Clinical and Biological Implications of the Tumor Microenvironment

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Received: 11 January 2012 / Accepted: 11 January 2012 / Published online: 3 February 2012
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Abstract In normal tissues and organs, the activities of the constituent cells are strictly restricted to the tasks assigned to them during development. In addition they (with the exception of leukocytes) remain inflexibly confined to their territorial domains by regulatory interactions with their neighbors. This creates specialized local micro-environments in which structure and function are orderly, stable and tightly controlled by feed-back loops, within interacting regulatory networks. This system has considerable ability to adapt to changing conditions. In contrast, the microenvironment in regions where tumors are forming and expanding is characterized by progressive loss of specialized or differentiated cellular functions, disorderly molecular signals, degeneration of microscopical organ structure. This, coupled with the traffic of cells into and out of the tumor, often culminating in local invasion and metastasis to other organs. The nature of these disturbed molecular and cellular interactions is, by definition, highly unstable and increasingly unpredictable as time passes. It also varies between different tumors, sometimes even leading to regression. However, systematic analysis of this dysfunction in the tumor microcosm, using multiple modern research techniques, has revealed that all actively growing primary and secondary neoplasms share an absolute dependency upon support from adjacent non-neoplastic cells of the host. This support, in turn, continuously depends upon dynamic interplay between tumor and host cell populations, via signaling molecules and surface receptors in the tumor microenvironment. Such interplay determines the fate of the growing neoplasm. Such information, described and evaluated in

this article, provides important new insights into the etiology of carcinogenesis and how tumor growth, invasion and metastasis might be therapeutically arrested. The facts and concepts assembled below, regarding the cancer microenvironment, demonstrate how modern molecular findings reveal the impact of the wide range of cancer diseases upon the internal cellular, tissue and organ environments of the whole individual and how this applies to designing new work to improve human cancer diagnosis and treatment. The article discusses several specific types of experimentally-induced and clinically common cancers to derive principles useful for interpreting events in the tumor microenvironment, which apply to cancers in general and especially to human malignant disease.

Keywords Metastasis · Histopathology · Epithelial stromal interactions · Embryonic development · Tissue interactions · Cell interactions · Inappropriate gene expression · Cancer diagnosis · Cancer treatment · Inflammation · EMT · Dysplasia · Carcinoma in situ

Terminology

The following brief lexicon of common pathological and clinical terms is provided for clarity of description and to aid understanding:

Connective Tissue/Stroma These terms are used interchangeably to refer to non-epithelial tissue containing sparse cells and much extra-cellular material consisting of collagen, elastin and reticulin fibres embedded in a featureless ground substance or matrix traversed by blood vessels, nerves and lymphatics. It provides the structural framework of organs and tissues.

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Epithelium This describes tightly packed cuboidal or columnar shaped cells with virtually no extra-cellular material which covers external surfaces or lines internal spaces. It exercises protective, secretory and absorptive functions.

Tumor/Neoplasm These terms are used interchangeably to denote a disorganized growth of tissue. The term cancer denotes a tumor that has become invasive into adjacent tissues and may have metastasized to other organs. Cancers are therefore a sub-category of tumors.

Malignant/Benign These are imprecise clinical terms which denote the likely impact of the disease on the patient's survival. Malignant implies that the tumor is invasive and/or metastatic.

Parenchyma This term denotes the cells which perform the specific functions of an organ (eg the kidney or liver epithelium), as distinct from the supporting stromal cells and the fibrous tissues of the capsule, septae and blood vessels of the organ.

Introduction

The term the cancer micro-environment includes all aspects of the four dimensional space in which primary and secondary tumors live and grow. It is important to understand this specialized environment as being *four* dimensional, because it is dynamic, interactive and constantly changing. The multiple fluctuating patterns of exchanges which occur here, between the many different cell populations composing, entering and leaving the tumor, determine its behavior, its effects upon other organs systems and the eventual outcome of the disease. Approaches to investigate, manipulate, or navigate through the complicated signaling processes located here, at the interfaces between tumor and host cell populations, for therapeutic purposes, therefore need to be aware of its plasticity and that information about a given tumor that is correct now may not apply at a later date.

Recognition of the existence of the entity known as the cancer microenvironment emerged from studies on the histopathological sequence of changes at the interface between putative tumor cells and the surrounding non-neoplastic tissues during carcinogenesis [1, 2] and contemporaneous parallel studies on embryonic organogenesis. These embryological studies revealed the critical importance of reciprocal interactions between different cell lineages during normal organ formation and in maintenance of orderly cell behavior and tissue architecture in post embryonic life [3, 4]. Sequential electron microscopical and histological studies on carcinogenesis in skin and mammary glands [5, 6] unequivocally positioned the most active visible changes at

the tumor-host interface and therefore just at the locus where dynamic interplay between different components of an organ would be expected. Although tumors had been studied with microscopes for many years, the pioneering work of Orr [1] provided the most thorough examination of the process with the light microscope and led to his formulation of the idea that the changes in the neighboring normal tissues might be functionally [7, 8] relevant to cancer formation in the target tissue. Combination of the information from all of these different areas of investigation with further evidence obtained with the electron microscope led to the conclusion [2, 3] that disturbance of normal regulatory interactions between different tissues in the area of a developing neoplasm is responsible for the progressive disorderly behavior characteristic of carcinomas, irrespective of the nature of the causative agent (e.g. viruses, chemicals or excess hormonal stimulation). Subsequent further investigations provided evidence indicating that tumors of mesenchymal tissues (sarcomas) could also result from interference with interactions between the constituent cell populations of the tissue. In this work, the interference was caused by an impermeable barrier of plastic film inserted in the tissue. If the film was first ground into a powder before implantation, no tumor resulted [9, 10] demonstrating that the plastic material was not chemically carcinogenic.

Hence, it emerged from this large body of work that tumors are not just collections of disorderly tumor cells but are maladjusted living entities composed of neoplastic cells and incorporated non-neoplastic cells, recruited by their neoplastic neighbors to provide essential support for the progressive parasitic growth of the neoplasm. Collectively, this evidence was recognized [10] to indicate that, during neoplasia, major structural and functional changes appear and develop at the interface between tumor cells and adjacent host cells, that is to say, in the cancer microenvironment. The consistent presence of this sequence of changes at the interface between tumor and host cells in tumors of different organs, induced by different carcinogenic agents, in different animal species, led to the conclusion that they are physical manifestations of important disturbances in tumor host interactions involved in the cause and progression of carcinogenesis.

For the first 20 years after it was revealed, this information was regarded as peripheral to the advances in molecular biology, which were finding mutations, rearrangements and reduplications in the genetic machinery of the tumor cells themselves. The prevailing opinion in cancer research was, therefore, firmly in favor of regarding these nuclear changes in the neoplastic cell population as paramount in creating tumors and the changes in the adjacent tissues as being incidental bystander effects. However, the work of Judah Folkman [11] and others on angiogenesis in the vicinity of tumors, eventually established that the expanding mass of tumor cells also needed some external support from the host.

Even so, the awakening interest did not extend to the roles of other cell populations and the extra-cellular matrix in the area, as being additional important participants in the process of primary tumor formation or in cancer metastasis, until relatively recently.

This article provides an analysis of data, which aims to shape the reader's understanding of the *significance* of biologically and clinically relevant events in the microenvironment, during primary and secondary tumor formation. The text will discuss evidence demonstrating its critical role in growth, progression, invasion and metastasis. As we shall see, all of these characteristics of cancer are absolutely dependent on the interactions between the tumor and its host. Indeed tumors, benign or malignant, cannot come into existence or grow, unless they can induce or subordinate surrounding host cells to cooperate with them in forming the neoplasm. The data presented below indicate that the supportive and nurturing role of the microenvironmental stroma is *the* most important finding in cancer biology, cancer pathology and cancer medicine to date, as its activities determine the outcome of the disease in living subjects. Consequently, research on mechanisms by which the tumor cells interact with their non-neoplastic neighbors to attract necessary support provides major new opportunities for advances in cancer treatment.

