Immunology Letters 156 (2013) 110–117

Contents lists available at ScienceDirect

Immunology Letters

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

Re-evaluation of the involvement of NK cells and C-type lectin-like NK receptors in modulation of immune responses by multivalent GlcNAc-terminated oligosaccharides

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ARTICLE INFO

Article history:
Received 2 September 2013
Received in revised form
16 September 2013
Accepted 16 September 2013
Available online 25 September 2013

Keywords:
Anti-tumor immunity
Carbohydrate dendrimer
NK cells
NKR-P1 receptors
NKT cells
Macrophages

ABSTRACT

Recognition of glycosylation patterns is one of the basic features of innate immunity. Ability of C-type lectin-like receptors such as NKR-P1 to bind saccharide moieties has become recently a controversial issue. In the present study, binding assay with soluble fluorescently labeled recombinant rat NKR-P1A and mouse NKR-P1C proteins revealed apparently no affinity to the various neoglycoproteins. Lack of functional linkage between NKR-P1 and previously described saccharide binder was supported by the fact, that synthetic *N*-acetyl-D-glucosamine octabranched dendrimer on polyamidoamine scaffold (GN8P) did not change gene expression of NKR-P1 isoforms in C57BL/6 and BALB/c mice divergent in the NK gene complex (both *in vitro* and *in vivo*). Surprisingly, *N*-acetyl-D-glucosamine-coated tetrabranched polyamido-amine dendrimer specifically binds to NKT cells and macrophages but not to NK cells (consistently with changes in cytokine patterns). Despite the fact that GN8P has been tested as an immunomodulator in anti-cancer treatment animal models for many years, surprisingly no changes in cytokine profiles in serum relevant to anti-cancer responses using B16F10 and CT26 harboring mouse strains C57BL/6 and BALB/c are observed. Our results indicate possible indirect involvement of NK cells in GN8P mediated immune responses.

1. Introduction

C-type lectin-like receptors are predominantly expressed by cells of the innate immune system. Some of them recognize pathogen-associated and/or endogenous carbohydrate structures, including aberrant glycosylation patterns of cancer cells [1,2]. One of the tumor-associated carbohydrate antigens expressed at high levels in melanomas and other cancers is the β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) [3]. Bezouska et al. reported that monosaccharide GlcNAc as well as GlcNAc-terminated glycodendrimers bind with high affinity to rat recombinant natural killer receptor protein 1 A (NKR-P1A) [4–6]. However, these results were not confirmed upon independent re-examination [7]. Moreover, it was demonstrated that NKR-P1 receptor family possess structural motifs optimized for the protein–protein, but not saccharide–protein interactions and protein ligands for some of them were identified [8–15].

Nevertheless, articles claiming interactions of C-type lectin-like receptors with oligosaccharides continued to be published, using various recombinant proteins and various *in vitro* and *in vivo* models. Despite the fact that original binding experiments were performed using rat derived recombinant proteins, the concept was broadened to mouse model, although binding of mouse C-type lectin-like receptors to the saccharide structures has never been published. Importantly, marked anti-tumor effects of synthetic glycoconjugates were observed in mice, which was interpreted in terms of activation of NK cells (an extrapolation of the rat-based data) [16–20].

Because of these controversies, we decided to clarify the basic facts, *i.e.* examine multivalent saccharide binding to (a)

Abbreviations: Clr, C-type lectin related protein; GlcNAc, *N*-acetyl-D-glucosamine; GN4P: GlcNAc4-PAMAM, *N*-acetyl-D-glucosamine-coated tetrabranched polyamidoamine dendrimer; GN8P: GlcNAc8-PAMAM, *N*-acetyl-D-glucosamine-coated octabranched polyamidoamine dendrimer; GN4P-A: GlcNAc4-PAMAM-ATTO 565, *N*-acetyl-D-glucosamine-coated tetrabranched polyamidoamine dendrimer fluorescently labeled with ATTO 565; GN4P-NH_2-$GlcNAc_4$-PAMAM, *N*-acetyl-D-glucosamine-coated tetrabranched polyamidoamine dendrimer with free NH_2 group; Gzmb, granzyme B; NKG2D, natural killer group 2, member D; NKR-P1, natural killer receptor protein 1; PAMAM dendrimer, polyamidoamine dendrimer; PMA, phorbol 12-myristate 13-acetate; Prf, perforin; SBA, soybean agglutinin; SMC, spleen mononuclear cell.

