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Colorectal Cancer Immunotherapy

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Abstract

Antitumor immunotherapy for colorectal cancer has been studied at the bench and bedside for decades. Some clinical trials of cancer immunotherapy have demonstrated a potential benefit for patients with colorectal cancer, yet immunotherapy remains only an experimental option for this disease. Here, we review the major immunotherapeutic approaches currently under investigation for colorectal cancer, including cancer vaccines and adoptive cell therapy. Weakness and advantages of each strategy and progress in clinical trials will be described. Examination of previous and ongoing research in colorectal cancer therapy should define a path towards identification, approval and mainstream adoption of colorectal cancer immunotherapeutics.

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the United States, and more than half of patients with this disease will eventually die of tumor metastasis. It is estimated that in the US there are ~75,000 new cases for men and ~70,000 new cases for women annually (Siegel *et al.*, 2012). Factors that increase the risk of developing CRC include age, diet, race and family history. Surgical resection results in a 90% 5-year survival rate for patients with disease localized to the mucosa, but absent from lymph nodes or distant sites (stage I and II). However, survival decreases to 12% for patients with distant metastasis, while patients with regional spread have an intermediate survival rate (Siegel *et al.*, 2012). Non-surgical therapeutics such as chemotherapy are approved for the treatment of regionally metastatic colorectal cancer; however, these have only modest efficacy and are ineffective against distant metastases (Gallagher & Kemeny, 2010; Sharif *et al.*, 2008). Moreover, these treatments generate side effects that can limit their use (Sharif *et al.*, 2008). The presence of chemotherapy-resistant cancer cells also limits the efficacy of these interventions. Thus, there is a clinical unmet need for therapies that eliminate regional and distant colorectal cancer metastasis. In recent years, greater emphasis has been placed on developing immunotherapies, especially after FDA approval of the cancer vaccine, sipuleucel-T, in 2010 and the immunomodulatory antibody, ipilimumab in 2011 (Callahan *et al.*, 2013; DeFrancesco, 2010). Generally, immunotherapies targeting CRC take one of two approaches: cancer vaccines or adoptive cell therapy.

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Colorectal cancer-associated antigens

Immunotherapy has the potential to eradicate cancer by eliciting immune responses through the recognition of specific antigens on tumor cells. However, the lack of antigens that are truly tumor-specific limits the development of immunotherapy. The targeting of tumor-associated self-antigens poses the risk of developing autoimmune toxicities against normal tissues from which the cancer is derived, while self-tolerance may also restrict immune responses to these antigens. The identification of a suitable tumor-specific antigen is one of the most important steps in developing immunotherapeutic treatments. Carcinoembryonic antigen (CEA) has been the most widely studied antigen in CRC since the 1960s (Gold & Freedman, 1965). CEA is a plasma membrane-associated glycoprotein, which plays a role in several processes, including cell adhesion (Hammarstrom, 1999). CEA promotes the aggregation of colorectal carcinoma cells and also may facilitate metastasis by acting as L-selectin and E-selectin ligands (Thomas *et al.*, 2008). CEA is expressed by multiple adult tissues and can be detected at low levels in healthy adult blood (Benchimol *et al.*, 1989; Thomas *et al.*, 2008). However, CEA is overexpressed by adenocarcinomas of the colon, rectum, breast and lung and is “shed” into the serum at high levels by these malignancies (Hammarstrom, 1999). Therefore, a CEA blood test was developed as a diagnostic marker to monitor CRC disease progression and post-surgical recurrence. However, its expression also is found in other normal tissues such as prostate, uterus, bladder and spleen and at low levels in blood, producing CEA-specific tolerance, which limits specific immune responses elicited by cancer vaccines. MUC1 is another widely targeted antigen in CRC immunotherapy. MUC1 mucin is a transmembrane glycoprotein, expressed on the apical surface of secretory epithelial cells (Hollingsworth & Swanson, 2004). MUC1 can bind to pathogens to limit bacterial invasion, and may also regulate cell motility and survival (Linden *et al.*, 2009; Singh & Hollingsworth, 2006). However, its overexpression on adenocarcinomas, including breast, lung, colon, pancreas, stomach, prostate, and ovary results in a loss of polarization and altered glycosylation, making it a potential target for multiple types of cancer (Beatson *et al.*, 2010; Ho *et al.*, 1993). The overexpression and abnormal glycosylation of MUC1 characterizes many colorectal adenomas and is associated with a poor prognosis by regulating tumor-promoting signaling pathways such as β-catenin and ras (Ajioka *et al.*, 1997; Singh & Hollingsworth, 2006).

