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Commentary

Acute and chronic in vivo therapeutic resistance

Beverly A. Teicher *

Genzyme Corporation, 49 New York Avenue, Framingham, MA 01701-9322, United States

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ABSTRACT

The response and engagement of host normal tissues in malignant disease are major factors in therapeutic resistance. Physically, solid tumors have regions of hypoxia and acidosis. These physical stresses can lead to a more aggressive malignant phenotype through activation of HIF, GLUT-1, carbonic anhydrase IX, and subsequent alterations in cellular metabolism and secretion of pro-angiogenic factors. Soluble factors released from malignant and normal cells into the tumor microenvironment provide signals promoting tumor cell growth and survival, and development of tumor stroma and vasculature. Transforming growth factor-beta is a key factor in malignant disease since it is pro-angiogenic, stimulates development of extracellular matrix, and promotes immune tolerance and epithelial–mesenchymal transition. Multiple nearby and distal host cells directly support tumor growth or become passive contributors to malignant disease. Endothelial cells and endothelia precursor cells, mesenchymal stem cells, macrophages and other infiltrating cells actively contributor to tumor growth. Treg and other immune cells maintain a tolerant environment for tumor growth. These varied aspects of malignant disease which are not readily recapitulated in cell cultures, contribute to in vivo resistance to anticancer therapies. Although some drug resistance developed in vivo is not genetically based or indefinitely stable, this form of therapeutic resistance may be critically important in the clinic.

Cancer is rarely cured by current therapies. Shortfalls in therapeutic benefit occur due to the nature of malignant disease prior to therapy and due to decreased benefit of therapies after multiple courses of administration. The causes of the therapeutic resistance prior to and during treatment may be different and thus may require different approaches to lead to improved outcomes.

Therapeutic resistance has many interdependent origins. It is likely that in clinical situations, therapeutic resistance frequently is not a permanent state that is; it is not due to a genetic change in the malignant cells (Table 1). The properties of the host and the response of the host normal tissues to the malignancy are major factors in therapeutic resistance. Conversely, the capacity of the malignancy to mobilize the host normal tissues to support and protect the tumor is an integral facet of response to therapy.

This review will discuss physical parameters, growth factors and cytokines, cells of varied types, and disease location in the host in relation to therapeutic response and resistance.

1. Physical factors affecting therapeutic response

It has been recognized for more than 50 years that the physiology of tumors is abnormal. The dysregulated proliferation resulting in a solid tumor mass is accompanied by aberrant signaling producing poor quality vasculature. The solid tumor mass has regions of hypoxia and acidic pH [1]. The low oxygen, acidic microenvironment in which the malignant cells reside is compensated by alterations in cellular biochemistry allowing tumor cells to cope and thrive (Fig. 1). Clinical studies in breast, uterine cervix, vulva, head and neck, prostate, rectum, pancreas, lung, and brain cancers, soft tissue sarcomas, non-Hodgkin's lymphomas, malignant melanomas, metastatic liver cancers and renal cell cancers show that 50–60% of these have regions of hypoxia (areas with oxygen tensions (pO_2 values) $\leq$ 2.5 mmHg) [2]. Hypoxic regions are distributed heterogeneously throughout tumor masses. The stress of hypoxia can lead to the development of a more aggressive malignant cell phenotype with expression of HIF-1, GLUT-1, and carbonic anhydrase IX and consequently secretion of pro-angiogenic factors. In addition, in about 30% of human cancers, the MYC oncogene is deregulated. When oxygen is decreased and normal cells become hypoxic, the HIFs are expressed and the level of MYC expression is decreased. The normal physiologic HIF-1 response can inhibit the activity of normal MYC; however, in cancer the deregulated oncogenic MYC collaborates with HIF to confer a metabolic phenotype that includes aerobic glycolysis and contributes to tumor progression [3].

2. Hypoxia

Data accrued over more than 50 years have shown that hypoxia decreases response to radiation. In recent years tumor oxygenation

* Tel.: +1 508 271 2843; fax: +1 508 620 1203.
E-mail address: beverly.teicher@genzyme.com.

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Table 1
Characteristics of acute and chronic therapeutic resistance of malignant tumors.

'Acute' In Vivo Resistance	'Chronic' In Vivo Resistance
Treatment/drug induced	Physiologic Change
Epigenetic	Genetic
Host changes	Microenvironment
Enzyme induction	Hypoxia
Immune depletion	Immune suppression
Transient over weeks or months	Characteristic of malignancy

has been measured in the clinic using the Eppendorf polarographic needle electrode which measures oxygen tension in very small regions of tissue allowing many measurements to be made in a pass through a tumor [2]. The data collected from clinical studies of superficial tumors validated the many years of preclinical observations in that radiation therapy patients with poorly oxygenated tumors had decreased local control, disease-free survival and overall survival. Many strategies have been tested preclinically and clinically to improve hypoxic tumor response to radiation either through improved oxygen delivery, treatment with small molecule oxygen mimics (radiation sensitizers) or hypoxic cell selective cytotoxic agents [4]. Clinically increasing oxygen delivery has proved cumbersome and, generally, blood levels of the small molecules sufficient to achieve sensitization have been difficult to achieve; thus, these strategies have not reached FDA approval. Even in the earliest stages of tumor development, there is an important interaction between free radicals, HIF-1-induced gene expression and hypoxia [5]. The distribution of hypoxia regions in tumors is dynamic especially in response to cytotoxic therapy. HIF-1 activity leads to the production of proteins that can act on the free radicals resulting from hypoxia-reoxygenation cycling and from immune cell infiltration to promote the survival of endothelial cells and tumor cells. As the understanding of the intracellular pathways activated in response to hypoxic stress has been elucidated, the number of potential therapeutic targets available has increased.

