Immunology Letters 162 (2014) 132–139

Contents lists available at ScienceDirect

Immunology Letters

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

Murine carcinoma expressing carcinoembryonic antigen-like protein is restricted by antibody against neem leaf glycoprotein

Arnab Das[a,c], Subhasis Barik[c], Anamika Bose[c], Soumyabrata Roy[c], Jaydip Biswas[b], Rathindranath Baral[c], Smarajit Pal[a,*]

[a] Clinical Biochemistry Unit, Chittaranjan National Cancer Institute (CNCI), 37, S. P. Mukherjee Road, Kolkata 700026, India
[b] Department of Surgical Oncology and Medical Oncology, Chittaranjan National Cancer Institute (CNCI), 37, S. P. Mukherjee Road, Kolkata 700026, India
[c] Department of Immunoregulation and Immunodiagnostics, Chittaranjan National Cancer Institute (CNCI), 37, S. P. Mukherjee Road, Kolkata 700026, India

ARTICLE INFO

Article history:
Received 12 June 2014
Received in revised form 31 July 2014
Accepted 4 August 2014
Available online 13 August 2014

Keywords:
Colon cancer
Carcinoembryonic antigen
CEA-like protein
Polyclonal antibody
Neem leaf glycoprotein
Antibody-dependent cellular cytotoxicity

ABSTRACT

We have generated a polyclonal antibody against a novel immunomodulator, neem leaf glycoprotein (NLGP) that can react to a specific 47 kDa subunit of NLGP. Generated anti-NLGP antibody (primarily IgG2a) was tested for its anti-tumor activity in murine carcinoma (EC, CT-26), sarcoma (S180) and melanoma (B16Mel) tumor models. Surprisingly, tumor growth restriction was only observed in CT-26 carcinoma models, without any alteration in other tumor systems. Comparative examination of antigenicity between four different tumor models revealed high expression of CEA-like protein on the surface of CT-26 tumors. Subsequent examination of the cross-reactivity of anti-NLGP antibody with purified or cell bound CEA revealed prominent recognition of CEA by anti-NLGP antibody, as detected by ELISA, Western Blotting and immunohistochemistry. This recognition seems to be responsible for anti-tumor function of anti-NLGP antibody only on CEA-like protein expressing CT-26 tumor models, as confirmed by ADCC reaction in CEA^+ tumor systems where dependency to anti-NLGP antibody is equivalent to anti-CEA antibody. Obtained result with enormous therapeutic potential for CEA^+ tumors may be explained in view of the epitope spreading concept, however, further investigation is crucial.

1. Introduction

Neem leaf glycoprotein or NLGP is an immunomodulator, recently reported as a therapeutic regimen to restrict murine sarcoma [1,2] and melanoma [3,4] growth. Involvement of cellular immune system, especially $CD8^+$ T cells, in such tumor growth restriction is widely discussed [1–4]. We have reported earlier that NLGP matures dendritic cells in the context of tumor, thereby, efficiently presents tumor antigens to T cells [5–8], resulting activation, proliferation, and acquisition of the cytotoxic instinct to kill tumor cells by $CD8^+$ T cells in antigen specific manner [1,2,7]. At the same time, systemic administration of immunogenic NLGP either as a whole molecule or fragmented peptides may raise antibody response within the host. However, antibody-based therapeutic aspects in context of NLGP are not examined.

Antibody-based therapy for cancer has become established over the past 20 years and is now one of the most successful and important strategies for treating cancer patients [9,10]. The fundamental basis of antibody-based therapy in cancer lies chiefly on recognition of antigen expressed on cancer cells by antibody, e.g., herceptin, rituximab, cetuximab etc. [11–13], thereby initiating of one or more effector functions including phagocytosis, opsonization, complement mediated cytotoxicity or antibody-mediated cellular cytotoxicity (ADCC) [14]. Introduction of the concept of epitope spreading [15] raises the possibilities of cross reactivity by recognizing one antigen by an antibody raised against other [16,17]. Another practical application to induce broad-spectrum responses is making use of multispecific antigen recognition by antibodies and T cells [18]. Context of wide acceptability of NLGP as an immunomodulator by most of the immune cells [5,7,8,19,20], prompted us to review such multi-specific antigen recognition, by the anti-NLGP antibody.