Interactions at the Tumor-Host Interface and Their Role in Normal Tissue Homeostasis and Carcinogenesis

Malignant tumors are disorganized masses of living material composed of different tissues in which cancerous *and normal* components are intimately intermingled as shown in Figs. 1 and 2. Collectively, these components constitute the physical microenvironment in which the interplay between tumor and host advances over time. Their microscopic and cellular architecture resembles that of the organs from which they are derived (Fig. 1a–d) but is irregular and disorderly and becomes progressively more deranged as the parasitic growth expands into the host (Figs. 1e–g and 2b & c). This breakdown of orderly cellular relationships, which characterizes neoplasia in whole animals and plants, cannot be seen in two dimensional cell culture and can only be truly appreciated in samples excised from intact tumors growing in vivo. Similarly, the far reaching hormonal and metabolic impact of the tumor upon the host, sometimes markedly deranging the function of distant unrelated organs (paraneoplastic syndromes [12, 13]), is only evident when one studies the whole organism in which the tumor is growing. The environment within and around the tumor is therefore crucial to its growth, survival and impact upon its host. To understand the fundamental nature of how such disorder can originate and propagate within the highly organized

spatial arrangement of different cell lineages forming the tissues of a normal organ and exert its effects, one first needs to consider how normal order is created among the trillions of descendants of the original fertilized egg and maintained throughout life.

i) Embryological organ formation

Initially, all of the cells in the cell mass, created by the early cleavage divisions of the zygote, are totipotent and if separated from the others can each create a whole new individual [14]. Soon afterwards, an organizing center emerges in this collection of cells and directs the developmental fate of different regions of the cell mass. It achieves this by a process known as primary embryonic induction [14], which specifies the rostro-caudal (sagittal) and dorso-ventral (coronal) axes of the future body and the position of the brain and spinal cord. Subsequently, the developing nervous system triggers the formation of other organs (eg the eyes, ears, limbs and kidneys) in their correct body locations, by a series of secondary inductions. In the 1950's and '60s it was found that a series of further hierarchical inductive interactions between epithelial and mesenchymal cell lineages specify the formation of even the detailed micro-anatomical structure of each individual organ including the salivary glands, teeth, lungs, breasts, skin appendages, pancreas, among others. It also emerged that these interactions continue into and throughout adult life to maintain and repair organ structure [15]. The detailed evidence for these conclusions is too extensive to provide here and can be reviewed in excellent articles published previously by other investigators [4]. These data demonstrate that the formation and maintenance of multicellular organisms depends upon reciprocal interactions between different cell lineages composing each organ. For example, recent work has shown that 40 different cell lineages interact in the formation of the lung [16]. The coordinating interactions in forming, maintaining and repairing such an organ are therefore numerous and complicated. Subsequent research has shown that these interactions are mediated by *context-dependant* signaling mechanisms that are spatially and temporally precise and specific [17]. Expression of a particular mediator in a slightly inaccurate location or time can result in biochemical or structural defects and this applies to many different organ systems [18]. Conversely, certain molecules, such as osteopontin and CD44 (a cell surface transmembrane glycoprotein) can, via alternatively spliced or glycosylated isoforms, mediate completely different interactions among separate cell lineages in different tissues and organs. Thus, osteopontin plays a role in lactation [19, 20], bone morphogenesis, wound healing [21], signaling between gonadotrophs and other cell populations in the pituitary [22], and other processes, whilst CD44 is involved in lymphocyte homing to lymph nodes, epithelial cell adhesion to

Fig. 1 Normal (a–d, above red line) and cancer (e–h) micro-environment. **a** Normal human mammary ducts (solid arrow) and glands composed of secretory acini (open arrow). Whole-mount of human mammary tissue stained with hematoxylin before fixation and viewed through a dissecting microscope (x20). **b** Histological section through terminal breast ductule and secretory acini to show vimentin staining of myoepithelial layer of the epithelium (brown stain) and to show other resident cell populations composing the normal organ. The circle outlines the acini and the diverse cell types (fibroblasts, macrophages, endothelial cells, lymphocytes etc.) in the cellular community living in the collagenous stroma around the glandular acini (x 100). **c** View of normal human mammary tissue stained with labeled antibody to CD8⁺ lymphocytes (brown) to show the organ also contains cells which are transiting through its tissues (x250). **d** Electron micrograph of the boundary between epithelium (E) of a normal breast duct and the surrounding stroma (S). The arrow marks the basement membrane (B) which provides support and attachment for the epithelium. Note the orderly arrangement of the epithelial-stromal junction compared to Fig. 1h below. (x14,000). **e** Infiltrating lobular carcinoma of the breast showing cancer cells invading as strings and columns (arrow) of adherent epithelial cells through dense fibrous connective tissue

containing few non-malignant stromal cells. A residual duct containing apoptotic cells is present centrally (x100). **f** Magnified view of invading columns of malignant cuboidal cells of the carcinoma in Fig. 2f, still displaying pathognomonic epithelial characteristics, including production of mucin droplets (white arrows), which is an exclusively epithelial property. The intervening stroma is collagenous and contains occasional non epithelial, non-malignant host mesenchymal cells (black arrowheads). No evidence of EMT (x250). **g** Infiltrating ductal carcinoma of the breast. Note that the tumor is invading as adherent malignant epithelial cells, still in distorted glandular formation. These are passing through sparse cellular connective tissue stroma containing non-malignant cells, lying between the malignant glands. There is no significant evidence of any inflammatory cell infiltrate, nor of EMT. (x100). **h** Electron micrograph of an area at the invading front of a malignant gland comparable to that within the rectangle in Fig. 1g. Invading epithelium of the gland is labeled (E). The basement membrane normally present along the whole of the epithelial stromal boundary (between the broad arrows) is completely dissolved. Also, the fibres and background matrix of the stroma (S), ahead of the invading glands, is disorganized and disintegrating, showing the disorder in the tumor microenvironment (x 2,000)

the substratum, hemopoiesis and macrophage activation amongst many other roles [reviewed in [23]]. Hence, the structure and behavior of tissues composing normal organs depends upon integrated parallel and serial communications between differing organ cell populations and the messengers and receptors involved in these signals can vary, depending upon the identities of the living players in a specific context at a given time.

ii) Cancer formation: Microscopic and cellular aspects

In the first few days after treating an organ with a carcinogenic chemical the treated area begins to show characteristic reproducible changes in the target cells and in the

adjacent tissues [2, 5]. In experimentally-induced skin carcinogenesis, the epidermis rapidly thickens due to cell multiplication and many new cells infiltrate into the underlying dermis from deeper layers and from the dermal blood vessels. Gradually, over the next 10–15 days the dermis changes from being sparsely populated to being crowded with many different cell types including fibroblasts, plasma cells, eosinophils, mast cells and endothelial vascular sprouts. The tightly packed collagen fibres composed of smaller fibrils of uniform diameter, characteristic of normal skin, become dispersed by increasing amorphous material, rich in acidic mucopolysaccharides, and the constituent fibrils become strikingly variable in diameter. Meanwhile

the epidermal cells loosen from each other and from the underlying basement membrane, but show much fewer distinctive changes than the adjacent dermis. These changes progress over the next few weeks and include i) the departure of the inflammatory cells whilst fibroblasts and capillary vessels remain ii) gradual disintegration of the basement membrane between the epidermis and the underlying stroma iii) disorderly reduplications of the basement membrane followed by its complete disappearance and iv) release of epidermal exosomes (cytoplasmic vesicles), accompanied by dissolution of the collagen fibres by their rupture and v) invasion of the epidermis into the disorganized milieu of the dissolving dermal stroma. Direct visual evidence of this sequence of changes at the tumor-stromal interface was obtained by electron microscopy [2, 5, 6] and this was the first insight revealing the existence of the entity known as the tumor microenvironment. Similar changes were found in the boundary zones between the constituent tissues of various organs, eg skin, mammary gland, mouth, larynx [10] during carcinogenesis and in neoplasms induced by different etiological agents (such as chemicals, viruses and hormones), demonstrating that they are a consistently recurring feature of the carcinogenic process. The progressive nature of these changes, their location at the boundary between the neoplastic and non-neoplastic cell populations and their chronological relationship to the loss of the characteristic microscopical structure of the organ combine to indicate that important events relevant to the mechanism of neoplasia are occurring in the epithelial/stromal interface zone of the tumor microenvironment, even before recognizable tumors appear.

