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Commentary

Monitoring circulating tumor cells in cancer vaccine trials

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The presence of circulating tumor cells (CTC) from various cancers has provided a wealth of information and possibilities. As the role of CTC detection in the treatment assessment of metastatic breast cancer becomes standard, there is interest in applying this tool in cancer vaccine development and clinical trial monitoring. Since we lack a proven immunologic assay that correlates with clinical response, CTC detection, quantification and phenotypic characterization may be a useful surrogate for clinical outcome. The Cancer Vaccine Development Program is involved in the development of HER2/*neu* peptide based vaccine development for the prevention of recurrence in HER2/*neu* expressing cancers like breast cancer. The CellSearch System (Veridex, LLC, Warren, NJ) has been used by our lab in conjunction with in vivo and/or in vitro immunologic measurements to define a monitoring tool that could predict clinical response. Once validated, this assay could significantly shorten clinical trials and lead to more efficient assessment of potentially promising cancer vaccines.

Introduction

The advent of vaccines over two centuries ago, beginning with Edward Jenner's smallpox vaccine, has led to improved public health in the realm of infectious disease.[1,2] The lessons learned from these endeavors have stimulated research into vaccination of a different kind, one against cancer. The Cancer Vaccine Development Program (CVDP) is involved with the development and implementation of novel vaccines targeting tumor associated antigens (TAA), like HER2/*neu*. Currently these vaccines are administered in the adjuvant setting to prevent disease recurrence in high risk patients rendered disease free after standard therapy. The results of our initial trials are encouraging with respect to prolonged disease-free survival and overall survival. Going forward with our vaccine program, one challenge that remains is the need to identify an acceptable immunologic assay or monitoring tool that correlates with clinical response in vaccinated patients.[3]

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The predominant immune mechanism for the recognition of antigens by T lymphocytes is the presentation of short MHC Class I (8–10 amino acids) and long MHC Class II (16–24 amino acids) peptides from TAA to CD8 and CD4 T cells, respectively, by antigen-presenting cells such as dendritic cells, B cells and macrophages. Upon activation, these lymphocytes elicit cell-mediated effector functions including lysis of tumor cells and/or provide helper factors for the generation of humoral immune responses.

HER2/*neu* is a proto-oncogene in the epidermal growth factor family of tyrosine kinases which encodes a transmembrane glycoprotein, highly expressed in many epithelial derived cancers.[4] The HER2/*neu* protein has been shown to be an immuno-reactive TAA.[5-7] Several immunogenic peptides within the HER2/*neu* sequence, including E75 and GP2, are recognized by cytotoxic T lymphocytes (CTLs).[8,9] E75, derived from the extracellular domain of the HER2/*neu* protein (369–377: KIFGSLAFL),[10] is the most studied HER2/*neu*-derived peptide.[11-15] GP2 is derived from the transmembrane portion of the HER2/*neu* protein (654–662: ISAVVGIL) and has been investigated to a lesser degree. GP2 has a lower predicted binding affinity but similar capacity for CTL induction.[16,17]

In phase I trials performed by the CVDP, E75 and GP2 have been shown to be safe, well-tolerated and capable of stimulating a vaccine-specific immune response. A Phase II trial of the E75 vaccine has shown a decreased recurrence rate, an alteration in recurrence patterns, and a trend towards a survival benefit. These results will require validation in a Phase III trial that is currently under development. While these initial clinical results are promising, improvements, such as optimal biologic dosing and periodic booster inoculations, may be required for the induction of a durable vaccine-specific immune response. Furthermore, the use of MHC Class II helper peptides capable of stimulating CD4 helper T lymphocytes may be required to enhance the efficiency of vaccine-specific immune response induction as well as the creation of a durable memory response.[18,19] The CVDP is currently testing one such CD4 helper peptide from HER2/*neu* called AE37. AE37 is a hybrid peptide consisting of the native HER2/*neu*-derived MHC class II epitope AE36 (776–790: GVGSPYVSRLLGICL) being

directly linked with a sequence of four amino-acids (LRMK) termed Ii-Key. Ii-Key fusions to peptides have been reported to increases potency ≥250 times compared to the unmodified class II epitopes in vitro.[20-22] A phase I trial investigating AE37 has been completed and demonstrates the vaccine to be safe, causing minimal toxicity, and capable of eliciting a robust HER2/*neu*-specific immune response even in the absence of an immunoadjuvant.[20]

