Vaccine Immunotherapy Strategies in Colorectal Cancer Treatment

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Abstract

The colorectal cancer (CRC) is a most frequent cause of cancer-related deaths. Surgical tumor resection remains the primary curative treatment for CRC but nearly 50% of patients with macroscopic clearance of the tumor by surgery relapses. For this purpose new complementary treatments, such as immunotherapy, are being explored. In this article, we analyze and discuss the clinical immunotherapy vaccine studies performed in patients treated for colorectal cancer.

Introduction

Depending on gender, colorectal cancer (CRC) is the second (for female) and third (for male) most frequent cause of cancer-related deaths [1]. Surgical tumor resection remains the primary curative treatment for CRC but, despite the fact that 80% of CRC patients have complete macroscopic clearance of the tumor by surgery, 50% of them will relapse [2].

Avenues for the clinical testing of rationally designed vaccination strategies, including immunotherapy, are being explored as complementary treatments.

Different investigation findings [3-5] suggest a role for the immune system in the cancer treatment and particularly, positive correlation between tumor infiltrating T cells and patients survival has been observed [6].

As spontaneously as therapy-induced, tumor specific immune responses need to be robust in order to attack and eliminate tumor cells. For this purpose, it's very important that the collaboration between cells of the innate immune system and cells of the adaptive immune system (B and T cells) [7]. Tumor-infiltrating T cells recognize the tumor antigens that are presented in the MHC context while B cells serve as local antigen presenting cells (APC), supporting and enhancing T-cell responses by cytokines and chemokines [8]. Immunotherapy should initiate responses against tumoral antigens and, about them, a significant consideration is whether to use vaccines based on defined antigens or tumor cell derivatives.

Many clinical trials have been conducted using active specific immunotherapy (ASI) in CRC, including autologous tumor cell vaccines, defined-tumor protein vaccines, monoclonal antibodies (MoAbs), anti-idiotype vaccines, multi-peptide vaccines, viral vector vaccine, DC (Dendritic Cells) vaccine, and naked DNA vaccine [9]. However, despite an abundance of preclinical data, relatively little is known regarding the efficacy of ASI in CRC.

The aim of this review is to provide an overview of clinical immunotherapy studies performed in CRC patients and speculate on the development of therapeutic and prophylactic vaccination for patient with colorectal cancer.

Vaccines using Defined Antigens

All antigens used in CRC vaccination studies consist of TAA (tumor-associated antigens) and consequently are likely to be expressed by normal cells [10-12]. Different TAA, such as p53, carcinoembryonic antigen (CEA), MUC1, Sialyl-Tn, 5T4, SART3 and MAGE have been applied in clinical trials to vaccinate patients with CRC [10-14].

The use of antigens potentially expressed by normal cells increase the risk of immune tolerance. Murine models [15,16] in agreement with human vaccination with the p53-SLP (synthetic long peptide) [17] indicated that the p53-specific CD8+ (not CD4+) T-cell repertoire is strictly restricted by self-tolerance and might only consist of lower affinity p53-specific CD8+ T cells. By contrast, a blunted CD4+ T-cell repertoire was found for the T-cell response against the CEA in animal models [18]. Also, studies in a MUC1-transgenic murine model indicated that low antibody secretion and CTL responses to MUC1 peptides are due to CD4+ T-cell tolerance [19-22]. In conclusion, these data suggest that tolerance forms a potential obstacle for cancer immunotherapies with TAA. To bypass tolerance and to induce tumor specific T-cell responses, a solution is to plan vaccines that stimulate responses against different TAA, able to induce complementary reactivity of non-tolerized CD4+ and CD8+ T cells.

Essentially three different strategies have been used to immunize patients against the above mentioned TAA:DC vaccination, recombinant viruses and peptide vaccination.

(a) Dendritic cells play a central regulatory role in tumor immunity through several mechanisms: recognition of tumor molecules by DC precursors, (b) direct and IFN-γ-mediated killing of transformed cells by NK/NKT cells activated by DCs, (c) capture and cross-presentation of released-TAA by immature DCs, (d) selection and activation of TAA specific T cells as well as nonspecific effectors including macrophages and eosinophils, and (e) homing of TAA specific T cells to the tumor site and recognition elements leading to the elimination of tumor cells [23].

