A STRATEGY FOR THE *IN VITRO* ANALYSIS OF THE METASTATIC PROCESS

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The process of metastasis can be broken down into ten separable components. Some of these components can be studied profitably *in vitro*. A series of questions is posed of each component in the process of metastasis, yielding some simple predictions about the likely course of future work, and the possible role of tumor viruses.

Metastasis is complex, but not impossibly so. *In vitro* cell culture has been successfully used in the past to resolve highly complex biological processes. I have been asked to summarize recent developments of *in vitro* cell culture in relationship to the process of metastasis. To avoid being redundant in the presence of this distinguished group, I have chosen to retreat from recent developments to first principles. That is, I propose to analyze metastasis as a process of many steps, and to discuss how best to transfer each step to *in vitro* conditions (Table I).

As a tool, *in vitro* cell culture depends on the persistence outside the whole animal of those pieces of physiological normality or abnormality which are under study. In order to relate the steps in metastasis to *in vitro* cell culture, I have arranged a series of five consecutive questions (Table II). When put to each step in the process of metastasis, these questions serve to reveal in an orderly way, the steps that are ripest for *in vitro* study (Table III).

The Strategy

Let us consider the strategy outlined in Table II. First, we must note the minimum number of cell types necessary to reconstruct each event *in vitro*. Obviously, the smaller this number the better, and work will be easiest if it is 1. Second, we want to know if the event involves proliferation of at least one cell type. Here, too, we hope for a positive answer for as few cell types as possible, for if the answer is yes, then it is easier to ask question 3, the nature of restrictive culture conditions in which only the cell that has undergone the event in question will proliferate. If no proliferation occurs in a given step, selection will have to depend on a cell surviving and proceeding to the next proliferative step.

Given a selective system, we can then ask question 4: Is the event due to a mutation? Either answer to this question is interesting. If it is a mutagenic event, we can hope to find the mutated gene and, thereby, its product (*48*). If the event does not require a change in the base sequence of any cell, then we can hope to reverse it without

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TABLE I. Separate Steps in Development of a Metastatic Tumor

Step No. and event	Location	Result and precursor
1 Transformation	Tissue A	Microtumor in A
2 Angiogenesis	Tissue A	Tumor in A
3 Detachment	Tissue A	(a) Single tumor cell in A or
		(b) Clump of tumor cells in A or
		(c) Clump containing normal and tumor cells in A
4 Penetration	(a) Capillary or vein of A	(a), (b), or (c) in vasculature
	(b) Lymphatics distal to A	(a), (b), or (c) in lymph node, thence to general circulation
5 Travel	General circulation	Embolism consisting of (a), (b), or (c) above.
6 Thrombosis	General circulation	Embolism consisting of (a), (b), or (c) above with platelets and fibrin.
7 Trapping	Capillary in tissue B	Embolism in tissue B
8 Penetration	Tissue B	(a), (b), or (c) in tissue B
9 Growth	Tissue B	Micrometastasis in B
10 Angiogenesis	Tissue B	Metastatic tumor in B

TABLE II. An *in Vitro* Strategy for Analysis of Each Step in the Process of Metastasis

(a) Minimum number of cell types?
(b) Proliferation?
(c) Selection systems?
(d) Genetic change? If (d) is yes
(e) Role for virus or mutagen?

TABLE III. Available Systems for the Study of Metastasis: The Strategy of Table II Applied to the Steps of Table I

Step No.	Answer to questions				
	a	b	c	d	e
1	1	Yes	Yes	Yes	Yes
2	⩾2	Yes	Yes	?	?
3	⩾1	?	Yes	?	?
4	⩾2	?	Yes	?	?
5	?	?	?	?	?
6	?	?	?	?	?
7	⩾2	?	?	?	?
8	⩾1	?	?	?	?
9	1	Yes	Yes	?	?
10	⩾2	Yes	Yes	?	?

the need for mutation (*51*). Finally, if the answer to question 4 is yes, then we can ask the fifth and last question in our plan: Can a tumor virus gene fill the mutational requirement?

