CHAPTER

7 Growth and Spread of Cancer

The present chapter covers basic concepts on the growth and spread of cancer which are considered the two cardinal features that characterize malignancy. Growth kinetics are discussed both at cellular level, as well as, at cell population level, with a detailed account on the parameters of measurement. The different pathways of spread of cancer are outlined, but metastasis, in view of its vital importance, is presented in more details.

THE CELL CYCLE

Phases of the cell cycle

The cell cycle is defined as the period that extends from one mitosis to the next mitosis and is divided into 4 phases, namely: Gl (gap l), S, G2 (gap 2) and M. The cell cycle includes two functional phases and two preparatory phases. The functional phases are: (a) the precise copying of DNA, known as S phase or DNA replication; and (b) the accurate segregation of duplicated sets of chromosomes between daughter cells in M phase (Fig7-1). The cell prepares itself biochemically for S phase in a preparatory phase known as Gl and prepares for mitosis in G2 phase. Cells that are not actually dividing may be either permanently removed from the cell cycle by terminal differentiation and apoptosis, or be temporarily arrested in a non-cycling quiescent state known as G0 (Fig7-2). The tumor volume is determined by the balance between the cycling and the non-cycling cell populations.

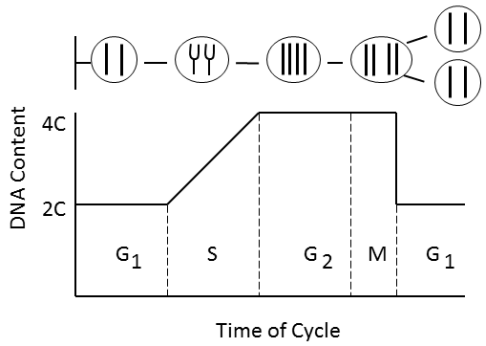

Fig 7-1 *Change in DNA* ***content*** *in relation to phases of cell cycle and mitosis*

The cell cycle contains two checkpoints or restriction points Rl and R2 which are located by the end of Gl and G2 respectively, in addition to the spindle check-point (Fig 7-2). Check points are pauses in the cell cycle during which the fidelity of DNA duplication and accuracy of chromosomal segregation are monitored. These cell cycle delays permit editing and repair of genetic information.

The normal cell cycle time (Tc) of humans has a wide range with a median value of 24 hours. The duration of the S-phase is about 10 hours, and G2 about 5 hours. Mitosis is the shortest phase with a duration of approximately one hour.

Regulation of cell cycle

The principal regulators of the cell cycle are two families of proteins called the cyclins and the cyclin-dependent kinases (cdks). Cyclin is a regulatory protein subunit, whereas cdk is an enzyme or a catalytic unit. Cyclin binds and activates the kinase which is then able to phosphorylate other protein substrates. Eventually, a protease (ubiquitin system) degrades the cyclin and hence the kinase is rendered inactive. The level of cdk does not change during the cell cycle, but the activity of the enzyme fluctuates following the variation in cyclin level.

So far, five main classes of human cyclins are identified (A to E) which act at specific phases of cycle. Gl cyclins (D and E) together with their corresponding cdks drive the cell during G1 phase and G1-S transition respectively. Cyclin A drive the cell during S phase. G2-M cyclins (A and B) together with cdk-1 drive the cell during mitosis (Fig7-2). The kinase cdk-1 (previously known as cdc-2) was first discovered as a component of the maturation promoting factor (MPF) which accumulates in the cell during mitosis. Eventually, it was characterized as a complex of cyclin B/cdk-1, a major regulator of G2-M transition. Logical candidates as substrates for cdk-1 would be nuclear lamins, chromosome proteins, microtubule cycloskeleton and actin attachment proteins. Conversely, logical candidates for cyclin E/cdk-2 would be the enzymes required for DNA synthesis and the enzymes needed for the production of DNA precursors.

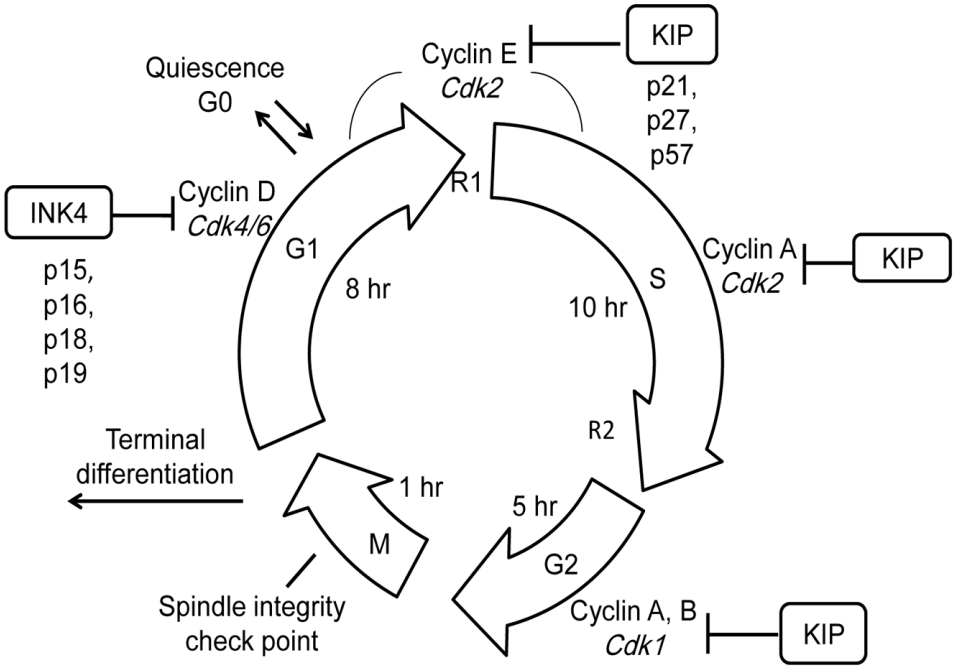

Fig 7-2 *Phases of normal cell cycle and their regulation by cyclins and cyclin-dependent kinases. Also shown are cell cycle check points (R1, R2 and spindle integrity). Cells generated from cell cycle are either terminally differentiated cells or reversible quiescent cells in G0 phase.*

Positive Regulation

Progression through the cell cycle is under the control of various positive and negative growth signals (Fig 7-3). When mitogenic signals (e.g. EGF, IGF, PDGF) stimulate a cell to enter the cycle from quiescence, the expression of cyclin D is stimulated. This regulation of cyclin D by extracellular signals is unique and is not observed with other cyclins. The activation of RAS (either by EGF or mutation) also results in activation of cyclin D/cdk-4 or 6 complex. The latter then phosphorylates retinoblastoma protein (pRb) with loss of its growth-suppressive function and release of bound E2F. The released E2F transcription factor is thus free to activate the transcription of genes whose products are required in the S phase. Gene products that appear to be activated by E2F include: cyclin E and A, PCNA, DNA polymerase and thymidine kinase.