Murine studies have shown that *ex-vivo* generated DCs can induce antigen-specific T-cell immunity and are superior to other types of

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tools [24]. These studies promoted DC-based anti-cancer vaccines in clinical trials.

To date, various clinical studies used the injection of autologous DCs pulsed with MHC class I-restricted peptides derived from different TAA: CEA [25-29], MAGE2/3 [26,30], Her2/*neu* [26]; alternatively DCs expressing antigen via recombinant techniques (viral vectors) [31] or mRNA encoding the CEA antigen [32]. DC vaccine has the advantage of including patients irrespective of their HLA type, in contrast, as will be explained later, to the use of defined HLA class I-restricted peptide-epitopes. A potential disadvantage is the possibility that the viral vector contains more immunogenic epitopes that compete for binding and presentation with the TAA epitopes, thereby weakening the induction of tumor-specific immunity.

A series of Phase I/II trials in CRC patients showed that DC vaccines are safe and able to induce a T-cell response. An overall immune response evaluating show that DC vaccines induce peptide-specific IFN-γ-producing T cells with variable efficiency. It is difficult to compare the outcomes of peptide vaccines with peptide-pulsed DC vaccines as generally the assays have not been harmonized and no gold standard exists.

Interestedly, Fong et al. [29] found that objective clinical responses correlated with the expansion of tetramer CD8^{+}T cells [29]; the other studies described only cases [26-32]. A remarkable study reported the use of a vaccine with multiple targets [26] in order to prevent immune escape. Unfortunately, only immune reactivity against CEA was tested by the authors, limiting the interpretation of trial results [26].

Over the years, viruses have been optimized as vectors for the TAA delivery. The vectors most used in treatment of CRC patients are: canarypox virus (ALVAC) and Modified Vaccinia Ankara (MVA) virus.

In general, these vaccines are not toxic and have been used to induce immunity against p53 [33,34], CEA [35-40], 5T4 [41-44] and EpCAM/KSA [45]. The use of these vaccines not only yielded antibody and T-cell responses against tumor antigens but frequently also against viral components. In most cases, the immune response to the tumor antigens was somewhat disappointing. The foreign viral components may have formed a more attractive target for the immune system. Indeed, whereas only low p53-specific T-cell reactivity in patients vaccinated with ALVAC-p53 was detected, a strong T-cell response against the viral vector was found [34]. By contrast, the response to p53 was much stronger and broader when patients were vaccinated with p53 overlapping long peptides [17]. Interestingly, the MVA-5T4 vaccine did induce strong 5T4-specifc immune responses and also, the group of patients with stronger 5T4-specific proliferative responses showed significantly longer survival [43,44]. Unfortunately, to date, only Phase I and II studies have been performed. Therefore, mainly descriptive effects on survival and tumor mass have been reported.

As previously documented [46,47], an elegant vaccine approach is to inject synthetic TAA peptides: minimal peptide vaccines [48] or SLP (synthetic long peptide) vaccines [49].

Minimal peptide vaccines comprise the minimal cytotoxic T-cell peptide-epitope sequence that can bind directly to its presenting HLA class I molecule. Because each peptide can only bind to one specified HLA class I subtype, the vaccination is limited to the patients expressing that specific HLA type.

Direct injection of such peptide vaccines into patients have met with limited clinical success, probably because the induced CD8^{+}T-cell responses aren't very strong and effector. Also, in many times the response is transient because of the lack of T-cell help. In some vaccination strategies, these minimal peptide vaccines may induce immunological tolerance rather than immunity [48]. To date, different Phase I and II vaccine studies used peptides derived from various TAA: SART3 [50], survivin [51], CEA [52], and a personalized mixture of antigens [53-55]. These minimal peptide vaccines were all well tolerated with mainly grade I/II adverse events at the injection site and induced or boosted antigen-specific T-cell or IgG responses in the majority of vaccinated patients.