In this perspective, we have screened anti-tumor activity of anti-NLGP antibody, against a panel of murine carcinoma,

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; CEA, carcinoembryonic antigen; pAb, polyclonal antibody; NLGP, neem leaf glycoprotein.

* Corresponding author. Tel.: +91 033 2476 5101x362; fax: +91 033 2475 7606.
E-mail addresses: rathindranath.baral@cnci.org.in (R. Baral), psmarajit@hotmail.com (S. Pal).

http://dx.doi.org/10.1016/j.imlet.2014.08.004
0165-2478/

sarcoma, and melanoma tumor models. Obtained results confirm the cross-reactivity of the generated anti-NLGP antibody towards carcinoembryonic antigen (CEA), thereby inhibiting the growth of tumor expressing CEA by inducing ADCC.

2. Materials and methods

2.1. Reagents and media

Human CEA was procured from Aspen Bio Inc., CO, USA. Anti-human CEA antibody, anti-mouse peroxidase labeled IgG and fluorescent stain DAPI were procured from Sigma, St. Louis, USA. Goat anti-mouse immunoglobulin isotypes/subclasses labeled with alkaline phosphatase were purchased from Southern Biotechnology, Birmingham, AL, USA. Different plant derived components (such as palcitaxel, vinblastin, quercetin dehydrate), KLH, BCG, OVA, and BSA were procured from Sigma, St. Louis, USA. Flow cytometry antibody, like, anti-mouse IgG-FITC was procured from e-Biosciences, San Diego, USA. TMB substrate solution (OptEIA^{TM}) was purchased from BD-Pharmingen, San Diego, USA. LDH release kit for cytotoxicity detection was procured from Roche Diagnostics (Mannham, Germany). AEC chromogen solution was purchased from VECTOR laboratories Inc (Burlingame, CA). Different culture media, including RPMI 1640/DMEM, FBS, penicillin-streptomycin, L-glutamine (2 mM), HEPES and sodium pyruvate were procured from GIBCO-Life Technologies, NY, USA. Chemiluminescence detection kit was purchased from Pierce (Rockford, IL, USA). Protein G, poly-L-lysine, Tween-20, Freund's adjuvant, p-nitrophenylphosphate (p-NPP) etc. were procured from Sigma, St. Louis, USA.

2.2. Neem leaf glycoprotein (NLGP)

Extract from neem *(Azadirachta indica)* leaves was prepared by the method described [21]. Briefly, pulverized leaf powder was soaked overnight in PBS, pH 7.4; supernatant was collected by centrifugation at 1500 rpm. Neem leaf preparation (NLP) was then extensively dialyzed against PBS and concentrated by Centricon Membrane Filter (Millipore Corporation, Bedford, MA, USA) with 10 kDa molecular weight cut off. The active portion of this NLP was identified subsequently, termed neem leaf glycoprotein or NLGP. NLGP appeared as a single protein in non-denatured PAGE and HPLC, however, three bands in SDS-PAGE [2,5,22].