The rapid arrival of new blood and lymphatic vessels in the vicinity of a developing neoplasm, which can easily be demonstrated by the implantation of tumor cell suspensions or of small fragments of tumor in the subcutis or the sclera of the eye, compellingly attests to the ability of malignant tumors to exert effects upon and recruit other cell lineages in the vicinity to their aid. There is a voluminous literature upon the topic of tumor angiogenesis and upon the molecules involved, which space does not permit us to review here. However, the indisputable deduction to be derived from this well documented phenomenon [24] is that tumors which achieve exponential growth have also attained the ability to interact with, recruit and shape the living and inert components in their microenvironment for their own advantage.

iii) *Matricellular molecules, miRNA and other signaling entities*

In the boundary zone between different tissue components of an organ (epithelium, stroma, vessels etc.) many different attachment molecules, cell surface receptors and signaling mediators have been identified as important participants in the dynamic interactions between different cell

lineages necessary for normal structure and function. The precise identities of these molecules differ, according to the organ and the cell types and the interactions necessary to fulfill the functions of the organ. However, various proteins including the integrins, CD44, various growth factor receptors, osteopontin, thrombospondin, and their many isoforms are frequently found to be mechanistically involved in the molecular dialog in this zone. Many of these families of molecules capture and present growth factors, secreted by adjacent cells, to cognate cell surface receptors on other cell lineages, or activate precursors by enzymatic cleavage and have therefore been termed matricellular agents [25]. Such anchored presentation of active factors to the cell surface serves to focus their effects and limit diffusion. Overproduction or disorderly expression of one or more of this class of molecules is a characteristic feature of carcinogenesis and metastasis in many types of cancer. For example, excessive production of many aberrant isoforms of CD44mRNA is characteristically seen in many different types of human cancer [26]. Serum osteopontin is also elevated in many cancer patients and levels have been related to prognosis [reviewed in [27]]. Knockdown of osteopontin by shRNA inhibited secondary tumor formation by xenografted metastatic human breast cancer cells and its upregulation in a non-metastatic sister cell clone from the same patient resulted in copious spontaneous metastasis formation [27].

Lately miRNA has also become implicated in signaling between stromal and epithelial cells and some recent reports [28, 29] have provided evidence suggesting that traffic of miRNA subtypes between disseminated cancer cells and bone marrow stromal cells can influence whether the cancer cells establish metastatic colonies or remain in dormant state. If these findings are corroborated, it will show that non-proteinaceous molecules also convey signals between interacting cell populations in the tumor microenvironment, which can determine whether tumor cells can fulfill an already activated, intrinsic potential towards malignant behavior.

These data illustrate the fundamental importance of cellular and molecular events in the tumor microenvironment, for tumor growth and progression of the disease, or its regression. It is vital to also emphasize that, while the molecules involved are essential mediators of the normal and abnormal interactions in this zone, the identities of the cell lineages which constitute a given tumor, and their status of receptiveness to instructive or inhibitory signals, are *the* decisive factors in determining the outcome of the nascent tumorigenic process.

A Note of Caution

Modern biomedical research, especially cancer research, extensively uses molecular biological tools to study cultured cells to investigate questions related to mechanisms

underlying pathological processes. Sometimes such questions are studied in cultures containing mixed populations, such as epithelial and stromal cell lineages, or even in cultured fragments of tissues and organs in efforts to approximate to natural conditions in the living organism. More

often studies are performed in monolayer cultured cells in plastic dishes. The information obtained is useful in understanding what cells and molecules *can* do in defined isolated conditions, but it cannot be extrapolated directly to explain how normal and pathological processes *do* work in vivo. For

◀ **Fig. 2** Carcinoma in situ, absence of EMT and tumor cell dissemination. **a** An area of severe dysplasia bordering upon carcinoma in situ in a urinary bladder biopsy. The epithelium is grossly thickened and irregularly arranged. Marked overexpression of CD44 (*brown stain*) is seen in its deeper layers. The stroma (S) shows increased cellularity and loosening and disorganization of collagen fibres. Stained with Hermes 3 antibody, which reacts with all isoforms of CD44 (x250). **b** Areas of intra-glandular carcinoma in situ (*encircled*) of the breast within a larger field of infiltrating lobular carcinoma. In carcinoma in situ, the neoplastic cells proliferate within the boundaries of the epithelial domain and have not yet acquired invasive properties. A gland which is partially converted to carcinoma in situ, is also present (*asterisk*) in this field of view and the arrow marks invading carcinoma cells in the adjacent stroma (x100). **c** Prostate cancer: Within a sea of unstained invading malignant glands (*asterisk*) in the stroma of this tumor there are occasional residual normal glands in which the peripheral ring of myoepithelial cells is stained with an antibody to high molecular weight cytokeratins (HMWK903). Some of the glands have ragged partial investment with myoepithelial cells (*broad arrow*) and focal invasion of epithelial cells, bursting into the stroma through the gaps, can be seen (*thin arrow*). No evidence of EMT. (x100). **d** Disseminating adherent clumps of cancer cells within veins in the liver. These show clear epithelial differentiation and some are still forming glands with central lumina (*white arrows*). No evidence of EMT. Occasional dissociated carcinoma cells (*black arrows*) are also disseminating in this patient (x250). **e** Survey view of an autopsy of a mouse with a red fluorescent protein-labeled human mammary carcinoma (P) xenograft in the mammary gland, generated by orthotopic inoculation of the human mammary carcinoma cell line MDA MB 435 LM3. This cancer metastasizes copiously to lymph nodes (*thin green arrows*) and lungs (*open white arrow*) but not to any other organs. The liver (L,) spleen(S) and kidneys (K) are not colonized, but do contain disseminated single living cells (Figs e and f below). A lymphatic enormously distended with tumor cells, draining the tumor is seen at bottom left (*solid green arrow*). **f** Isolated single tumor cells labeled with RFP in the liver of the mouse shown above, visible by inspection of the liver surface with a dissecting microscope under illumination with light of wavelength which excites RFP fluorescence (x 250). **g** Scattered labeled single tumor cells in the spleen (x250).

evaluating the relevance of findings made in vitro, it is essential to conduct correlated investigations in living animals and/or humans to test whether the findings in vitro apply in the more complicated conditions operating in vivo. Even the work done on transgenic animals is often misleading, as the introduction of a genetic defect into the germ line often has diffuse and unknown cascades of downstream effects in the pathways with which the products of the gene normally participate. The results are very interesting, but show only how experimental genetic manipulations *can* lead to disease but not how “wild-type” cancer actually starts and progresses.

Unfortunately, because comprehensive, correlative work is difficult, time consuming and expensive, most investigations produce interesting laboratory data, which are claimed to be significant, but are unconfirmed in vivo. Therefore, it is necessary to stress that studies in living animals and humans to test the significance of findings in cultured cells, tissues and organs are indispensable for reaching reliable conclusions about the causation, mechanisms and treatment of cancer

and its effects on the human body. Direct extrapolation of inferences from studies in vitro to whole animals can lead to significant mistakes, as for instance in assuming that epithelial to mesenchymal transition (EMT) occurs in the pathogenesis of human cancers (see below) and that it may be a suitable therapeutic target.