Most interesting are the therapeutic effects, in fact a reduction of tumor load was described in nearly all studies. Hattori et al. [53] described a positive relation between the presence of vaccine-induced IgG and overall survival [53], whereas others studies observed reduction of tumor mass or a decrease in the level of serum tumor markers in individual patients.

To improve the peptide immunogenicity, it was proposed to use synthetic long peptides (SLP) that can be produced by chemical linkage of multiple immunogenic epitopes including both HLA class I and class II epitopes [56]. Different trials using multipeptide vaccines for the treatment of solid malignancies have been performed showing promising results. Moreover, the combination of these polyspecific, peptide-based vaccines with reagents that modulate the immune response by increasing its strength or duration (e.g. anti-CTLA4, anti-PD-1) or impairing immune suppressive circuits (anti-Tregs and or anti-MDSC) is generating effective clinical responses in this new wave of clinical trials that will impact on the clinical outcome of cancer therapy [57,58].

Long-peptide vaccines used in Phase I or II vaccination trials to vaccinate CRC patients represented the TAA: p53 [17], hCG-b [59], MUC1 [60] and mutation specific Ras [61]. Only limited toxicity has been found in these trials and two of these four studies determined vaccine-induced immunity. In particular, Khleif et al. [61] after vaccination with mutation-specific Ras peptide demonstrated a mutated Ras-specific immune T response (CD4^{+}/CD8^{+}) [61].

A latest study on p53-SLP vaccination revealed in 9/10 CRC patients long-lasting vaccine-specific responses [17] and, in addition, the p53-specific T cells were able to secrete both Th1 and Th2 cytokines after P53 peptide stimulation [17]. However, multiparameter flow-cytometry revealed that only a minor population of the p53-specific CD4^{+} T cells was optimally polarized, suggesting that the vaccine strategy used was not optimal for the induction of p53-specific Th1 cells. These results emphasize the importance of immune monitoring to further optimize vaccine strategies and to better understand clinical responses after vaccination.

About the patient survival, most interesting are the data of Moulton et al. [59]; they using a hCG-b long peptide vaccine, found that patients with high levels of anti-hCG-b antibodies exhibited significantly longer survival compared with patients who developed low anti-hCG-b Ab levels [59]. Unfortunately, no efficacy study was performed to determine the true effect of vaccination on survival. The immunogenicity of peptide vaccines may be further increased by using adjuvants (incomplete Freund's adjuvant), or immunomodulators (cytokines and/or agonists of innate immune receptors).

Despite many years of work, the number of identified antigens recognized by tumor-infiltrating lymphocytes (TIL) of CRC is very limited [62-65]. Consequently, vaccines to date have been developed on the basis of proteins that are selectively expressed by tumor cells but

for which immunity can be blunted, such as p53 where the CD8^{+} T-cell component suffers from central tolerance, or may lead to autoimmunity such as has been observed with CEA [16,18].

The exception includes microsatellite instable (MSI-H) tumors that, owing to numerous of frameshift mutations in microsatellites, express neo-antigens. MSI-H is a molecular feature of tumors associated with the familial Lynch or hereditary non-polyposis colorectal cancer (HNPCC) syndrome, accounting for approximately 5% of all colorectal cancer cases [66-68] and for approximately 15% of all sporadic colorectal, gastric and endometrial cancers [69-73].

Since frameshift-mutated products (FSPs) are foreign to the immune system, they represent a unique group of tumor-specific antigens. No tolerance and consequently strong T-cell responses are expected against this FSPs. Unfortunately, relatively little is known on immunogenic behavior of such FSPs [65]. Few studies have been performed to predict the immunogenic behavior of a selection of frameshift-mutated genes that are frequently detected in MSI-H cancers [65,74,75]. Speetjens et al. (2008) [74] recently developed a methodology for predicting their immunogenic behavior that is based on accumulation and MHC class I presentation. Their results indicated that, out of the 15 FSPs examined, four (TGF-bR2–1, MARCKS-1, MARCKS-2 and CDX2–2) are of primary interest [74] and four additional antigens (TAF1B-1, PCNXL2-2, TCF7L2-2 and Baxa+1) are of moderate interest for further tumor immunological research [74]. Other results suggested that FSP-specific T cells may be present in the circulation of patients with MSI-H CRC, healthy HNPCC syndrome mutation carriers, but not in patients with microsatellite stable CRC or in healthy donors [75,76].

