Fighting Fire with Fire in Cancer

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Abstract Cancer will not be cured until we understand and target the unique alterations that distinguish tumor cells from normal cells. This chapter briefly describes four new approaches to anticancer therapy based on boosting the immune system's response to tumor cells, countering the metabolic adaptations that allow tumor cells to thrive under conditions that kill normal cells, manipulating the increased oxidative stress associated with the tumor environment, and exploiting the aneuploidy characteristic of many advanced tumor cells. The long-term goal is to devise biomarkers and novel therapeutic agents able to more effectively fight aggressive cancers.

Keywords Warburg effect • Metabolism • CTLA-4 • IL-7 • CPT1C • IDH • BRCA

Introduction

Why has it been so difficult to cure cancer? The answer lies in our inability to define all of the elements that contribute to the transformation and survival of tumor cells, and challenges in dissecting the body's responses to these malignant growths. As far back as 1924, Otto Warburg proposed that "The cause of cancer is the replacement of the respiration of oxygen in normal body cells by a fermentation of sugar," a concept now widely known as the "Warburg effect" [1, 2]. Because this change would not alter the "antigenic face" of a cancer cell, the immune system would not be able to recognize these aberrant cells and remove them, necessitating externally imposed therapies. Oncologists of the day therefore focused on eliminating all fast-replicating cells, normal or cancerous, by radiation or chemotherapy, a trend that remained firmly in place into the 1990s (Table 1).

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Table 1 Chemotherapeutic agents approved

Year approved	Agent
1942	Nitrogen mustard
1948	6-Mercaptopurine
1958	Methotrexate
1959	Cyclophosphamide
1975	5-Fluorouracil
1978	Cisplatin
1992	Paclitaxel
1996	Gemcitabine
1996	Topotecan
2004	Pemetrexed

Table 2 Targeted agents approved

Year of approval	Target	Drug name
1998	Her-2	Herceptin
2001	Bcr-abl	Imatinib
2003	EGFR	Gefitinib
2003	Proteasome	Bortezomib
2004	VEGF	Bevacizumab
2006	HDAC	Vorinostat
2007	mTOR	Temsirolimus
2011	CTLA-4	Ipilimumab
2011	ALK	Crizotinib
2011	B-Raf	Vemurafenib
2012	Hedgehog	Vismodegib
2013	Btk	Ibrutinib

In a talk at a 1966 Nobel Laureates' meeting, Warburg reiterated his belief that cancer cells ultimately arise when a switch from normal respiration to fermentation is caused by (for example) damage to the enzymatic machinery required for respiration (see http://www.mediatheque.lindau-nobel.org/videos/31517/on-the-primary-causes-and-on-the-secondary-causes-of-cancer-german-presentation-1966/laureate-warburg). However, in the 1970s, Warburg's hypothesis was again sidelined as scientists became convinced that the underlying cause of cancer was aberrant function of either oncogenes or tumor suppressor genes. After the discovery of Src in 1976 [3], a multitude of other oncogenes were identified, including EGFR, Her2, Abl, Raf, Alk, Btk, PI3'K, Tor, and many others. This focus on a genetic origin for cancer initiation was then reflected in the development of anticancer therapeutics. Since 1998, numerous therapeutic agents targeting specific oncogenes or other relevant molecules or structures have been approved (Table 2).

Although anti-oncogene agents have proven undeniably helpful, it has unfortunately become clear that the cancer cell genome is too varied and oncogenes are too numerous for these strategies to be able to eradicate all tumors. As Robert Weinberg

said, “There are more paths to developing tumors than there are stars in the sky.” Pathways now known to be deregulated in cancer include the Hedgehog, Wnt/Notch, TGFβR, FAS, and VEGFR pathways, as well as those involving receptor tyrosine kinases [4, 5]. Concomitantly, the development of new chemotherapeutics has slowed down significantly, with no new agent being approved in the last 10 years. Researchers have instead returned to Warburg’s hypothesis and are exploring ways of disrupting tumor cell metabolism and mitosis, as well as seeking means of boosting anticancer immune responses. The long-term goal is to develop novel anticancer therapeutics that differ from existing classes of agents and so may be more effective. In the following sections, we will describe our work on four novel avenues that show promise as future anticancer strategies: (1) boosting the immune system; (2) targeting cancer cell metabolism; (3) targeting reactive oxygen species; and (4) exploiting cancer cell aneuploidy.