Specific Examples of Clinical Conditions Showing Disturbed Microenvironmental Conditions: Dysplasia Versus Neoplasia

Clinico-pathological disease entities that are known to carry a high risk of subsequent malignancy among the affected patient population, provide valuable clues to the role of the microenvironment in the origin and subsequent behavior of tumors. This section of this article will discuss specific histological features of dysplasia and carcinoma in situ in surgical biopsies of the bladder, uterine cervix, breast, esophagus and pigmented lesions of the skin. These, when evaluated with an experienced eye, reveal useful information relevant to understanding the train of events at the boundary between unstable pre-neoplastic epithelium and host stroma in early human carcinogenesis. This constitutes the primordial tumor micro-environment. The earliest diagnostic features suggesting the possibility of impending neoplasia in surgical tissue biopsies include cytological atypia and architectural irregularity in an epithelial population, a condition known as dysplasia. The boundary between epithelium and stroma remains apparently intact but the epithelium has become thicker and the arrangement of cells in the affected zone becomes progressively disturbed (Compare Figs. 1b & c with 2a & b). Concomitantly, increasing overexpression of many CD44 gene isoforms is observed (Fig. 2a) in the dysplastic cells [30], which can be diagnostically helpful in recognizing early or borderline lesions.

Soon afterwards, the most superficial region of the underlying stroma shows gradually increasing rarefaction and birefringence of the collagen fibers and increasing density of stromal cells per unit area. Electron microscopic examination of this area demonstrates thinning and disorganization of collagen fibers, loss of uniformity of diameter of their constituent fibrils and increasing interfibrillary stromal ground substance [2]. At this stage the boundary between the epithelium and the stroma becomes increasingly difficult to define, whether by conventional light microscopy or by electron microscopy, or by specialized stains for basement membranes and diagnosticians are confronted with complicated decisions as to the prognostic implications of the changes in individual patients. In pigmented lesions, the interpretation can be sometimes even more difficult because proliferation of melanocytic cells in the junctional zone between epithelium and stroma (junctional activity) are common (as in benign compound and

junctional nevi) and can sometimes be exuberant but orderly, as in a Spitz nevus [31], making interpretation complicated and difficult. In such melanotic lesions, the background clinical information including to the age of the patient, the number of pigmented lesions and their anatomical locations, as well as their macroscopic appearances are helpful in reaching decisions as to therapy. It is important to emphasize that such contextual information will continue to be valuable in evaluating the clinical significance of microscopic and molecular changes in all types of cancer.

Ultimately, in tumors which progress to invasive and metastatic malignancy, the proliferating neoplastic cells penetrate into the adjacent stroma and the identification of individual infiltrating tumor cells (Figs. 1e–h and 2b and c) is the decisive factor used, in difficult cases, to diagnose a lesion as unequivocally malignant and needing wide excision. Conversion to aggressive behavior is also signaled by greatly increased numbers of capillary vessels and stromal cells in the vicinity of the stromal/epithelial boundary, as this indicates the emergence in the neoplastic cell population of the ability to recruit host tissue components to support its growth.

In glandular malignancies (such as those of the breast, pancreas, prostate etc.), the cardinal signs of disorderly epithelial-stromal relationships are also present. In these carcinomas, however, focally increased cellularity of the local stroma, thinning of collagen fibres, increase of interstitial matrix and breakdown and reduplication of the basement membrane before invasion[6], are accompanied by an additional characteristic feature indicative of abnormal relationships between different cell lineages in the tumor microenvironment. This consists of a gradual disappearance of the myoepithelial (outer) cell layer of the two layered epithelium allowing the neoplastic luminal epithelial cells to contact and invade the stroma. (Figure 2c) These observations indicate that the neoplastic cells outcompete and replace their non-neoplastic myoepithelial neighbors, thus upsetting the balanced equilibrium between stromal and epithelial lineages, which maintains a two layered epithelium lining in normal glandular units

Hence, the features seen at the epithelial stromal junction in biopsies of early human neoplastic lesions of several organs (eg bladder, esophagus, oropharynx, skin, breast pancreas, prostate, etc.) reveal that the progressing disturbance in communications between cells undergoing neoplastic transformation and their non-neoplastic neighbors seen in experimentally induced cancers are also seen in naturally occurring human cancers. They are also critical microscopic indicators used by surgical pathologists to determine whether suspect lesions are malignant and need to be treated. The microscopic pathologic data from human tumors, therefore, confirm that substantial and progressive changes occur in the tumor microenvironment during malignant progression. In order to obtain fundamental new understanding of

critical events in malignancy, it is now essential to complement molecular studies with parallel observations at the microscopic level using new tools such as fluorescent labeled cell lineages, so that the context of the molecular events can be interpreted more effectively (see below for more information).

Why is the Histopathological Tissue Context Important?

When this changing scene, described above, is viewed as a narrative process, in which different cellular participants arrive and leave, while permanent residents radically change their relationships, it becomes evident that profound changes are occurring in the tumor microenvironment. These cannot be seen at the molecular level alone and the identities of the cell populations which produce the molecules that exert important effects need to be ascertained, as well as the specific locations in the tumor at which they are synthesized and secreted. The effects of bioactive molecules are dependent upon spatio-temporal factors (ie where and when they are produced) as well as their affinity, avidity, concentration and specificity for their targets. So, in order to understand the significance of changes in a disorganized and poorly coordinated, mixed, cellular population during tumor induction, growth, invasion and metastasis, *and how they lead to independence from regulatory controls*, it is essential to study events at the cellular and tissue level, as well as from the genomic and transcriptomic perspective.

Tumors are composite cellular societies incorporating many different cell lineages of different developmental origins as well as extra-cellular materials such as collagen, elastin, basement membranes, complex carbohydrates etc. which provide support and structure. In the corresponding normal tissues, from which the tumor arose, these mixed cellular and extra-cellular components existed in dynamic harmony and the balanced equilibrium created the morphological structure of the organ. The cooperative interactions of these components led to the multiple complicated physiological activities which the organ performed for the host and far exceeded the capabilities of any of the cell lineages on their own. Such interactions, in which the interacting components (whether cells, whole animals or even whole societies) create an entity in which the whole is far greater than the sum of the parts, are now recognized in many different scientific fields as emergent phenomena. Emergent behavior is a combinatorial, interactive process in which a population displays new traits which cannot be achieved by individuals acting separately and which subside when the specific population mix disaggregates or ceases to interact. It is essential to realize that tumors represent areas in which this wholeness has been disturbed and that the causes of neoplasia therefore cannot be effectively studied by examining each of the interacting components in isolation

because the separation of the component parts makes it impossible to ascertain the cause of the breakdown of the emergent phenomenon.

It is for these reasons that understanding of the pathological context in which molecular and cellular interactions occur in a tumor is vitally important for research on cancer and its treatment. In the next sections we shall examine important practical implications of these statements for clinical and preventative cancer medicine.

Small Cancers Can Regress...Cancer Screening Follow Up Studies and Death Rates

Briefly, in this discussion of the pathological context in which interactions in the tumor microenvironment occur, we should recall that the life history of tumors does not always and inevitably progress to accelerating growth and progression to invasion and malignancy. Evidence from meta-analyses of several long-term follow-up studies on large scale cancer screening programs, for a variety of cancer types, have indicated that, whilst there is definitely an increase in detection of early stage cancers, the reduction of mortality from corresponding cancers is lower than was expected [32]. For example, while it is generally agreed that screening does prevent some cancer deaths, it is estimated that, for every breast cancer death averted 838 women must undergo mammographic screening for 6 years. This indicates that a significant proportion of tumors found are indolent, do not advance or can regress. Other even more direct evidence that early stage carcinogenic processes can slow down, stop or reverse comes from follow up studies on histologically low-grade ductal carcinoma in situ of the breast in patients, who were treated by biopsy only, or declined all treatment. These showed that this pre-malignant condition only progressed to invasive carcinoma of the breast in approximately 30% (11/29) of patients followed for 30 years [33]. Fully established metastatic cancer can also regress completely, as seen in patients with stage IV-S neuroblastoma, in whom tumor deposits in multiple organs shrink and eventually become undetectable [34]. These findings all agree well with experimental data on the reimplantation of naturally occurring lung metastases from breast cancers in mice, which showed that after transplantation of such secondary tumors into the mammary glands of syngeneic mice, all formed local tumors but only 30% of these metastasized again in the new hosts[[35]-see below]. Hence the available evidence shows that the balance of interactions between malignant and non-malignant cell populations within the nascent neoplasm sometimes changes so that the tumor cells lose properties, such as the ability to grow in ectopic organs, which they had previously acquired.