Analysis of the Process of Metastasis

*1) Transformation (tissue A)**

This is the initial event in the process of metastasis. It has been extensively studied *in vitro* for at least 25 years. Clearly it can occur in a single cell. The consequence of passage through this step is proliferation of the transformed cell and its descendants. At least three different selective assays are available in which transformed cells but not untransformed cells grow (*46*) (Table IV). Recent work has led to the suggestion that growth in the most restricted *in vitro* selective assay, growth without anchorage (Table V), is also most well correlated with tumorigenic transplantability *in vivo* (*50*).

There is a constellation or syndrome of nonselective *in vitro* cellular alterations specifically correlated with this selective assay and thereby with simple tumorigenicity. The syndrome includes in addition to growth without anchorage, a change in the internal distribution of actin, and increased secretion of an activator of the serum protease plasmin (Table V). The binary relationships among the parts of this syndrome are currently under study (Table I).

TABLE IV. Step 1 of Metastasis Is Itself a Hierarchy of Changes in Growth Control

Selective system	Growth of cell type			
		Transformant		
	Normal →	Minimal →	Intermediate →	Full
Low serum	−	+	+	+
High density	−	−	+	+
No anchorage	−	−	−	+
Reference	REF (*37*), 3T3 (*36*)	F1SV (*36*) SVR 63 (*45*)	SVRE 12 (*37*) SVRE 17 (*37*)	SV101 3T3 (*36*) SVRE8 (*37*) SVRE9 (*37*)

TABLE V. *In Vitro* Correlates of Tumorigenesis

A. Phenotypic syndrome of full transformation (Step 1) *in vitro*

1) Growth without anchorage
2) Loss of internal actin-containing cables
3) Production of plasminogen activator
4) Tumorigenicity

B. Binary linkages within syndrome

	Reference
Anchorage-actin	*38*, *40*
Anchorage-plasminogen activator	*37*, *44*
Anchorage-tumorigenicity	*50*
Actin-plasminogen activator	*39*, *40*
Actin-tumorigenicity	*50*
Plasminogen activator-tumorigenicity	*8*, *26*, *33*

* Tissue A is the primary, and tissue B the metastatic site of the tumor. See Table I.

Proliferation is limited by the availability of nutrients and the absence of capillary penetration in all three selective *in vitro* assays for transformation. Hence, there is no reason to expect any part of this syndrome of transformation to correlate with any steps in Table I beyond the first.

Transformation can be the result of a genetic change in a single cell, since carcinogens are mutagens (*29*, *30*) and cells in the transformed state may genetically revert (*2*, *9*, *31*, *34*, *57*, *58*). At least for some tumor viruses, the genetic alteration in transformation is known. It is the insertion of a functioning viral gene into the cellular genome. This has been shown unambiguously for the papova virus SV40. Here, conditional lethal virus mutants yield conditional (temperature sensitive) transformed cells (*22*, *47*, *55*, *60*).

2) Angiogenesis (tissue A)

At least two cell types are required for this event, the completely transformed cell from step 1 and the host capillary endothelial cell. The tumor cell secretes a compound of unknown structure, tumor angiogenesis factor (TAF), which, as Folkman and others have shown, causes capillary endothelial cells nearby to proliferate so that capillary growth is directed toward the microtumor (*16*). This is followed by continued vascularization of the microtumor, providing an inexhaustible supply of nutrients. Selective systems *in vitro* would be of two types: For TAF-secreting tumor cells and for TAF-requiring endothelial cells. Neither system has been reported, but there is no difficulty in principle, since both transformed cells (from step 1) and capillary mural (*7*) and endothelial cells (*18*) grow well in culture. Indeed the entire normal microvasculature has now been beautifully described at the electron microscope level (*52*).

Questions that should be asked with such systems include: How are TAF secretion and synthesis regulated? How can they be blocked (*4*, *13*)? Do endothelial cells ever transform genetically in response to TAF, gaining the hereditable ability to proliferate in the absence of the compound? Is tumor-secreted TAF tumor-specific, or is it identical to the factor(s) necessary for maintenance of capillary density in normal tissues?

