Biochemical and Biophysical Research Communications xxx (2018) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Natural antibody against neuroblastoma of TH-MYCN transgenic mice does not correlate with spontaneous regression

Naonori Kawakubo [a, b], Yui Harada [a, *], Minori Ishii [a, b], Ryota Souzaki [b], Yoshiaki Kinoshita [b], Tatsuro Tajiri [c], Tomoaki Taguchi [b], Yoshikazu Yonemitsu [a]

[a] R&D Laboratory for Innovative Biotherapeutics, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan
[b] Department of Pediatric Surgery, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan
[c] Department of Pediatric Surgery, Kyoto Prefectural University of Medicine, Kyoto, Japan

ARTICLE INFO

Article history:
Received 5 July 2018
Accepted 19 July 2018
Available online xxx

Keywords:
MYCN
Neuroblastoma
Natural antibody

ABSTRACT

The mechanism underlying the spontaneous regression of neuroblastoma is unclear. Although it was hypothesized that this regression occurs via an immunological mechanism, there is no clinical evidence, and no animal models have been developed to investigate the involvement of immune systems, especially natural antibodies, against neuroblastoma. We performed an immunological analysis of homo- and heterozygous TH-MYCN transgenic mice as a model of aggressive neuroblastoma. Mice with no or small (<5 mm) tumors showed higher antibody titers in plasma than mice with large (>5 mm) tumors. A significant negative correlation was observed between the tumor diameter and the titer of antitumor antibody. This antibody had complement-dependent cytotoxicity but not antibody-dependent cellular cytotoxicity against neuroblastoma cells. Moreover, B-cell depletion had no effect on the tumor incidence *in vivo*. We revealed that TH-MYCN transgenic mice have a natural antibody against neuroblastoma that correlate with tumor size. However, this antibody does not correlate with the spontaneous regression of neuroblastoma. Thus, the function of the natural antibody is limited.

Neuroblastoma (NB) is the most common extracranial solid malignant tumor in children [1]. In some cases of infant neuroblastoma (stage 4S), this tumor undergoes spontaneous regression and has a favorable prognosis with a greater than 90% survival rate [2]. Complete regression is also seen in some stage 1 tumors [3]. However, despite the enormous amount of basic and clinical research conducted on this disease entity, neuroblastoma patients with MYCN amplification (>10 copies per cell) show particularly poor prognoses [4].

Brodeur et al. introduced four major pathways that may explain, together or in part, the spontaneous regression of NB [5]: (1) immune-mediated cell killing by antibody-dependent cellular cytotoxicity (ADCC) or by natural killer (NK) cells, (2) deprivation of neurotrophin and the activation of programmed apoptosis, (3) apoptosis caused by low levels/absence of telomerase, and (4) epigenetic changes in gene expression. However, there has been no clear evidence in support of these or any other mechanisms of spontaneous NB regression.

TH-MYCN mice, a model in which the human MYCN gene is integrated under the control of a rat tyrosine hydroxylase, develop NB tumors [6]. The histological and pathological aspects of these tumors are similar to those of human NB [7,8], and the mouse tumors show genomic aberrations with some similarity to those in human NB [9]. The mouse tumors have many of the molecular characteristics of human NB [10]. There have been very few reports on TH-MYCN mice from an immunological viewpoint. In the few studies that have been performed, infiltrations of T cells, macrophages and dendritic cells have been observed in the tumor microenvironment [11,12]. Myeloid-derived suppressor cells (MDSCs) also infiltrate to the tumor and exhibit immune suppressive activity [12]. In a xenograft model of TH-MYCN primary culture cells, treatment with anti-GD2 and NK cells achieved a good prognosis [13].

Based on these findings, we hypothesized that spontaneous regression occurs via an immunological mechanism, and we investigated this possibility by analyzing the immune response in TH-MYCN mice. Our focus is whether there is a causal relationship

* Corresponding author. R&D Laboratory for Innovative Biotherapeutics, Graduate School of Pharmaceutical Sciences, Kyushu University, 6th Floor, Collaborative Research Station I, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan.
E-mail address: rkfraile@med.kyushu-u.ac.jp (Y. Harada).

https://doi.org/10.1016/j.bbrc.2018.07.097

between spontaneous regression and the natural antibody.

1. Methods

1.1. Mice

TH-MYCN heterozygote mice [6] were kindly provided by Professor K. Kadomatsu at Nagoya University. Genotyping was carried out as described by Haraguchi et al. [14]. The tumors were detected by ultrasound using a VEVO770 Micro-Imaging System (FujiFilm VisualSonics, Toronto, ON, Canada).

Animal experiments were reviewed and approved by the Ethics Committee on Animal Experiments at the Faculty of Medical Sciences, Kyushu University. The experiments were carried out under the conditions indicated in the Regulations for Animal Experiments of Kyushu University and Law 105 and Notification 6 of the Government of Japan.

1.2. Cells and antibodies

IMR-32 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI-1640 medium supplemented with 15% fetal bovine serum (FBS) under a humidified atmosphere containing 5% CO_2 at 37 °C.