In general, most FSPs consist of a relatively small number of amino acids downstream of the frameshift mutation, suggesting that the FSPs may contain a sequence that can only be presented by a limited number of HLA class I or HLA class II molecules. In order to treat patients, knowledge of which HLA class I and II molecules can present epitopes comprised by the FSPs should be obtained. Although MSI-H tumors comprise only about 15% of all colorectal tumors, patients with a MSI-H tumor are very interesting vaccination candidates because strong effector responses are expected after vaccination using nonself-antigens. Notably, the amino acid length of the FMPs make them perfect candidates for overlapping SLP-based vaccines that have been shown to be highly immunogenic in human [49].

Strategies of Cell-Derived Vaccination

The TAA are poorly characterized and many remain to be identified. It is believed that the best source of antigens is the tumor cell itself, and a possible benefit of tumor cell derived vaccines is the ability to induce responses against various and unidentified targets, may be minimizing the tumor immune escape.

Problems related to these vaccines are: a) relevant TAA might be under-expressed in the tumor cells and thus may result in feeble immune responses compared with vaccination by defined antigens; b) the poor possibility to monitor the patient immune response, because the antigens which are presented to the immune system are unknown [77]. As autologous as allogeneic tumor cell-derived samples have been used in vaccination studies.

Vaccine preparations based on autologous tumor tissue frequently consist of autologous single tumor cell suspensions, obtained from tumor tissue. Before reinjection, the tumor cells are irradiated and usually combined with immune-stimulating agents: heat shock proteins

[78], Ulster strain of the Newcastle disease virus (NDV) [25,79,80], BCG [81-85] and IL-2-transfected fibroblasts [86].

An assured advantage of using autologous tumor cell preparations is that all antigens are relevant for the immune recognition of the tumor. However, the preparation of these vaccines is time consuming, relatively expensive and, therefore, clinically difficult to apply outside a clinical trial setting.

The use of autologous cancer cells doesn't generate severe side effects, but it's very hard to perform an extensive immune monitoring. Only a study reported a T-cell response that was either *de novo*-induced or boosted in 15 of 29 patients [78]. The majority of the studies used the delayed cutaneous hypersensitivity skin reaction as an indirect parameter to measure vaccine-specific immunity and in two studies a positive correlation was found between a positive skin test and survival [80,84].

Six out of the eight clinical Phase II [25,80,83] and Phase III [80-85] studies, determined the effect on prognosis and specifically, three studies reported that tumor cell reinjection combined with BCG had an effect on survival in two subgroups: stage II patients [81,82] and patients with colon cancer [85]. Furthermore, one randomized Phase III trial (where NDV-infected autologous tumor cells were injected) reported a significant effect on survival when compared with non vaccinated patients [80]. Other Phase II and randomized Phase III trials using NDV-infected autologous tumor cells, described a positive effect on survival of vaccinated patients when compared with historical controls or in subgroups of only colon cancer patients [25,79]. Despite these results with autologous tumor cell-derived vaccines, no new clinical trials have been initiated. This is possibly due to the problems to optimize the vaccines based on limited immunological data.

A different vaccination approach is the use of ex vivo antigen-pulsed dendritic cells (DCs). Animal models support this strategy showing that such DCs administered to tumor-bearing hosts were able to elicit a successful antitumor T-cell response.

The dendritic cells are the most powerful professional APC at the interface between innate and adaptive immunity with the ability to activate many effector cells (NK, T, B and NKT cells), that have a key role against cancer.

