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Characterization of sentinel node-derived antibodies from breast cancer patients

Girja S. Shukla, Stephanie C. Pero, Yu-Jing Sun, Chelsea L. Carman, Seth Harlow, David N. Krag*

Department of Surgery and Vermont Cancer Center, University of Vermont College of Medicine, 89 Beaumont Avenue, Burlington, VT 05405, USA

ABSTRACT

Autoantibodies to breast and other cancers are commonly present in cancer patients. A method to rapidly produce these anti-cancer autoantibodies in the lab would be valuable for understanding immune events and to generate candidate reagents for therapy and diagnostics. The purpose of this report is to evaluate sentinel nodes (SNs) of breast cancer patients as a source of anti-cancer antibodies. Radiotracer lymphatic mapping in 29 patients with breast cancer confirmed the identity of the SNs which provided source cells for this study. Flow cytometry demonstrated ~28% of the MNCs were B cells and ~44% of the B cells were class switched memory B cells. EBV-induced proliferation of B cells yielded tumor binding antibodies from 3 wells per 1000 but cultures were too unstable for detailed evaluations. Hybridomas generated by electrofusion produced IgG (48%), IgM (34%) and IgA (18%) antibody isotypes which were screened for binding to a panel of breast cancer cells of the major molecular subtypes. Tumor lysate binding was observed in 28% of the hybridoma clones and 10% of these bound whole tumor cells with unique binding patterns. More detailed evaluation of selected clones showed binding to the patient's own tumor. SNs are removed from more than 100,000 breast cancer patients in the US per year. Samples from these lymph nodes represent a substantial opportunity to generate anticancer antibodies.

1. Introduction

Lymph nodes are highly unique secondary immune mini-organs that receive lymphatic fluid drawn from large areas of the body. Immune cells contained within the lymph are delivered from a wide region of the body to the interior of a lymph node. These regionally delivered immune cells interact with multiple other types of immune cells that are systemically delivered by blood to the lymph node. The lymph node serves as a cross road of multiple types of immune cells that arrive from different locations of the body. Lymphatic fluid also contains a vast array of material representing dissolved and particulate matter. In this capacity, lymph nodes receive lymphatic fluid directly from a breast cancer. Such material may contain cellular debris, exosomes, cytokines and living metastasizing cancer cells. The lymph node creates a complex environment for an immune response to antigens derived from a cancer. Our research group is interested in using the tumor-draining lymph node to obtain B cells that have undergone an adaptive response to cancer antigens and produce anti-cancer antibodies.

For decades the strategy of developing new cancer drugs has been to identify druggable targets on or within cancer cells that are common to all or a majority of cancer patients. In contrast to the goal of focusing on single cancer cell targets, recent groundbreaking clinical results with checkpoint inhibitors and with adoptive cell therapy have shown that the immune system targets multiple, unique mutated antigens (Parkhurst et al., 2017; van Rooij et al., 2013). Only a fraction of these mutated antigens are shared or public antigens (Angelova et al., 2015). Successful responses, including eradication of metastatic cancers are associated with immune targeting of a personalized set of mutated cancer proteins (Tran et al., 2017). This information provides motivation to develop additional methods of generating personalized immune treatments of cancer.

Autoantibodies to cancer commonly occur (Disis et al., 1994; Kotlan et al., 1999; Alsoe et al., 2008) and hundreds of tumor-directed autoantibodies have been identified using serological identification of antigens by recombinant cDNA expression cloning (SEREX) (Fortner et al., 2017). Although SEREX can identify the presence of anti-tumor antibodies it does not provide the methods to generate candidate antibodies. A method to rapidly produce anti-cancer autoantibodies in the lab would be valuable for understanding immune events and to generate candidate reagents for therapy and diagnoses.

In the present study, we used mononuclear cells from surgically removed cancer-draining lymph nodes of breast cancer patients and characterized the B cell repertoire for their phenotypes. We analyzed their secretary antibody products for their isotypes and binding to a

* Corresponding author.
E-mail address: David.Krag@uvm.edu (D.N. Krag).

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panel of breast tumor cell lines and to the patients' own tumors.

2. Methods

2.1. Patients

Patients with breast cancer were enrolled as part of protocol approved by The University of Vermont Committees on Human Research. Patients had invasive tumors at least one centimeter in diameter and were undergoing breast surgery and removal of a sentinel lymph node.

2.2. Localization of lymph nodes

Within 4 h prior to surgery, 1 to 4 ml of sulfur colloid labeled with 1.0 mCi Technetium-99 m was injected into the breast around the primary tumor. The lymph node receiving drainage directly from the tumor was identified with a hand held gamma detector as the lymph node became radioactive. These first draining lymph nodes are called SNs. The accuracy of this method and the use of this limited biopsy as a preferred technique has been established through a large randomized trial (Krag et al., 2010).

2.3. Sentinel lymph node processing

The cells were harvested from the nodes by scraping the cut surface or removing a thin slice of sentinel lymph nodes. Lymph node slices were mechanically disaggregated and sieved before Ficoll gradient centrifugation for the separation of mononuclear cells (MNC) as a buffy coat. The yield of lymphocytes varied from 0.5 to 50 × 10 (Kotlan et al., 1999) cells per patient.

