Targeting the tumor microenvironment

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TABLE OF CONTENTS

1. Abstract

Despite some notable successes cancer remains, for the most part, a seemingly intractable problem. There is, however, a growing appreciation that targeting the tumor epithelium in isolation is not sufficient as there is an intricate mutually sustaining synergy between the tumor epithelial cells and their surrounding stroma. As the details of this dialogue emerge, new therapeutic targets have been proposed. The FDA has already approved drugs targeting microenvironmental components such as VEGF and aromatase and many more agents are in the pipeline. In this article, we describe some of the "druggable" targets and processes within the tumor microenvironment and review the approaches being taken to disrupt these interactions.

2. Introduction

Cancer was long viewed as a cell-autonomous process, in which successive acquisition of mutations in oncogenes and tumor suppressor genes resulted in progressively enhanced proliferation and resistance to cell death. This is now widely viewed as an oversimplification and the recognition that additional cell types and the soluble factors they produce make a vital contribution to epithelial tumorigenesis.

Carcinomas are complex organs (1) consisting of tumor epithelial cells, in a microenvironment rich in multiple non-malignant, albeit altered, cell types – fibroblasts, endothelial cells and leukocytes – all of which interact, either physically or via the secretion of paracrine signaling molecules. In this respect, the tumor organ is no different from all of the other organs, the form and function of which are the product of an exquisite dialogue between the epithelial and stromal cells, and an understanding of the processes driving its biogenesis should prove to be the making of its undoing.

While this paradigm shift has caused a fundamental reevaluation of how cancer biologists think about tumorigenesis, it is really "old news" for developmental biologists who, for almost a century, have realized that cues from adjacent cell types can induce important changes in cell fate during development. Here, the intricate cross-talk of soluble factors between epithelial and mesenchymal cells plays a vital role in tissue morphogenesis and function. This field was first illuminated by the work of Spemann and Mangold in salamanders (2), which showed that ectopically engrafted tissue could modulate the differentiation of adjacent structures. Later, co-culture experiments were used to define some of the reciprocal interactions between epithelium and stroma which are necessary for the induction of the differentiated state (3, 4), and these principles have been shown to hold true in many organs, including the mammary gland, kidney and prostate (for reviews see 5, 6, 7).

A broad appreciation of the importance of heterotypic cell-cell interactions in tumor induction and progression lagged considerably behind, despite the intermittent publication of compelling papers on this subject. The demonstration by Judah Folkman (8) that tumors had the power to stimulate the growth of new blood vessels provided further convincing evidence that tumorigenesis could not proceed without active cooperation from non-malignant cells. DeCosse and coworkers showed that a mammary tumor could be induced to differentiate by co-culture with normal mammary stroma (9). Leland Chung and his colleagues showed that co-injection of

either normal or transformed fibroblasts could enhance the tumorigenicity of several different human cancer cell lines in xenograft experiments (10). Similarly, Cunha and co-workers showed that fibroblasts from reactive stromal regions of tumors had the capacity to transform otherwise non-malignant prostate epithelial cells (7, 11). More recently, evidence is emerging that mutations in proto-oncogenes and tumor suppressor genes (12) as well as distinctive epigenetic changes (13) are not always restricted to the tumor epithelium and that clones of altered stromal cells been observed further supports the contention that tumor-stromal co-evolution can be an important driver of tumorigenesis.

It is also clear that only a proportion of familial cancers are explained by the inheritance of strongly predisposing mutant alleles, such as BRCA1, BRCA2 and APC. A significant fraction of the remaining familial component is likely to arise from the contribution of lower penetrance alleles which, while having weak effects alone, may lower the threshold at which tumorigenesis is likely to occur. Interestingly, SNPs altering the expression level or activity of a number of microenvironmental proteins have been reported to predispose to cancer (reviewed in 14).

Following up from Beatrice Mintz's demonstration that transformed embryonal carcinoma cells injected into blastocysts could contributed to tumor-free tissues in adult mice (15), work in the 1980's from our laboratory showed that expression of the viral Src oncogene did not result in cellular transformation in the developing chick embryo, yet dissociated cells were demonstrably transformed in culture. These studies led to the realization that even expression of a potent oncogene was not sufficient for transformation and that additional factors, such as disruption of tissue architecture by wounding, may be necessary (16, 17). These studies were consistent with the ideas of Dvorak on the similarities between tumors and wounds (18) and also the growing appreciation of the relationships between inflammation and tumorigenesis (see below). In the chick model, TGF-beta was implicated as a key mediator in this process (19) a finding which conflicted with the then dominant theory of TGF-beta as a proliferation suppressor; subsequent studies established that disruption of the microenvironment by excessive production of matrix metalloproteinases (MMPs) can lead to genomic instability, epithelial-to-mesenchymal transition and malignant transformation of mammalian cells *in vitro* and *in vivo* (20-22).