3) Detachment (tissue A)

At its simplest, this step would be the result of a change in adhesion by a single cell (*12*, *20*). However, if the tumor cell detaches from other cells, the event *in vitro* would require mixed cultures. Current *in vitro* systems can select for cells that have reduced adhesion to each other or to a solid substrate (*6*). Among the physiologically significant agents known to affect both adhesion and motility is the serum protease, plasmin. This raises the possibility of combining some of the selective assays of step 1 with the cell populations of step 2 in order to study step 3.

Two critical unknowns exist in this step. First, we do not know the number and possible heterogeneity of the detached cells (Table I). Understanding of further steps will depend on knowing whether the precursor of those steps is (a) a single tumor cell, (b) more than one tumor cell, or (c) a clump containing at least one tumor cell plus any number of host cells of different types (endothelial, stromal, epithelial, circulatory). Second, we do not know whether detached cells proliferate before the next step. Obviously the two questions are linked, since immediate proliferation of a single detached cell will yield a clump as precursor to subsequent steps.

4) Penetration (tissue A)

The possible participating cell types here are many: The endothelial and mural cells of the capillary, the viable cells of the blood, the cells of the lymph ducts and nodes, all may join the detached cells in expediting penetration of blood vessel walls by at least one tumor cell from tissue A. Available *in vitro* selective assays include those dependent upon ease of deformation of cell shape (*17*, *49*), the ability of cell surfaces to support active cell movement (*11*), and ease of separation of endothelial intercellular connections.

If the tumor cell activator of plasminogen is membrane-bound (*41*), it would be profitable to examine the ability of this protease, among others, to affect normal intercellular bonds (*20*). Indeed, the possibility must be considered that capillaries responding to TAF are thereby rendered more easily penetrable.

5) Travel

The same uncertainty about cell types exists for this step as for the previous one, as does the uncertainty about proliferation. It should be possible to determine the extents of proliferation and clumping by the cells entering from step 4. Almost all animal experimental studies of metastasis join the main sequence of events here, since they involve injection of suspensions of tumor cells into the general circulation (*14*, *15*). Fidler and Nicolson (this volume) have in fact shown that formation of clumps by suspended tumor cells after injection into the main circulation is a critical precursor to the remaining steps leading to metastatic growth.

6) Thrombosis

The surviving cells of step 5, together with platelets, fibrinogen, and thrombin, must participate. The requirement for other cells in the thrombus has not been demonstrated. Major unknowns here include (a) the half-life of a thrombus, (b) the optimum thrombus size for a precursor of later steps, and (c) whether any proliferation of normal or tumor cells occurs in this step (*28*, *59*).

No selective systems exist for the cell types involved in prothrombin activation. In a sense such selective systems would be the mirror image of the ones dependent upon plasmin, since thrombosis would seem to be blocked by sufficient activity of tumor-specific plasminogen activator (*56*). That is, the optimal level of tumor-associated plasminogen for metastasis may be less than the optimal level for tumor growth.

7) Trapping (tissue B)

Trapping is the first observed event in most animal studies (*3*, *21*, *32*). It is not surprising therefore that the ability of injected suspensions to form clumps correlates with success in this step, since clumping would appear to model the events up to and including thrombosis.

Trapping involves interaction between the vascular endothelium of the target tissue and the thrombus. While *in vitro* cell-cell adhesion between tumor cells and epithelial cells is easier to study in the absence of platelets and fibrin, it may be necessary to include serological cofactors in studies of this step.

8) Penetration (tissue B)

In direction, this is the reverse of step 4, but there is no reason to expect a completely different mechanism. Again the operative cells must include at least one tumor cell, the capillary cell types, and whatever host cells in the thrombus are viable and motile (*10, 23*). Here plasmin would seem to be once again a boon, loosening the thrombus and freeing the tumor cells. To the extent that this is so, techniques assaying fibrinolysis by single cells are available for selective systems (*1 ,24*). However, for this step, as for the previous two, we know very little about proliferation and the necessity for genetic change.