Boosting the Immune System

The objective of immunotherapy is to strengthen the body’s ability to recognize and attack tumor cells—that is, fight the fire of cancer with the fire of an aggressive immune response. Activation of the T lymphocytes that underpin any adaptive immune response requires two stimuli: (1) binding of the T cell receptor to a complex of antigenic peptide plus MHC (pMHC), which delivers an antigen-specific signal; and (2) binding of CD28, a receptor expressed on the T cell surface, to CD80 or CD86 ligands expressed on the surface of antigen-presenting cells (APCs), which delivers a co-stimulatory signal. In 1995, our group reported that T cell activation is negatively regulated by cytotoxic T lymphocyte antigen-4 (CTLA-4) in a manner vital for the control of lymphocyte homeostasis [6]. Subsequent work demonstrated that CTLA-4 exerts its inhibitory activity by binding to CD80 or CD86, thereby preventing the binding of CD28 to these surface proteins and blocking the co-stimulation needed for optimal T cell activation [7]. Several other negative regulators of T cell activation, including PD1, have since been discovered [8, 9].

There are multiple reasons why the immune system is often unable to completely eradicate a cancer without help from targeted therapeutics. First, there are very few truly tumor-specific antigens, since healthy and malignant tissues are identical in most of their components. Second, the negative homeostatic regulation imposed by CTLA-4 and PD1 decreases the activation of antitumor T cells. Third, like chronic viral infections, cancers often induce T cell exhaustion, in which hyperactivated T cell clones display specific profiles of transcription factor and inhibitory receptor expression that eventually suppress the antitumor response [10]. Fourth, the general lack of co-stimulation characteristic of malignant tissue prevents the activation of antitumor T cells [11]. Fifth, cancers impede the homing of naïve T lymphocytes into tumor-draining lymph nodes, decreasing the probability of the rare interaction between T cells and APCs displaying the relevant pMHC complex [12]. This plethora of difficulties indicates that strengthening the antitumor response in vivo will

require a battery of complementary approaches. Accordingly, immunotherapies used to treat cancer patients have been devised that are antibody based, cytokine based, or cell based. In the following sections, we discuss some exciting results based on the first two approaches.

Targeting CTLA-4

Because CTLA-4 is a negative regulator of T cell activation, agents have been designed to block CTLA-4 and thereby hopefully sustain antitumor T cell responses. In a phase 3 study of a human anti-CTLA-4 antibody (ipilimumab), a cohort of melanoma patients treated with this agent gained a significant survival advantage [13]. Suppression of the inhibitory signals generated by CTLA-4 apparently allowed prolonged activation of antitumor T cells. The success of this trial has led to renewed efforts to determine why antitumor $CD8^+$ T cells do not routinely attack and kill cancer cells on their own, and how their activities can be boosted.

IL-7 Treatment

Several groups have attempted to strengthen antitumor T cell responses in vivo by vaccination with tumor antigens, but clinical trials of this approach have not shown the expected efficacy [14]. In searching for ways to improve this result, our group discovered that vaccine-induced immune responses to tumors could be augmented and sustained by providing exogenous IL-7 [15]. In this study, we employed the RIP-TAG2 transgenic mouse model, in which mice expressing the SV40 large T antigen (TAG) under the control of the rat insulin promoter (RIP) develop pancreatic β-islet cell tumors [16]. We crossed these mice with transgenic mice expressing the lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) under the control of RIP [17] to produce RIP(GP x TAG2) mice. Tumors arising in these mice were not cleared by the immune system even though they expressed the foreign GP antigen [18]. Even after LCMV infection, which mimics administration of a live antitumor vaccine, only a limited increase in overall mouse survival occurred and no sustained antitumor response was observed [18]. However, if IL-7 treatment of LCMV-vaccinated tumor-bearing RIP(GP x TAG2) mice was initiated at 8 days after LCMV infection—a point that coincides with the peak of the $CD8^+$ T cell response—the virus was eliminated, and mouse survival was prolonged by over 100 days [11, 15].