2.4. Flow cytometric analysis of tumor-draining lymph node B cells

Briefly, mononuclear cells from the tumor-draining lymph nodes in PBS containing 2% fetal bovine serum were stained with fluorophore-conjugated Abs (Becton, Dickinson and Co.) against specific cell-surface markers (CD19, CD27, IgD, IgM, and CD38) to identify different categories of B cell sub populations. Cells were stained in 2 batches; one batch of the cells was stained for CD19, CD27, IgD and IgM and the other was for CD19, CD38 and IgM. Multiple controls including fluorescence-minus-one (FMO) samples were run in parallel. The stained cell samples were run on BD LSRII (BD Biosciences, San Jose, CA) flow cytometer equipped with BD FACSDiva™ version 8 software for data acquisition. The data were analyzed using FlowJo™ v10.1 software (FlowJo, LLC, Ashland, OR) following required gating with the help of proper controls including fluorescence minus one. The analysis provided an insight into the tumor-primed B cell development in draining lymph nodes.

2.5. Memory B cell separation and EBV transformation

Memory B cells were isolated from mononuclear cells in two steps. In the first step, we used a kit (Stem Cell Technologies) that is designed for enriching B cells by magnetic separation of non-B cells. This kit uses antibodies bound in bispecific tetrameric complexes that are directed against human blood cell surface antigens (CD2, CD3, CD14, CD16, CD36, CD43, CD56, CD66b, Glyphorin A) and dextran. In the second step, $CD27^+$ cells were positively selected using magnetic particles. The isolated memory B cells were immortalized during 2 weeks in the presence of allogeneic irradiated mononuclear feeder cells, Epstein-Barr virus (EBV) from B95-8 cell culture supernatant (American Type Culture Collection), and TLR-9 ligand CpG 2006 by the procedure described earlier (Traggiai et al., 2004). We have used this method of immortalization routinely in our laboratory and have successfully adapted it for 384-well plates. In the experiments presented here, we plated ~50 memory B cells per well and observed the cell immortalization in 70%–85% of the wells with positive IgG or IgM secretion. In our laboratory, this cell plating number has been found to consistently result in monoclonal cell expansion in most of the wells. Similar observations have been reported by other researchers.

2.6. Hybridoma generation

We generated hybridomas from the cells obtained from tumor-draining lymph nodes of 29 breast cancer patients. The MNC were stimulated for 3 to 5 days with phytohemagglutinin-L (PHA-L, 2.5 μg/ml) at 37 °C/5% CO_2 before fusion. The hybridoma fusion partner to MNC was murine plasmacytoma P3X63.Ag8.653.(Kearney et al., 1979) Mononuclear cells were fused under hypo-osmolar condition (Zimmermann et al., 1990) using a Multiporator/Helix chamber (Eppendorf, Westbury, NY) or BTX Electro Cell Manipulator ECM 2001/microslides (Harvard Apparatus, Inc., Holliston, MA). The fused cells were suspended (25,000/ml) in the culture medium and 100 μl were added to each well of 96-well plates. This density of fused cells distribution in 96-well plates has been consistently found to yield approximately 0–1 growing hybridoma per well in our laboratory. Distributing the fused cells at a density that yields single clones is more time-consuming up front but makes downstream screening more rapid and reliable. Following HAT (hypoxanthine-aminopterin-thymidine medium; ATCC, Manassas, VA) selection, hybridomas were shifted to 1xHT medium (Hypoxanthine-Thymidine, ATCC) and eventually to a complete growth medium.

2.7. Dot-blot analysis of antibody secretion by EBV-immortalized B cells

Immunoglobulin secretion in a 384-well plate was determined by dot blot analysis on nitrocellulose membrane which involved arraying the supernatants from EBV-immortalized B cell cultures as individual spots on a nitrocellulose membrane. The antibody-arrayed membranes were blocked for 2 h at room temperature and the antibody secreting wells were detected with anti-human IgG/IgM/IgA antibody–HRP conjugates (Thermo Fisher, Waltham, MA) and ECL reagent (Amersham).

2.8. ELISA of antibody secretion by hybridoma clones

The hybridoma supernatants were screened by ELISA for identifying the immunoglobulin-secreting wells and their isotypes (IgM, IgG and IgA). Briefly, hybridoma supernatants were added to the wells of clear Maxisorp 96well plate (Nunc A/S, Roskilde, Denmark) and incubated overnight at 4 °C. The wells were washed 5×, 3 min each, with TBST (TBS containing 1% Tween-20). The wells were blocked with 1% casein for 2 h at room temperature. Following incubation with anti-human IgG/IgM/IgA antibody–HRP conjugates (Thermo Fisher, Waltham, MA) and washing, TMB soluble substrate (Calbiochem, Billerica, MA) were added for identifying the presence of immunoglobulins and their subclasses in the hybridoma supernatants. The human immunoglobulin standards were run in parallel for measuring and normalizing the supernatant Ab concentrations in certain experiments.

2.9. Binding studies

Each immunoglobulin secreting clone was evaluated for binding to a panel of breast tumor cell lines representing Triple Negative (ER-negative, PR-negative, HER2-negative; BT20 and SUM159), HER2-positive (HER2+, ER-negative, and PR-negative; AU565 and SKBR3), Luminal A (ER-positive and/or PR-positive and HER2-negative; T47D and MCF7), and Luminal B (ER-positive and/or PR-positive and HER2-positive; MDA-MB361 and BT474) subclasses of breast cancer. The studies with non-cancer breast epithelial cells (MCF10A and MCF12A) were performed in parallel for a comparison.

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