour to distant sites. As this invasion and spreading is usually the cause of mortality in cancer patients, this area of research is

Department of Biological Chemistry, School of Medicine, Johns Hopkins University,
725 North Wolfe Street, 408 WBSB, Baltimore, Maryland 21205, USA
Tel: +1 410 614 2016; Fax: +1 410 614 9573;
E-mail: dmontell@jhmi.edu

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Fig. 1 | Tumours are complex structures that contain many cell types. The figure shows some of the main components in tumour invasion and metastasis. The mammary tumour (purple) is surrounded by a stroma that is densely populated with macrophages (green) and laced with blood vessels (red). Factors that regulate macrophage recruitment and function, as well as those that stimulate angiogenesis, help to determine the metastatic ability of the tumour. These host factors and cells provide therapeutic targets in addition to the more conventional ones in the tumour cell. (Image kindly provided by E.Y. Lin and J.W. Pollard.)

factors that TAMs secrete are probably responsible for their ability to promote metastasis. These studies suggest that therapeutic compounds that target M-CSF receptors specifically, or TAMs more generally, might be useful in cancer treatment.

Angiogenesis and combination therapy. The ability of tumours to grow beyond ~1 mm in diameter is thought to depend on their ability to attract endothelial cells and to induce them to form new blood vessels, which then supply the tumour with nutrients. This process is known as angiogenesis and has been at the centre of much excitement about new therapeutic compounds because of the theoretical advantages of targeting a normal cell type—the endothelial cell—rather than the highly mutable and evasive tumour cell. G. Bergers (San Francisco, CA, USA) described two cancer models that her group is studying to test the potential of angiogenesis inhibition *in vivo*. Vascular endothelial growth factor (VEGF) is a potent chemoattractant for endothelial cells, and antagonists of this factor have anti-angiogenic properties. In pancreatic-islet-cell carcinogenesis, treatment with a VEGF antagonist (SU5416) caused tumour regression when the inhibitor was delivered early in disease progression, but was much less effective when administered to mice at a later stage. Another drug, SU6668, which inhibits the platelet-derived growth factor (PDGF) receptor and, to a lesser extent, fibroblast growth factor (FGF) and VEGF signalling, was less effective at blocking the early stages of angiogenesis, but more effective at disrupting established blood vessels in mature tumours. Moreover, combining SU5416 and SU6668 produced synergistic effects. PDGF receptors were not present on tumour cells, but were found on the surfaces of perivascular cells, which are mesenchymal cells in close contact with blood vessels. These findings highlight the importance of pericytes in tumour angiogenesis and as therapeutic targets. They also indicate the

different drug sensitivities of newly forming blood vessels compared with established vessels, as well as the therapeutic benefit of combining several drugs.

R. Benezra (New York, NY, USA) described the contribution of Id proteins, which are negative regulators of basic helix–loop–helix transcription factors, to angiogenesis and metastasis. Mice that lack either Id1 or Id3 are viable and fertile. However, double knockouts haemorrhage and die during embryogenesis due to defective angiogenesis. Reducing the gene dosage of both Id1 and Id3 by half resulted in a reduction of the density of blood vessels in tumours that form in mice overexpressing the breast-cancer-associated oncogene *HER2/Neu*. However, some tumours still progressed due to the presence of a viable rim of cells that surround a necrotic core. Remarkably, an experimental chemotherapeutic compound that is not normally effective against *Her2/Neu*-induced tumours caused tumour regression in mice with reduced Id1 and Id3 gene dosage. This result suggests that combinations of angiogenesis inhibitors and conventional chemotherapeutics may be more effective than either treatment alone, and studies are under way to identify small-molecule inhibitors of Id proteins.