Because DCs can be simply generated from the patient blood, this approach was translated to clinical trials planned to evaluate their capacity to prime tumor-specific T cells (CD8^{+}/CD4^{+}) and clinical efficacy. The advantage of using allogeneic tumor cells to pulse DCs is that the allogeneic tumor cell component can become an off-the-shelf product. A potential disadvantage is that the immune system will be aroused against tumor antigens that are not present in the patient's tumor and as such are irrelevant. Because, just about 40% of all CRC expressed at least one of MAGE A-1 to -6 antigens [87-90], in some trials, DCs were pulsed with an allogeneic melanoma cell lysate (rich in the MAGE antigens) [91,92]. No severe toxic effects were found in these clinical trials but the clinical effect has been limited to single cases [91,92].

Combinatorial Treatments: Role of Cytokines in Vaccine Therapies

In vaccination strategies for cancer, cytokines play a dual role. They are critical for the *ex vivo* generation of the cell populations used in vaccines and adoptive cell therapy, and they are also important *in vivo*

as adjuvants to these therapies to augment the potency and duration of anti-tumor response.

While cancer treatment vaccines have shown only modest activity with simpler regimens, an area of intense focus has been the use of cytokines as adjuvants to augment the immune response elicited by the vaccine [93,94]. The Cytokine Working Group conducted a study of high-dose IL-2 plus an HLA-A2-restricted gp-100 peptide in HLA-A2-positive patients with metastatic melanoma. The initial results have been mixed [95,96] and current therapies that appear to have a superior therapeutic index and to be more widely available (not requiring a specific HLA type as peptide vaccines do) are likely to temper enthusiasm for this approach.

As mentioned previously, DC-based vaccination therapy for CRC is a very promising strategy and the cytokines are used in several aspects of dendritic cell-based vaccine strategies, including their elicitation from peripheral blood monocytes obtained by leukapheresis, most commonly with IL-4 (Interleukin-4) and GM-CSF (Granulocyte-Macrophage Colony Stimulating Factor) and their maturation to potent antigen-presenting cells [for example, with TNF-α and IL-1β] [20,97]. The safety of using autologous DC vaccines has been reported in clinical trials enrolling over 1,000 cancer patients exposed to a wide variety of types of DC product, route and schedule of administration [98]. The only cancer vaccine that has been approved thus far—sipuleucel-T for prostate cancer—relies on the fusion of a prostate cancer antigen to GM-CSF, which is then loaded into autologous peripheral blood monocytes thought to be predominantly dendritic cells.

The "built-in" GM-CSF provides a way to activate the dendritic cells away from the cancer's immunosuppressive microenvironment so the dendritic cells can then present the cancer antigen to the T cells and elicit an immune response.

A similar approach to incorporate cytokines into vaccine therapy has been to transfect tumor cells or DC used in vaccination with the gene for cytokines such as IL-2 or GM-CSF to localize the cytokine effects to the sites of tumor and T cell activation. For this purpose the results recently obtained in murine colon carcinoma are most impressive, where murine DCs of an established JAWS II cell line were transduced with a retroviral vector carrying murine IL-2 gene (JAWS II/IL-2). JAWS II/IL-2 cells demonstrated slightly decreased tumor antigen (Tag) uptake capacities. However, this modification resulted in enhanced ability of the cells to migrate *in vivo*. The multiple injection of vaccines containing JAWS II/IL-2 cells caused MC38 tumor growth delay and prolonged mice survival. The immunological response was manifested as cytotoxic natural killer (NK) and T cell activation and tumor tissue infiltration by CD8+ and CD4+ cells, accompanied by increased IFN-γ production by spleen cells [99]. The authors propose application of IL-2 transduced DCs as an adjuvant in immunotherapy as well as chemo-immunotherapy but in these cases must be very careful because the passage from animal models to patients in many times generates discrepancies in the obtained results and this often depends on an inappropriate choice of the experimental model.