Expression of c-myc is another important positive growth signal. In normal mammalian cells, expression of the c-myc gene is strictly dependent on mitogenic stimuli and recipro-

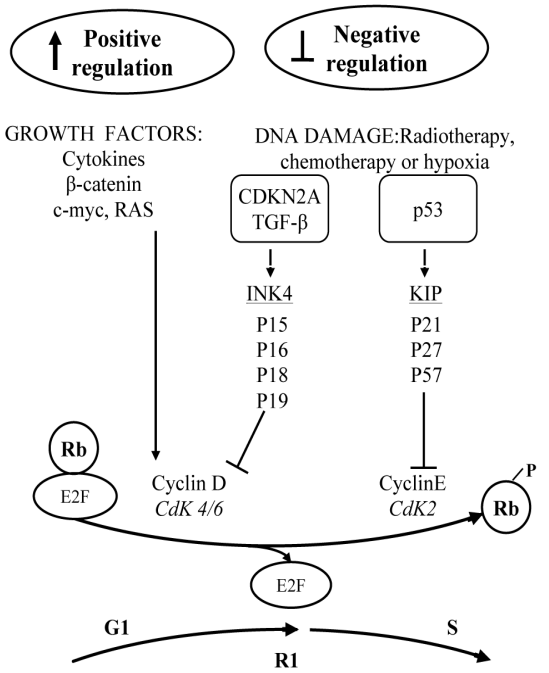

Fig 7-3 *Regulation of cell cycle through control of G1-S transition. Cyclin/cdk-Retinoblastoma pathway is a key regulatory mechanism, but , p53, INK4 and KIP proteins are the main inhibitors of cell cycle.*

cally, is suppressed by growth inhibitory signals. Oncogenic activation of c-myc occurs through genetic alterations, such as, gene amplification or translocation. It was recently verified that c-myc prevents cell cycle arrest by interfering with the growth inhibitory function of p27. Viral oncoproteins (as HPV E6 and E7) inhibits p53 and Rb proteins respectively with activation of cell cycle.

Negative Regulation

Cyclin/cdk complexes are inactivated by several inhibitory proteins collectively termed cyclin kinase inhibitors (CKIs), resulting in arrest of cell cycle (Fig7.3). There are two classes of CKIs: the KIP family including p21, p27 and p57, and the INK4 family, including the tumor suppressor p16 and three closely related proteins pl5, pl8 and pl9. The KIP family inhibits cyclin E/cdk 2, whereas, INK4 family inhibits cyclin D/cdk4-6. Cyclin kinase inhibitors mediate cell cycle arrest in response to a variety of growth inhibitory stimuli such as TGF beta, mitogen withdrawal or contact inhibition.

The tumor suppressor gene p53 is a potent negative regulator of the cell cycle through p53-p21-pRb pathway. Thus wild p53 after radiation damage of DNA, activates WAF-1 gene to produce p21 which inhibits cyclin E/cdk-2 or 6 phosphorylating activity. As a result pRb is hypophosphorylated with binding of E2F and arrest of cell cycle in Gl-S transition.

CELL POPULATION STRUCTURE OF CANCER

A fundamental problem in cancer research is the identification of the cell type which initiates and sustains the growth of the tumor. Two theories were proposed (Reya, 2001, Wang and Dick, 2008), namely: (1) the "*stochastic model*" suggests that every cell in the tumor is a potential cancer initiator and this behavior develops at random and cannot be predicted, and (2) the *hierarchy model* postulates the existence of a functionally distinct subset of tumor cells (cancer stem cells, CSC) which have the ability to initiate and sustain tumor growth. Cancer stem cells are capable of self-renewal, produce differentiated cells, resist apoptosis (immortal) and may enter a reversible phase of quiescence (G0) as a protective mechanism. CSC also require a supportive microenvironment (niche) to grow. The CSC theory is at present generally accepted. It explains many biologic phenomena (cancer initiation, progression, dormancy), as well as, may help in future development of more effective cancer therapy (Kakarala, 2008).

According to the cancer stem-cell model, the cell population of malignant tumor includes the following 2 main compartments: proliferating (cycling) and nonproliferating (non-cycling cells). The latter includes differentiated cells, dead cells and quiescent cells in G0 phase (Fig 7-4). The proportion of cycling stem cell or growth fraction (GF) is measured by the Ki-67 index using im-

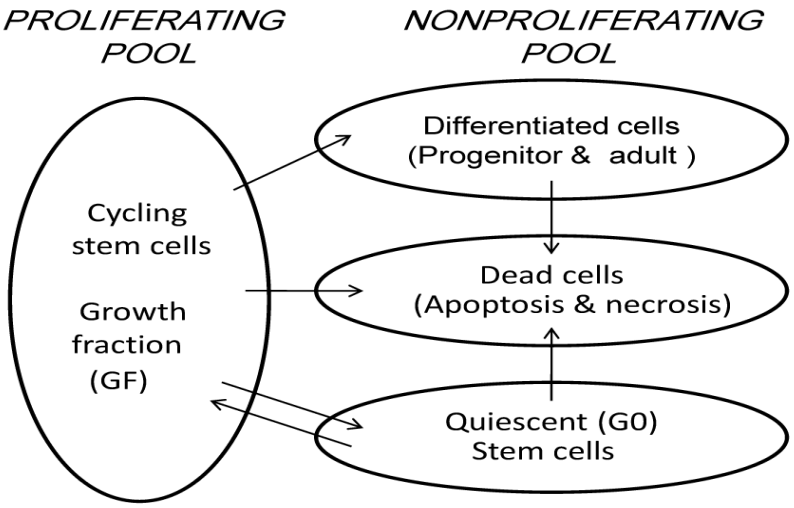

Fig 7-4 *The four cell population compartments of a malignant tumor. The growth fraction is the main factor that affect tumor growth and response to chemotherapy.*

munohistochemical or flow cytometry methods. The KI index varies considerably from <2% in slowly-growing benign tumors, 2-40% for most malignant tumors, to almost 100% in rapidly-growing tumors such as Burkitt lymphoma.

The cell loss compartment in cancer cell population includes cells with the two different modes of cell death, namely: apoptosis and necrosis. At present, the method of choice to quantitate both apoptotic and necrotic cell populations is flow cytometry after labeling with fluorescein conjugated annexin V (AV) and phosphatidyl serine (PS). Viable cells are negative for both (AV) and (PS), apoptotic cells are (AV+) and (PS-) and necrotic cells are (AV-) and (PS+). Another flow method to quantitate apoptosis is the TUNEL test (TDT-mediated duTP nick-end labeling) by adding a fluorescein-labeled nucleotide deoxyuridine triphosphate to the nick in the DNA strand.

PARAMETERS OF TUMOR GROWTH

The tumor growth rate which is clinically observed is the net outcome of two opposing processes namely: cell production and cell loss. The rate of cell production depends mainly on the proportion of proliferating cells in a tumor and to a lesser extent on their cell cycle time. Cell proliferation is an important parameter to predict response to therapy and prognosis of cancer.

The following is a discussion of the main seven growth parameters of tumors.

Cell cycle time (Tc): The cell cycle time (Tc) of human tumors has a wide range with an average of about 2 days (double the time of normal cells). The cell cycle time is an unreliable parameter of proliferation of human tumors in view of the wide variations among cells in the same tumor, and hence, its estimation has little clinical relevance. It is determined by the percent labeled mitosis method in which a radioactive labeled nucleic acid precursor (thymidine) is injected into the patient and serial biopsies of the tumor are taken at different intervals to determine the fraction of cells with labeled mitoses by autoradiography.

S-phase fraction (SPF): This refers to the proportion of tumor cells in the S-phase. It can be measured using different techniques including thymidine labeling index (TLI), flow cytometry, Bromodeoxyuridine (BrdUrd) or lododeoxyuridine (IdUrd) labeling, expression of histones 3 and 4 and Cyclin-A expression.

The growth fraction (GF): This is defined as the proportion of newly formed proliferating cells in the total number of newly produced cells, hence, representing the proportion of cycling cells in a tumor. The growth fraction is about 40% in a typical malignant tumor. Approximately, GF is three times the S-phase fraction.