Metastasis

When Stephen Paget conducted his landmark studies on autopsy records of 735 women with breast cancer [36] he laid the first firm scientific foundation for the existence of microenvironmental conditions which influence the growth of tumors. From his data on the non-random distribution of metastatic deposits in the organs of these patients he inferred that the host is not passive in determining the location of metastatic tumor deposits. This conclusion was based upon the reasoning that, after the disseminating cancer cells enter the systemic blood circulation, they are distributed to all organs, but only grow in some locations. This remarkable leap in inductive logic was conducted without the aid of microscopes or any sophisticated equipment, other than the human brain. Paget could not have foreseen that his work would have such far reaching significance for understanding tumor growth, but his conclusions opened new ways to view the relationship between the tumor cell population and the host. The existence of site specific preferences in location of secondary tumor growths has since been confirmed many times by autopsy series on patients with many different types of cancer and formed the basis for the “seed and soil” hypothesis first formulated by Paget in 1889 [36]. However, for obvious ethical reasons the hypothesis could not be confirmed by experiment on humans until a unique clinical opportunity allowed humane collection of data by Tarin et al. [37], which established the concept beyond reasonable doubt, nearly a century later.

This new information was obtained from autopsies on patients who had been treated with peritoneo-venous shunts to alleviate symptoms caused by intractable ascites due to inoperable carcinomas within the abdomen. The resulting steady infusion of peritoneal fluid, via the shunt, carried desquamated cancer cells floating in the ascitic fluid, into the systemic circulation, for months or even years. Our measurements established that billions of living cancer cells entered the blood each day and the viability and tumorigenicity of these circulating cancer cells was demonstrated by reinoculation into nude mice. This treatment was considered ethically acceptable because the patient’s quality of life was so impaired by the accumulating fluid, that the risk of shedding cancer cells directly into the bloodstream was acceptable to the patient in return for a large relief from discomfort. The autopsies showed that some patients did not develop any metastases, while others developed multiple small metastases of almost uniform size in some but not all organs. Amongst this second group, there were some patients who were known, from radiological studies, to have metastases in some organs before insertion of the shunt and they had more deposits in the *same organs but not anywhere else*, although millions of viable cancer cells were distributed to every organ. In both groups, scattered small, collections of cancer cells could be

identified in metastasis-free organs, but there was no evidence of immune cell aggregation around these quiescent cells (Fig. 3b), indicating that the failure of these unequivocally malignant cells to form expanding secondary deposits was *not* due to immune cell attack and destruction. This work, therefore, confirmed that the disseminating cancer cells were capable of establishing secondary tumor colonies in *some* anatomical sites in a given patient but could not do so in other organs. These data convincingly validate the conclusion that the microenvironment in which a disseminating cancer cell is deposited by the circulation influences whether it can form a secondary tumor. They are further corroborated by data from directly comparable experiments in animals, showing that metastasizing cancer cells preferentially grow in some organs and not others [38, 39].

Subsequent studies on spontaneous metastasis from tumors generated by intra-mammary inoculation of matched metastatic [40] and non-metastatic [41] clones derived from a human breast cancer cell lines labeled with red or green fluorescent protein (RFP/GFP) provided more detailed information. The metastatic variant routinely colonized the lungs and lymph nodes (Fig. 2e) but not any other organs, yet labeled solitary cells (Figs. 2f and g) retrieved from *metastasis-free* organs of these animals were viable and proliferative in culture and tumorigenic, when reinoculated in the mammary glands of fresh mice. Additionally, the tumors formed by these labeled, reinoculated cells were once again metastatic, but secondary deposits occurred only in the lungs and lymph nodes, although scattered individual fluorescent cells were seen in all organs. Animals hosting tumors generated by the non-metastatic human breast cancer clone were also found to contain scattered fluorescing single cells in all organs, but no metastases. These dormant solitary tumor cells also proliferated again when cultured in vitro and formed tumors upon orthotopic inoculation but although disseminated labeled cells could be seen in the organs of the new hosts, no metastases were formed. This evidence combined with the other data provided above provides decisive confirmation that tumor cells which have unequivocal metastatic capability must still interact cooperatively with a suitable local organ microenvironment in order to accomplish secondary tumor formation. In this respect secondary tumor formation recapitulates primary tumor growth in requiring cooperation and support from neighboring host cells and tissue components. Direct confirmation of the need for stromal recruitment to support tumor growth and progression is provided by observations on different stages in the formation of human metastatic tumor deposits sampled at autopsy. Comparison of the changes in various small metastases within individual patients enables reconstruction of the train of events in their formation. The pictures in Fig. 3a–d show the early events in the life history of deposit as it attracts surrounding neighbors into its interior as it grows.

Detailed microscopical studies conducted on large numbers of freshly excised large and small metastases from several histopathological types of human tumors during diagnostic clinical work indicate that:

- Recruitment and incorporation of non-neoplastic host organ stromal cells and vessels is essential for growth. Small colonies, below 1 mm diameter can exist as pure collections of tumor cells, by using diffusion to obtain oxygen and nutrients and dispose of waste. However, above this limit such simple mechanisms are inadequate to sustain tumor growth and larger colonies routinely contain blood vessels and supporting stromal cells. Figure 3c shows the penetration of such components into a very early metastasis.
- During expansion, tumor cells kill host parenchymal cells and contact is necessary for this. For example hepatocytes adjacent to infiltrating metastatic tumor cells forming liver metastases can be seen undergoing apoptosis when contact occurs [42].
- Tumor cells at the periphery of the infiltrating metastasis then utilize pre-existing stromal architecture, at least initially, to support continuing growth.[42]
- Inflammatory cell infiltration is rare
- Metastases can grow very large (sometimes >5 kg) and the continued incorporation and growth of host stromal components is essential for this expansion
- Mitotic activity in host stromal cells exceeds that in tumor cells in many parts of the tumor (For corroboration see Fig. 1d in Tarin [12])
- If tumor cell growth outstrips stromal growth, regional tumor necrosis occurs
- Vascular leakage and hemorrhage is common and vessel walls are incomplete and this leakiness can be therapeutically exploited as shown by Yasunaga and colleagues [43], as discussed below
- The host stromal component can vary considerably from very cellular to highly fibrous (Figs. 1e–h and 2a–c) indicating that stromal tumor interaction is extremely variable

These observations collectively show that the establishment and progressive growth of secondary tumors is absolutely dependent upon interactions of tumor cells with many different types of host stromal and epithelial cell lineages comprising the tumor microenvironment. Further work in vitro [44, 45], provided evidence suggesting that the signaling between different populations can be mediated by soluble mediators which inhibit the survival of disseminated cells in some locations but not in others. Autopsy studies on humans [46, 47] and animals [35] indicate that normal cells do not survive for more than a few days in ectopic locations, when disseminated by accident or by experimental design via the circulation and it seems likely that the local