Subsequent work focused on determining how IL-7 can overcome immune inhibitory networks during a chronic viral infection, which mimics the continuous production of a tumor antigen by a cancer [19]. Although IL-7 treatment had already shown significant therapeutic promise [20–22], and had been successfully used in several nonhuman primate SIV infection models [23–25], the efficacy of IL-7 in

promoting viral clearance had not been fully explored [19]. We administered IL-7 to mice infected with LCMV clone 13, which establishes a chronic infection and generates massive viral antigen levels [26]. In part by enhancing thymic output, IL-7 treatment of LCMV-13-infected mice increased both the magnitude of the immune response and the size of the entire naive T cell pool, including T cell clones directed against non-LCMV epitopes [19]. LCMV-13-specific $CD8^+$ T cells showed enhanced degranulation kinetics and cytokine production upon IL-7 exposure, resulting in PD-1 downregulation on effector T cells and efficient viral clearance [19]. IL-7 treatment also induced a cytokine milieu favoring leukocyte activation and production of the cytoprotective cytokine IL-22 [19]. At the molecular level, IL-7 led to a reduction in Socs3 expression in T cells that was FoxO dependent [19]. These model system data indicate that a combination of IL-7 and a cancer vaccine may result in an antitumor response capable of benefitting cancer patients.

Targeting Cancer Cell Metabolism

"Oncogene addiction" is a concept devised to explain the observation that the inhibition of crucial oncogenes, or the reconstitution of previously lost or repressed tumor suppressor genes, can have a broad antitumorigenic effect. However, in addition to their genetic and epigenetic alterations, cells undergoing transformation implement specific metabolic adaptations that are induced by their altered microenvironment. These adaptations lead to upregulation of the stress response and other pathways that are not inherently tumorigenic but allow developing tumor cells to survive under conditions that would kill normal cells [27]. This "metabolic addiction" of pre-cancerous and ultimately cancerous cells provides new opportunities for specific therapeutic intervention, since normal cells, which have not had to endure the same constant internal and external stress, should be unaffected by agents targeting cancer cell metabolic adaptations.

Targeting Carnitine Palmitoyltransferase-1C

Our group has discovered that carnitine palmitoyltransferase-1C (CPT1C), a brain-specific metabolic enzyme, may be involved in tumor cell metabolic adaptation to heightened environmental stress [28]. Expression of CPT1C, but not the ubiquitous CPT1A or heart-specific CPT1B, correlated inversely with mTOR pathway activation in tumor cells, indicating that CPT1C may act in a pathway parallel to mTOR-enhanced glycolysis [28, 29]. CPT1C contributes to rapamycin resistance in murine primary tumors and is overexpressed in human non-small-cell lung carcinomas (NSCLC) [28]. CPT1C overexpression in a human cancer cell line led to increased fatty acid oxidation and ATP production, and resistance to glucose deprivation or hypoxia [28]. Importantly, siRNA-mediated depletion of CPT1C reduced tumor

growth in an in vivo xenograft mouse model [28], and delayed tumor development and increased mouse survival in a neurofibromatosis type I tumor model [30–32]. Subsequent studies have established that CPT1C is a p53 target gene, and that CPT1C expression is induced by metabolic stress factors such as hypoxia and glucose deprivation in a p53- and AMPK-dependent manner [32]. These results indicate that p53 initially protects cells from metabolic stress via induction of CPT1C but that excessive CPT1C expression can promote carcinogenesis [32]. Because CPT1C expression is normally restricted to the brain, and most drugs cannot penetrate the blood–brain barrier, CPT1C may be an ideal candidate for specific small-molecule inhibition as a treatment for hypoxic and otherwise treatment-resistant cancers [29, 32].