Lymphangiogenesis. Angiogenesis refers to the growth of new blood vessels, but a second vascular system, the lymphatic system, is also important in metastasis. Lymph nodes are a common site of early metastasis, and it therefore seems logical that lymphatic vessels could provide another route for the widespread dissemination of tumour cells (Stacker *et al.*, 2002). Moreover, lymphatic vessels are more permeable than blood vessels because they lack a basement membrane and tight junctions between the endothelial cells. VEGFs are known for their ability to stimulate endothelial-cell proliferation, migration and vessel formation. K. Alitalo (Helsinki, Finland) provided an analysis of the role of VEGFC and its receptor, VEGFR3, in the development of the lymphatic vasculature. After an initial role in blood-vessel formation, *VEGFR3* expression becomes limited to lymphatic endothelia during embryogenesis. Alitalo described the phenotype of *VEGFC*-knockout mice, which have defects in their lymphatic vasculature. He also described mice that overexpress VEGFC; they undergo increased lymphangiogenesis and show signs of intra-lymphatic tumour growth and lymph node metastasis in several tumour models. A soluble form of VEGFR3 blocked lymphatic metastasis of breast and lung cancer, suggesting that inhibition of lymphangiogenesis could be another suitable therapeutic target.

U. Cavallaro (Basel, Switzerland) suggested that lymphatic vessels may have a more active role in tumour metastasis than had been previously appreciated. In addition to providing a passive conduit for tumour cells, lymphangioblasts may actively invade tumours that have reduced cell–matrix adhesion, thus allowing tumour cells to passively enter newly forming lymphatic vessels, and to ‘stumble’ into the vasculature.

Breast-tumour–bone interactions. Tumour cells interact not only with the vasculature, but also with cells in the target tissues in which new colonies form. For example, breast cancers frequently metastasize to bone (Sloan & Anderson, 2002). Normal bone density is maintained by a delicate balance of the opposing activities of two types of bone cell: the bone-forming osteoblasts and the bone-degrading osteoclasts. When tumour cells take up residence in bone, they can upset the balance, resulting in either excessive bone

degradation (osteolytic metastases) or excessive bone deposition (osteogenic metastases). The phenotype depends on which resident cell type becomes hyperactivated, which in turn depends on the particular growth factors and cytokines that are produced by the tumour cells. T. Guise (Charlottesville, VA, USA) described the contribution of parathyroid-hormone-related protein (PTHrP) to the bone destruction that accompanies osteolytic breast cancer. Transforming growth factor- β (TGF- β), which is stored in the bone matrix, stimulates tumour cells to produce PTHrP (Fig. 2). PTHrP then stimulates bone resorption by altering the production of specific factors by osteoblasts. Thus, an amplifying paracrine signalling loop develops that leads to further tumour growth and bone loss.

Molecular mediators of metastasis

Met: a promiscuous protein partner. An important event in the initiation of tumour metastasis is the conversion of stationary epithelial cells into migratory and invasive cells. Most tumours form from epithelial cell types, and to escape the epithelium of origin, they must break down cell-cell junctions, breach the basal lamina, dismantle apical-basal polarity and become migratory. It has been known for more than a decade that treatment of Madin-Darby canine kidney (MDCK) epithelial cells in culture with a single molecule, known as hepatocyte growth factor (HGF) or scatter factor (SF), can cause all of these effects and more. In addition, changes in the expression level and/or sequence of Met, which is the receptor for HGF/SF, are correlated with metastatic disease. Therefore, there has been, and continues to be, much research into the function of HGF/SF, its receptor (Met) and the many other molecules that associate with Met.

When treated with HGF/SF, MDCK cells undergo an entire programme of changes that are collectively known as 'invasive growth' (Trusolino & Comoglio, 2002). The cells break apart from one another, scatter, proliferate, migrate and then form branching tubules. Met is a receptor tyrosine kinase, and on ligand binding, the enzyme is activated and the receptor becomes tyrosine phosphorylated, which creates docking sites for proteins such as Shp2 (Src homology-2-domain-containing tyrosine phosphatase), Grb2 (growth-factor-receptor-bound protein 2), phosphatidylinositol-3-OH kinase (PI(3)K) and Gab1 (Grb2-associated binder 1). Among these effectors, Gab1 seems to be important, as W. Birchmeier (Berlin, Germany) presented results indicating that mice lacking Gab1 have many of the same abnormalities as mice lacking HGF/SF or Met.