In cell culture and in vivo the stress produced by exposure to hypoxia leads to increased genetic instability and a mutator phenotype in cancer cells [6]. Hypoxia forces metabolic adaptations throughout the cell including in the activity of several genes involved in DNA repair and damage response. Decreases in the DNA mismatch repair (MMR) pathway proteins MLH1 and MSH2 and in homologous recombination (HR) proteins, increase genetic instability. Acute reoxygenation of hypoxic cells is associated with increases in DNA strand breaks and activation of ATM/ATR, Chk1/Chk2 and BRACA1 DNA damage response pathways. Often models cannot distinguish between the effects of acute hypoxia, cycling hypoxia and reoxygenation and chronic hypoxia on transcription and translation of proteins involved in maintaining genetic stability and cell survival [6]. Genetic instability is a function of hypoxia-mediated resistance to apoptosis and decreased DNA repair, leading to increased mutagenesis rates and altered chromatin biology. A subpopulation of malignant cells adapted to low oxygen levels and continue to proliferate despite compromised DNA repair. The malignant cells with mutations and altered gene expression that survive and progress under nutrient deprivation, often metastasize and expand to produce more aggressive, therapeutically resistant disease [2]. To understand the mechanism(s) of intrinsic and acquired resistance, a subline of DU-145 prostate carcinoma was made resistant to camptothecin topoisomerase I inhibitors and the gene expression profiles analyzed [7]. Two pathways were altered in the resistant cells. The first reflected a decrease in apoptotic susceptibility through changes in apoptosis control via Bcl-2 and caspases and anti-apoptotic pathways operating through Akt/PKB. The second was changes in nuclear factor κB and TGF-β pathways that contribute to malignant cell proliferation.

3. Angiogenesis

As malignant cells proliferate, the number of cell layers surrounding from existing host vasculature increases. The resulting hypoxia and metabolic changes trigger secretion of angiogenic factors [8]. Subcutaneously implanted syngeneic and xenograft tumors in mice have demonstrated the capacity of tumor cells to mount an angiogenic response in the host and the requirement for an angiogenic response to promote continued tumor growth. The transcription factor HIF-1 is critically involved in promoting transcription of genes that allow cells to cope with hypoxia and produce secreted angiogenic factors that stimulate local endothelial cells and recruit distal endothelial and endothelial precursor cells to grow a tumor vascular supply. Hypoxic tumors are resistant to both chemotherapy and radiation therapy. The efficacy of radiation therapy is directly related to the oxygenation and reoxygenation of the tumor [9]. Radiation therapy requires oxygen radicals to generate double-strand breaks in DNA resulting in cell death. Anticancer drugs must be able to reach tumor cells in concentrations sufficient to be cytotoxic. Poor drug delivery to hypoxic regions of tumors is related directly to the distance of the cells from vasculature and to the leakiness and malformation of tumor vessels. Most chemotherapeutic agents are most cytotoxic to proliferating cells and a higher portion of the hypoxic cell population is not actively cycling. Many chemotherapeutic agents are dependent on cellular oxygen and oxygen radicals for cytotoxicity. Antitumor alkylating agents, anthracyclines, etoposide, bleomycin and other cytotoxic agents are markedly diminished in effect in hypoxic conditions [10]. Human tumor cell lines that were chronically conditioned to cycling hypoxia exhibited altered response to cytotoxic agents in culture and exhibited more aggressive growth as xenografts [11]. Multi-drug resistance efflux pump (p-glycoprotein, ABC transporters, MDR) expression can be induced by hypoxic stress [12,13]. The inhibition of angiogenesis as an anticancer therapy has progressed primarily by blocking endothelial cell response to angiogenic factors produced by malignant cells and host cells in the tumor mass.

4. Gylcolysis

Under normally oxygenated conditions the primary energy source for cells is glucose metabolism via mitochondrial respiration; however, under hypoxic conditions glucose metabolism via glycolysis becomes dominant with diminished but optimized mitochondrial respiration [14]. This increase in glycolysis is not limited to hypoxic cells and is not limited to solid tumor cells. Leukemia cells in normally oxygenated cell culture also have high levels of glycolysis. Normally oxygenated cultures of tumor cells produce as much as 60% of their ATP through glycolysis [15]. The aerobic glycolysis common in most malignant cells is known as the Warburg effect. Although this observation has been known and validated for more than 80 years using it to therapeutic advantage has been difficult; however, it is the basis of ^{18}F-2-deoxyglucose PET widely used for cancer diagnosis.

Glycolysis produces lactic acid, in addition, intracellular balance of acid with bicarbonate and engagement of the pentose phosphate shunt release carbon dioxide from the cells [16]. To cope, expression of carbonic anhydrase IX (CA9) on the surface of tumor cells which catalyzes the hydration of carbon dioxide produced by the cells to form carbonic acid is increased contributing to the acidic extracellular microenvironment of tumors. A low extracellular pH triggers the activation of primary