Specifically, the following monoclonal antibodies (mAbs) were used in this study: Alexa488-conjugated anti-mouse IgM (Abcam, Cambridge, UK), Dylight488-conjugated anti-mouse IgG (Abcam), FITC-conjugated anti-mouse CD3 (BioLegend, San Diego, CA), FITC-conjugated anti-mouse IgG1 (BioLegend), FITC-conjugated anti-mouse IgG2a (BioLegend), FITC-conjugated anti-mouse IgG2b (BioLegend), FITC-conjugated anti-mouse B220 (BioLegend), PE-conjugated anti-mouse CD8a (BioLegend), FITC-conjugated anti-mouse IgG3 (Santa Cruz Biotechnology, Dallas, TX), PerCP/Cy5.5-conjugated anti-mouse CD4, PerCP/Cy5.5-conjugated anti-mouse CD49b, PerCP/Cy5.5-conjugated anti-mouse CD19, FITC-conjugated anti-human IgM (BioLegend), and Alexa-488-conjugated anti-human IgG Fc (BioLegend).

1.3. Flow cytometric analysis

The cells reacted with appropriate mAbs were analyzed using a FACSCalibur™ cell analyzer with CellQuest software (Becton Dickinson, Tokyo). Dead cells were excluded by staining with propidium iodide. The data analyses were performed using FlowJo 7.6 software (Tree Star, Ashland, OR).

1.4. In vivo depletion

The administration schedule was as follows: For NK cell depletion, anti-asialo GM1 (Wako, Osaka, Japan) was given intraperitoneally (i.p.) (100 μg/dose) to mice 3 × /week beginning when the mice were 3 weeks old until they were 22 weeks old. $CD4^+$ or $CD8^+$ cells in the mice were eliminated by an i.p. injection of mAbs (Clone GK1.5 for $CD4^+$ cells depletion, Clone 53–6.72 for $CD8^+$ cell depletion; Bio X Cell, West Lebanon, NH) 2 × /week beginning when the mice were 3 weeks old until they were 22 weeks old.

For the depletion of peritoneal B cells, 100 μl of 2% thioglycolic acid was i.p.-administered to newborn mice, and the next day, 250 μg of anti-CD20 mAb (Clone 5D2, Genentech, San Francisco, CA) was i.p.-injected. The schedule of administration was days 0, 7, 14, 42, and 70. For the depletion of the mothers' peritoneal B cells, 100 μl of 2% thioglycolic acid and anti-CD20 mAb was i.p.-administered to the mother before pregnancy 1 × /week for 3weeks.

1.5. Detection of anti-neuroblastoma antibody

The tumors obtained from TH-MYCN mice were dissociated using a Tumor Dissociation Kit (Miltenyi Biotec, Cologne, Germany). The tumor cells were incubated with mouse plasma obtained from TH-MYCN mice at 4° for 30 min, then washed three times with D-PBS and stained with secondary antibody (anti-mouse IgG/IgG1/IgG2a/IgG2b/IgG3/IgM).

For the detection of human anti-NB antibody, IMR-32 was incubated with human plasma obtained from healthy adult volunteers at 4° for 30 min, washed three times with D-PBS, and stained with secondary antibody (anti-human IgG/IgM). All participants gave informed consent for the use of their data, and ethical approval was obtained from the Ethics Committee of the Institute of Health Science at Kyushu University.

1.6. Purification of human IgG

Purification of IgG from human plasma was done using Dynabeads™ Protein G (Veritas, Tokyo). A 10-μl aliquot of human plasma was diluted in 200 μl of D-PBS with Tween-20, then incubated with Dynabeads™ Protein G for 10 min at room temperature. The beads-IgG complex was eluted by elution buffer, and the purified IgG was obtained.

1.7. Complement-dependent cytotoxic assay

First, 4×10^4 TH-MYCN primary cells suspended in 15 μl of RPMI-1640 containing 1% FBS were mixed with 5 μl of mouse plasma. Then, 20 μl of human complement and 60 μl of RPMI-1640 containing 1% FBS were added and the mixture was incubated at 37 °C for 5 min. The viable cells were then immediately counted using a hemocytometer and trypan blue staining.

1.8. CDC assay

First, 4×10^4 IMR-32 cells suspended in 90 μl RPMI-1640 medium containing 15% FBS were cultured with 10 μl of human plasma for 1 h at 4 °C. Then, 10 μl of human complement (Sigma-Aldrich Japan, Tokyo) was added and the mixture was incubated at 37 °C for 60 min. The viable cells were then immediately counted using a hemocytometer and trypan blue staining.

1.9. ADCC assay

For the evaluation of ADCC, 1×10^5 TH-MYCN primary cells were labeled with DiOC18 (Sigma-Aldrich Japan) according to the manufacturer's protocol. Labeled target cells were washed twice, suspended in 100 μl RPMI-1640 medium containing 10% FBS, and mixed with 20 μl of mouse plasma for 30 min at 4°. NK cells were obtained from spleens of wild-type mice using EasySep™ Mouse NK Cell Isolation Kit (Stemcell Technologies, Vancouver, Canada). NK cells were added at 1:1 and 5:1 effector:target cell ratios. After 4 h of coincubation, all cells were harvested and stained with propidium iodide according to the manufacturer's protocol. Duplicate samples were assessed by flow cytometry, and the numbers of dead cells (DiOC18-positive and PI-positive) were determined. The percentage of specific killing was calculated using the following formula: (percentage of total dead target cells^{target} − percentage of total dead target cells^{control})/(100 − percentage of total dead target cells^{control}) × 100.

1.10. Statistical analysis

All data are expressed as the mean ± SEM. The statistical

Please cite this article in press as: N. Kawakubo, et al., Natural antibody against neuroblastoma of TH-MYCN transgenic mice does not correlate with spontaneous regression, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.07.097