Collectively, these studies established that heterotypic cell-cell interactions were critical to tumor induction and progression, but the identity of many of the molecular mediators of this communication remained to be determined. In the past few years, considerable progress has been made toward identifying some of the key signaling processes which underlie epithelial-stromal interactions. This newfound understanding has opened the way to the design of rational therapies which target the microenvironment of the tumor, as a means of destroying the tumor itself.

3. Heterotypic cell signaling mediators

The extent to which soluble factors which are secreted by one cell type and act by binding receptors on other cell types influence the malignant process is now widely appreciated. This has provided a profusion of therapeutic leads, some of which are already approved drugs and many more of which are in the pipeline. One example of a signaling molecule targeted in this way is tumor necrosis factor alpha (TNF-alpha) which, although originally believed to have predominantly anti-tumor activity, has more recently been shown to exert many pro-tumorigenic functions (23). One approach has led to the generation of an anti-TNF-alpha monoclonal antibody, Infliximab, which is approved in the US for the treatment of Crohn disease and ulcerative colitis, both of which predispose to colorectal cancer. An alternative strategy, to build a decoy receptor which will sequester soluble TNF-alpha is marketed as Etanercept and is approved for the treatment of rheumatoid arthritis. Monoclonal antibodies against TNF-alpha have shown efficacy in a series of preclinical models (23) and both of these drugs are in clinical trials to test efficacy in cancer. This paradigm of using decoy receptors and blocking antibodies is a recurrent theme in microenvironment-directed therapies.

Cells of the immune system have long been observed in association with tumors (24). This was thought to reflect the body's response to the tumor organ, but several studies have now shown that such lymphocytic infiltrates actually correlate with poor prognosis (25). Tumor cells secrete a series of chemokines which actively promote macrophage recruitment (26, 27). Pollard and co-workers have delineated an important feedback loop by which tumors recruit cells of the immune system, which then provide the tumors with mitogenic stimuli. CSF1, produced by tumor cells is a potent chemoattractant for macrophages. Macrophages are rich in growth factors, such as EGF, and proteases, which can promote tumor cell proliferation, angiogenesis and matrix remodeling, all of which can facilitate progression (28). Treatments which have shown promise

against tumor-associated macrophages include Trabectedin (29) and a blocking antibody against CSF1 which has shown significant efficacy in a breast cancer xenograft model (30).

Instead of targeting macrophages, another exciting approach has been the proposal to use macrophages (or, more likely their monocyte precursors) as vehicles for gene therapy (27). This builds on the approach pioneered by Rosenberg of gene transfer into tumor infiltrating lymphocytes (31). Because they accumulate preferentially in hypoxic areas of tumors, macrophages may prove an effective means of drug delivery to regions of the tumor which are difficult to target by other means and which are believed to contain cells which are more resistant to chemotherapies. As proof of principle, it was shown that macrophages transduced with CYP2B6 could infiltrate tumor spheroids grown *in vitro* and activate cyclophosphamide, resulting in death of adjacent tumor cells (32). Similarly, Dubinett, Sharma and colleagues demonstrated that introducing dendritic cells transduced with CCL21 into the tumor microenvironment in a transgenic mouse model of broncheoalveolar cell carcinoma results in the recruitment of many endogenous dendritic cells, T-lymphocytes and natural killer cells, which significantly extended the survival of the mice (33). Vinel and co-workers have shown efficacy of fibroblasts transduced with IL-12 in mouse models of pancreatic and hepatocellular carcinoma (34, 35).

Transforming growth factor beta (TGF-beta) has complex autocrine and paracrine roles in tumor progression. This factor is a potent inhibitor of epithelial cell growth, but sensitivity to these effects of TGF-beta is frequently lost during tumor progression and, in later stages, TGF-beta signaling has pro-oncogenic functions which is reflected by the poor prognosis of tumors of many tissues which overexpress TGF-beta (36). Compelling evidence for a role of this pathway in stromal-epithelial crosstalk emerged from the work of Moses and colleagues, who showed that deletion of the TGF-beta type II receptor in stromal fibroblasts resulted in the transformation of adjacent epithelia in the prostate and forestomach (37). Fibroblasts from these animals have upregulated expression of growth factors and increased the proliferation of mammary cancer cells when co-injected beneath the kidney capsule (38). Existing strategies to target this pathway include small molecule inhibitors of the receptors, and antibodies and decoy receptors which prevent ligand-receptor interactions (39).