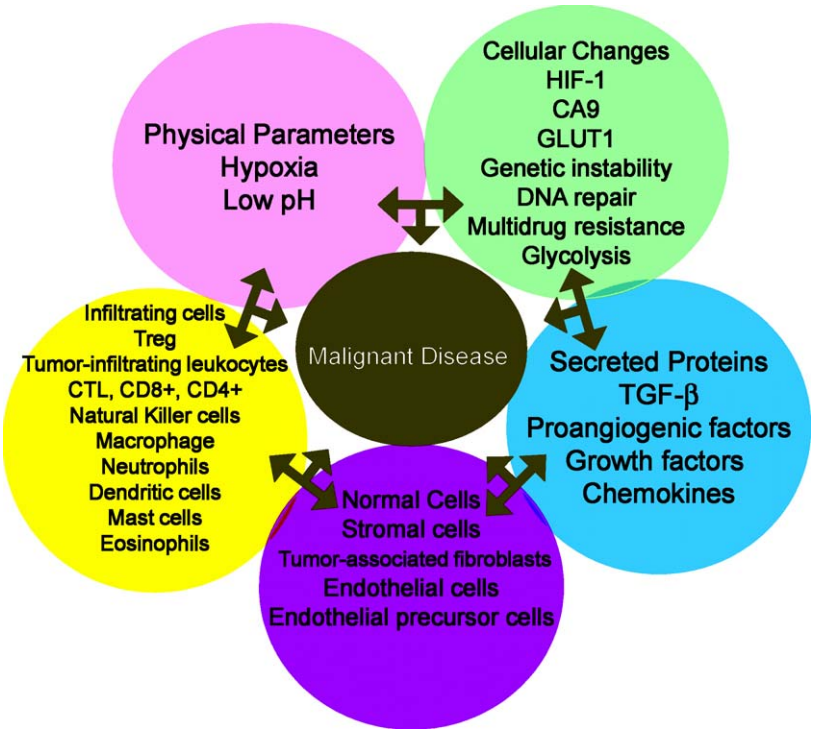

Fig. 1. In vivo therapeutic resistance results from an interplay of factors characteristic of the abnormal physiology of malignant tumors.

and secondary active transporters in the membranes of tumor cells to protect the cytosol from acidosis. In normal tissues, the extracellular pH is maintained around 7.4; however, in malignant tumors the extracellular environment pH can be as low as 5.5–6. Gillies and Gatenby hypothesize that the frequent observation of a high rate of aerobic glycolysis in cancer indicates that adaptation to hypoxia and acidosis must be a major component of the carcinogenic sequence of malignant cell evolution [17]. As tumor cells proliferate hypoxia and acidosis occurs in tumor regions distal from vasculature thus selecting for cells that can up-regulate glycolysis and are resistant to acid-induced toxicity. They further hypothesize that these phenotypic changes are critical late steps in carcinogenesis that confer proliferative advantages in normoxic and hypoxic conditions allowing the malignant cells to produce an acid environment that is toxic to neighboring normal cells. The HIF family and associated proteins offer potentially promising target for development of cancer therapeutics [18]. Alternatively, the aberrant hypoxia in solid tumors may offer an opportunity for the development of tumor selective therapeutics, for example prodrugs that are activated intracellularly under hypoxic conditions. While no oxygen delivery agent, radiation sensitizer or hypoxic cell selective cytotoxic agent has yet reached approval, a recent analysis of 86 clinical trials found that modification of tumor hypoxia significantly improved the effect of radiation therapy [19].

5. Growth factors and cytokines affecting therapeutic response

Increasing evidence supports a role for the tumor microenvironment in drug resistance as a major cause of relapse in hematological malignancies as well as solid tumors [20]. Tumor–tumor cell interactions, tumor–stromal cell interactions and tumor cell–extracellular matrix interactions contribute to contact mediated drug resistance (Fig. 1). Soluble factors released in the tumor microenvironment provide signals promoting tumor cell growth and survival [21]. Environment mediated-drug resistance (EM-DR) includes cell adhesion mediated-drug resistance (CAM-DR) and soluble factor mediated-drug resistance (SM-DR) produced by tumor–host interactions. In hematologic malignancies including leukemias, myeloproliferative disorders, lymphomas and multiple myeloma as in solid tumors, mutation or deletion of members of the TGF-β signaling pathway are frequent [22]. Elevated levels of TGF-β promote myelofibrosis, angiogenesis and immune system suppression in hematologic malignancies as with solid tumors. B cell chronic lymphocytic leukemia (B-CLL) cells frequently have mutations in the signal sequence of the TGF-β type I receptor (TβR-I) gene that may be a prognostic factor in B-CLL. The expression of TGF-β type II receptor is similar on B-CLL and normal B-cells. However, B-CLL cells are not responsive to TGF-β or to interleukin-4 (IL-4) while normal B-cells are growth inhibited. A group of Burkitt's lymphoma lines that express the full range of latent Epstein–Barr virus (EVB) genes and selected EBV-transformed lymphoblastoid cell lines were completely refractory to TGF-β and appeared to lack expression of TGF-β type II receptor [23].

Tumor cells are protected from immune system attack by the hypoxic, immunosuppressive tumor microenvironment which inactivates anti-tumor T cells [24]. Under hypoxic conditions the tumor produces extracellular adenosine which inhibits anti-tumor T cells via G_s-protein-coupled and cAMP-elevating A2A and A2B adenosine receptors (A2AR/A2BR). Extracellular adenosine increases in the tumor microenvironment due to changes in the activity of enzymes involved in adenosine metabolism. T-cell receptor activated and/or tumor hypoxia-exposed anti-tumor T cells in hypoxic regions express HIF-1 which induces changes in the T cells resulting in tumor-protective immunosuppressive effects. Normal human foreskin keratinocytes transfected with human papillomavirus type 16 DNA, designated HKc/HPV16, progress toward malignancy through reproducible steps including loss of sensitivity to growth inhibition by all-trans-retinoic acid and TGF-β [25]. Inhibition of growth and HPV16 early gene expression in HKc/HPV16 cells by all-trans-retinoic acid is mediated by TGF-β and loss of all-trans-retinoic acid sensitivity is linked to TGF-β resistance.