The Role of Regulatory T Cells

In contrast to animal models, the history of constant interactions between tumor and immune system shapes both the tumor and the immune system of an individual patient in a way that is difficult to mimic in animal tumor models. It is very crucial that vaccines only boost the reactivity of immune cells that mediate an antitumor effect and not that of immune cells that support tumor growth, such as the regulatory T cells (Tregs). Therefore, to gain a thorough understanding

of the immunological events occurring in patients in vaccination trials, it is essential to comprehensively perform immune monitoring during vaccination trials. The results from immune monitoring make it possible to understand possible clinical effects and to guide the optimization of vaccination strategies [77]. Unfortunately, most immunotherapeutic vaccine trials frequently report on one particular aspect of the desired immune response (e.g. IFN-γ-producing cells). They do not include more detailed analyses of the total vaccine-modulated immune response. CRC are infiltrated by both CD4+ and CD8+ Foxp3+ Tregs, and the number of Foxp3+ Tregs negatively correlates with disease stage and survival in colorectal cancer [100-102]. Notably, the analyses of the antigens recognized by CRC infiltrating Tregs revealed that they recognized CRC-associated antigens, especially Mucin, Her-2/neu and CEA [63]. Hence, therapeutic vaccination with these antigens may not only boost CD4+ and CD8+ effector T cells but also the Treg population. Vaccine-induced expansion of such antigen-specific Tregs has been observed as in animal models [103] as in humans [104]. More specifically, the magnitude of the vaccine-enhanced antigen-specific Treg response was related to clinical failure of an otherwise successful therapeutic vaccine for premalignant disease [105]. In a recent trial, in which CRC patients were vaccinated with overlapping p53 long peptides, strong p53-specific CD4+T-cell responses were found but this did not coincide with the expansion of p53- specific CD4+Foxp3+T cells [17]. This fits with the observation that the spontaneous T-cell response to p53 in CRC patients is not under control of Tregs [63]. In humans, several approaches have been used to delete Tregs [106]. Notably, decreases in CD4+CD25+Foxp3+cells have been detected when patients with hepatocellular cancer were treated with low cyclophosphamide [107], as well as in metastatic melanoma patients treated with the anti-CD25 Ab daclizumab [108], or after using denileukin diftitox [109]. Whereas, the use of daclizumab did not enhance the efficacy of the peptide-pulsed DC vaccine [110], multiple injections of denileukin diftitox did result in enhanced CEA-specific T-cell responses [109].

Remarkable Considerations

Despite of advances both in diagnosis and in therapeutic management, the prognosis of colorectal cancer remains poor but encouraging results have shown that in colorectal cancer a favorable clinical outcome, as assessed by disease free and overall survival is associated with a coordinated Th1/cytotoxic memory T cell infiltration [110].

For that all, it is essential to stop ignoring the immune control as a prognostic factor [110] and to introduce the immune score as a marker to classify cancers [111,112]. This marker has a dual advantage: firstly, it appears to be the strongest prognostic factor for disease free and overall survival particularly in early stage cancers and secondly, it provides a tool or a target for novel therapeutic approaches such as immunotherapeutic protocols. In this review, we have reported the different immunotherapy vaccine-strategies experimentally used to treat patients with colorectal cancer.

Analyzing the data obtained from the evaluated clinical trials, we noted that good results were obtained in different studies especially with peptide-vaccinations, where the authors observed reduction of tumor mass or a decrease in the level of serum tumor markers in individual patients [51,52]. In particular, most recent studies described a positive relation between the presence of vaccine-induced antibodies and overall patient survival [53,59].

At present, the major problem to make immunotherapy approach successful for CRC remains the immune evasion of tumor cells.

Understanding the hierarchical status of different tumor-immune escape mechanisms at different stages of tumor progression will guide the design of efficacious therapeutic strategies.

Thus, it will be of particular interest to investigate the kinetics of the interactions between different inhibitory molecules and endogenous factors that influence the expansion and trafficking of Tregs and tolerogenic DCs within tumor-draining lymph nodes and the tumor surroundings.

The current wealth of available data promises a future scenario in which inhibition of tumor escape strategies and of cancer immune-inhibitory signals will be successful in combination with other therapeutic strategies to overcome immunological tolerance and promote tumor rejection.

To conclude, successful innovative therapies for colorectal cancer must involve combined approaches, which should involve systemic chemotherapy and transplantation to reduce the burden or to eliminate immune suppressive cells, together with tailor-made immunotherapies customized to each single patient.

Acknowledgements

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