A key feature in developing successful vaccines and running vaccine trials is the ability to monitor the effect using in vivo and in vitro measurements that can act as surrogates for clinical response. Currently our lab utilizes delayed type hypersensitivity (DTH) reactions to monitor in vivo efficacy in vaccinated patients. In vitro immune responses are determined using assays that measure the number (flow-cytometry based dimer assay) and functional activity (cytokine secretion by ELISPOT assay) of vaccine specific T-cells. As set forth by the Cancer Vaccine Clinical Trial Working Group (CVCTWG), the goals of vaccines include inducing biologic activity upon the immune system and/or against the target disease as evidenced by a clinical, molecular or immune response. Cancer vaccines should generate sufficient immunogenic responses at consecutive follow-up time points after baseline assessment. The CVDP has fulfilled these basic requirements as described by the CVCTWG when performing our vaccine studies, and we continue to search for the best way to determine effectiveness of our vaccines.[23] As confirmed by the CVCTWG, it is widely recognized that as a field we lack a proven immunologic assay correlating with clinical response, therefore we continue to look for emerging in vivo monitoring techniques of peptide-vaccine-specific immune response that could prove beneficial in predicting clinical outcomes. Such assays could shorten preventive cancer vaccine trials and improve the efficiency of cancer vaccine development.

One such potential tool is current technology developed for the detection, quantification and phenotypic characterization of circulating tumor cells (CTC). CTC in cancer have been a source of curiosity from the time, over a century ago, when Paget discussed distant dissemination of breast cancer.[24] The detachment of cells from a primary tumor site and subsequent dissemination to colonize a secondary tumor site is part of the metastatic process. This theory does not address completely the metastatic process, which is complex and multi-factorial in nature.[25] However, the ability to detect and quantify CTC is an important area of research in clinical oncology, as it holds significant potential in terms of estimating patient prognosis, predicting response to treatment and developing systemic and immunotherapy adjuvants. The CVDP is currently exploring the potential use of CTC detection for the monitoring of cancer vaccine efficacy in patients receiving immunotherapy.

Numerous techniques have been explored for the enrichment (density gradient separation, filtration and immunomagnetic separation) and detection (direct microscopy and fluorescent-activated cell sorting cytometric techniques, manual immunocytometry, and PCR based methods) of CTC.[25] The CVDP is currently using the CellSearch system (Veridex, LLC, Warren, NJ), which combines immunomagnetic separation and flow cytometry. This technology has previously been shown to be reliable at detecting and assessing CTC in patients with metastatic breast and colorectal cancer.[26,27]

Method for Detection, Quantification and Phenotypic Characterization of CTC

The CellSearch (Veridex) method is a semi-automated system designed to enrich, identify and enumerate epithelial tumor cells in peripheral whole blood. This system uses epithelial cell surface adhesion molecule (Ep-CAM)-based immunomagnetic separation, intracellular cytokeratin labeling and morphological assessment. The CellSearch Circulating Tumor Cell Kit contains a ferrofluid-based capture reagent and immunofluorescent reagents. The ferrofluid reagent consists of nanoparticles with a magnetic core surrounded by a polymeric layer coated with antibodies against the EpCAM antigen (anti-Ep-CAM ferrofluid) that bind to and immunomagnetically enrich and capture CTC. Thus, antibody-coated magnetic beads are utilized to separate CTC from whole blood. After immunomagnetic capture and enrichment, reagents are added for identification, and enumeration of CTC. Reagents include wash buffers, a cell permeabilization reagent, fluorescently labeled antibodies, and cell fixative. Fluorescently labeled antibodies (anti-CK-Phycoerythrin (PE)) target the following: cytokeratins 8, 18 and 19 characteristic of epithelial cells. To exclude non-nucleated cells, the nucleus is fluorescently labeled with 4', 6-diamidino-2-phenylindole (DAPI) dye. Next leukocytes are identified with an anti-CD45-Allophycocyanin (APC) to distinguish them from epithelial cells. Lastly buffers are used for cell washing and re-suspension.[28] The identification and enumeration of CTC is performed with a semi-automated fluorescence-based microscopy system that permits computer-generated reconstruction of cellular images. CTC are defined by morphological features including positivity for EpCAM, intracellular epithelial CK and DAPI, but lack expression of CD45; hence, a phenotype characterized by $EpCAM^+$, CK^+, $DAPI^+$, $CD45^-$. The accuracy, precision, linearity and reproducibility have been established for this clinically validated FDA-approved system.[29] The specificity of this system is consistently high as circulating epithelial cells are rarely identified in healthy patients (mean = 0.1 ± 0.2/7.5 ml of blood) and in patients with benign disease (mean = 0.1 ± 0.9/7.5 ml of blood).[29,30]