Targeting Mutated Isocitrate Dehydrogenases

Isocitrate dehydrogenase (IDH)-1 and IDH2 are metabolic enzymes that govern the important NADP/NADPH ratio in the cytoplasm and mitochondria, respectively. Oncogenic mutations to these enzymes have recently garnered much interest since their discovery during cancer genome sequencing projects [33–35]. The normal function of IDH1/2 is to convert isocitrate to α-ketoglutarate (αKG) while reducing NADP to NADPH and liberating CO_2 [36]. To date, mutations of IDH1 altering a single arginine residue (R132) in the enzymatic active site have been found at high frequency in glioblastoma multiforme (GBM) [34], acute myeloid leukemia (AML) [33], cholangiocarcinoma [37, 38], and chondrosarcoma [39]. IDH1 R132 mutations occur less frequently in other types of cancers such as melanoma, NSCLC, and prostate and colon cancers [40]. IDH2 mutations, predominantly R172K and R140Q [36, 41], have been identified in cholangiocarcinoma [37, 38], myelodysplastic syndrome (MDS) and myeloproliferative disorder (MPD) [42–44], AML [33], chondrosarcoma [39], angioimmunoblastic T cell lymphoma (AITL) [45], and D2HG aciduria [46].

In 2009, scientists at Agios Pharmaceuticals used a metabolite profiling strategy to make the breakthrough discovery that the tumorigenic effect of IDH1/2 mutations is not due to a loss of function of these proteins. Instead, the mutant IDH enzymes acquire a neomorphic activity in which the normal product αKG is converted to 2-hydroxyglutarate (D2HG) in a reaction that consumes, rather than produces, NADPH [36, 47].

To examine the pathophysiological consequences of IDH mutations in the most relevant in vivo system possible, our group generated a conditional knock-in mouse model using the lox-stop-lox (LSL) system. In the absence of Cre recombinase (Cre), neither the LSL IDH1 R132 mutant allele nor the wild type IDH allele is expressed, but when Cre is present, a stop codon is excised and the mutant IDH1 protein is expressed from the endogenous locus [48]. Initial characterization of various mouse strains revealed that IDH1 knockout mice were viable and fertile but that expression of the mutant IDH1 enzyme and its consequent D2HG production were

embryonic lethal [36]. Mutant IDH1 enzyme expressed solely in the myeloid compartment (LysM promoter) resulted in splenomegaly, decreased bone marrow cellularity, and extramedullary hematopoiesis by age 6 months [48]. LysM IDH1 knock-in LSK cells showed an increase in highly methylated CpG sites and histone hypermethylation [48], consistent with the DNA methylation changes observed in human IDH1- or IDH2-mutant gliomas [49] and AML [50].

The available data indicate that mutant IDH enzymes exert their tumor-promoting function through their novel enzymatic activity, which generates massive quantities of D2HG. Mechanistic studies of D2HG have focused on its competitive inhibition of 2-OG-dependent dioxygenases (2OGD), which use αKG as a cosubstrate [36]. In mammalian cells, there are more than 60 2OGD involved in collagen biosynthesis, fatty acid metabolism, DNA repair, RNA and chromatin modifications, and hypoxia detection [51]. The general enzymatic reaction performed by 2OGD converts αKG to succinate and CO_2, and requires oxygen, ascorbate, and iron as cofactors [51]. D2HG has been shown in vitro to competitively inhibit 2OGD [52], and the high concentration of D2HG measured in cells and tissues of IDH-mutant tumors makes it very likely that D2HG impairs the activity of this class of enzymes in vivo as well [36]. Additional potential targets of D2HG inhibition include the TET proteins involved in DNA methylation, the JumonjiC domain-containing histone demethylases, the prolyl hydroxylases (PHD) and lysyl hydroxylases (LHD) required for collagen folding and maturation, and the PHDs that regulate hypoxia-inducible factor (HIF) signaling [36].

In early 2014, an oral, potent, reversible, and selective inhibitor (AG-221) of the mutated IDH2 protein underwent evaluation in a clinical trial of patients with advanced IDH2-mutant hematologic malignancies. Encouragingly, AG-221 treatment reduced D2HG levels and demonstrated a dose-dependent survival benefit [53]. Pursuit of a similar compound to combat IDH1-mutant cancers is ongoing.