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0165-2478/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.imlet.2013.09.009

recombinant rat NKR-P1A and mouse NKR-P1C proteins and (b) to various leukocyte subpopulations. As a model oligosaccharide compound we used *N*-acetyl-D-glucosamine-coated octabranched polyamidoamine dendrimer (GN8P) and *N*-acetyl-D-glucosamine-coated tetrabranched polyamidoamine dendrimer fluorescently labeled (GN4P-A), as already described in the literature [16–19,21].

An important implication of previously published experiments was possible role of the putative lectin–saccharide interactions in NK-cell based anti-tumor mechanisms. Therefore, in the present study we included also an *in vivo* syngeneic tumor model.

In mouse, the NK receptor complex is diversified with variable protein expression patterns. Seven different members of NKR-P1 receptor family have been identified [22–24]. Immunological studies of NKR-P1C function have largely been dependent on the use of PK136 mAb, which identified NKR-P1C (NK1.1) as a key activation antigen of mouse NK cells [25,26]. For a long time mice that were NK1.1-negative (*i.e.* BALB/c, DBA, C3H, AKR, CBA, and 129) were considered to be deficient in the expression of one or more members of the NKR-P1 family. Genetic studies demonstrated that the prototype NK1.1-negative BALB/c strain expresses all *Nkrp1* transcripts, including the one for *Nkrp1b* (homolog of *Nkrp1d* in C57BL/6 mice) and *Nkrp1c.* Surprisingly, a single amino acid substitution within the extracellular domain determined whether the anti-NK1.1 antibody was able to bind to the individual NKR-P1C isoform or not. Therefore two representative strains, C57BL/6 and BALB/c, were tested [27].

In the present study we examined mRNA expression of relevant receptors (NKR-P1), along with the corresponding gene isoforms in spleen-derived mononuclear cells (SMC) and sorted NK cells. Furthermore, we evaluated NK cell cytokine synthesis after GN8P treatment in healthy or syngeneic tumor-bearing mice and followed with the question whether NK cells are the major/the only cell population binding GlcNAc-terminated glycodendrimers. For cytofluorometric quantification of the oligosaccharide binding to various splenocyte subpopulations we used GN4P-A as an optimal tool.

As stated above, several previous studies interpreted *in vivo* effects of polyvalent dendrimers on an assumption that they were based on stimulation of NK cells (*via* NKR-P1) by these synthetic complex saccharides [16–19]. However, our present data indicate that these effects are probably due to interactions with other cell types (*via* so far unidentified receptors).

2. Materials and methods

2.1. Glycoconjugates

Generation 1 of the polyamidoamine dendrimers with 8 terminal β-linked *N*-acetyl-β-D-glucosamine moieties (GN8P) was described previously [16,17]. The structure of GN8P does not allow direct linking to a fluorescent label without structural changes [21]. Therefore, to determine the binding to spleen mononuclear cell subpopulations, we used *N*-acetyl-D-glucosamine-coated tetrabranched polyamidoamine dendrimer with free NH_2 group ($GN4P\text{-}NH_2$) fluorescently labeled with ATTO 565 (GN4P-A), kindly provided by Prof. Vladimír Křen.