4. Nonsteroidal anti-inflammatory drugs (NSAIDs)

As many as 1 in 6 epithelial cancers are believed to arise at sites of inflammation (40, 41), suggesting that the disruption of tissue architecture, the influx of immune cells and the tissue repair processes might allow pre-existing neoplastic cells to escape the constraints imposed on them by their microenvironment. Large observational studies of hundreds of thousands of individuals have associated frequent aspirin use with a significantly reduced incidence of colorectal cancer (42, 43), and evidence is also increasing that NSAID use may help in the chemoprevention of cancers of the breast, ovary, prostate and lung (44, and references therin). NSAIDs suppress the activity of the cyclooxygenases, COX1 and COX2, the latter being the primary inducible form which produces prostaglandins in inflammation and in tumors. Prostaglandins activate a diverse array of signaling pathways with pro-tumorigenic roles (45).

5. Angiogenesis inhibitors

The recruitment of a vasculature to supply blood and oxygen is an essential transition which must occur if a tumor is to grow larger than a couple of mm^3, which is the diffusion limit for oxygen *in vivo*. Angiogenesis is regulated by a delicate balance between a series of pro- and anti-angiogenic factors. Because the vasculature is genomically stable, it was hypothesized that targeting this key aspect of the malignant phenotype might prove effective therapeutically while having less risk of developing somatically acquired resistance. Several angiogenesis inhibitors are in development and some have already been approved. Bevacizumab (Avastin^{TM}), a humanized monoclonal anti-VEGF antibody has been approved in combination with 5-Fluorouracil for the treatment of metastatic colorectal cancer (46), and following this validation many other anti-VEGF agents are at various stages of development (47). The arsenal of anti-angiogenic drugs has been further expanded following the realization that the receptor tyrosine kinases which are activated by these ligands are also inhibited by existing drugs. BAY 43-9006, a Raf inhibitor, also potently inhibits VEGFR2 and VEGFR3 (48). Gleevec, which is approved for inhibition of cKIT in gastrointestinal stromal tumors and BCR-ABL in chronic myelogenous leukemia, is also a potent inhibitor of the PDGF Receptor (49). Targeting PDGFR on pericytes, with consequent disruption of the tumor vasculature, may prove an unforeseen bonus of using Gleevec, and may indeed contribute to its effectiveness *in vivo*. In some cases, it seems, the "off-target" effects of a drug might be beneficial!

The new interest in angiogenesis has led also to the rehabilitation of an older drug, Thalidomide, which was removed from sale in 1962 when it was realized it caused birth defects in children. Thalidomide is a potent inhibitor of angiogenesis (50), and clinical efficacy has been demonstrated in multiple myeloma (51). In 2005 it was approved in Australia for the treatment of this disease, and the US FDA followed suit in 2006.

As our understanding of the role of the extracellular matrix in the maintenance of tissue homeostasis has improved, it has been realized that many extracellular matrix proteins possess cryptic fragments which, when released by cleavage, can exert potent biological effects. Examples of such fragments include Canstatin and Tumstatin (from Collagen IV), endostatin (from Collagen XVIII), Angiostatin (from plasminogen) and Restin (from Collagen XV), all of which exert antiangiogenic effects (52). Intriguingly, Collagen XVIII, from which Endostatin is derived, is located on Chromosome 21, the chromosome of which three copies are found in individuals with Down syndrome. When controlled for age, individuals with Down syndrome develop epithelial cancers at one tenth of the rate of unaffected individuals (53). Transgenic mice engineered to overexpress endostatin experience a significant reduction in the rate of tumor growth (54). In clinical trials (55, 56), recombinant human endostatin failed to live up to the dramatic promise it showed in earlier pre-clinical studies (57), however a new more soluble variant called Endostar (58) was approved in China in 2005 (59) and we eagerly await the further evaluation of this peptide in additional studies.

6. Aromatase inhibitors

For ERα-positive breast cancers, estrogen provides a crucial mitogenic cue and estrogen receptor antagonists, such as tamoxifen, have had a very significant impact in the clinic. Estradiol is synthesized in the ovaries and at extragonadal sites, with extragonadal synthesis being dominant after the menopause. In postmenopausal women with estrogen-dependent breast cancer, estrogen produced locally within the breast stromal fibroblasts and adipocytes is the primary source of this factor (60, 61). Aromatase in these cells is a key enzyme in estradiol biosynthesis and so represents an excellent target for microenvironment targeted therapy. This is a clear example of a crucial growth factor being synthesized in the breast tumor microenvironment by a “druggable” target. The third-generation aromatase inhibitors – anastrazole, letrozole and exemesthane – can inhibit whole body aromatization by up to 98%.