CTC Detection in Metastatic Disease

The process of isolating, identifying and enumerating CTC has been proven feasible and clinically relevant in common epithelial malignancies such as breast and colorectal cancer (CRC). In patients with metastatic breast cancer an elevated level of CTC, defined as ≥5/7.5 ml of blood, prior to beginning treatment and at first follow-up visit has been proven to be an independent adverse prognostic factor.[30] Moreover, after initiation of systemic chemotherapy the elevated level of CTC at time of re-staging was not only independently associated with adverse progression-free (PFS) and overall survival (OS), but was also predictive of reduced chemotherapy efficacy in advanced breast cancer.[31] Importantly, elevated levels of CTC at any time during systemic treatment of metastatic breast cancer were a reliable predictor of disease progression and mortality.[32]

Circulating tumor cell number at baseline and during therapy has been shown to be an independent prognostic factor for PFS and OS in patients with metastatic CRC.[33] A recent study stratified patients with advanced CRC into favorable and unfavorable prognostic groups on the basis of <3 or ≥3 CTC per 7.5 ml peripheral blood.[27] CTC level <3/7.5 ml of blood was an independent predictor

of favorable PFS and OS in metastatic CRC irrespective of clinical characteristics (age, performance status, liver involvement), line of therapy (1st, 2nd or 3rd) or type of therapy (Oxaliplatin, Irinotecan, Bavacizumab).

Outside metastatic breast and CRC, CTC enrichment and analysis has shown a potential role in urological disease. Moreno et al. demonstrated in a pilot study an OS benefit for patients with metastatic prostate cancer who demonstrated CTC ≤5/7.5 ml of blood.[34] Recently, Naoe et al. found that CTC could be detected in urothelial cancer patients with distant metastasis.[35]

CTC Detection in Adjuvant Therapy

With the establishment of CTC monitoring in metastatic breast cancer and the emerging role of CTC monitoring in metastatic CRC, there is continued work to show their presence in the non-metastatic setting and a role for their quantification as an indicator of response to adjuvant therapy. Pachmann and colleagues recently described the detection and quantitative response of CTC by laser scanning cytometry of anti-Ep-CAM-stained epithelial cells in non-metastatic primary breast cancer patients before, during and after adjuvant chemotherapy.[36] The different patterns of response (decreasing, marginal or increasing CTC numbers) were statistically significant in terms of predicting relapse-free survival. Further research is ongoing in the adjuvant setting with encouraging results pending from a large European study enrolling >1,500 breast cancer patients that will be presented at the American Society of Clinical Oncology annual meeting this year.

CTC Detection in Immunotherapy

The concept of CTC detection in immunotherapy has been introduced using PCR based methods in lymphoma and metastatic prostate cancer.[37,38] With growing evidence demonstrating the presence of detectable CTC in non-metastatic breast cancer patients, the CellSearch System (Veridex) was used in a pilot study by our group to quantify and phenotype CTC in a peptide vaccine trial using E75 + GM-CSF in clinically disease-free, node-negative and node-positive breast cancer patients who had completed standard breast cancer therapy.[39] The results demonstrated that CTC are readily identified in these patients and a reduction in CTC could be detected in peripheral blood samples taken from pre- and post vaccination time-points. Since this initial pilot study, further data has been collected on 25 vaccinated patients in this E75 peptide vaccine trial showing the presence of CTC in 76% of patients overall with 68% of CTC^+ patients exhibiting ≥2 CTC (mean 4.8 ± 1.0 CTC/20 ml of blood). Not only do CTC appear to decrease from pre- to post vaccination, but 6 months following vaccination, 9 of 10 patients have either stable or decreasing CTC. In addition, with the administration of booster doses of the E75 vaccine, when a matched comparison of 8 patients pre- and post-booster is evaluated there is >50% reduction in CTCs/20 ml (pre-1.1 ± 0.5 vs. post-0.4 ± 0.2, $p = 0.14$).[40] These results are encouraging; however, the clinical significance of these findings has yet to be determined.

Conclusion

As described by the CVCTWG, biologic agents used for cancer vaccines are safer than conventional cytotoxic drugs in the treatment of cancer.[23] The growing evidence of CTC detection in the metastatic setting has led us to investigate CTC enrichment and enumeration for determining vaccine efficacy. The use of CTC detection has been clinically validated in the metastatic setting, suggested in the adjuvant setting, and shown feasible in immunotherapy. This is a promising tool that, once validated, may provide a surrogate for clinical response. If proven effective, this would take us closer to the goal of shorter clinical trials and, therefore a more efficient assessment and discovery of potentially promising cancer vaccines.

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