It turns out that Met interacts with a diverse array of partner proteins, some of which contain domains of homology with Met itself. For example, P. Comoglio (Torino, Italy) reported that semaphorins and their plexin receptors, which both contain such homologous domains, form a ternary complex with Met. Met seems to function as a co-receptor for semaphorin, binding to plexin after the semaphorin does so. Moreover, semaphorins alone can cause scattering of MDCK cells, suggesting that Met can transmit semaphorin signals independently of HGF. Met also forms a constitutive complex with $\alpha 6 \beta 4$ -integrin receptors, and signalling through Met requires $\alpha 6 \beta 4$ -integrin, but does not require the presence of the usual integrin ligands, such as laminin or fibronectin. Comoglio suggested that Met receptors are able to produce cell-type-specific responses to semaphorins and HGF by combinatorial signalling through different partner proteins.

P. Herrlich (Karlsruhe, Germany) described yet another Met partner, the CD44 molecule. CD44 is a transmembrane protein that is subject to extensive alternative splicing. One particular

Fig. 2 | Tumour-bone paracrine interactions. The interactions between tumour cells (pink nuclei) and bone are shown here. Left-hand side, an osteolytic interaction in which parathyroid-hormone-related protein (PTHrP), which is produced by tumour cells, stimulates the activity of bone-eating osteoclasts (multinucleate cell). Osteoclasts in turn produce transforming growth factor- β (TGF- β), which stimulates the tumour cells. Right-hand side, osteoblastic interactions. Tumour cells produce many factors, including endothelin 1 (ET1), PTHrP and platelet-derived growth factor (PDGF)-BB, which stimulate the activity of bone-producing osteoblasts (blue nuclei). Osteoblasts in turn produce factors that stimulate the growth of the tumour cells. (Adapted from a figure by T. Guise.)

splice variant, v6, associates with Met, and the resultant heteromultimer forms a complex with HGF/SF. Antibodies against the v6 isoform of CD44 interfere with Met signalling, as does the expression of a form of v6 that lacks its cytoplasmic tail. It remains to be determined just how many partners Met has and what combinations of signalling complexes exist in a single cell, as well as the range of combinations between different cells. As the list of interacting proteins increases, it is becoming clear how a single molecule can have such a panoply of effects.

A sticky problem. As mentioned above, tumour cells that exit an epithelium must downregulate cell-cell adhesion. One prominent epithelial cell-cell adhesion molecule is E-cadherin, which can be downregulated in tumours in various ways. In some tumours, the gene that encodes E-cadherin is mutated, whereas in others, E-cadherin messenger RNA expression is reduced. In another part of his talk, Birchmeier described a post-transcriptional mechanism for eliminating E-cadherin from cells. A protein called Hakai interacts with both Met and E-cadherin. Homologous to E3 ubiquitin ligases, Hakai stimulates ubiquitylation and endocytosis of tyrosine-phosphorylated E-cadherin, and thereby leads to its removal from the cell surface.

There are many ways in which cell adhesion molecules might contribute to metastasis or protect against it. For example, leukocytes are known to use adhesion molecules such as selectins and selectin ligands, as well as integrins and integrin ligands, to adhere to and extravasate between the endothelial cells that line blood vessels. Tumour cells might use the same molecules for extravasation during metastasis, and thus selectins and integrins may promote metastasis. Alternatively, such molecules might hinder metastasis by facilitating the transit of leukocytes that attack and destroy tumour cells.