Guanylyl cyclase C (GUCY2C, GCC), an emerging target in CRC immunotherapy (Snook *et al.*, 2007; Snook *et al.*, 2011), is a receptor for the endogenous hormones guanylin and uroguanylin and exogenous bacterial heat-stable enterotoxin (Lucas *et al.*, 2000). Upon ligand-induced activation, GUCY2C produces the second messenger cGMP, which activates the cGMP-dependent protein kinase (PKG) (Lucas *et al.*, 2000). GUCY2C is primarily expressed on the apical surfaces of intestinal epithelial cells, regulating numerous physiological and pathophysiological processes (Kim *et al.*, 2013). Importantly, GUCY2C expression persists through all stages of colorectal tumorigenesis from premalignant polyps to distant colorectal cancer metastases (Cagir *et al.*, 1999; Carrithers *et al.*, 1996; Waldman *et al.*, 1998). Moreover, its expression is maintained in greater than 95% of metastatic colorectal cancers (Carrithers *et al.*, 1996; Schulz *et al.*, 2006). These observations suggested that GUCY2C could have utility as a biomarker for metastatic colorectal cancer, a

hypothesis that has been supported by several retrospective and prospective clinical trials (Cagir *et al.*, 1999; Carrithers *et al.*, 1996; Waldman *et al.*, 2009). Additionally, immune compartmentalization separates the mucosal and systemic immune systems, and limits their cross-talk suggesting that GUCY2C-specific systemic immunity is unlikely to cause autoimmunity in intestinal mucosa (Snook *et al.*, 2007). Beyond the above antigens, other antigens, such as Her2/neu, Sialyl-Tn, survivin and others, as well as mutated antigens, including p53 and K-ras, also have been studied in colorectal cancer, though without great success.

Cancer vaccines

Cancer vaccines are active therapeutic approaches designed to trigger the immune system to respond to one or more tumor-specific antigens and attack cancer cells through the recognition of these antigens. The challenges in developing a cancer vaccine include 1) identifying a suitable antigen target and 2) designing an appropriate vaccine mechanism to elicit immune responses against cancer cells expressing that antigen. Cancer vaccine approaches include tumor cell vaccines, peptide vaccines, dendritic cell vaccines, DNA vaccines, and viral vector-based vaccines.

Autologous tumor cell vaccines

Autologous tumor cell vaccines are produced from tumor cells isolated from patients, engineered into a vaccine *ex vivo*, and re-administered to the patient. Before the identification of tumor-specific antigens, autologous tumor cell vaccines were the major option for cancer immunotherapy. The advantage of whole cell vaccines is that autologous tumor cells comprise all tumor antigens, and as such can potentially elicit adaptive antitumor immunity to multiple antigens. However, a significant disadvantage to this approach is the difficulty in generating a ‘universal’ vaccine that could be applicable to all patients with a given cancer. Rather, a personalized approach is often employed, requiring the generation of vaccine material from each patient. Additionally, the efficacy of whole tumor vaccines is poor, reflecting low immune responses, potentially due to the low abundance of tumor-specific antigens within the vaccine (Lokhov & Balashova, 2010). Only a small proportion of the proteins expressed by a cancer cell are specific to tumor cells, while the vast majority of antigens in the vaccine are shared among normal cells. Moreover, the expression level of tumor-specific antigens by tumor cell vaccines is likely to be lower than vector-based and DNA vaccines. Therefore, the immune response generated by whole cancer cell vaccines has been largely insufficient to provide benefit to patients.

