Journal of Immunological Methods xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Autologous antibodies that bind neuroblastoma cells

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ARTICLE INFO

Article history:
Received 18 February 2015
Received in revised form 15 July 2015
Accepted 16 July 2015
Available online xxxx

Keywords:
Neuroblastoma
Phage display
Antibody
Hybridoma
Bone marrow

ABSTRACT

Antibody therapy of neuroblastoma is promising and our goal is to derive antibodies from patients with neuroblastoma for developing new therapeutic antibodies. The feasibility of using residual bone marrow obtained for clinical indications as a source of tumor cells and a source of antibodies was assessed. From marrow samples, neuroblastoma cells were recovered, grown in cell culture and also implanted into mice to create xenografts. Mononuclear cells from the marrow were used as a source to generate phage display antibody libraries and also hybridomas. Growth of neuroblastoma patient cells was possible both in vitro and as xenografts. Antibodies from the phage libraries and from the monoclonal hybridomas bound autologous neuroblastoma cells with some selectivity. It appears feasible to recover neuroblastoma cells from residual marrow specimens and to generate human antibodies that bind autologous neuroblastoma cells. Expansion of this approach is underway to collect more specimens, optimize methods to generate antibodies, and to evaluate the bioactivity of neuroblastoma-binding antibodies.

1. Introduction

Neuroblastoma is the most common extracranial solid tumor in children, accounting for 8–10% of all childhood cancers. Most patients with neuroblastoma are young (median age at diagnosis is 18 months) and commonly present with metastatic disease. More than 60% of patients have high-risk tumors with a poor prognosis (Navid et al., 2009). Anti-GD2 antibody therapy in patients that have minimal residual disease following stem cell transplantation has led to improvement of survival (Fish and Grupp, 2008; Yu et al., 2010). Recently, dinutuximab (a GD-2-binding monoclonal antibody), in combination with granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin-1 (IL-2), and 13-cis-tetinoic acid (RA) was approved by FDA for pediatric patients with high-risk neuroblastoma (Dhillon, 2015). Anti-GD2 antibody therapy can be associated with considerable pain and its efficacy has only been observed to date in patients with minimal residual disease (Cheung and Dyer, 2013). Antibody therapy is clearly biologically active but it is important to develop new and better tolerated antibodies to improve the outcomes of patients with neuroblastoma. A multiplicity of antibodies may need to be developed to effectively treat neuroblastoma. At the time of initiating treatment, variable target antigen density on neuroblastoma cells indicates that sensitivity to a single antibody would not be equal for all tumor cells. In addition, regrowth following an initial response may lead to tumor cells that no longer express sufficient target antigen to be inhibited. Therefore, additional treatments with the initial antibody would be ineffective for this new population of tumor cells. Antigen density can be readily assessed with immunohistochemistry and could guide selection of suitable antibodies to treat neuroblastoma if a multiplicity of antibodies were available. Our goal is to use a patient's own set of antibodies that have been generated in response to their tumor to identify new therapeutic antibodies. Therapeutic antibodies generated from a patient to their own tumor may also have applicability to subsequent patients.

Phage display is a powerful method of generating and screening large numbers of antibodies for binding to targets. Using patient-derived B cells to generate phage antibody libraries has the potential advantage of including antibodies that have been made by the patient in response to their own cancer. Phage antibody libraries have been generated from patients with breast cancer, colorectal cancer and multiple tumor types (Rubinstein et al., 2002; Belimezi et al., 2006; Graus et al., 1998; Figini et al., 2009; Roovers et al., 2001; Baskar et al., 2009; Li et al., 2001; Dantas-Barbosa et al., 2009) including neuroblastoma (Uttenreuther-Fischer et al., 2006). Application of these libraries has been primarily to screen for ligands to cancer cell lines or heterologous cancer tissue. Very few reports have described screening an antibody library against autologous tissue (Novinger et al., 2014). Using high-resolution liquid chromatography/tandem MS proteomic analyses of serum antibodies coupled with next-generation sequencing of the V gene repertoire in peripheral B cells, recent analysis of the serum antibody repertoire generated in response to a tetanus toxoid vaccine indicates very little overlap of antibody sequences between individuals

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http://dx.doi.org/10.1016/j.jim.2015.07.009
0022-1759/

(Lavinder et al., 2014). This indicates that the range of antibodies between patients will be highly variable even to a common antigenic target. Given the complexity of an endogenous tumor and the molecular variation of tumors between patients, even greater variability of antibodies can be expected. Indeed, between patients there may be little to no overlap of antibodies targeting heterologous tumors. Seeking antibodies that target autologous tumor may be the most productive way to identify therapeutic antibodies.

In this study we took advantage of the availability of residual bone marrow aspirates that were obtained for clinical purpose in pediatric patients with neuroblastoma. We used bone marrow B cells as a source for generating antibodies, using phage display and hybridoma technologies. We also recovered neuroblastoma cells from the same marrow aspirates that allowed screening for the antibodies to autologous cancer cells.

2. Material and methods

2.1. Patients and tissue collection

Tissues from pediatric patients with neuroblastoma were obtained after an IRB approved consent obtained from each subject or subject guardian. Marrow samples were obtained as part of regular clinical evaluation and residual marrow used for this study.

2.2. Generation of low passage cultured tumor cells and mouse xenografts

Bone marrow aspirates were passed through a 40 μm filter to collect neuroblastoma cells as clusters called neurospheres (Fig. 1, step 1). Retained neuroblastoma cells were cultured with media optimized for the recovery of neuroblastoma cells (Fig. 1, step 3) (Hansford et al., 2007). After neuroblastoma cells attained logarithmic growth in tissue culture, cells were expanded. At low passage number, aliquots were cryopreserved. For each sample, the neuroblastoma cell type was confirmed by immunofluorescence using the neuronal markers nestin, synaptophysin, GD2, p75, tyrosine hydroxylase (TH), and NB84. Cultured neuroblastoma cells were implanted subcutaneously in the flanks of NOD-scid IL2Rnull mice (Fig. 1, step 3). Tumors that grew successfully were engrafted serially in additional mice and also plated for post-xenograft in vitro culture.