One definitive way to tease apart the various contributions of specific adhesion molecules to tumour growth or metastasis is to study mice in which a specific molecule has been removed genetically from a particular tissue. Although the genetic 'gymnastics' that are required to carry out such studies are difficult, several speakers reported elegant experiments of this nature. For example, R. Hynes (Boston, MA, USA) discussed the contribution of selectins and α v-integrins to tumour growth and metastasis, on the basis of studies using human cancer cells introduced into mouse knockouts. Single-gene mouse knockouts of either P-selectin or L-selectin, but not of E-selectin, impaired the metastasis of human cancer cells after their injection into the mouse tail vein. The decrease in metastasis was only observed for tumour cells that express selectin ligands, indicating that it is a direct interaction between these molecules and endothelial cell selectins that promotes metastasis. These results suggest that small-molecule inhibitors of selectins may prevent metastasis.

In a second assay, human tumour cells were injected subcutaneously into wild-type mice or mice that lack selectins. Surprisingly, much larger tumours formed in the selectin-knockout mice, although little metastasis was observed. Bone marrow transplantations indicated that selectins normally function in bone-marrow-derived cells, not in endothelial cells, to suppress tumour growth. Thus, selectins suppress the growth of primary tumour cells, but enhance the metastasis of tumours that express selectin ligands.

W. Muller (Hamilton, Canada) presented compelling evidence of a requirement for β 1-integrin in middle-T-antigen-mediated transformation. He discussed results from studies in mice in which middle T antigen was overexpressed in the mammary gland at the same time that β 1-integrin function was specifically lost from this tissue. This was achieved by breeding mice that, first, express the middle T antigen under the control of the MMTV promoter; second, carry a β 1-integrin gene flanked by *loxP* DNA sequences; and third, express Cre recombinase under the control of the MMTV promoter. In such mice, Cre recombinase is expressed specifically in mammary tissue, where it causes excision of the β 1-integrin gene, and thus elegantly circumvents the problem that complete loss of β 1-integrin is lethal to the organism. The resulting mice showed a 50% reduction in tumour mass compared with mice that overexpress middle T antigen in the mammary gland but that have normal β 1-integrin gene function. Muller speculated that the residual tumours that formed may have been due to incomplete excision of the β 1-integrin gene. These studies show the level of sophistication that cancer genetic techniques have reached in the mouse.

The TGF- β tug of war. Another molecule that figured prominently at the meeting was TGF- β . It has become clear that TGF- β has dual and opposing roles in cancer. On the one hand, it has inhibitory effects on the growth of epithelial cells and therefore functions as a tumour suppressor. On the other hand, once tumour cells escape these inhibitory effects on growth, the same signalling pathway can activate invasion and metastasis.

Endoglin is a transmembrane protein that forms a complex with the TGF- β receptor and has been suggested to be a receptor subunit. M. Quintanilla (Madrid, Spain) presented evidence that endoglin is actually a negative regulator of TGF- β signalling. Mice that are heterozygous for a loss-of-function allele of endoglin were more resistant than control mice to the induction of benign skin papillomas, but showed a higher frequency of conversion to malignancy. These

phenotypes bear a close resemblance to those seen in mice that overexpress TGF- β specifically in the skin.

Mining for motility molecules using microarrays. Although the study of cell motility has generally focused more on events that take place at the cell membrane than those in the nucleus, it is clear that transcriptional regulation is important for the cellular response to secreted growth factors such as HGF/SF and TGF- β . Therefore, the use of DNA microarrays to compare the gene-expression profiles of cells in the absence or presence of growth factors should shed light on the patterns of transcription that are associated with cell motility, invasiveness and metastasis. Various purification strategies to obtain populations of motile or invasive cells were presented by several investigators, and their patterns of gene expression were compared with those of a non-invasive control population. For example, Comoglio described the genes induced by HGF; Condeelis collected motile tumour cells from mice and compared their gene expression with that in cells of the primary tumour.