Autocrine motility factor (AMF), a multifunctional secreted cytokine, is abundant at tumor sites [26]. AMF promotes local tumor growth and metastasis and has effects on cell migration, invasion, proliferation and survival and possesses phosphorglucose isomerase activity. AMF can catalyze the first step in glycolysis and gluconeogenesis. During tumor progression Fas ligand (FasL)-expressing tumor cells can kill tumor-infiltrating lymphocytes (TILs) [27]. Soluble FasL in tumor masses increases with tumor progression and blocks Fas-mediated tumor killing by cytotoxic T lymphocytes (CTLs) and natural killer cells (NK). Increased production of FasL by tumor cells is associated with decreased Fas expression, thus facilitating escape from immune surveillance and promotion of tumor progression and metastasis. Simultaneously, increased secretion of TGF-β by both tumor cells and, in a paracrine manner, stromal cells, leads to enhancement of tumor invasion and metastasis as well as immuno-suppression. Increased FasL and TGF-β secretion contribute to the tumor immune privilege. Chen et al. examined the blockade of tumor-specific CD8+ T cells immune response by Ag-specific CD4+ CD25+ regulatory T cells (Treg) by monitoring the homing, expansion and effector function of both cell types in draining and non-draining lymph nodes [28]. The CD8+ cells expand similarly and produce similar levels of interferon-γ in the presence of Ag-specific Treg; however, the Treg abrogate CD8+ T cell-mediated tumor rejection by suppressing the cytotoxicity of expanded T cells. The molecular mechanism of suppression specifically involves TGF-β.

TGF-β is a key factor in malignancy. Frankly malignant disease is not responsive to TGF-β growth inhibitory effects. The TGF-β pathway including receptors and intracellular signal transduction protein (Smads) is a hot-spot for mutation in cancer [29]. Frequently malignant cells and some normal cells involved in the malignant process secrete large amounts of TGF-β perhaps as a remnant of attempting to rectify the aberrant biology of the malignancy. The secreted TGF-β has local and distal effects on host tissues. TGF-β prompts stromal cells (fibroblasts) to secrete extracellular matrix, induces angiogenesis locally and recruitment of endothelial precursors from the bone marrow, and suppresses immune activity of infiltrating cells inducing Treg differentiation and recruiting macrophage into the malignant process [30]. In rat thyroid cells, K-ras-transfected-induced carcinogenesis resulted in reduced expression of TGF-β type II receptor and consequent loss of sensitivity to TGF-β. Transfection of these cells with a vector to express TGF-β type II receptor partially restored the receptor protein and partially restored response to TGF-β. In human gastric cancer cell lines which express very low levels of TGF-β type I receptor, there is evidence of hypermethylation of the CpG island in the 5′-region of the TGF-β type I receptor gene [31]. Although exposure of the cells to azacytidine increased expression of TGF-β type I receptor, growth inhibition by TGF-β did not occur. TGF-β is an attractive target for cancer therapy. However, the TGF-β signaling pathway defect in cancer is sometimes not readily apparent [32]. Several strategies including the use of TGF-β2 antisense oligonucleotides, pan-TGF-β neutralizing antibodies, dominant negative TGF-β receptor II and TGF-β R1 kinase small molecule inhibitors are in preclinical or early clinical development [33].

TGF-β was shown to be a critical factor in the therapeutic resistance of the EMT-6 cisplatin-resistant and EMT-6 cyclophosphamide-resistant mouse mammary tumor lines [34]. Both drug resistant tumor lines had increased extracellular matrix, increased intratumoral vessel density and increased metastatic potential than EMT-6 parental tumor line. Hosts bearing the resistant tumors had higher circulating levels of TGF-β than mice bearing the parental tumor. However, upon administration of cytotoxic anticancer agents, the acute rise in circulating TGF-β was greater in mice bearing the parental tumor than in mice bearing the drug resistant tumors. Treatment of tumor-bearing mice with the naturally occurring TGF-β inhibitor decorin did not change the sensitivity of the parental tumor to cyclophosphamide or cisplatin; however, treatment with decorin increased the sensitivity of the drug resistant tumors to the same agents such that the drug resistance was nearly abrogated [35]. Similar results were obtained with a TGF-β neutralizing antibody [36]. Similar patterns of resistance and restoration of sensitivity were observed in the bone marrow of the tumor bearing mice indicating that the presence of the malignancy altered the physiology of the host whole-body [29]. Similarly, human MCF-7 breast carcinoma cells resistant to doxorubicin had decreased expression of TGF-β type I and type II receptors compared with the parental MCF-7 cells [37]. Exposure of both cell types to radiation resulted in increased expression of both TGF-β receptors at the mRNA level. The therapeutic resistance of the MCF-7 doxorubicin-resistant cells was caused, at least in part, by the defects in TGF-β signaling. Although the proliferative response of human LCC2 tamoxifen-resistant breast cancer cells to tamoxifen in culture was not affected by exposure to a TGF-β neutralizing antibody, when nude mice bearing subcutaneous LCC2 tumors were treated with TGF-β neutralizing antibodies and tamoxifen, sensitivity to the tamoxifen was restored. Tamoxifen-stimulated secretion of TGF-β may explain treatment failure in some breast cancer patients [38]. Murine L1210 leukemia cells resistant to cisplatin had a decreased expression of TGF-β type I receptor and increased expression of the inhibitory Smad 6 compared with parental L1210 cells as determined by Western blot [39]. Two doxorubicin-resistant human myelomonocytic leukemia cells lines, ME-F2/ADM100 and ME-F2/ADM200, express multidrug resistance genes mdr1 and P-gp in proportion to resistance to doxorubicin. These cells were grown in culture in the presence of 10 different cytokines, of these only TGF-β changed multidrug resistance gene expression by the cells markedly increasing expression of P-gp and mdr1 [40].