2.3. Isolation of marrow mononuclear cells (MNCs)

The marrow mononuclear cells were separated from the marrow filtrates using Ficoll density gradient centrifugation. The cells were cryopreserved for the generation of antibodies using phage display and hybrodoma techniques.

2.4. Generation of phage display scFv library

To conserve limited material, phage libraries were constructed from pooled patient MNCs. Library NB2 was constructed from MNCs pooled from four patients and NB3 library was constructed from MNCs pooled from two patients. Total RNA was extracted from cryoperserved marrow MNCs and pooled from multiple patients for the construction of phage display libraries (Fig. 1, step 2). cDNA was synthesized from 20 μg total RNA using the Superscript III first-strand synthesis system (Invitrogen. Grand Island, NY) with a combination of random hexamers and oligo dT primers to ensure broad representation of antibodies. Each V family of variable regions (VH or VL) was amplified by independent PCR, with a total of 45 different reactions according to previously published methods (Andris-Widhope et al., 2001). A PCR-overlapping

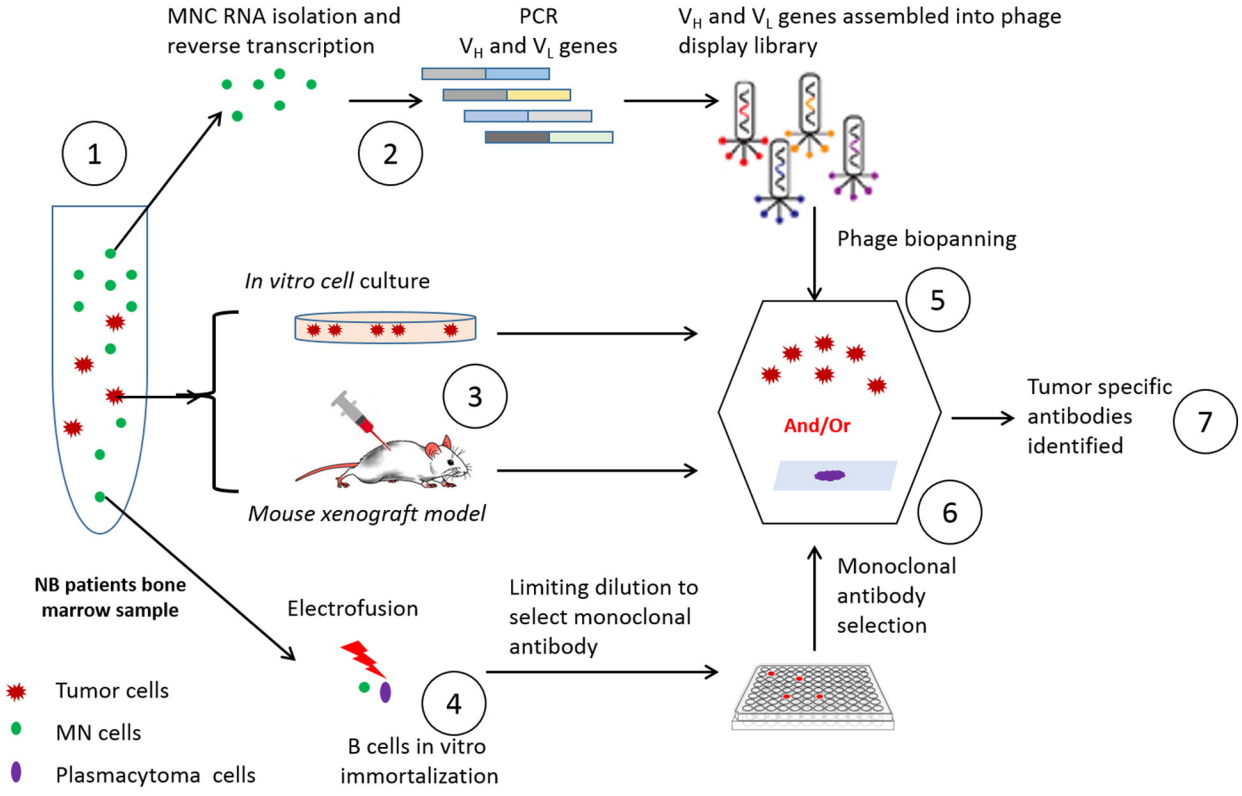

Fig. 1. Schematic presentation of methods used in this project. Neuroblastoma patients' bone marrow samples (step 1) were used for tumor cell isolation and mononuclear cell (MNC) separation. Selected MNC population was used for phage display scFv library construction (step 2) and human hybridoma cell generation (step 4). Isolated tumor cells (step 3) were used for in vitro tissue culture and mouse xenograft modal establishment. The generated phage libraries were used for panning and screening on matching patients' primary cultured tumor cells and/or mouse xenograft tissue sections using immunofluorescence (IF) staining (step 5). Monoclonal human antibodies selected from hybridoma work were tested on matching patients' in vitro cultured tumor cells and/or mouse xenograft tissues (step 6). Tumor specific antibodies were identified in step 7 by screening on normal human tissues by IF stain.

Please cite this article as: Sun, Y., et al., Autologous antibodies that bind neuroblastoma cells, J. Immunol. Methods (2015), http://dx.doi.org/10.1016/j.jim.2015.07.009