The growth fraction (GF) is an important parameter since it correlates with the natural history of the tumor including: its growth rate, probability of recurrence, time to first recurrence and behavior of the recurrence. The growth fraction is also an important guideline for the response of tumor to treatment. Thus, cancers with a high growth fraction have a short natural history, but, are more responsive to radiotherapy and chemotherapy.

The GF can be measured by the use of IdUrd with flow cytometry or immunohistochemistry and application of the equation GF = 3 x SPF. It can be also calculated from DNA frequency distribution curves by adding the G2M area to the SPF area. GF can also be measured by using Ki-67 and PCNA immunostaining.

The potential doubling time (Tpot): This is defined as the time required for the tumor cell population to double its number in absence of cell loss. Human tumors have an average Tpot of about 5 days. Estimation of the Tpot is clinically relevant procedure since it gives an index of clinical behavior and may be used as a guide for the choice of therapy. Burkitt's lymphoma, for example, has the shortest Tpot amounting to 26 hours. Embryonic tumors of childhood and high grade lymphomas have values in the range of 2-4 days. Mesenchymal sarcomas in general tend to have long Tpot values with an average of 23 days. Squamous cell cancer has an intermediate value with an average of 7 days.

The Tpot can be calculated from the formula ($Tpot = \lambda Ts/SPF$) where λ is a constant and is usually taken as 0.75, Ts is the duration of the S-phase and may be taken as 15 hours or determined directly by using a special cytometry procedure.

The volume doubling time (Td): It is the actual clinically observed doubling time and depends on both the Tpot and the cell loss factor. Human tumors have a median Td of 28-630 days and for a typical tumor it is about 60 days. Td is determined by sequential tumor volume measurements using clinically applicable methods such as direct

measurements of superficial tumors, or radiological measurement of internal tumors.

Cell loss fraction: This is an important parameter that tends to slow down the growth of tumors, particularly when they attain large sizes. Cell loss is responsible for the difference between Td and Tpot.

Cell loss is high in human tumors and amounts to 40-95% of the total cell production. Sarcomas have a lower cell loss factor (amounting to about 40%) than in the case of carcinomas with a mean value of about 80%. This accounts for the relatively lesser degree of discrepancy between Td and Tpot in sarcomas compared to carcinomas. High grade lymphomas and embryonic tumors of childhood have the highest cell loss factor and can be as high as 95%.

It is possible to estimate the cell loss factor in a given tumor if both the Td and Tpot are independently measured, and using the relationship: cell loss = 1 - [Tpot / Td]. Alternate methods for determining cell loss is the mulitparameter cytometry and the TUNEL assay (measure apoptosis).

Turnover index (TI): The turnover rate of a tumor denotes the percentage of tumor cells renewed per day. It is estimated by the turnover index which is the sum of the mitotic index (MI) and apoptotic index (AI). The TI is a valuable parameter since it includes information on both cell proliferation as well as cell loss. The TI concept may also help to interpret the kinetics of tumors in terms of cell proliferation versus cell accumulation. Counts of mitotic figures and apoptotic bodies are made microscopically on Azur A-stained tumor tissue sections. Recently, quantitation is rapidly made by flow cytometry.

Approximate values of kinetic parameters of a typical human tumor are presented in (Table 7-1).

Table 7-1 Kinetic Parameters of a Typical Malignant Tumor

Parameter	Value
Cell cycle time	2 days
S-phase fraction	7%
Growth fraction	40%
Cell loss	90%
Potential doubling time	5 days
Volume doubling time	60 days
Turnover index (T1)	1%

Temporal Growth of Tumors

Tumor growth can be determined by measuring tumor volume as a function of time. Tumor volume (V) is calculated from the measured shortest and largest diameters (d_1 and d_2) by using Chamber's and Scott formula

$$(V=\sqrt{(d_1 d_2)^3})$$

Malignant tumors show an early lag phase, an intermediate constant growth phase (exponential) and a final progressively decreasing (Gompertzian) growth rate (Fig 7-4). The observed growth rate is the outcome of cell production (Tpot) and cell loss. Exponential growth implies that the volume doubling time of the tumor is constant, and often leads to the false impression that the rate of tumor growth is accelerating with time. The terminal deceleration of growth of large tumors is probably due to increase of cell death and a decrease of cell proliferation as tumor vascularity and nutrition deteriorate. Slowing of tumor is not observed (and may be reversed) with lung metastases or with indolent lymphomas which progress to aggressive ones. The majority of studies on tumor growth have been based on examination of lung metastases using serial radiographs. However, lung metastases tend to grow more rapidly than primary tumors.

Three important clinical events are related to tumor size, thus, 2 mm is the critical size to produce metastases, 5 mm is the smallest size of radiologic detection, and 1 cm is the size of earliest clinical diagnosis by palpation, at such a time, the tumor already contains about one billion cells. The natural history of cancer is rather a long one, measured in years (Fig 7-5). Thus, in case of breast cancer, taking a median doubling time of 100 days, a total natural history of 10 years is estimated, of which 5 years are pre-clinical (tumor size <1 cm).

Measurement of Cell Proliferation

Eleven basic methods are now available to study the proliferation rate of tumors. With recent advances in immunohistochemistry, and flow cytometry, these new methods tend to replace previous more laborious methods. A good correlation was reported between the results obtained with

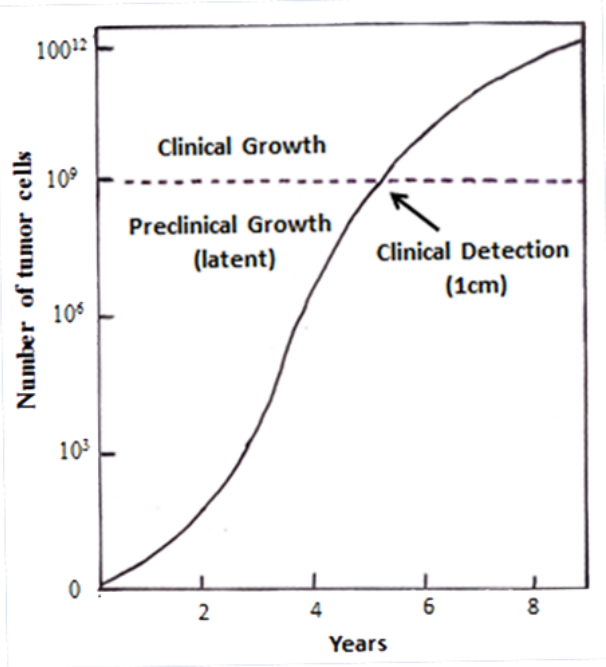

Fig 7-5 *Hypothetical growth curve of a human tumor showing an early lag phase, exponential phase, then terminal slowing of growth at large size. Note the long latent period (5 years).*

BudR, PCNA, MIB-1 and other non-immunohistochemical methods. Tumor cell proliferation studies also correlates with the survival of patients. Thus, such methods may supplement routine histologic studies, as a mean of improving the accuracy of predicting the biologic behavior of tumors.

Mitotic counts

Counting the number of mitoses within a given tumor section, with the help of an eyepiece grid, provides a rough indication on the rate of tumor proliferation. The increase of mitotic figures in a tumor is partly due to an increase in growth fraction and partly due to prolongation of metaphase. The mitotic activity is assessed by one of two methods, namely: (a) the mitotic index expressed as the number of mitoses per 1000 cells, or (b) mitotic counts in 10 microscopic high power fields. Limitations of the method are: its subjectivity, difficulty to distinguish mitosis from apoptosis, and the artifactious reduction of mitotic rate caused by delay in tissue fixation.