Targeting Reactive Oxygen Species

An important cellular stress factor increased in cancer cells is the level of reactive oxygen species (ROS). ROS regulation is critical for normal cellular functions and survival, and the accelerated growth of tumor cells generates increased ROS. Cancers therefore need to adjust signaling pathways linked to ROS regulation to cope with their enhanced ROS. Elevated ROS are generated by hypoxia, defective metabolism, endoplasmic reticulum (ER) stress, and oncogene activity [54]. Conversely, ROS are eliminated routinely via NADPH, glutathione, and dietary antioxidants, and under stress conditions through the activation of transcription factors such as NRF2 and the activity of tumor suppressors such as BRCA1, p53, PTEN, and ATM [54]. During carcinogenic progression from normal tissue to neoplastic transformation to carcinoma in situ and finally to invasive carcinoma, cellular ROS levels progressively increase because of metabolic aberrations acquired following transformation. Unlike normal cells, cancer cells can cope with this inexorable rise

in ROS by upregulating the above antioxidant pathways and transiently lowering ROS levels. Targeted therapeutics that interfere with this upregulation may therefore result in ROS increasing to the point where the apoptotic death of the cancer cell is induced. Alternatively, agents that increase ROS production beyond the capacity of the upregulated antioxidant mechanisms to cope may kill the tumor cell while sparing normal cells in which these pathways are not activated.

We recently explored whether altered ROS regulation could explain the tissue specificity of BRCA1-related cancers, which occur almost exclusively in the breast and ovary. We found that BRCA1 deficiency enhanced ROS levels in breast cancer cells and that Nrf2-driven antioxidant pathways were defective [55]. Further analysis revealed that BRCA1 directly interacts with Nrf2 and that this interaction affects Keap1-mediated Nrf2 ubiquitination, stability, and activation [55]. Interestingly, estrogen treatment partially restored Nrf2 levels and enhanced tumor growth in the absence of BRCA1 [55, 56]. We hypothesize that loss of BRCA1 in heterozygous carriers of somatic BRCA1 mutations has different effects depending on the tissue. In tissues without an estrogen-rich environment, BRCA1 deficiency impairs Nrf2 antioxidant signaling, leading to an accumulation of ROS in the BRCA1-deficient cells that kills them. However, in the breast and ovary, estrogen activates Nrf2 via a mechanism that depends on PI3K–AKT and protects BRCA1-deficient cells from ROS-induced death. If a BRCA1-deficient breast or ovarian cell also loses PTEN function, the PI3K–AKT pathway may be further stimulated and may reinforce estrogen-mediated Nrf2 signaling. Mitogenic and antioxidant pathways acting downstream of AKT, coupled with the genomic instability caused by a lack of BRCA1-mediated DNA repair, might then eventually drive the complete malignant transformation of the BRCA1-deficient cells [56]. Exploitation of the altered ROS regulation in these cells may serve as the basis for an effective therapy in the future.

Exploiting Cancer Cell Aneuploidy

A long-term goal of our laboratory is to develop novel anticancer therapeutics that differ from existing classes of agents. We have recently taken advantage of an alteration shared by many advanced cancer cells but not found in normal cells: aneuploidy.

By using a systematic approach that combines RNAi screening with gene expression analysis in human breast cancers and cell lines and focusing on cancer cell aneuploidy, we have identified polo-like kinase-4 (PLK4), an enzyme critical for aneuploidy maintenance, as a promising therapeutic target [57]. A drug discovery program culminated in the isolation of CFI-400945, a potent and selective small-molecule PLK4 inhibitor [57]. In vitro treatment of human cancer cells with CFI-400945 results in effects similar to those of siRNA-mediated PLK4 kinase inhibition, including mitotic defects, centriole duplication, and cell death [57]. In in vivo mouse models based on human ovarian or breast cancer xenografts, tumor growth was significantly inhibited by CFI-400945 in a manner influenced by the PTEN status of

the tumor [57]. PTEN-deficient xenografts showed a greater response to CFI-400945 than xenografts expressing wild type PTEN, making PTEN status a potential predictive biomarker for therapy with this first-in-class agent [57].

Conclusion

This chapter has briefly outlined four innovative anticancer approaches under investigation in our laboratory. Our belief is that by concentrating on unique aspects of tumor biology, we can identify strategies and targets that are applicable to a broad range of cancers and less likely to induce damaging side-effects in normal tissues. By fighting the fire of malignancy with the fire of creative thinking, we hope to indeed conquer cancer in our lifetime.

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