The aromatase inhibitors have been shown to be even more effective than tamoxifen at preventing breast cancer recurrence in post-menopausal women (62). This remains, to our knowledge, the most successful example yet of a therapy targeted at a component in the microenvironment resulting in strong efficacy against tumors in a clinical setting.

7. Bisphosphonates

More than a century ago, Paget observed that tumors of different tissues exhibited an apparent preference in the distant sites at which metastases were established, likening tumor cells to seeds which could only flourish if they fall on a congenial soil (63). Metastasis to bone is a frequent event in advanced cancer, leading to pain, a loss of bone mineral density and an increased susceptibility to fractures. The microenvironment of the bone is particularly hospitable to the cells of prostate, breast and renal tumors and multiple myeloma, all of which can adapt very well to this niche. Bisphosphonates, such as Zoledronic Acid, Ibandronate, Pamidronate and Clodronate, achieve high local concentrations by binding to bone hydroxyapatite, leading to loss of osteoclasts and, consequently, an attenuation of bone resorption. These pyrophosphate analogs are effective inhibitors of the mevalonate pathway resulting in a defect in the prenylation of signaling proteins, leading to osteoclast apoptosis. Inhibition of the same pathway by Zoledronic Acid in tumor cells might also elicit direct anti-tumor effects (64).

Zoledronic acid has shown considerable promise in the treatment of tumors metastasizing to bone (reviewed in 65). For example, if treatment is started when bone loss due to multiple myeloma is first detected, this significantly reduces the incidence and delays the time of onset of skeletal complications. Preliminary data from a number of trials using bisphosphonates less potent than Zoledronic Acid (reviewed in 66) suggest that this treatment may also prevent breast cancer metastasis to bone. A number of trials are now underway to test the more potent compound in the adjuvant setting.

8. Conclusions

We are now coming to the appreciation that the complexities of cancer will rarely be solved by single agents and that rational combinations of targeted therapies chosen on the basis of the particular lesions in individual tumors will be necessary. The scientific arguments for this are clear and the moral arguments are compelling. It is frustrating, however, that the financial

interests of pharmaceutical companies are often at odds with the interests of patients, and that the legal and regulatory barriers to testing combinations of patented drugs in large trials are depressingly prohibitive (67). Nevertheless, we remain greatly encouraged that so many colleagues now appreciate the importance of targeting the tumor microenvironment and, as our collective understanding of the complexities of tumor ecology deepens, we hope that patients will soon benefit from drug combinations targeting the whole tumor organ and not just its epithelial component.

9. Acknowledgements

The work in the authors' laboratory is supported by grants from the U.S. Department of Energy (DE-AC03-76SF00098 and a Distinguished Fellow Award), the NIH (CA64786; and CA57621 to Zena Werb and MJB), and by an Innovator award from the U.S. Department of Defense Breast Cancer Research Program.

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Table 1 Microenvironmental processes targeted by existing and developing treatments

Microenvironmental target	**Therapeutic agent**
Estrogen biosynthesis	Aromatase inhibitors (anastrozole, letrozole and exemesthane)
Macrophage-Tumor interactions	Anti-CSF1 monoclonal antibody
	Trabectedin
Prostaglandin production	Cox2 inhibitors (e.g. Non-steroidal anti-inflammatory drugs)
Metastatic microenvironment in bone	Osteoclast targeting agents (e.g. zoledronic acid, clodronate)
Stromal-epithelial interaction	TGF-beta inhibitors (e.g. SB-431542, LY580276, Lerdelimumab)
	TNF-alpha inhibitors (e.g. Etanercept, Infliximab)
Angiogenesis	Anti-VEGF monoclonal antibody (Bevacizumab)
	VEGF Receptor inhibitors (e.g. BAY-43-9006)
	PDGF Receptor inhibitor (e.g. Gleevec)
	Pro-angiogenic integrins inhibitors (e.g. endostatin and other ECM fragments)
	Thalidomide (several possible mechanisms)

Abbreviations: CSF: Colony stimulating factor, NSAID: Non-steroidal anti-inflammatory drug, PDGF: Platelet derived growth factor, SNP: Single nucleotide polymorphism, TGF: Transforming growth factor, TNF: Tumor Necrosis Factor, VEGF: Vascular endothelial growth factor

Keywords: Tumor microenvironment, cancer, cancer therapy, stromal-epithelial interaction

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Running Title: Targeting the tumor microenvironment