The *Stathmokinetic technique* was introduced in order to improve sensitivity by measuring the rate of entry into mitosis. Cells reaching the M-phase are blocked by metaphase blockers (vincristine, vinblastine, or colcemid). The rate of entry into mitosis is estimated using two biopsy samples taken within a fixed interval. The need to take two biopsies and the incompleteness of metaphase blockage by the usual drug doses are two shortcomings.

Thymidine labeling index (TLI)

Autoradiography is used to detect the presence of radioactive thymidine (3HTdR) in cellular DNA. The proportion of labeled cells in tissue at a short interval (usually one hour) after injection of 3HTdR into a patient is called the labeling index (LI). It represents the proportion of cells that were undergoing DNA synthesis (S-phase) at the time of injection, hence a crude measure of the overall rate of cellular proliferation in a tissue. The labeling index can also be estimated by preparing autoradiographs after short incubation of fresh biopsy with 3HTdR in vitro. Although thymidine labeling index is a well-established technique that also permits histologic correlation, yet it is tedious, and requires the administration of radioactive DNA precursor, and several weeks are required for the results to be obtained.

Flow Cytometry (FCM)

Flow cytometry has the advantage of speed and automation. In this technique, a single cell suspension is prepared from the tumor, and cells stained with a fluorescent dye (propidium iodide) whose binding is proportional to DNA content. Cells are then directed in a single file through a laser beam to excite the DNA-specific dye, and the fluorescence emission is collected and analyzed by photosensors. Cells then can be sorted according to their DNA content, which is proportional to the intensity of the fluorescent light. The proportion of cells in the different phases of cell cycle can be quantitated. The S-phase and growth fraction are important parameters of cellular proliferation. However, the principal disadvantage of flow cytometry is the contamination of tumor nuclei by stromal cell nuclei and inflammatory cells.

Multiparameter cytometry is now available and has solved many problems in tumor cell kinetics. This was possible through multiple labeling of the investigated tumor cells. Thus, by double labeling of cells with propidium iodide (DNA marker) and

phycoerytherin (a cytokeratin marker), a precise quantitation of the cytokeratin-labeled carcinoma cell from stromal cells (devoid of cytokeratin) can be achieved. By similar approach, it is possible to quantitate the amount of dead cells in a tumor. Since acridine orange (AO) stains undenatured DNA in viable cells, whereas, ethidium bromide (EB) labels dead cells, a combination of both can help to measure the proportion of dead cells in a tumor. This may provide a means of assessing tumor response to cytotoxic agents.

Proliferating cell nuclear antigen (PCNA)

Proliferating cell nuclear antigen (PCNA) is a polymerase-delta auxiliary protein that accumulates in the nucleus mainly during the S-phase. Several monoclonal antibodies, such as PC 10, recognize PCNA in formalin-fixed, paraffin-embedded sections and give an estimate of the cells engaged in replicating DNA. Three methods are available to quantitate labeled nuclei and to calculate the labeling indices; namely : manual counting, automated image analysis, and flow cytometry. Microscopic methods permit visual analysis of the tissue, so that viable tumor areas could be selected for evaluation, whereas, necrotic zones are avoided. Therefore, more information can be obtained by using a slide-based system in which the observer can simultaneously evaluate tumor cell kinetics and histology. The main disadvantage of using anti-PCNA antibodies for determining cell proliferation potential is the relatively long half-life of the protein, being about 20 hours. This may result in false positive identification of cells that are no longer cycling.

Ki-67 / MIB1

Ki-67 is a monoclonal antibody that reacts with a nonhistone nuclear antigen detected in all dividing cells. The antigen is present in the nuclei of cells in the Gl, G2, S and M phases, but not in G0 cells. One initial major disadvantage of Ki-67 was that the method works only on frozen sections and fresh cytology smears. At present, several monoclonal antibodies such as MIB1 and MIB3 have become available and can demonstrate Ki-67 in routinely fixed and embedded tissues, needing only simple antigen-retrieval techniques such as microwave oven processing.

Bromodeoxyuridine (BrdUrd)

BrdUrd or iododeoxyuridine (IdUrd) are incorporated into DNA during the S-phase of cell cycle. Monoclonal antibodies specific for BrdUrd or IdUrd are used to estimate the S-phase fraction of cycling cell populations. BrdUrd or IdUrd are injected intravenously prior to tumor biopsy or resection. Hematoxylin and Eosin-stained tissue sections may be screened to select the best tissue blocks for evaluating BrdUrdin corporation. Labeled cells are detected by means of standard immunohistochemical methods using monoclonal antibodies directed against BrdUrd. The labeling index is obtained by determining the percentage of labeled nuclei out of the total number of tumor nuclei.

Nucleolar organizer region -associated protein (Ag NORs)

Nucleolar organizer regions (NORs) are loops of DNA found in the nucleoli which encode ribosomal RNA. In association with the NoRs are found several distinct argyrophilic nonhistone proteins. Silver chloride histologic techniques on paraffin sections are available that differentially stain these NOR-associated proteins. This technique provides an indirect measure of tumor growth potential but it is not linked to any specific phase of cell cycle.

DNA polymerase alpha

It is a critical enzyme in eukaryotic DNA replication. Accordingly, the gene for DNA polymerase alpha is constitutively expressed in all phases of the cell cycle for actively cycling cells, but not in quiescent (G0) cells. Using monoclonal antibodies directed against DNA polymerase alpha, a correlation was found with other proliferation markers.

Cyclins

Cyclins (and CDKs) play an important role in progression along the cell cycle and control of the main cell cycle checkpoints. Thus, cyclin Dl controls Gl phase, cyclin E controls entry into S phase, cyclin A acts throughout S phase and cyclin B controls entry into M phase. Monoclonal antibodies to many of these cyclins are now available and may be used (with the help of immunohistochemistry or flow cytometry) to detect their overexpression. Cyclins are markers of both tumor cell proliferation, as well as, tumor aggressiveness. Thus, overexpression of cyclin D1 has been shown to be associated with a poor prognosis.

Markers of quiescent and non-proliferating cells: Statin

An alternative approach to estimate proliferative activity is to determine the fraction of cells not participating in the cell cycle. This stimulated search for genes and gene products associated with cell quiescence. Statin is an example of such a gene product. It is a component of the nuclear envelope of senescent and quiescent cells. It exists in two forms according to solubility in detergents. The detergent insoluble form is present in senescent or quiescent cells while both proliferating and quiescent cells express the detergent-soluble form. Stimulation of quiescent cells leads to rapid decrease in the detergent insoluble statin association with G0 to S-phase transition. The appearance of statin appears to be associated with the process of differentiation. This marker has not been widely tested in clinical studies.

Histones 3 and 4

Histones 3 and 4 (H3 and H4) are components of the core particle around which the DNA string is wrapped to form the nucleosome. They can, therefore, be used as a marker of the S-phase during which their synthesis is activated to provide the core around which newly formed DNA molecules are wrapped. In situ hybridization techniques can be used to detect H3 mRNA. This involves the use of commercially available fluorescein-labeled specific probes that hybridize to the entire mRNA transcript of human H3 gene. The hybridization signals are then detected with standard immunofluorescence or immunoperoxidase procedures. A correlation exists between H3 labeling index and Ki-67 immunostaining in a number of human tumors.

A summary of the available methods for measuring the various growth parameters of human tumors is presented in (Table 7-2). Unfortunately, most methods of assessing cell kinetics do not give information about proliferative rates of the stem cells in tumors, which are the important targets for curative therapy.