Early clinical trials of tumor cell vaccines included whole tumor cell lysates combined with bacillus Calmette-Guérin (BCG) or bacterial cell wall products as adjuvants. However, most studies find no significant difference in post-surgical clinical outcomes [disease-free survival (DFS) and overall survival (OS)] between vaccine and negative control groups (Gray *et al.*, 1989). OncoVAX (Vaccinogen, Inc.) is a personalized antitumor vaccine utilizing irradiated, non-tumorigenic autologous tumor cells with adjuvant BCG (Uyl-de Groot *et al.*, 2005). In stage II colon cancer patients, OncoVAX increased the 5-year survival rate by 15% and 5-year disease-free survival rate by 16% compared to control treatment (Hanna *et al.*, 2001). Moreover, the recurrence rate was reduced by 44% in

patients with stage II and stage III colon cancer (Hanna *et al.*, 2001). However, localized ulceration and systemic reactions including fever and chills have been seen in some patients receiving OncoVAX immunization (Harris *et al.*, 2000). To date, five clinical trials for OncoVax have been completed and a pivotal Phase III trial under an FDA Special Protocol Assessment (SPA) classification will start in 2013.

Another tumor cell vaccine approach utilizes virus-infected, irradiated tumor cells as autologous colon cancer vaccines. In this case, virus-infection of tumor cells produces an adjuvant effect, eliminating the need for BCG. A vaccine comprised of autologous tumor cells infected with Newcastle disease virus (ATV-NDV) produced a 97.9% two-year survival rate in patients with resected colorectal cancer, compared to 66.7% when treated with autologous tumor cells+BCG (Ockert *et al.*, 1996). In a phase II clinical trial, 23 patients with CRC were treated with ATV-NDV after surgical resection of liver metastases. During an 18-month follow-up period after surgical resection, 61% of ATV-NDV-vaccinated patients developed tumor recurrence, compared to an 87% recurrence rate in the control group that received surgery alone (Schlag *et al.*, 1992). However, a randomized phase III study of 50 patients with resectable CRC liver metastases demonstrated that ATV-NDV-vaccinated patients had no significant improvement in overall survival, disease-free survival or metastases-free survival, though subgroup analyses suggested some benefit from ATV-NDV (Schulze *et al.*, 2009).

Peptide vaccines

In contrast to the whole autologous tumor cell approach in which no antigens are identified, a peptide vaccine employs the smallest possible unit of a vaccine: the 8–11 amino acid epitope of an antigen that is recognized by effector T cells. A peptide vaccine is based on the identification and synthesis of epitopes, which can induce tumor antigen-specific immune responses. Peptide vaccines are typically used in combination with various immunological adjuvants to increase immunogenicity and enhance antitumor immune responses. Compared to other vaccine approaches, peptide vaccines have a few advantages: easy production and storage and low cost (Parmiani *et al.*, 2002). Peptide vaccines also may have a lower risk of inducing autoimmunity, since they are usually derived from truly tumor-specific antigens, such as mutated peptides. However, peptide vaccines also have some disadvantages limiting their development. These include poor immunogenicity, HLA-restriction limiting the peptide vaccines to specific HLA haplotypes, and cancer recurrence due to antigenic escape (Bartnik *et al.*, 2013; Parmiani *et al.*, 2002).

Recently, several peptide vaccines for CRC have reached phase I clinical trials. A synthesized peptide vaccine derived from HLA-A2402-restricted epitopes of RNF43 (ring finger protein 43) and TOMM34 (34-kDa translocase of the outer mitochondrial membrane) has been studied with adjuvant chemotherapy (Okuno *et al.*, 2011). RNF43 and TOMM34 are tumor-associated antigens whose expression is increased in more than 80% of CRC specimens compared to normal mucosal tissues (Shimokawa *et al.*, 2006; Yagyu *et al.*, 2004). In this clinical trial, eight of 21 patients had a positive CTL response against both RNF43 and TOMM34, while one patient had no CTL response and 12 patients had CTL responses against one of the peptides (Okuno *et al.*, 2011). Three of 19 patients with

metastatic colorectal cancer had progressive disease, while 16 had stable disease (Okuno *et al.*, 2011). Another peptide vaccine in phase I trials was synthesized based on novel HLA-A24-restricted peptides from RNF43, TOMM34, KOC1 (K homology domain-containing protein overexpressed in cancer), and VEGFR1 and VEGFR2 (vascular endothelial growth factor receptors 1 and 2). One of 18 patients with metastatic CRC had a complete response against lymph node metastasis for 3 years, while 6 of them had stable disease for 4 to 7 months (Hazama *et al.*, 2011). These peptide vaccine approaches were well tolerated because no severe adverse events caused by the vaccines were observed in patients (Hazama *et al.*, 2011). Future phase II and III studies are needed to define the efficacy of these approaches compared to established treatments for colorectal cancer.