The accumulation of genetic and epigenetic alterations results in oncogenic transformation forming malignant progenitor cells. Highly malignant progenitor cells express stem-like markers such as Sca-1, CD133, CD44, Oct-3/4, c-KIT and multidrug resistance efflux pumps [41]. Diverse tumor-associated factors are up-regulated in these cells and their progeny through activation of developmental signaling pathways such as epidermal growth factor (EGF)/epidermal growth factor receptor (EGFR), hedgehog, Wnt/β-catenin, notch, TGF-β and integrin cascades that contribute to sustained growth and survival. These growth factor networks participate in events leading to disruption in cell–cell adhesion contacts and dissociation and migration during epithelial–mesenchymal transition (EMT). The increased expression of mesenchymal proteins during EMT contributes to migratory and invasive behavior of malignant progenitor cells.

6. 'Normal' cells affecting therapeutic response

Multiple cell-types are required for malignant tumor growth. Some such as endothelial cells and stromal cells act directly to support the tumor growth; others such as immune system cells become passive in the face of malignant disease or become active contributors to the malignant process (Fig. 1). Immune homeostasis is a balance between immune defense against foreign pathogens and suppression of the immune system to maintain self-tolerance and prevent autoimmune disease. TGF-β controls initiation and resolution of inflammatory responses through the regulation of chemotaxis and activation of leukocytes in the periphery such as lymphocytes, natural killer cells, dendritic cells, macrophages, mast cells and granulocytes. Dominant tolerance mechanisms prevent inappropriate immune responses. CD4+ CD25+ regulatory T (Treg) cells are central constituent of the dominant tolerance mechanism [42]. Malignant cells often secrete

large amounts of TGF-β that acts on non-transformed cells present in the tumor mass as well as distal cells in the host to suppress antitumor immune responses producing an environment of immune tolerance, augmenting angiogenesis, invasion and metastasis and increasing extracellular matrix deposition. Cells of the innate immune system contribute to the high concentrations of TGF-β found in tumor masses. Dendritic cell subpopulations secreting TGF-β contribute to the generation of Treg cells that actively inhibit the activity of other T cells. Elevated plasma TGF-β is associated with advanced stage disease and may separate patients into prognostically high risk populations. Anti-TGF-β therapy could reverse the immunosupressive effects on the host, decrease extracellular matrix formation, decrease angiogenesis, decrease osteolytic activity and increase the sensitivity of the malignant cells to cytotoxic therapies and immunotherapies [43].

7. Lymphocytes

Although many properties of cytotoxic T lymphocytes remained intact upon exposure to Treg cells, granule exocytosis which is dependent upon the responsiveness of cytotoxic T lymphocytes to TGF-β was impaired [44]. The cytotoxic T lymphocytes regained full killing ability when the Treg cells were removed. Treg cells have a major impact on the cytolytic action of specific CD8+ T cells that target the tumor [45]. In addition to local secretion of TGF-β and interleukin-10, direct cell contact through binding of cell surface molecules such as CTLA-4 on Treg cells to CD80 and CD86 molecules on cytotoxic T lymphocytes occurs [46]. Treg cells act in a dominant trans-acting manner to actively suppress immune activation and to maintain immune tolerance [47]. Treg cell development and function critically involves the forkhead transcritption factor Foxp3 which is a Treg cell lineage specification factor. Foxp3 forms a DNA-binding complex with NFAT (nuclear factor of activated T cells) to regulate the transcription of target genes. Although not exclusive, Foxp3 binding suppresses activation of target genes involved in T cell stimulation [48]. Foxp3 suppression of gene targets is crucial for Treg cell function. Malignant tissue protection from CD8+ and CD4+ T cells (antitumor T cells) limits the therapeutic potential of immunotherapies. Extracellular adenosine in the tumor microenvironment and Treg cell-produced extracellular adenosine has a role in tumor protection from immune damage in a hypoxic microenvironment [49]. Adenosine triggers immunosuppressive signaling via intracellular AMP-elevating A2A adenosine receptors (A2AR) on antitumor T cells. Extracellular adenosine is increased in tumors due to the changes in activities of enzymes involved in adenosine metabolism [50]. T cell receptor activated and/or hypoxia-exposed antitumor T cells may be inhibited in tumor microenvironments by HIF-1 activity in T cells. Thus, the protection of hypoxic malignant cells from antitumor T cells is mediated by the same mechanisms that protect normal tissues from immune cell damage during acute inflammation. Murri et al. examined the interrelationship between systemic inflammatory response (white cell count, C-reactive protein and albumin concentration), standard clinicopathological factors, tumor T-lymphocyte (CD4+ and CD8+) and macrophage (CD68+) infiltration, proliferative (Ki-67) index and microvessel density (CD34+) by IHC and assessed their prognostic value in 168 potentially curative early stage breast cancer resection patients [51]. Increased tumor grade and proliferative activity were associated with greater tumor T-lymphocyte and macrophage infiltration and microvessel density. However, only tumor microvessel density was independently associated with poorer survival.

8. Endothelial cells

Hypoxia is one of the most potent stimuli of VEGF expression. This effect is mediated by hypoxia-inducible factor-1 (HIF-1), a master regulator of angiogenesis [52]. While HIF-1 is a major factor, HIF-1 independent pathways are also involved in angiogenesis control. In large multicellular tumor spheroids reactive oxygen species generation decrease and multidrug resistance development, that is increased expression of the MDR transporter P-glycoprotein (P-gp), is related to expression of HIF-1 and intracellular redox state. Interestingly, Manning et al. found that treatment of immune competent mice bearing Her2/neu expressing tumors with anti-VEGF receptor-2 antibody DC101 caused an antitumor immune response [53]. There was in induction of tumor-specific T cell responses as a consequence of exposure to DC101.