Fig. 3 Necessity for recruitment of stromal cells for tumor formation and growth: Life history of metastatic deposits. **a** The arrival of the tumor cells in the lung via the blood stream. Loose epithelial tumor cells (*arrow*) are seen in the lumen of a pulmonary arteriole lying in thin septum between alveolar air sacs (*asterisks*). x250. **b** In a different patient, cancer cells had been disseminating for years from a carcinoma in the ovary but there were no metastases in any organ at autopsy. A small clump of malignant cells forming a rudimentary glandular structure with a lumen can be seen in a lung capillary (*open arrow*). Other carcinoma cells are extravasating singly from the small vessels and lying free in the extravascular tissues (*solid arrows*), but there is evidence of recruitment of adjacent stromal cells into an organized structure. There is also no sign of any inflammatory cell response near these tumor cells. (x250). **c** This picture shows the first stage in recruitment of non-malignant stromal cells into the structure of an early deposit in the lungs of the same patient as in the lesions in Fig. 3a above and 3 d below. Host mesenchymal cells and a small capillary

blood vessel (*open arrow*) have passed through the narrow base of a papillary frond covered with malignant epithelium into its core, to form its supporting framework. Two parallel endothelial cell nuclei resembling a diagonally aligned equals (=) sign lie to the left of the open arrow. These line a vascular space which extends upwards and to the right within this space there are two nucleated blood cells. Outside the white space, stromal cell nuclei (*blue/black*) are visible amongst pink stromal collagen which they have recently produced and assembled. The frond projects into the lumen of a large glandular space lined by malignant epithelial cells. Asterisks mark alveolar air spaces (x250). **d** A later stage in the formation of a pulmonary metastasis in the same patient as the lesions in Fig. 3a and c. The tumor deposit is much larger and recruitment of host stromal cells is more advanced. This metastasis has formed many more papillary structures, containing mesenchymal cores (*arrow*), projecting into the central lumen of the malignant gland (x100).

inhibitory mediators act as homeostatic regulators preventing formation of colonies in inappropriate locations. It follows that metastatic tumor cells have clearly found ways to circumvent these controls. However, in the case of metastasis, it is still unknown whether the failure to form metastases in some organs is routinely due to active suppression of the metastasis competent cells population, which has already made deposits in other sites, by the new host organ or to passive failure of the stroma of the organ to respond to recruitment signals from the tumor cells. It is also possible that it is due to active suppression in some places and to passive unresponsiveness in others. Conversely tumor cells which succeed in forming metastases clearly succeed in actively inducing the neighboring host stroma of a compliant organ to cooperate. These questions are now accessible to exact experimental analysis using tumor cells and in host animals in which host cell populations (eg

bone marrow cells, stromal cells of an organ) can be labeled with different genetically programmed fluorescent proteins (Green FP, Red FP, Yellow FP, Blue FP) all within the same animal.

The marked instability of the neoplastic process should also be included in this consideration of tumor stromal interactions in metastasis. This and its implications for the clinical and biological manifestations of malignancy has been discussed in depth elsewhere [13] but, briefly, the molecular interactions discussed above, upon which metastasis and tumor growth depend, should be regarded as accidental or chance results of chaos and disorganization and not as orderly systematic processes. Many disseminated cancer cells from a metastatic cancer do not survive or remain dormant in distant sites even though they are viable and tumorigenic, as discussed above. This indicates that,

among the billions of cells shed from metastatic cancers, only a small proportion manage to mobilize the *full* complement of necessary pathways and networks to grow continuously in a foreign environment, although all of them have obviously activated processes that enabled them to escape and disseminate. Also, even when a metastatic deposit has succeeded in establishing itself in a new site, its constituent cells can lose the capacity to metastasize again. This can be illustrated by transplantation of growing deposits into a new host [35]. In studies performing this maneuver, lung metastases re-implanted in the breast all grew relentlessly to form local tumors, but only 30% of these tumors metastasized again in the new host. Hence, the constituent cells had retained the ability to recruit *mammary* stromal cells to aid their growth but had lost the capacity to recruit lung stroma. This variability and unpredictability clearly complicates the experimental analysis of the metastatic process, but these difficulties can be effectively overcome by appropriately designed experiments *in vivo*, such as the work with different labeled cell populations proposed in the previous paragraph.

Inflammation and Cancer

In recent years the idea that inflammation is an important factor in the causation of cancer has become fashionable [48–50], based largely on the detection of some “inflammation related mediators” and cytokines in tissues and fluids. It is alleged that inflammatory processes in the tumor micro-environment contribute to the induction and progression of neoplasia, but histopathological proof of this assertion is almost invariably absent in the articles claiming these connections. Although a number of publications have presented data from animal studies that seem to support the notion of an inflammatory contribution to cancer formation, clinical observations on many premalignant conditions and tumors in humans do not lend strong support to this possibility. Also, it is important to note that there is no convincing evidence in the publications that postulate a connection between inflammation and neoplasia, which distinguishes whether acute (characterized by infiltration by large numbers of polymorphonuclear leukocytes) or chronic inflammation (ie long-standing infiltration of the lesion by large numbers of lymphocytes and plasma cells) is the responsible factor. Hence, the claim is remarkably vague about an important aspect of inflammatory processes.

It is true that a small proportion of patients with chronic inflammatory bowel diseases such as Crohn’s disease (~10%) and ulcerative colitis (<10%) develop small intestinal or colonic carcinomas after many years. The data are complicated to evaluate because they vary from study to study, and in different countries and in different severities of disease. However, the meta-analyses show that the incidence/

prevalence/risk of cancer in these diseases is very low [51]. Likewise, a similarly small proportion of patients (<5%) with infectious hepatitis develop hepatomas and <5% of patients with chronic pancreatitis develop pancreatic ductal adenocarcinoma [52]. It is also recognized that long standing reflux esophagitis is sometimes (~10% of patients with gastro-esophageal reflux) associated with Barrett’s esophagitis [53] and dysplastic columnar metaplasia of the squamous epithelial cell lining, which can progress to invasive carcinoma (approximately 5% of the patients with Barrett’s esophagitis, ie less than 0.5% of patients with long-standing esophageal inflammation). Therefore, the number of cases of reflux esophagitis with inflammation that do *not* develop malignant tumors vastly outweighs those that get cancer of the corresponding organs.

It should also be noted that these estimates of the prevalence of cancer in patients with these diseases come from specialist tertiary medical referral centers and therefore may overestimate the true prevalence of cancer in the general population, because the population who get less severe manifestations of inflammatory bowel disease and infectious hepatitis are not referred to these specialist hospitals and do not have a raised prevalence of bowel or liver cancer. Therefore, it is important to recall that much more than 90% of patients with these types of chronic inflammatory disease do *not* develop cancer and it is consequently important to ask why the vast majority of the patients, being exposed to the same high degree of inflammatory cell infiltration over prolonged periods do not develop malignant disease. In the case of hepatomas and uterine cervical carcinomas it is much more likely that the viruses infecting the epithelial cells, rather than the inflammatory cell infiltrates, are initiating the aberrant epithelial gene regulation that leads to disturbed tissue interactions causing the cancer.

Some types of cancer, such as medullary carcinomas of the breast, characteristically contain a large number of lymphocytes and plasma cells and macrophages. However, these lesions are only a very small proportion (~2%) of the total number of breast cancers, occur in a relatively older population and are relatively indolent in behavior. Large collections of lymphocytes and/or plasma cells are rare in biopsies and resection specimens of other types of breast carcinomas, comprising 98% of the total. Therefore, it is difficult to use such data to reason that inflammatory cell infiltrates represent a significant causal factor in general human breast cancer causation or progression to metastasis.