Table 7-2 Methods for Measurement of Growth Parameters

Method	Parameter
Mitotic index	M-Phase
Thymidine-labeling	S-Phase
MIB-1	Growth fraction
PCNA	Mainly S-Phase
DNA polymerase alpha	Growth fraction
Histones	S-Phase
Cyclin D1	G1-Phase
Cyclin E	Entry to S-Phase
Cyclin A	S-Phase
Cyclin B	M-Phase
DNA Flow cytometry	S-Phase and Growth fraction
Iododeoxyuridine DNA analysis	S-Phase and T_{POT}
Direct tumor measurement	Volume doubling time
Statin labeling	Non-proliferating fraction

Cell Proliferation in Cancer

The following are the 6 cardinal features of aberrant proliferation in malignant cells:

1. Usually there is *increase in growth fraction* (GF) due to overexpression of cyclins and CDKs and loss of inhibitory proteins (p16, p21 and p27). The GF may be scored as low, intermediate or high. A growth fraction exceeding 40% is considered high. Tumors with high GF are most sensitive to cytotoxic therapy, but prognosis is generally unfavorable, since any residual tumor cells will rapidly repopulate the tumor and cause a recurrence. Tumors with low GF are indolent and resistant to treatment.

2. There is usually an *increase of cell cycle time* in malignant tumor to about 2 days, in comparison with the normal cell cycle of 16 hours. However, the high growth rate of tumors is due to a high growth fraction in spite of long cell cycle time.

3. Failure of cell cycle clock results in *unscheduled DNA synthesis* outside the normal S-phase. Aneuploidy or abnormal DNA amounts are the ultimate result, and is defined as variation of more than 10% of the normal DNA peak (either increase or decrease). Hypodiploidy is rare and is mainly due to excessive apoptosis.

4. *Loss of the p53* defensive mechanisms of DNA results in failure of cell cycle check points. This leads to increase of mutations and genetic instability.

5. *Heterogeneity of cell proliferation* in different areas of tumors due to additional mutations and the

development of subclones with different proliferative capacities.

6. Large tumors generally outgrow their blood supply; hence, *tumor anoxia, apoptosis and necrosis* are common features. This results in slowing of growth rate in advanced malignant tumors.

CLINICAL RELEVANCE

Chemotherapy and cell cycle

The antineoplastic mechanism of action of chemotherapeutic drugs is through either the cell cycle or protein synthesis. Such irrepairable molecular damage will ultimately lead to cellular apoptosis. The mechanism of action of chemotherapy drugs may be through S-phase, M- phase or m-RNA (blockage of protein synthesis).

1. *S-phase agents*:

a. Antimetabolites: These compounds can inhibit important enzymes necessary for DNA synthesis or serve as a false substrate leading to aberrant incorporation of molecules into DNA, e.g. methotrexate inhibits the enzyme dihydrofolate reductase necessary for thymidine synthesis; and 5-fluorouracil binds to the enzyme thymedylate synthase in place of the normal substrate uracil leading to incorporation of aberrant molecules into DNA.

b. DNA adducts and crosslinks: alkylating agents (cyclophosphamide, chlorambucil, melphalan and nitrosourea) have active alkyl groups which bind to electrophilic sites in DNA and other biologically active molecules forming adducts. Bifunctional alkylation of DNA can result in crosslinks between the strands of DNA which impedes replication. Cisplatin has a similar mode of action.

c. Topoisomerase inhibitors: the enzyme topoisomerase normally function to reduce the torsional strain of DNA during replication. Anthracyclines inhibits topoisomerase, leading to tortional strain damage to DNA.

d. DNA breaks: Bleomycin combines with ferrous ion and oxygen forming a metal-bleomycin-oxygen complex which actively cleaves DNA producing both single and double strand breaks.

2. M-Phase agents: Vincristine and vinblastine are antimicrotubules agents that disrupt the microtubules of the mitotic spindle leading to arrest of mitosis in metaphase.

3. Anti-RNA agents: Actinomycin inhibits RNA leading to block of protein synthesis.

THE SPREAD OF CANCER

Malignant tumors, contrary to benign ones, have the property of spreading away from their original primary site of origin. This is accomplished through 5 main pathways, namely: local, lymphatic, hematogenous, implantation and intraepithelial spread.

Local Spread

Local spread of cancer is typically permeative, destructive to normal tissue and associated with desmoplasia. The invasive tumor focus is named according to its size, thus, tumors <1 mm are called microinvasive, whereas, tumors <1 cm are called minimally invasive. Larger tumors may be subclassified into organ confined or invading adjacent structures.

Local invasion may result in serious complications (e.g. malignant fistulas), or even ends fatally if the tumor arises in a vital site (e.g. brain or intestine). Moreover, since tumor stroma is rich in lymphatics and blood vessels, metastases may develop in lymph nodes or distant locations.

Lymphatic Spread

In carcinomas, lymph node metastases are only possible after penetration of basement membrane. However, in gastrointestinal carcinomas, since the mucosa lacks lymphatics, lymph node metastases are only possible after invasion of submucosa. Lymph node stations are classified into regional (removed in radical surgical operations) and distant nodes (determine inoperability if positive).

A sentinel lymph node is the first node to receive lymphatic drainage from a primary tumor, hence, the first to develop metastases. At operation, it is identified as the first node to take on radionucleide or blue dye injected into the tumor. Intraoperative study of sentinel node for metastases is a helpful guide to the extent of lymphadenectomy, especially in breast cancer and melanoma. Midline organs, such as thyroid, pancreas and urinary bladder, do not have a sentinel node. *A skip metastases* are those which develop in distant nodes, sparing the proximal or sentinel ones. *In-transit metastases*, or satellite nodule, is a metastatic tumor in a lymphatic located between the primary tumor and sentinel node.

Histologically, the extent of metastases in lymph nodes are classified into 4 stages with increasing intensity, namely: (1) Immunohistochemically-detected (CK) malignant cells, (2) micrometastases (size <2 mm), (3) macrometastases but confined to lymph node, and (4) invasion of lymph node capsule with involvement of extranodal soft tissue or lymphatics.

Hematogenous spread

Malignant cells may reach the blood either directly by invading thin-walled capillaries or venules at tumor margin (angioinvasion), or indirectly through lymphatic invasion which ultimately drain to the thoracic duct and venous system. The site of metastases may be explained on a hemodynamic basis (e.g. liver metastases from gastrointestinal cancer), but, the specific organ tropism of metastases in some cancers is only explained on molecular biological basis.

Implantation spread

This mode of spread occurs across body cavities (e.g. pleural, pericardial and peritoneal spread). It is also observed in subarachnoid space (medullo -blastoma), joint cavities, and surgical wounds after cancer operations (seedling).

Intraepithelial spread

In this process, malignant cells infiltrate between normal epithelial cells, but without invasion of basement membrane. It is best seen in Paget disease of nipple, as well as, extra-mammary Paget (in axillary and genital skin). This mode of spread is also observed in carcinoma in situ (CIS) with pagetoid spread, superficial spreading melanoma, and invasion of breast ducts in lobular carcinoma (cancerization of ducts). Since the epithelium lacks lymphatics and blood vessels, metastases cannot occur with intraepithelial spread of cancer.

METASTASIS

Metastasis refers to the transfer of malignant cells from one site to another not connected to it. In other words, the secondary tumor is discontinuous from the primary tumor. It is estimated that about 30% of cancer patients have metastases at initial presentation, and at least 50% of patients die as a result of metastases. Metastases usually resemble the primary tumor histologically and biologically if they develop at initial presentation of patients, but differ if they develop late in the clinical course of the disease due to the phenomenon of the tumor progression with time. Significant changes in genotype and phenotype of cancer is expected due to the accumulation of additional mutations.