Aberrant, dysfunctional tumor vasculature is a barrier to effective cancer therapy [54]. Evidence supports the notion described by Hellmann [55,56] and popularized by Fukumura and Jain [57] that antiangiogenic treatments such as VEGFR2 inhibitors and anti-VEGF can produce a transient 'normalization' of tumor vasculature, thereby improving tumor perfusion and delivery of systemic therapies for a period of time [58]. VEGF-targeted therapies originally developed on the theory that inhibition of blood vessel growth would starve the tumor by preventing the delivery of nutrients and oxygen. It is now apparent that the antitumor activity associated with VEGF neutralization derives from multiple effects on the tumor and host. Bevacizumab VEGF neutralization alters tumor vessel physiology and allows improved delivery and efficacy from chemotherapy if drug timing and scheduling is optimized [59].

Upon recruitment and activation by the tumor, bone marrow-derived myeloid cells including macrophages, neutrophils, eosinophils, mast cells and dendritic cells along with endothelial precursor cells participate in formation and maintenance of tumor blood vessels [60]. TGF-β has varied effects on hematopoietic progenitor cells. TGF-β markedly inhibits the proliferation of megakaryocyte progenitors (CFU-MK), and high-proliferative potential progenitors (HPP-CFC) but has no effect on erythroid (BFU-E) or granulocyte-macrophage progenitors (CFU-GM). In most preclinical and clinical settings the benefits of antiangiogenic therapy are transitory. Two modes of resistance to antiangiogenic therapies can be envisioned: evasive resistance, an adaptation to circumvent the specific angiogenic blockade; and intrinsic or pre-existing indifference [61]. Antiangiogenic and vascular disrupting therapies eventually result in glucose and oxygen deprivation conditions in the tumor that trigger pro-survival responses such as expression of glucose-regulated protein GRP78, an endoplasmic reticulum chaperone, associated with resistance to several anticancer drugs [62].

Angiogenesis and lymphangiogenesis are regulated by the integrin family of cell surface receptors and their ligands which are extracellular matrix proteins and immunoglobin superfamily members [63]. Specific integrins promote endothelial cell migration and survival during angiogenesis and lymphangiogenesis. Other integrins promote pro-angiogenic macrophage trafficking to tumors. Semaphorins and their receptors, neuropilins and plexins, are most commonly associated with axon guidance during development of the central nervous system. Recently, various semaphorins have been associated with the promotion or inhibition of tumor angiogenesis, metastasis and survival [64]. The role of semaphorins in drug resistance remains an active area of investigation. Browder et al. found that administration of continuous low dose chemotherapy (cyclophosphamide) to mice bearing syngeneic tumors provided a better therapeutic effect than intermittent higher doses of the same agent [65]. The improved therapeutic outcome was associated with increased tumor endothelial cell apoptosis and decreased drug resistance development by malignant cells. Thus, an angiogenesis-directed treatment regimen was more efficacious than the traditional regimen focused only on

tumor cell killing. Antiangiogenic therapy combinations with chemoradiation regimens could be beneficial for treating many cancers [66].

9. Stromal cells

Metastasis, the hallmark of malignant disease, requires numerous biological functions that enable malignant cells to disseminate from a primary tumor to establish disease in distant tissues. Gupta et al. found that epidermal growth factor receptor ligand epiregulin, cyclooxygenase 2 (COX2) and matrix metalloproteinases 1 and 2 expressed by human breast cancer cells promoted new blood vessel formation, shedding of malignant cells into circulation and breaching of lung capillaries by circulating tumor cells to initiate pulmonary metastasis [67]. Small cell lung cancer (SCLC) cells express high levels of CXCR4, the receptor for the chemokine stromal-cell-derived factor 1 (SDF-1/CXCL12). Adhesion of SCLC cells to extracellular matrix or stromal cells in the tumor microenvironment produced chemotherapy resistance via integrin signaling mechanisms [68]. Stromal cells protect SCLC cells from chemotherapy induced apoptosis. Activation of integrins and CXCR4 generated adhesion and survival signals for SCLC cells. One hypothesis is that 'cancer stem cells', a small subpopulation of self-renewing, multipotent cells, are responsible for driving tumor progression. Hypoxia has profound effects on cancer stem cells including activation of dedifferentiation pathways, stem cell identity maintenance and increased metastatic potential. A highly tumorigenic fraction of side population (SP) cells/tumor stem cells localizes in the hypoxic zones of solid tumors [69]. A highly tumorigenic SP fraction migrates to the hypoxic area similar to migration of normal bone marrow SP cells to areas of injury/hypoxia. Thus, the hypoxic microenvironment is a niche for highly tumorigenic SP cells. Aggressive melanoma and breast carcinoma express the embryonic morphogen Nodal which is essential for human embryonic stem cell (hESC) pluripotency [70]. Metastatic tumor cells do not express the inhibitor of Nodal, Lefty, and overexpress Nodal. Exposure of tumor cells to a hESC microenvironment rich in Lefty resulted in decreased expression of Nodal, decreased tumorigenesis and increased apoptosis. In preclinical models, combining agents that target the tumor microenvironment resulted in improved efficacy [71]. The combination of lenaliodomide, an immunomodulatory drug, sunitinib, a tyrosine kinase inhibitor, and low-dose cyclophosphamide comprised a highly effective regimen in a variety of human tumor xenografts suggesting that producing an inhospitable microenvironment improves outcome.