It can be shown with antibody-labeled staining that some human tumors contain a greater number of macrophages than the corresponding normal tissues. Others contain less elevated levels and some contain very few. Macrophages are also reported by several investigators to be common in experimentally-induced tumors and several publications have claimed a causal relationship between these cells and tumor

progression to metastasis [54]. However, detailed histopathological studies on 1,322 human breast cancers did not find conclusive evidence to support these claims [55]. Lately, some authors have built upon this work implying that tumor associated macrophages play a significant role in carcinogenesis and metastasis. On the basis of intra-vital microscopy experiments in GFP labeled animals, it has been claimed that macrophages facilitate cancer cells to invade local tissues and enter blood vessels [56]. These findings were obtained in relatively short-term video studies on living tumors and do not convincingly exclude the real possibility that the observations resulted from chance co-localizations of jostling macrophages, tumor cells and macrophages in the turbulent and crowded tumor microenvironment. Further studies on histological sections of human breast cancer specimens indicated that the density of areas of direct apposition of tumor cells, endothelial cells and stained macrophages were more numerous in 30 patients with systemic metastases than in 30 patients who did not yet have any. It should be noted that these are relatively small numbers of patients on which to base the conclusion of macrophage involvement in intravasation. Also, the study did not find any association between the density of such areas and the occurrence of lymph node metastases.

These considerations leave cautious investigators with an open mind about the involvement of macrophages in cancer progression or control. Moreover, detailed studies on the activated macrophage content of *spontaneously-occurring* mammary carcinomas in mice showed no correlation with either spontaneous metastatic capability of the tumor nor with the capacity of its cells to colonize downstream organs after intravascular injection [57]. However, it should be mentioned that recently published data [58] have indicated that tumor associated macrophages (TAM) can diminish the efficacy of chemotherapeutic agents and that drugs blocking the cathepsin activity TAM improved the anti-tumor activity of the cytotoxic agents.

Mast cells are common infiltrators of carcinogen-treated mouse skin antecedent to tumor formation [1, 2], but they soon disappear and are not present in the invasive cancers which appear later. Mouse mammary carcinomas of various etiologies, that I have studied, did not contain significant numbers of mast cells. Some reports describe mast cells in human cancers [59] but the illustrations show only modest numbers and this accords with my own surgical pathology clinical experience. There is no convincing evidence that this cell lineage is causally involved in the carcinogenic or metastatic processes. Its transitory presence in the vicinity of some types of developing cancers, therefore, more likely represents a detoxifying reaction to carcinogenic chemicals or a response to mediators released by chemical irritation of the tissues. On the other hand, CD8(+) T cells are a known active component of cell mediated immunity and a recent histopathological study of 1,334 breast cancer patients [60]

has provided some support for a link between better survival and the total lymphocyte count in the tumor and in the surrounding tissues. Higher peri-tumoral B lymphocyte counts were also associated with better prognosis, but the numbers cited were not impressively high (12 cells per field) and the intra-tumoral content was found to be unrelated [61]. However, the same authors found no evidence for an association of prognosis with intra-tumoral FOXP3 regulatory T cells [62] and so the role (if any) of inflammatory cells in human cancer remains unconfirmed although potentially contributory to progression in some circumstances and to regression in others. The recent review by Talmadge [63] presents a balanced and fair assessment of inflammatory cell infiltration of tumors and its significance.

Other investigators, report biochemical data indicating that interleukins, chemokines and signaling pathways associated with inflammatory mediators are elevated in tumor formation. They claim that this is evidence of reciprocal signaling between tumor cells and local inflammatory cells and demonstrates that such signaling is essential for enhancing an inflammatory infiltration of the area as well as promoting invasion and metastasis [48, 49, 54]. These findings may be correct for the experimental models used, but the conclusions drawn from them are not compatible with clinical pathological observations. The data from human cancer pathology described above shows that histopathological evidence of acute or chronic inflammation is not commonly seen in local or metastatic cancers, thousands of which are being biopsied, excised and examined every day in the USA. Granted, special immunohistochemical stains sometimes reveal more macrophages, lymphocytes and polymorphonuclear leukocytes than are detected with routine hematoxylin and eosin staining, but the proportions of such cells detected with such special techniques are not impressive, relative to other cell types and are insufficient to support a convincing case for inflammation being a significant factor in human neoplasia in general. Moreover, it is important to emphasize that the biochemical detection of inflammatory *mediators* in or near a tumor is not convincing evidence that a coordinated local inflammatory process, with accumulation of acute or chronic inflammatory cells, is histopathologically detectable in the corresponding tissue or organ. Such mediators exert many other effects and may well be detectable in the tumor because of inappropriate expression of the corresponding genes in a dysfunctionally regulated tumor cell population [13]. Unless such direct microscopic evidence is visible, it is incorrect to speak of inflammation being present.

It remains possible that chronic inflammation may play a contributory role in a small proportion of neoplastic lesions, but the clinical evidence does not, as indicated above, support the conclusion that it is a major causal factor in cancer formation or metastasis. In general, therefore the data from experimental animals are conflicting about whether immune

cells and mediators might play a role in cancer progression or regression and, in humans, the clinical data are either inconclusive or oppose the concept.

Epithelial Mesenchymal Transition

In a discussion of the cancer microenvironment it is necessary to touch upon the subject of epithelial-mesenchymal transition (EMT) and its counterpart termed mesenchymal-epithelial transition (MET). In brief, it has been claimed by some investigators [50, 64, 65] that, in order to invade and metastasize, epithelial cells must i) undergo phenotypic conversion into spindle-shaped mesenchymal cells without firm attachments to their neighbors and that ii) after vascular dissemination, they undergo a reverse conversion to epithelial phenotype. This second concept was constructed in order to explain how metastases routinely reconstitute the histopathological structure of the tumor from which they came. Claim i), above, ignores the frequently seen clinical reality of sheets of mutually adherent malignant cells and glands invading local tissues as a collective force destroying normal tissues in its path (Fig. 1g) and disseminating via the blood as glandular structures with well formed lumina (Figs. 2d and 3b). Dissociated single cells are also seen within vessels (Figs. 2d and 3a), of course, but these retain epithelial characteristics and are rarely “mesenchymal” in appearance. The associated contorted suggestion, posed in claim ii), that cells which have undergone EMT then perform MET to revert to epithelial lineage identity after vascular dissemination, in order to form the plainly epithelial structures in the metastases they create [50, 65, 66], strains clinical pathological credulity [67]. Pathologists just do not see such transitions in routine diagnostic cancer metastasis specimens. The evidence presented by the proponents of these supposed transitions is, therefore, histopathologically very weak and is derived from a series of misinterpretations of histopathological and immunohistochemical observations on small numbers of samples and on tissue cultures.

The contradictory evidence, indicating that that these supposed transitions are figments of the imagination, comes from examination of hundreds of thousands of human tissue samples and is reviewed in detail elsewhere [67, 68]. Detailed investigations on diagnostic samples have confirmed that the proteins, such as vimentin and FSP1, claimed by the EMT proponents to be characteristic of mesenchyme and therefore demonstrative of EMT, are also expressed by the epithelial cells in normal organs (Fig. 1b) and are certainly not specific markers for mesenchymal cell lineages. Hence, the immunohistochemical observations, adduced as evidence supporting the claim, are indicative only of the expression of a set of proteins which is well known to be within the epithelial cell repertoire and is not

able to distinguish a genuine transition from one cell type to another. Any real transition must be accompanied by stable acquisition of the behavioral, biosynthetic and morphological properties of the supposed new cell type. For example, mesenchymal cells should be able to show some capabilities to make cartilage, bone, tendon or adipose tissue which the supposed EMT variants do not do. In addition only ~7% of invasive ductal carcinomas of the breast [69] and ~3% of lobular carcinomas express vimentin [70, 71], which is hardly convincing evidence for the hypothesis that this protein marks a transition to a mesenchymal cell lineage, especially when it well known that myoepithelial cells, which are indisputably of epithelial lineage, constitutively express this protein. Moreover, the morphological change to a spindle cell shape is a flimsy basis for assuming that a cell has changed its original character. Hence the claims of lineage transformation from epithelial to mesenchymal character are based upon misinterpretations and extrapolations from tissue culture observations and transgenic animals to human cancers, which do not withstand critical pathological or embryological analysis. Finally, the EMT/MET hypothesis does not explain why such transitions might be functionally necessary, because the pathological features of human tumors shows that they can readily invade and metastasize when they are in epithelial configuration as shown in Figs. 1e–g, 2d and 3b. EMT, therefore, currently lacks credibility as a tangible factor affecting events in the dynamic cancer microenvironment.