Dendritic cell vaccines

The maturation and licensing of dendritic cells (DCs) is critical for presentation of immunogenic peptides and activation of T cells. While traditional vaccines rely on DCs to acquire antigen, mature and present epitopes to, and activate, T cells *in vivo*, DCs also can be collected from patients, pulsed with tumor epitopes, matured *ex vivo*, and transferred back into patients as a cancer vaccine to elicit antitumor immunity. Several methods for loading tumor antigens onto DCs have been examined, including pulsing DCs with peptides derived from tumor antigens (Mayordomo *et al.*, 1995) or tumor cell lysates (Berard *et al.*, 2000) and physically fusing DCs with tumor cells (Gong *et al.*, 1997). To increase the ability of DCs to induce T cell responses, DCs can be engineered to express co-stimulatory molecules and cytokines by transfection with recombinant genes such as CD40L (Liu *et al.*, 2002). A phase I clinical study based on autologous human DCs pulsed with a CEA peptide was performed in 21 patients with metastatic cancers expressing CEA. While the vaccine was safe and well-tolerated, only one patient experienced stable disease after vaccination (Morse *et al.*, 1999). In another phase I clinical trial, 10 patients with gastrointestinal cancers were treated with autologous DCs pulsed with CEA652, a 9-mer peptide derived from CEA (Itoh *et al.*, 2002). Only two of the patients experienced stable disease, while the remaining 8 patients developed progressive disease (Itoh *et al.*, 2002). Yet another phase I clinical trial examined 12 patients immunized with CEA-derived peptide-loaded DCs with the adjuvant Flt3 ligand (Fong *et al.*, 2001). Two of twelve patients had disease stabilization for 3 months and 6 months respectively, while two patients had complete responses for more than 10 months and one patient had a mixed response with some regression of liver metastases (Fong *et al.*, 2001). Collectively, these results in the context of the FDA-approval of the DC vaccine, sipuleucel-T, suggest that DC vaccines warrant further investigation in colorectal cancer.

DNA vaccines

A DNA vaccine is naked plasmid DNA that induces expression of specific antigens upon delivery to mammalian cells. Since the 1990s, DNA plasmids have been utilized as vaccines to drive humoral and cellular immune responses against pathogens and tumor antigens in preclinical mouse studies. Compared to other cancer vaccines, DNA vaccines are usually well tolerated, safe, less costly, easy to produce and store and potentially induce both humoral and cellular immunity. However, there are also disadvantages limiting the utility of DNA vaccines. These include low transfection efficiency and poor immunogenicity. To

overcome these shortcomings, researchers have developed the gene gun and electroporation to increase transfection efficiency by delivering DNA plasmid directly into cells and increasing cell membrane permeability, respectively. Also, inclusion of inflammatory cytokines or co-stimulatory molecules as fusion products with the antigen or in separate plasmids can enhance immunogenicity. The mechanism of action of DNA vaccines to activate immune responses relies on several processes. The un-methylated CpG motifs of DNA plasmids derived from bacteria interact with Toll-like receptor 9 (TLR9) on APCs, inducing APC maturation (Hemmi *et al.*, 2000). APCs also present the DNA-encoded antigens that were acquired by either the direct or indirect presentation pathways (Howarth & Elliott, 2004; Shedlock & Weiner, 2000). During direct presentation, plasmid DNA is delivered directly into DCs, resulting in antigen expression and presentation by the DCs. During indirect presentation, plasmid is delivered to parenchymal cells (skeletal muscle), which express the antigen. The antigen is then acquired by DCs and presented through 'cross-presentation' to naive T cells in lymph nodes to induce adaptive immunity.