Tumor stromal inflammatory cells (lymphocytes, macrophages, mast cells) and stromal fibroblasts can make up 50–90% of a tumor mass. Tumor cells and stromal cells actively interact in the disease process. Malignant cells evolve a desmoplastic stroma that expresses an extracellular matrix including growth factors and cytokines that support tumor growth and invasion [72]. Tumor-associated stromal cells provide paracrine stimuli that further promote mesenchymal cell growth and survival. When J774 mouse macrophage were chronically exposed to severe hypoxia (<1% oxygen atmosphere) the cells increased heat-shock protein 70 kDa (HSP70), tumor necrosis factor-α (TNF-α) and nitric oxide production and exhibited death resistance to challenge with Leishmania parasite [73]. Finak et al. analyzed tumor stroma gene expression profiles (53 primary breast tumors) and derived a stroma-derived prognostic predictor (SDPP) gene signature that correlated with clinical outcome [74]. Genes in the SDPP indicated differential immune responses, angiogenic and hypoxic responses compared with normal comparator and highlighted the importance of stromal biology in tumor progression. San Francisco et al. compared fibroblasts cultured from normal human prostate and prostate carcinoma-associated fibroblasts (CAFs) [75]. TGF-β expression was higher in the prostate cancer carcinoma-associated stroma than non-malignant prostate stroma. The increased capability of CAF to form colonies in soft agar correlated with higher TGF-β expression and may explain CAF promotion prostate epithelial cell malignant progression [75]. Hu et al. examined the role of myoepithelial cells and fibroblasts in breast cancer progression using an in situ carcinoma model and found that invasion was promoted by fibroblasts and inhibited by normal myoepithelial cells [76]. Bone marrow stromal cells (BMSCs) from multiple myeloma patients produce more TGF-β than BMSCs from healthy donors [77]. Neutralization or blockade of TGF-β signaling decreased secretion of interleukin-6, a growth factor for multiple myeloma, and VEGF by BMSCs and blocked tumor cell growth triggered by adhesion of multiple myeloma cells to BMSCs.

10. Disease location and tumor growth

The organ/tissue environment in which malignant cells reside affects tumor growth and response to therapy (Fig. 1). Metastasis, orthotopic and genetically engineered models of cancer allow study of the biology and therapeutic response of tumors in varied locations bringing preclinical science into areas well known to clinical oncologists. Metastasis involves complex, redundant pathways in tumor cells and normal tissues that include survival, arrest, invasion and growth at site distant from the primary tumor [78]. Gene expression profiles of tumor cells capable of metastasis are being elucidated. When normal mouse mammary cells transfected with inducible oncogenic MYC and Kras, or polyoma middle T were injected intravenously into mice, the cells lodged in the lungs and grew nodules when the oncogenes were induced, thus demonstrating that proliferative thrust is critical for development of a mass [79]. In natural systems, the metastatic phenotype may arise by a stepwise selection process driven by hypoxia. Cell motility is increased by hypoxia-induced hepatocyte growth factor (HGF)-MET receptor signaling promoting migration toward blood and lymphatic microcirculation [80]. VEGF-induced changes in vascular integrity and permeability promote intravasation and extravasation as well as angiogenesis at the metastatic site.

11. Tumor penetration

The anticancer drug response of tumors is limited by the ability of drug molecules to penetrate through cell layers to reach cells distal from vasculature in cytotoxic concentrations [34–36,81]. Heterogeneous pH, oxygenation, and cellular proliferative status in the mass influence drug efficacy. Small cell lung cancer produces extensive extracellular matrix at primary and metastatic sites [82]. Integrin-β1-stimulated suppression of apoptosis through adhesion of small cell lung cancer cells to the extracellular matrix confers resistance to chemotherapeutic agents and enhances tumorigenicity. Gatenby and Gillies proposed a somatic evolution model of invasive cancer as a sequence of phenotypic adaptations that may be reached through multiple pathways to explain the diverse genotypes and phenotypes that comprise malignant disease [83]. The evolutionary dynamics that give rise to malignant cell phenotypic properties can be described within the context of microenvironmental selection forces.

Using an orthotopic pancreatic cancer model, Vonlaufen et al. found that pancreatic stellate cells contribute to pancreatic cancer progression by developing a very abundant stromal component [84]. Tumor–stromal interaction in a hypoxic microenvironment enhances pancreatic cancer cells invasiveness through hepatocyte growth factor (HGF)/c-Met signaling [85]. Clinical specimen

immunohistochemical data suggest that high HIF-1α and HGF/c-Met signaling indicate poor prognosis in pancreatic cancer patients; thus, targeting hypoxic tumor stroma may be a useful therapeutic approach. Gemcitabine-resistant sublines of L3.6pl and AsPC-1 human pancreatic tumor cell lines were developed by exposing the monolayers to increasing gemcitabine concentrations [86]. Gemcitabine-resistant cells were more invasive and migratory and had increased vimentin, decreased E-cadherin, nuclear localization of β-catenin, activated c-Met and increased CD24, CD44 and epithelial-specific antigen (ESA); all changes consistent with epithelial–mesenchymal transition [87].

12. Epithelial–mesenchymal transition

Epithelial–mesenchymal transition (EMT) occurs during normal development and pathological processes [88]. When previously immortalized human mammary epithelial cells were transfected with the transcription factors twist and snail, the cells expressed mesenchymal traits, stem cell markers, had an increased ability to grow as spheroids (mammospheres) and tumors in mice [89]. Human breast cancer lines genome-wide transcriptional profiling allowed identification of a sub-group that can be termed Basal B/Mesenchymal, with prominent invasive properties and a predominatly mesenchymal gene expression signature that is distinct from luminal (Luminal) or mixed basal/luminal (Basal A) sub-groups [90]. Interestingly, pancreatic and colorectal tumor cell lines insensitive to EGFR inhibition lost or mutated the epithelial junction constituents E-cadherin and γ-catenin, lost homotypic adhesion and often gained proteins associated with epithelial–mesenchymal transition such as vimentin, zeb-1, or snail providing a rationale for combining EGFR antagonists with agents that affect epithelial–mesenchymal transition for treatment of more mesenchymal-like tumors [91]. When basal gene expression profiles for clinical specimens of head and neck squamous cell carcinoma and non-small cell lung carcinoma were correlated with response to EGFR inhibitors, common markers of resistance between the two diseases included genes related to epithelial–mesenchymal transition [92]. Increased expression of vimentin, loss of E-cadherin, claudin 4 and claudin 7 as well as loss of the Ca^{2+}-independent cell–cell adhesion molecules EpCAM and TROP2 correlated with clinical resistance to EGFR inhibitors. An understanding of contextual cues and molecular mediators that control epithelial–mesenchymal transition is required to develop diagnostic tools and therapeutic agents directed toward this process [93].