J. Massagué reported another method for enriching populations of invasive and metastatic cells *in vivo*. He injected human breast-cancer cells into the left ventricles of mice, and at a low frequency, the tumours metastasized to bone. Metastatic cells that were collected from the bone, expanded *in vitro*, and re-injected into mice showed a much higher frequency of bone metastasis than was otherwise observed. Iterative rounds of purifying highly metastatic cells were carried out, and gene-expression profiling was then used on this population and compared with the starting population. Several genes that encode secreted growth factors such as osteopontin, interleukin 11 (IL-11), connective tissue growth factor (CTGF) and FGF5 were highly expressed in the metastatic cells compared with the parental population. CTGF and IL-11 are activated in response to TGF- β signalling, suggesting a mechanism for the known ability of TGF- β to stimulate the formation of bone metastases in this model system. When expressed in the parental cell line, no single gene was sufficient to increase its metastatic potential; instead, combinations of 2 or 3 genes were necessary.

The embryological origins of invasive cell behaviour

Met mediates migration in mouse myocytes. The molecules that promote invasion and metastasis do not exist solely to aid and abet cancer cells. These molecules have useful purposes as well, especially during embryonic development when cell invasion is a necessary part of the process. Therefore, investigating normal cell migration during embryonic development contributes to our overall understanding of cell motility and may also lead to a better understanding of tumour cell behaviour. C. Birchmeier (Berlin, Germany) described her laboratory's investigations of the phenotypes of mice lacking various proteins that promote embryonic cell migration. *Met*- and *HGF/SF*-knockout mice, for example, have a marked reduction in the number of myoblast cells, and therefore of differentiated muscles, in the limb. This observation was pivotal in showing that limb muscles actually develop from precursor cells in the somite, which migrate into the limb bud. A similar reduction in limb musculature was observed when the gene that encodes the Met adaptor protein Gab1 was knocked out in the mouse. However, the *Gab1*^{-/-} phenotype was not as strong as the *Met*^{-/-} phenotype, suggesting that additional downstream signalling molecules are able to promote some degree of myoblast migration, even in the absence of Gab1.

Drosophila as a model system. One of the technical advantages of studying embryonic development is the ability to carry out forward genetic screens to identify genes that are required for invasive cell behaviour. One of the organisms best suited to this type of approach is the fruitfly *Drosophila melanogaster*. D. Montell (Baltimore, MD, USA) described a population of cells, referred to as border cells, in the *Drosophila* ovary, which are initially epithelial but, as part of their normal development, separate from the epithelium and invade the neighbouring cluster of germline cells, known as the nurse cells. Three signalling pathways control this migration. A steroid hormone regulates the timing of cell migration, a paracrine cytokine signal that activates the JAK/STAT pathway dictates which cells migrate, and a growth factor related to PDGF and VEGF guides the cells to the oocyte. All of these pathways are known to be upregulated in human cancer and, thus, identifying new molecules that are required for border-cell migration may lead to a better understanding of invasive behaviour generally, and of tumour metastasis specifically. Montell found that the pointed-end-directed actin motor, myosin VI, is essential for normal border-cell migration. She went on to discuss a study of myosin VI expression in human ovarian cancer, showing a strong correlation between the aggressiveness of ovarian cancers and the expression level of myosin VI. On the basis of this, she suggested that myosin VI may contribute to the metastasis of ovarian cancer cells.

Future directions

The findings presented at this conference point to paracrine signalling between tumour-cell and host-cell populations as a crucial feature of cancer, and this could prove to be the disease's 'Achilles' heel'. It is equally clear that the specific molecular mediators of these paracrine signals are different for each type of tumour cell and

micro-environment. It is necessary to develop drugs against each of the signalling pathways that promote tumour growth, survival and motility. The future of cancer treatment may lie in pinpointing which growth factors are secreted by tumour cells, as well as which growth-factor and cell-adhesion receptors are abundantly expressed on tumour cell surfaces. Such knowledge might then allow oncologists to devise tailor-made combinations of chemotherapeutics that would optimally antagonize the tumour cells, TAMs and other cellular accomplices, while activating beneficial components of the immune system. Considering that it is unlikely that a single combination of drugs will be optimal for all patients, we should take advantage of the many possible synergistic effects that can be generated when combinations of drugs are used. Although the finishing line is not quite in sight, there is hope that the dissemination of new knowledge at meetings such as this will lead to the discovery of therapeutic approaches that will outpace the spread of disease.

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