A veterinary DNA vaccine expressing human tyrosinase was approved for canine oral melanoma in 2007 (Kutzler & Weiner, 2008). However, no DNA vaccines for human cancers have been approved. Also, there are relatively few studies with DNA vaccines for CRC in phase I clinical trials. In one example, the effects of a DNA vaccine expressing CEA and hepatitis B surface antigen were studied in 17 patients with metastatic colon cancer (Conry *et al.*, 2002). Although immune responses were observed in 4 patients, no objective clinical benefits were detectable (Conry *et al.*, 2002).

Viral-vector vaccines

The vertebrate immune system has evolved over millennia to respond to and repel infectious microorganisms, such as bacteria, viruses and fungi. Thus, it stands to reason that vaccines mimicking pathogenic microorganisms would be the most immunogenic and efficacious. In that context, viral vectors can be engineered to express tumor antigens and the natural immunogenicity of viral vectors acts as an adjuvant to help boost tumor antigen-specific immune responses. Thus, viral vectors both deliver the antigen and provide sufficient adjuvant effects to produce immune responses. Viral vectors for cancer vaccines include recombinant lentiviruses, poxviruses, adenoviruses and retroviruses, and others (Mosolits *et al.*, 2005), because of their high transfection efficiency and potent immunostimulatory ability. However, viral vector vaccines are limited by immune responses against the vector, high expense, potential pathogenesis, and potential insertional mutagenesis. In the context of these advantages and disadvantages, several viral vector vaccines have been explored for CRC treatment in clinical trials. Recombinant poxviral vectors expressing CEA have been explored since the 1990s. CRC patients were immunized with vaccinia virus or replication-defective avian poxviruses encoding CEA (Marshall *et al.*, 1999; Tsang *et al.*, 1995). Although CEA-specific cytolytic T cell responses were observed, the objective clinical responses in vaccinated patients were disappointing. Later, poxviral vectors expressing CEA as well as the co-stimulatory molecule B7.1 were explored (Horig *et al.*, 2000; von Mehren *et al.*, 2000). A heterologous prime-boost strategy including vaccinia and canary pox viruses expressing CEA induced CEA-specific T cell responses in patients (Marshall *et al.*, 2000). More recent clinical trials including vaccinia and fowlpox expressing CEA and the three

costimulatory molecules B7-1, ICAM-1, and LFA-3 have shown the best efficacy to date, inducing immune responses and producing prolonged disease stabilization in a majority of patients (Marshall *et al.*, 2005).

MUC1 was one of the first CRC antigens identified and has been examined in clinical trials for >15 years (Karanikas *et al.*, 1997). While some preclinical studies suggested tolerance to MUC1 is too robust to allow the induction of MUC1-specific responses after immunization (Chen *et al.*, 2000; Turner *et al.*, 2007), other vaccine approaches have demonstrated strong antitumor immunity despite tolerance (Mukherjee *et al.*, 2007). Recent studies revealed interesting results regarding the role of the premalignant tumor microenvironment in shaping MUC1-specific immune responses. In an animal model of inflammatory bowel disease (IBD), a known risk factor for colorectal tumorigenesis, inflammation was associated with the systemic induction of myeloid-derived suppressor cells (MDSCs) which compromise adaptive immunity and antitumor immunity (Beatty *et al.*, 2010). Interestingly, MUC1-specific immunization reversed these effects, delaying IBD induction and colorectal tumorigenesis. More recently, a phase I clinical trial examined MUC1 vaccination in cancer-free patients with a history of premalignant colon lesions (Kimura *et al.*, 2013). Many of these patients produced robust MUC1-specific immune responses; however, non-responders possessed high levels of circulating MDSCs prior to vaccination. Thus, like the animal models, these patients may have a pre-existing immunosuppressive microenvironment limiting immune responses. It will be interesting to see if a MUC1-immune response can reverse the premalignant microenvironment in these patients and protect them from developing cancer in the future.

In contrast to MUC1 and CEA, GUCY2C-targeted vaccination is in early stages of development. An adenoviral vector expressing the extracellular domain of GUCY2C (Ad5-GUCY2C) induces GUCY2C-specific immune responses as well as prophylactic and therapeutic immunity against metastatic colorectal cancer in mice without adverse effects (Snook *et al.*, 2008a; Snook *et al.*, 2009; Snook *et al.*, 2008b). These results have led to the design of a phase I clinical trial examining Ad5-GUCY2C in early-stage colorectal cancer patients. The study is scheduled to begin in 2013.