In vivo resistance was characterized in cells that were non-responsive to chemotherapeutic agents when grown as tumor and were sensitive to the same agent when are grown in culture. Many proteins expressed by cells, in culture and in vivo, are pro-survival only in the tissue/host context. TGF-β is a factor in vivo therapeutic resistance that does not provide a survival benefit in monolayer culture. The human prostate cancer lines, DU-145, LNCaP and PC-3 are sensitive to melphalan, cyclophosphamide and doxorubicin in culture but are not responsive to these agents when grown as xenografts. TGF-β in the tumor-bearing mice may protect the malignant cells from the cytotoxic therapies [94]. Cancer cell adhesion confers a transient de novo drug-resistant phenotype described as cell-adhesion-mediated drug resistance (CAM-DR) [95]. When exposed to melphalan, human multiple myeloma cells adhered to fibronectin coated wells had similar DNA damage as suspension cells but were relatively protected from melphalan-induced mitochondrial perturbations and caspase activation. The EMT-6 mouse mammary tumor was used to examine the effects of sequential treatment with chemotherapy on the response of tumors [96]. After treatment with melphalan, both 7 and 12 days later, the tumor was resistant to a second drug treatment. When the interval between drug treatments was increased to 14 and 21 days, the tumors remained resistant to a second treatment with a 14-day interval but were less resistant with a 21-day interval. Thus, epigenetic changes induced by the first dose of chemotherapy persisted for some time and markedly decreased the tumor cell killing effect of a second drug dose producing acute in vivo resistance.

Although in early in the active investigation of therapeutic antiangiogenic agents, the assumption was that development of resistance of 'normal' endothelial cells to drugs would be a very rare event, data from preclinical and clinical studies show that endothelial cells under sustained stress from targeted antiangiogenic agents undergo epigenetic changes that decrease dependence on the targeted growth factor pathway or increase production of the targeted growth factor or receptor. Many strategies have been developed in an attempt to overcome or prevent the development of in vivo therapeutic resistance. One general strategy is targeting the cytotoxic molecule to the tumor via a carrier molecule that is expressed selectively in the tumor mass either by the malignant cells or in the extracellular matrix. Wu et al. found that targeting cell-impermeable prodrug activation to proteases selectively expressed in tumor microenvironment reduced toxicity to normal tissues [97]. The activated prodrug was available to tumor cells and stromal cells as a 'bystander effect'. Hypoxia-inducible factors (HIFs) and proto-oncogene c-Myc regulate complex adaptations by tumor cells in hypoxia. These transcription factors reprogram metabolism, protein synthesis and cell cycle progression [98].

13. Conclusions

For many years the study of resistance to anticancer agents was carried out primarily using cells grown in culture. Frequently, the mechanisms by which the isolated populations of malignant cells developed resistance did not seem to be recapitulated in an important way in vivo and did not translate into new clinical strategies. Since that time a view of malignancy as a disease process that requires host involvement has moved the field to examine the role of varied cell types, secreted and matrix factors, physical conditions and tumor location in drug response and resistance. Pharmacologic changes in the host and tumor such as drug metabolism through induction of enzymes in the liver and tumor, drug tissue distribution and drug accumulation and retention in the tumor are important factors in response of the malignant disease to therapy. Changes in drug metabolism are induced by the therapeutic and can be epigenetic or genetic.

The epigenetic phenomenon of acute in vivo resistance is demonstrated by the decreased response of a tumor to subsequent chemotherapy doses whether the same agent administered repeatedly or a different agent. Epigenetic changes regulate molecules involved in apoptotic cell death control, thus survival-promoting mechanisms prevent drug-induced cell death resulting in pleiotropic drug resistance. A more stable resistance was observed when tumors were exposed to several doses of the same drug over a period of months. Clearly, malignant cells are in intimate communication with their microenvironment and with distant tissues in the host [99]. Transplantable tumor models have added knowledge of solid tumor physiology allowing elucidation of the high degree of heterogeneity in the environment in which tumor cells survive in vivo. However, much of the therapeutic response resistance research performed with transplantable tumor has focused on alternations in the tumor cells in isolation rather than on the tumor as a tissue interacting with involved host normal cells and distal normal cells [34–36,58,99]. Amongst imaging techniques, positron-emission tomography is most readily able to image drug resistance and is being more widely applied

preclinically to examine mechanisms of drug resistance even prior to initial clinical trial of new agents [100].

Some early in vivo investigators regarded drug resistance under-treatment of the disease. In the clinic this led to the development of high-dose chemotherapy regimens requiring bone marrow transplant, a strategy that has been widely explored and has been adopted in some diseases [101]. Frequently, cancer is not cured by anticancer therapies because the disease becomes less responsive to the therapy and/or their normal tissues reach a limit of tolerance to the therapy. One interpretation is that the malignant disease becomes tolerant to the cytotoxic therapy. In vivo a survival advantage to repeated cytotoxic insults may be achieved by the induction of factors that are operative in complex tissues and require normal cells [34]. Therapeutic resistance of a tumor in a host can evolve by phenotypic change in the tumor cells that does not confer resistance on isolated tumor cells but which alters drug handling by the host. Although a much broader spectrum of anticancer drugs can be envisioned addressing malignant, stromal, vascular, secreted proteins and extracellular matrix targets, cancer drug discovery remains an extreme therapeutic index challenge [102]. Near-term, personalized medicine approaches in oncology will remain focused primarily on targets expressed by the malignant cells [103].

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