Diagnostic, Prognostic and Therapeutic Implications of Understanding the Cancer Microenvironment

It has been observed in clinical [72] and experimental [73] investigations that metastases in different locations in the same patient do not respond uniformly to chemotherapy with a given drug. The reasons for this are not fully understood but it has been proposed that the differing tumor microenvironments created for themselves by metastases in different organs make them differentially susceptible/resistant to cytotoxic agents. At present there is insufficient information to derive mechanistic explanations for these differences although some good evidence suggests that environmental regulation of the multidrug resistance (MDR) phenotype can play a role in some circumstances [73]. However, it is clinically very useful to recognize that, chemotherapeutic agents are not necessarily equally effective against residual primary cancers as well as all their distant deposits, and that the continued growth of resistant colonies in some sites can result in failure of treatment overall. Thus clinical trials on the efficacy of treatment regimens should include assessment of the response rates of metastases as well as of primary tumors.

The absolute dependence of cancer cells on neighboring non-neoplastic host components of the tumor microenvironment for survival described in several places in this article, opens new opportunities for detection and treatment of cancers. Increased local stromal vascularity stimulated by the tumor can be detected by various types of scans using ultrasound and or radionuclides. However, focally increased vascularity can also be caused by other pathological conditions such as arthritis, inflammation and tissue repair. Therefore, supplementary methods of investigation may be needed to confirm that the vascularity is associated with tumor growth. One of these approaches involves detection of increased metabolic activity in rapidly growing tissues, which can be revealed by PET (positron emission tomography) using labeled glucose, facilitating the imaging of the anatomic location of tumor recurrences and metastases. These concepts illustrate how the changes in the tumor microenvironment can be exploited clinically to detect small tumors that are otherwise difficult to localize. Other methods using labeled nanoparticles which can unravel to release their cargos in protease-rich tumor stroma [74] and/or antibodies to preferentially concentrate in areas where the tumor cells juxtapose to stromal components [43] are under active exploration in some laboratories.

At first, the targeting of the tumor vasculature by systemically administered drugs seemed extremely attractive as a therapeutic option but the modest results achieved with such agents including Avastin (Bevacizumab) have been disappointing, even to the extent that the Federal Drug Administration of the United States has withdrawn approval for use of the drug in the treatment of breast cancer, as trials showed no improvement in overall survival (OS) or progression free survival (PFS) [75–77]. The results with patients with cancers of other organs have also been disappointing although, anecdotally, occasional patients, especially some with glioblastoma, have shown improvement. However, the adverse side effects of the drug (hypertension and hemorrhage) give cause for close monitoring, if a trial of therapy is contemplated. Therefore, it seems that more sophisticated approaches to impeding stromal support for tumors are needed.

The well documented genetic instability of tumor cells coupled with their rapid replication results in the ongoing emergence of resistant clones under the selection pressure of cytotoxic therapy. Such resistant clones can enable tumor recurrence and failure of treatment, making effective permanent eradication of malignant tumors by current cytotoxic agents and long-term (over 10 years) survival a difficult and infrequently achieved goal. In contrast, the non-neoplastic stromal cells supporting the tumor population do not display marked genetic instability, although mutations do occasionally occur in tumor stromal cells [78, 79] and the cellular and extra-cellular components of the tumor stroma might, therefore, constitute a better target for anti-cancer therapy.

An example of how extra-cellular stromal components might be used to achieve tumor destruction is described in a recent report describing substantial reduction of tumor burden [43]. A high intra-tumoral concentration of a topoisomerase I inhibitor, SN 38, was achieved by conjugating it via an ester bond to a mAb targeted against collagen 4, a plentiful component of the tumor stroma. Polyethylene glycol (PEG) was utilized as a spacer, close to each bond, to maintain stability in the blood. These immunoconjugates selectively extravasated from leaky tumor vessels and minimally from normal vessels, because the immunoconjugates are too large to pass through normal vessel walls. Stroma targeting immunoconjugates bound to the stroma to create a scaffold, from which sustained release of cytotoxic agent occurred. The agent subsequently diffused throughout the tumor tissue to damage both tumor cells and vessels. Cancer-stroma-targeting immunoconjugate therapy was thus validated as a new modality of oncological therapy, especially for refractory, stromal-rich cancers. Another method of improving cytotoxic anti-tumor therapy by manipulation of the microenvironment involved blocking cathepsin activity in stromal TAM [58] as mentioned above.

A different method using drugs which inhibit stromal mediators of tumor cell growth was successfully used by Sumida et al. [80] to impair the growth of xenografted human gastric carcinoma by blockade of PDGF-R signaling. These investigators demonstrated that carcinoma-associated stromal fibroblasts, pericytes and lymphatic endothelial cells expressed high levels of PDGF-R whereas carcinoma cells did not and treatment with high-dose imatinib and irinotecan in combination inhibited tumor growth and lymph node and peritoneal metastases by disrupting stroma-tumor signaling via this natural mediator.

Options for cell-based therapy also command serious attention when it is fully appreciated that the human organism is a heterogeneous community of trillions of cells, of diverse specialized lineages, exquisitely coordinated by powerful short range (inductive) and long range (endocrine) signals, established in embryo and maintained throughout life. For example, it becomes necessary to explore how to harness specific components of these natural (non-immune) regulatory mechanisms to inhibit, control and subordinate rogue sub-populations into harmonious behavior. To investigate this approach, non-cancerous living cells from the same organ can be dispatched into the lesions via intra-vascular or direct intra-tumor injection, as self-replicating vectors able to synthesise and deliver high concentrations of biologically-active inhibitor molecules on site, and actively participate in local tissue reorganization. Modern technology already exists to color-code the effector cells with fluorescent tracker proteins and engineer them to produce molecules, already shown to be inhibitors of tumour growth and metastasis. It is predicted that the inoculated cells will

multiply and be incorporated into each tumor under the influence of the same signals that the tumor uses to summon adjacent normal stromal cells to enter its corrupted community, multiply and supply its needs. Being alive, the non-malignant cellular delivery vehicles can also co-adapt and evolve with their target population. The idea is to obstruct and undermine, from within, the support the cancer must necessarily derive from its stroma, in order to survive and grow, thereby instigating shrinkage of the lesion and the restoration of order.

Synthesis and Conclusions

The above evidence shows that the tumor microenvironment is a dynamically interactive zone in which the balance of events frequently, but not always, tips in favor of increasing disorder and advancement of malignant cells into deeper and more distant areas. Understanding of the diffuse spatio-temporal nature of the disturbances in the interactions between the heterogeneous tumor cells and adjacent non-neoplastic host cell lineages in tumors helps to design novel treatment methods that seek to penetrate the microenvironment and deliver agents which block support for the malignant cells derived from the stroma, directly to the tumor-stromal interface. This shift in emphasis should diminish deleterious side effects on rapidly dividing non-malignant cell populations in other organs such as the bone marrow, gut and skin, caused by current therapeutic efforts which seek to directly attack the tumor cells themselves with cytotoxic agents. For now, these are the best therapeutic modalities available and rightly constitute the best standard of care, but the data discussed above indicate that refocusing research and therapeutic efforts on blocking the interactions at the tumor host interface within the tumor microenvironment would be more effective at arresting malignant progression with less patient morbidity.

Acknowledgements It is a great pleasure to record sincere thanks to my colleagues GG Miklos PhD and DL Darling MD MRCP for comments, criticisms and suggestions which have been extremely helpful to me in preparation of this article.

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