2.3. Animals, cell lines and tumors

Female Swiss and C57BL/6 mice bred and maintained in "Animal Care and Maintenance Facility" of Chittaranjan National Cancer Institute (CNCI), Kolkata was used in this study. Female BALB/c mice were purchased from Indian Institute of Chemical Biology, Kolkata, India. Athymic nude (4–6 weeks old) mice were purchased from National Centre for Laboratory Animal Sciences (NCLAS), NIN, Hyderabad and maintained in a specific pathogen free facility. At the initiation of the experiment, their age was 4–5 weeks with average body weight of 25–27 g. Swiss, C57BL/6, BALB/c and athymic nude mice were maintained in a temperature controlled, pathogen free room at CNCI, Kolkata. Autoclaved dry pellet (Epic Laboratory Animal Feed, Government of West Bengal, Kalyani, India) and autoclaved water were given *ad libitum*. Human CEA$^+$ Colo-205, HCT-116, HT-29 (colon cancer) and CEA$^-$ SiHa (cervical cancer), SCC084 (oral cancer), MCF7 (breast cancer) along with murine CEA$^-$ B16 (B16MelF10) cell lines were maintained in their respective culture media for 8–10 days at 37 °C humidified incubator. S180 (Sarcoma 180), EC (Ehrlich's carcinoma), and CT-26 (colon cancer) cells are maintained at CNCI, Kolkata, by regular intraperitoneal passage in Swiss and BALB/c mice, respectively. The Institutional Animal Ethics Committee approved the experimental design.

2.4. Generation and purification of polyclonal antibody

Syngenic female BALB/c mice ($n = 6$ for each group) were immunized with NLGP (25 μg) coupled with KLH (1:1), along with incomplete Freund's adjuvant (1:1) subcutaneously (s. c.) once a week for 4 weeks and blood was collected weekly after each immunization by retro-orbital puncture and serum was separated and stored. Anti-NLGP polyclonal antibody (pAb) was purified from immunized mice serum using protein G as per manufacturer's protocol. The protein concentration was estimated using Folin-phenol reagent [23].

2.5. SDS-PAGE and western blotting

Purified NLGP and CEA were electrophoresed on 10% SDS-PAGE, transferred for western blotting as described [2] and incubated with anti-NLGP and anti-CEA antibodies. After washing, blots were incubated with HRP-conjugated secondary antibody for 2 hr at room temperature. Bands were detected using Western lighting chemiluminescence detection kit according to the manufacturer's manual.

2.6. In vivo tumor growth restriction

Viable mouse CT-26, S180, EC (2×10^6 cells/100 μL PBS) and B16Mel (2×10^5 cells/100 μL PBS) tumor cells were inoculated s.c. on the right hind leg quarter of 6 week old female BALB/c, Swiss and C57BL/6 mice, respectively. To develop subcutaneous solid tumors in athymic nude mice, viable Colo-205 (2×10^6) cells were inoculated in right hind leg quarters. In all tumor systems, after reaching the tumor volume of 24 mm^3 approximately, a group of mice ($n = 6$) in each set was administered s. c. with anti-NLGP antibody (25 μg/mice/injection) on the left hind leg quarter once per week for 4 weeks in total. Other group of mice received unrelated rat IgG antibody as isotypic control. Tumor growth was monitored twice a week by caliper measurement and tumor volume was determined using the formula: Tumor volume = $(\text{width})^2 \times \text{length}/2$. Survivability of two groups in mice bearing each tumor type was also monitored.

2.7. ELISA

Generated anti-NLGP antibody was confirmed for its CEA specificity by its reaction with a panel of antigens, consisting of: NLGP, palcitaxel, vinblastin, quercetin, KLH, BCG, OVA, and BSA by ELISA.

Isotypes (IgG, IgM, and IgA) and its subclasses (if it is IgG) were determined by ELISA. A panel of alkaline phosphatase conjugated secondary antibodies, e. g., anti-mouse IgG, IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3 were used for this purpose.

The specificity was also checked by its reaction to purified CEA$^+$, CEA$^-$ tumor cell lysates.

2.8. Immunohistochemistry

Sections (5 μm) from formalin fixed paraffin embedded colon cancer tissues ($n = 10$) and adjacent normal specimens were prepared as described [2] using anti-NLGP and anti-CEA antibodies. Negative controls were included in each staining step, where primary antibodies are replaced by mouse IgG.

Murine CT-26 tumors were harvested and snap-frozen in OCT compound to prepare sections (5 μm) using cryostat, air-dried, and fixed in ice-cold methanol for 20 min. After blocking with 5% BSA, cryosections were stained with both anti-NLGP and anti-CEA mAbs.