Adoptive cell therapy

Passive immunotherapy is a process in which immune effectors (cells or molecules) are transferred to the host, rather than activate the host's endogenous immune system (active immunotherapy). One form of this therapy is adoptive cell therapy (ACT). Most adoptive cell therapies focus primarily on T cell therapy, due to the highly specific nature and potent killing ability of T cells. In adoptive T cell therapy, autologous T cells are removed from patients, activated and expanded to large numbers *ex vivo* and transferred back into patients for a therapeutic effect. One advantage of ACT is that *ex vivo* reprogramming and activation of T cells may overcome some mechanisms of self-tolerance, which inhibit T cell activation *in vivo* (Restifo et al., 2012). Indeed, the administration of large numbers of T cells with high specificity to tumor antigens may lead to tumor regression. However, some disadvantages for adoptive cell therapy also need to be considered such as a possible lack of

immune memory, poor persistence of adoptive T cells *in vivo*, prohibitive expense and time to produce T cells (4–16 weeks), as well as risk for sever adverse effects.

The primary strategies for adoptive T cell transfer have utilized tumor infiltrating lymphocytes (TILs) or genetically engineered T cells. It is known that some tumors possess tumor-antigen-specific T cells within the tumor microenvironment (Dudley *et al.*, 2002). Unfortunately, these cells are suppressed or dysfunctional such that cancer cells overwhelm the response (Whiteside, 2006). However, T cells collected from the TIL can be re-stimulated *ex vivo* in a process that reverses their unresponsive state. Expanded TIL re-administered to patients with metastatic melanoma promote drastic reductions in tumor burden in early phase clinical trials (Dudley *et al.*, 2002; Rosenberg, 2011). However, the use of TILs is currently limited to patients with melanoma, potentially due to a higher immunogenicity of melanoma in comparison to other cancers. Alternatively, genetically engineered T cells expressing antigen receptors with predetermined affinity facilitate the targeting of virtually any tumor type. Indeed, T cells engineered to express high avidity T cell receptors (TCRs) target tumors of various histological origins. However, these TCRs would be limited to patients with the corresponding MHC haplotype. Alternatively, the use of antibody-based chimeric antigen receptors (CARs), which express a single chain variable fragment derived from a tumor antigen-recognizing monoclonal antibody, fused to intracellular T cell signaling domains, can be used universally across all patients since CARs target native antigens on the surface of tumors without MHC restriction.

In that context, a phase I trial in colon cancer examined human T cells engineered to express a high avidity CEA-specific murine TCR (Parkhurst *et al.*, 2011). Three patients with metastatic colon cancer were treated with these engineered T cells, all of which experienced decreased serum CEA levels and one of which experienced an objective clinical response. However, all patients developed a severe transient inflammatory colitis. Severe side effects also were seen in one patient treated with Her2-specific CAR T cells for metastatic colon cancer (Morgan *et al.*, 2010). Thus ACT has failed to demonstrate safety and efficacy in colorectal cancer patients and future studies will have to identify mechanisms that allow CAR-expressing T cells to selectively eliminate cancer cells, but leave normal tissues unaffected.

Conclusion

The limitations of surgery and adjuvant chemo/radio/antibody therapies to treat CRC patients necessitate the development of novel approaches, including immunotherapy. While some clinical trials utilizing cancer vaccines have demonstrated objective clinical responses in immunized patients with metastatic CRC, more work is needed. The approval of the first cancer vaccine, sipuleucel-T, should establish a new paradigm for the development, clinical testing and regulatory approval of future cancer vaccines for colorectal and other malignancies. ACT in clinical trials for CRC has resulted in severe toxicities; however, successes targeting melanoma and leukemia have demonstrated the feasibility of this approach. Alternative approaches to minimize toxicities in CRC patients by identifying appropriate antigen targets or interventions that reduce the severity of toxicities will be necessary for this therapy to achieve success. While it is unlikely that a single therapy will

be a universal cure for CRC, a future convergence of therapeutics including surgery, chemotherapy, immunotherapy, and possibly others may ultimately change patient outcomes in CRC.

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