2.2. Expression, refolding and purification of rat NKR-P1A receptor

The expression, refolding and purification of rat NKR-P1A encompassing residue A90-K215 were performed using the conditions described previously for the expression of mouse NKR-P1C [28]. For the protein production, construct pET-30a-rNKRP1A was transformed into *Escherichia coli* BL-21(DE3) Gold strain (Stratagene, Agilent Technologies, Santa Clara, CA, USA). Cells were grown at 37 °C in LB medium supplemented with kanamycin and expression of the recombinant protein was induced by isopropyl-β-D-thiogalactopyranoside when the cell density reached OD_{600} of 0.8. After induction, bacteria were grown for 4 h and then harvested by centrifugation at 6000 × *g* for 10 min. Inclusion bodies were isolated and then solubilized in 4 ml of 6 M guanidine-HCl (pH 8.0) containing 10 mM DTT, 1 μM leupeptin and 1 μM pepstatin and incubated at 40 °C for 1 h. Any insoluble material was removed by centrifugation at 50,000 × g for 30 min. The rat NKR-P1A protein was refolded by rapid dilution into a 100-fold excess of refolding buffer containing 50 mM Tris–HCl (pH 9.0), 1.0 M L-arginine, 100 mM $CaCl_2$, 9 mM cysteamine, 3 mM cystamine, 1 mM NaN_3 and 1 mM phenylmethanesulfonyl fluoride (Sigma–Aldrich, St. Louis, MO, USA). The refolding mixture was then dialyzed twice at 4 °C against 8 l of 15 mM Tris–HCl (pH 9.0), 9 mM NaCl and 1 mM NaN_3 for 6 h. The refolded protein was loaded onto a column of Q Sepharose FF (1.6 cm × 14.0 cm, GE Healthcare, Waukesha, WI, USA) equilibrated in the same buffer, and eluted by linear gradient of NaCl from 9 mM to 1 M. Fractions containing protein were pooled and finally purified by gel filtration on a Superdex 75 10/300 GL column (GE Healthcare) in a buffer containing 15 mM Tris–HCl (pH 9.0), 150 mM NaCl and 1 mM NaN_3. Protein concentration was measured using the Bradford assay (BioRad, Hercules, CA, USA) with bovine serum albumin as a standard. The protein identity and monodispersity were confirmed by mass spectrometry, and the evidence for compact fold of both proteins was verified by measuring of 1H-NMR spectra.

2.3. Neoglycoconjugate binding assay

Equal amounts (5 μg/well) of BSA-Glycoconjugates (*N*-acetylglucosamine$_{36}$BSA, *N*-acetylgalactosamine$_{28}$BSA, D-mannose$_{28}$BSA, L-fucose$_{36}$BSA, and D-galactose$_{32}$BSA) obtained from Dextra (Reading, United Kingdom) were separated by SDS PAGE using Nupage Novex 10% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA), transferred onto nitrocellulose membrane and incubated (2 h, 4 °C, dark room, blocked in 3% BSA) with soluble recombinant rat NKR-P1A and mouse NKR-P1C proteins, labeled with fluorescein (Sigma–Aldrich). Soybean agglutinin (SBA) was used as a positive control. The blots were developed by Molecular Imager PharosFX Systems (BioRad).

2.4. Animals and design of immunological experiments

Eight-week-old inbred male C57BL/6 and BALB/c mice were purchased from AnLab (Prague, Czech Republic). The mice were housed under natural day/night conditions (22 °C, 55% relative humidity), and fed on a commercial ST1 diet (Velaz, Prague, Czech Republic) *ad libitum.* Mice were subcutaneously inoculated in the lower back on day 0 with 10^6 syngeneic tumor cells, purchased from ATCC (*via* Teddington, UK) (B16F10 and CT26 cells into C57BL/6 and BALB/c mouse, respectively). Healthy or tumor-bearing animals were injected intraperitoneally with three doses of GN8P (0.15 mg/kg) or phosphate buffered saline (PBS) every three days beginning on day 11. Animals were bled on day 17, 2 or 24 h after the final injection; sera were collected and stored at −20 °C until analysis and spleens harvested. All procedures were approved by the Czech Animal Care and Use Committee and The Institutional Committee for Ethics of Animal Experiments.

2.5. Isolation of spleen mononuclear cells and NK cell sorting

Spleens were squeezed through a nylon mesh, and separated on a Ficoll-Paque density gradient (1.086) to obtain spleen mononuclear cells (SMCs). SMCs were counted, resuspended in