9) Growth (tissue B)

For the first time since step 2, we have a hope of easy access to *in vitro* conditions. The minimal number of cell types required here can be as low as 1: The single tumor cell that passes through step 8. Therefore, the first experiments to understand this step should be ones to determine whether or not the cell that can accomplish it is genetically identical to the cell that passes through step 1. Putative cells for selection can be obtained from host tissues after injection of metastatic cells. Proliferation is certain, so that the *in vitro* selective systems of Table IV, among others, are immediately available to purify away extraneous host cells.

10) Angiogenesis (tissue B)

This step is formally equivalent to step 2, providing that the capillary cells of tissues A and B are able to proliferate equally well in response to TAF's secreted by the tumor. A drug may be available to distinguish between steps 2 and 10. ICRF-159 is a drug that inhibits pulmonary metastases resulting from blood-borne dissemination of tumor cells, without affecting the growth rate of the primary implant. Apparently this drug alters the morphology of tumor-specific blood vessels (*27*).

General Observations

The process of metastasis laid out in this fashion shows an unexpected symmetry (Table III). The events in steps 1 and 9, and possibly 3 and 8, have the best chance of being studied *in vitro* with pure cultures derived from a single cell. Steps 2, 7, and 10 appear to require the participation of two or more different cell types, and the heterogeneity of cell types needed to study steps 5 and 6 remains to be determined. Thus, the end and the beginning of the process seem similar to each other and easier to study *in vitro* than the middle (Table III). However, the very symmetry of the overall process masks certain significant temporal asymmetries. For instance, a cell in later steps need not proliferate until it first interacts with endothelial cells, while a cell in steps 1 and 2 must first grow in isolation. Thus, selections that require growth in pure culture may not recover the tumor cell type participating in later steps, and mixed cultures may be essential for all late steps including 9.

Proliferations need occur only in steps 1, 2, 9, and 10. We have no clear data for or against proliferation in any of the other steps. We might ask whether any of these other steps are specifically sensitive to antineoplastic drugs. By the same argument, we might ask *in vitro* if the nonproliferative steps are specifically sensitive to drugs

such as cytochalasin B and colchicine, which disrupt cell shape without killing (*5*).

We must remain fluid in our choice of priorities for *in vitro* cultures. For instance, of all cell types, it is the vascular endothelium that is most intimately associated with the process of metastasis, being an obligatory participant in steps 2, 4, 5, 7, 8, and 10.

Work on the *in vitro* cultivation of cells from normal vascular endothelium and of the smooth muscle, and mural cells of the vasculature may at first seem more related to studies of atherosclerosis or diabetes than of malignancy, but this is naive. Work on cultivation of normal cell types (*18*, *19*, *42*, *43*) should be of interest to all who study the process of metastasis. Similarly, cell biologists interested in the maintenance of cell shape *in vitro* and the mechanism of change of shape provide critical information for steps 4 and 8. A last example: Clot formation and dissolution are essential parts of steps 6, 7, and 8, and at least one enzyme of the clotting system, plasmin, is specifically activated by many primary tumors and their metastases. Thus, *in vitro* culture of cells participating in the clotting system must also come within our purview.

By assuming that all genetic events occur in the tumor cell or its descendants, we overlook an exciting possibility. All steps that involve host cells, that is, all steps but 1 and 9, may in principle be enhanced or accomplished as a result of a host, rather than a tumor, cell mutation. Some recent observations fit this exciting class of genetic events. Normal skin biopsy fibroblasts from osteosarcoma patients are able to form tumors in antilymphocytic serum (ALS)-treated mice (*53*, *54*). Normal skin biopsy fibroblasts from patients with adenomatosis of the colon and rectum (ACR), an autosomal dominant disease, are able to grow in low serum, but not without anchorage (*25*, *34*, *35*). This suggests two novel approaches for future work on metastases *in vitro*: (a) A search for abnormalities in the "normal" vascular endothelium and other tissues of patients with metastases; (b) a search for viruses affecting the growth control of these "normal" cell types.

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