Metastases may result from hematogenous, lymphatic or implantation spread of cancer. Metastases of lymph nodes and tumor implants usually remain confined to their original site for some time, but, hematogenous metastases are more serious and needs a detailed discussion at cellular and molecular levels.

Histologic Structure of Stroma

Cancer microenvironment is composed of cellular component, as well as, extracellular matrix ECM (Fig 7-6). ECM is a product of stromal cells. The latter also produce several growth factors which affect tumor cells and ECM. Thus, fibroblasts produce collagen and fibroblast growth factors (FGF) which is fibrogenic, as well as, angiogenic promoting endothelial proliferation and migration. Tumor-associated macrophages (TAMs) produce VEGF and metalloproteinases (MMPs).

T-lymphocytes produce variable cytokines and chemokines. Vascular smooth muscle cells produce Osteopontin (OPN) which is a chemotactic agent. The extracellular matrix also acts as a store for these growth factors to be released with matrix degradation, thus promoting angiogenesis and tumor invasion.

The stroma or *extracellular matrix (ECM)* consists of three main components: (1) *fibrous proteins*: collagens (types I to IX) and elastins, (2) *adhesion glycocoproteins*: (laminin, fibronectin, thrombospondin and tenasin), and (3) *proteoglycans* (glycosaminoglycans and hyaluronic acid). Epithelial and endothelial cells are separated from the extracellular matrix (ECM) by the basement membrane, which is composed of a network of type IV collagen rich in laminin adhesive molecules. Cell adhesive molecules or receptors are also present on surface of epithelial cells and help to adhere the cells to each other (E-Cadherins), to vascular endothelium (integrin VLA-4 and glycoprotein CD44), and to extracellular matrix (laminin and fibronectin).

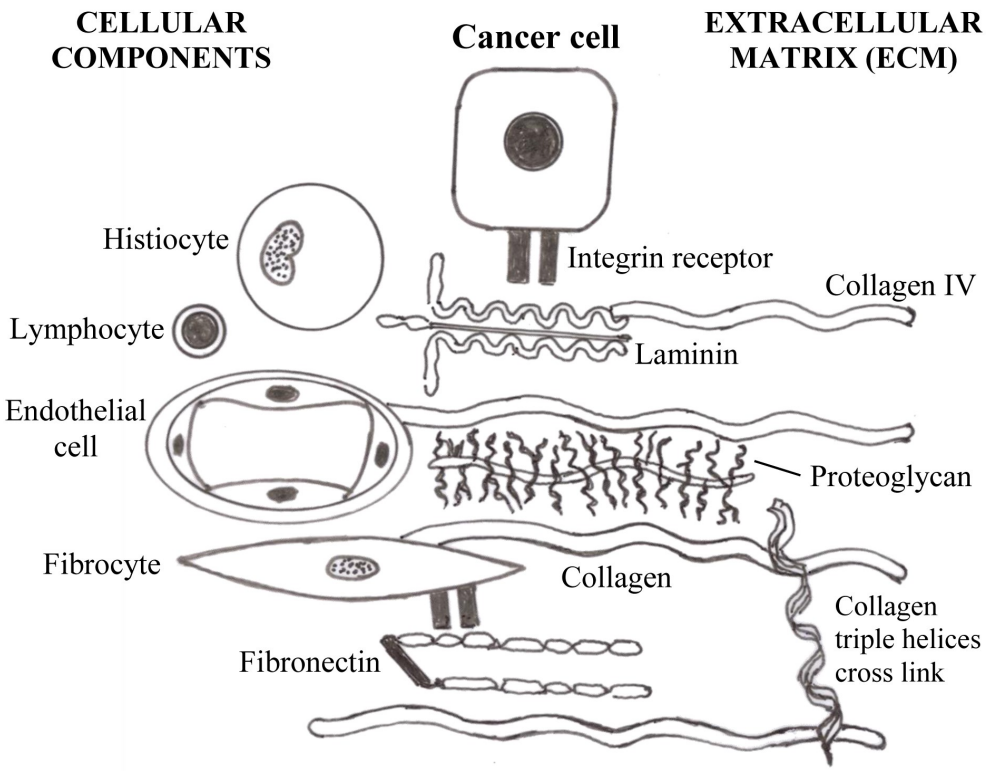

Fig 7-6 *The tumor microenvironment. It includes both cellular components and extracellular matrix (ECM) of protein nature produced by stromal cells.*

Steps of Metastasis

The numerous events of metastasis can be grouped together into three main steps: (1) Invasion of extracellular matrix (ECM),(2) Vascular dissemination and homing ,and (3) Angiogenesis and establishment of metastases (Fig 7-7).

Invasion of ECM

There is at first a down regulation of the expression of intercellular adhesive molecules (ICAM) and dissociation of tumor cells from each other. Actual local invasion involves three main steps, namely: attachment to ECM, matrix degradation by proteolytic enzymes, and cell motility.

1. Attachment is accomplished by the binding of laminin and fibronectin cell surface receptors with their corresponding adhesion glycoproteins in ECM.

2. Matrix degradation. Three classes of proteases have been identified which cooperate in the *degradation* of ECM, namely: (a) matrix metalloproteinases MMP (e.g. Collagenases).(b) cysteine proteases (e.g. cathepsin-D), and (c) serine proteases (e.g. plasminogen activator). Increased expression of these enzymes correlates with clinical aggressiveness of the tumor. Conversely, carcinoma in situ cells lack the enzyme collagenase type IV, hence, penetration of the basement membrane cannot be accomplished. The expression of these enzymes can be inhibited by retinoids, cortisone and TNF-β.

3. Tumor cell motility, or migration, is mediated through two mechanisms: (1) autocrine effect of cell motility factors (e.g. beta 15 thymosin), and/or (2) cleavage products of the matrix components which have chemotactic properties, as well as, growth promoting and angiogenic properties.

The epithelial-to-mesenchymal transition (EMT) theory was also proposed to explain local invasion of cancer (Thiery, 2002). Accordingly, the malignant epithelium is converted to a mesenchymal phenotype with different properties, including: loss of epithelial proteins (E-cadherin, cytokeratin 18 and MUC-1) and expression of mesenchymal proteins (vimentin, fibronectin and proteases), change of

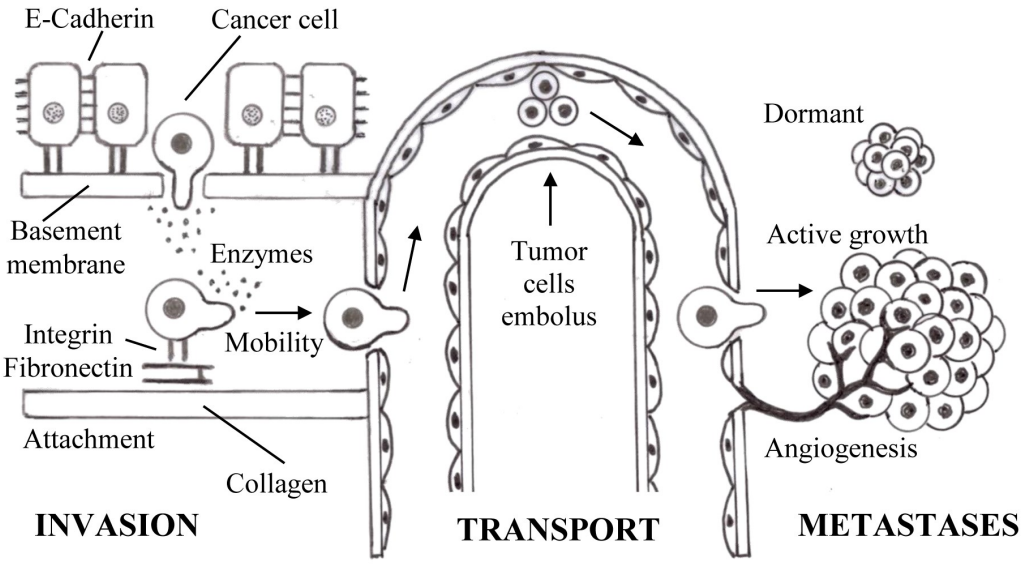

Fig 7-7 *The three main steps of metastasis. Local invasion depends upon cell attachment, matrix degradation and cell mobility: Successful homing and growth of metastases require angiogenesis and a favorable environment.*

cuboidal shape to a spindle shape, and loss of stationary property and acquiring cell mobility. These phenotypic transitions are reversible, and it is hypothesized that once tumor cells have reached their metastatic destination, they may transform back into an epithelial phenotype. A reversible epigenetic mechanism is probably involved in these changes. Thus, loss of E-cadherin will release beta-catenins from their complexed state leading to expression of snail signal pathway which is responsible of the shift of protein expression from epithelial to mesenchymal type. The concept of EMT was developed from in vitro experiments, but so far, not confirmed by clinical observations. However, it is probably operable in human cancer as a temporary reversible phenomenon.

Angioinvasion of venules and lymphatics requires degradation of basement membrane of endothelium, as well as, cancer cell motility. The result is intravasation of tumor cells into circulation.

Vascular Dissemination and Homing

Tumor cells in the circulation protect themselves from the hostile environment (both mechanical and immunologic) by aggregating in clumps in association with the platelets. Tumor arrest and extravasation at specific metastatic sites (organ tropism) is commonly (90%) accomplished by the binding of specific cell surface adhesive molecules to endothelium (e.g. integrins such as VLA-4 or glycoproteins as CD44), but rarely (10%) it is determined by hemodynamic vascular embolism (Fig 7-8). Moreover, organ tropism may also be related to the production of growth promoting or growth inhibiting factors at the metastatic site (favorable or unfavorable soil).

Establishment of metastases

It appears that metastasis is a very inefficient process. Thus, about 30% of breast cancer patients may have micrometastases in their bone marrow at initial presentation (i.e. 70% of metastases fail or die). However, only 40% of these patients will ultimately develop clinically evident metastases within 5 years (i.e. 60% of metastases re-

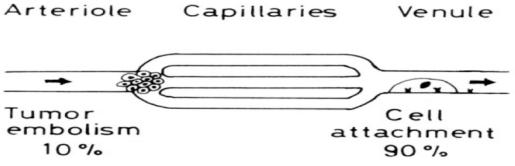

Fig 7-8 *The two mechanisms of arrest and homing of circulating cancer cells at the metastatic site: Organ tropism*

main dormant for some time). Accordingly, once individual tumor cells arrive in the secondary site, they may experience one of three fates: they may die, they may proliferate to form micrometastases, or they may remain viable but dormant.

Dormancy is defined as the property of malignant cells to remain nonproliferating but viable at the metastatic site for a long time (also known as occult micrometastases or minimal residual disease). This phenomenon is explained by 3 main theories: (1) cancer stem cells in the metastases enter into a quiescent G0 phase, (2) defective angiogenesis either due to a metastatic nonangiogenic clone of cancer stem cell, or an unfavorable microenvironment producing antiangiogenic factors. A balance may result between tumor proliferation and apoptosis, such that tumor size remains stationary and (3) antitumor immune reaction of the host. Regrowth of the dormant cancer cells to produce a late recurrence is probably induced by additional mutations which activate cell proliferation and angiogenic potential.

Angiogenesis is defined as the development of new blood vessels in tumor stroma to support increasing needs of the growing tumor, since 2 mm is the maximal critical distance of diffusion of oxygen and nutrients. The mechanism of angiogenesis is an example of tumor-stroma interaction. Thus, as a defense response to tissue anoxia, tumor cells produce vascular endothelial growth factor (VEGF). This angiogenic growth factor targets special receptors on endothelial cells (VEGFR) resulting in endothelial proliferation and formation of new vessels. There is parallel activation of Notch signaling pathway and the expressed products will modulate and organize the proliferating vessels. VEGF is also stored in the extracellular matrix (ECM), to be liberated again as a result of matrix degradation by matrix metalloproteinase (MMP) enzymes.

There are reciprocal interactions, in a paracrine fashion, between tumor cells, the extracellular matrix (ECM), and endothelial cells (EC). In a cooperative manner, tumor cells may either directly produce angiogenic factors, or stimulate fibrocytes and macrophages to induce them. Furthermore, the ECM may act as a reservoir for angiogenic stimulators or inhibitors. Thus the angiogenic factor b-FGF is sequestrated or stored in ECM, and is released by the action of proteases produced by the tumor. In contrast, the relation between tumor cell and EC is a symbiotic one (Fig 7-9). Thus, tumor cells produce angiogenic factors to EC, which pay back by producing polypeptide growth factors to the tumor (e.g. ILGF, PDGF, GM-CSF, and IL-1).

It is now becoming clear that angiogenesis, and hence tumor growth, is the result of balance between stimulators and inhibitors of this vascular phenomenon (called angiogenic switch). The main *natural angiogenic factors* are: (1) vascular endothelial growth factor (VEGF), (2) basic fibroblast growth factor (bFGF), and (3) platelet-derived growth factor. Hypoxia of tumors is associated with survival advantage. Hypoxia may induce not only VEGF but also EC receptors involved in angiogenesis. This reaction is mediated by the hypoxia- inducible factor (HIF-1) and hypoxia-regulated genes (e.g. VEGF gene and LDHA gene).

The most important natural inhibitors of angiogenesis *(antiangioenic factors)* are:(1) thrombospondin-1 (TSP-1), (2) angiostatin, and (3) endostatin. The first is expressed by activation of wild p53, and the last two factors are derived from proteolytic cleavage of plasminogen and collagen respectively.

Genetic Control

Three metastasis-suppressor genes have been discovered: (1) nm 23 in breast cancer, (2) KAI-1 in prostatic cancer and (3) KISS gene in melano-

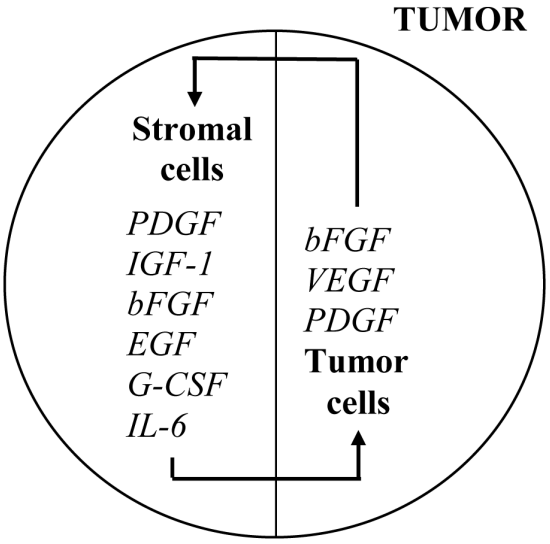

Fig 7-9 *Interaction between tumor cells and stromal cells. There is exchange of growth factors through a paracrine mechanism. Moreover, tumor cell necrosis will release several damage associated molecular proteins (DAMPs) and tumor antigens resulting in dendritic / T cell mediated immune response.*

ma. Mutation or loss of function of these genes is associated with increased risk of metastases. Genes that encode for E-cadherins or tissue inhibitors of metalloproteinases are also considered metastasis-suppressor genes. Loss of their function would reduce tumor cell adhesion and promote degradation of ECM, hence favoring tumor spread. Wild type p53 also has anti-angiogenic properties under normal conditions. Accordingly loss of function of p53, or activation of ras oncogene, stimulates angiogenesis due to increase of VEGF and inhibition of thrombospondin, and hence enhance the development of metastases.

Other genes may enhance the development of metastases. Thus, *Rho gene* is a recently discovered oncogene that belongs to the GTP-binding RAS family. It plays an important role in the onset, progression and spread of cancer. Activation of Rho/GTpase pathway results in cell dyscohesion, matrix degradation, tumor cell motility, angioinvasion and metastases. It also stimulates angiogenesis and inhibits apoptosis. Rho oncogene is upregulated by transforming growth factor beta (TGF-β), hypoxia, and stromal cell derived factor 1-alpha (SDF-lalpha). Rho gene is suppressed by nm23.

Inflammatory breast carcinoma has proved to be an ideal model for the study of Rho and Rho related proteins since this tumor exhibits marked intravascular permeation. However, Rho overexpression has also been reported in other various tumors as malignant melanoma, hepatocellular carcinoma and gastric carcinoma. The high incidence of the Rho family of GTPases in human tumors suggests that they may be potential candidates for targeted therapy.

CLINICAL PRESENTATION

Lymph Node Metastasis

Generally, the frequency of lymph node metastasis is more common in carcinomas than sarcomas. However, some carcinomas are incapable of producing lymph node metastasis (e.g. basal cell carcinoma and verrucous squamous carcinoma). Whereas, others are highly metastasizing (e.g. small cell undifferentiated carcinoma, malignant melanoma, breast and gastric carcinomas). The neuroendocrine tumor with the highest incidence of lymph node metastasis is neuroblastoma (25% of cases).

Hematogenous Metastasis

The incidence of distant metastasis varies markedly among different tumors. In some tumors, hematogenous spread is so frequent, that those tumors are considered as systemic diseases. Sarcomas belonging to this group are: non-Hodgkin's lymphoma, neuroblastoma, and osteosarcoma. The three carcinomas with the highest metastatic potential are: choriocarcinoma, cutaneous melanoma and small cell carcinoma of the lung. Conversely, brain gliomas and locally aggressive tumors of low malignant potential are not associated with metastatic spread.

In some organ sites, the frequency of distant metastasis varies according to the histologic type of tumor (e.g. lung and thyroid). Thus, small cell carcinoma of the lung and follicular carcinoma of thyroid are characterized by a relatively high incidence of hematogenous spread. In other tumors such as melanoma, the metastatic potential varies greatly according to the location, being high in cutaneous melanoma and low in ocular melanoma.

Patterns of Metastasis

The patterns of distant metastases are sometimes so characteristic to be almost diagnostic of the primary tumor. Thus, multiple metastases of extremely disseminated nature is characteristic of choriocarcinoma, malignant melanoma and small cell carcinoma. Whereas, a solitary metastasis is suggestive of primary carcinomas in the kidney or colon. Prostatic carcinoma preferentially spreads to bones, especially lumbar spine and pelvis, whereas ocular melanoma spreads to the liver and bronchogenic carcinoma favours the brain and adrenal gland.

In pediatric tumors, neuroblastoma favours three main metastatic sites, namely: a) the bones particularly the skull and orbit (Hutchinson's type), b) the liver (Pepper's syndrome) and c) the skin (blueberry muffin babies). Conversely, metastases from other pediatric sarcomas such as Ewing's tumor and rhabdomyosarcoma favour the lungs.

Metastases with Unknown Primary

Between 2% to 6% of cancer patients present with metastases without clinical evidence of any primary tumor. In 85% of cases, the primary tumor is small in size and occult. However, in 15% of cases the primary tumors are not identified, and probably have undergone spontaneous regression.

The location of metastases is helpful in searching for tumor source. Thus, in supra-diaphragmatic metastases, the head and neck region, lungs and breasts are the main sites of primary tumors, whereas, in infra-diaphragmatic metastases, the primary is usually the result of peripheral lung carcinoma, mesothelioma or ovarian carcinoma. Peritoneal adenocarcinoma without an obvious primary site is usually a primary peritoneal carcinoma (Mullerian origin). Identification of tumor markers in the serum is helpful to suggest the possible primary site (Chapter 5).

In a series of metastases with unknown primary (Greco, 2001), light microscopy revealed adenocarcinoma in 60% of cases, undifferentiated tumors in 35% and squamous cell carcinoma in 5%. In the undifferentiated group immunophenotyping on tumor sections revealed 80% carcinomas and 20% lymphomas, soft tissue sarcomas and melanoma. Helpful tumor tissue markers in this regard are: CK, EMA, Vimentin, LCA, S-100, Chromogranin and HMB-45. The prognosis is not always hopeless. Thus, a favorable outcome may be associated with non-Hodgkin's lymphoma, germ cell tumors, PNET and prostatic carcinoma.

CLINICAL RELEVANCE

Antiangionic therapy

Targeted antiangionic therapy is based upon the fundamental concept that tumor growth, invasion and metastasis are angiogenesis dependent. The most prominent targets for antiangionic therapy are the vascular endothelial growth factor (VEGF) and its receptor (VEGFR), but other potential therapeutic targets can include integrins and metalloproteinase enzymes. Two antiangionic drugs have been approved for clinical use, namely: (1) *Avastin*, a monoclonal antibody which inhibits (VEGF), indicated in colorectal carcinoma, and (2) *Iressa*, a small molecule antityrosine kinase which binds and inhibits (EGFR), indicated in non-small cell lung cancer. Clinical trials of these drugs revealed a limited therapeutic benefit (Devita, 2008). Thus, Avastin therapy prolonged the median survival by 5 months only, and with Iressa therapy, partial remission was observed in only 12% of patients.

Occult Metastases

Over the years, investigators have attempted to improve techniques for the detection of circulating tumor cells in blood, as well as, occult (dormant) micrometastases in lymph nodes and bone marrow. Occult metastases in bone marrow have been shown to be of prognostic significance in a variety of malignancies, especially breast, lung, colorectal cancer and neuroblastoma. Detection of occult malignant disease carries valuable data which may be included in TNM staging, and will help the design of more effective therapy.

REFERENCES

Awwad HK: Radiation Oncology: Radiological and Physiological Perspectives. Kluwer Academic Publishers, The Netherlands, 1990.

Chou CH and Figg WD: Antiangiogenesis Agents, in Cancer, Principles AND Practice of Oncology, Devita VT, Lawrence TS and Rosenberg SA (editors), 8th edition, Wolters-Kluwer /Lippincott, Philadelphia, p527-536, 2008.

Geisinger KR, Levine EA and Silverberg SG: Sentinel nodes. Pathol Case Rev, 13:85-121, 2008.

Kumar V, Abbas AK, fans to N and Aster JC (Editors): Pathologic Basis of Disease, 8th edition, Saunders / Elsevier, Philadelphia PA, 2010.

Lanza R: Essentials of Stem cell Biology. Elsevier Academic Press, Amsterdam, 2006.

Tannock IF, Hill RP, Bristow RG, and Herrington L (Editors): The Basic Science of Oncology, 4th edition, McGraw-Hill, New York, 2005.

Wang JCY and Dick JE: Cancer stem cells, in Cancer Principles and Practice of Oncology, Devita VT, Lawrence TS and Rosenberg SA (Editors), 8th edition, Wolters Kluwer/Lippincott, Philadelphia PA, p 135-143, 2008.