Preclinical studies of carbohydrate mimetic peptide vaccines for breast cancer and melanoma

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Abstract

Limited immune responses to tumor-associated carbohydrate antigens (TACA) are due in part to their being self-antigens. Immunization with xenoantigens of TACA provides an approach to break tolerance and augment responses to TACA. Carbohydrate mimetic peptides (CMPs) as xenoantigens can induce serum antibodies that target shared carbohydrate residues on differing carbohydrate structures. In preclinical studies, we observe that CMP immunization in mice induce immune responses that are effective in inhibiting the in vitro and in vivo growth of breast cancer and melanoma tumor cells expressing self-target antigens. CMPs of TACA can be further defined that induce IgM antibodies with broadened responses to both breast and melanoma cells. Consequently, CMPs are effective at generating a multifaceted carbohydrate-reactive immune response that should be clinically evaluated for their ability to amplify carbohydrate immune responses against circulating or disseminated tumor cells.

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1. Introduction

Cancer vaccines provide an avenue to generate sustained tumor specific immunity. Among the classes of tumor rejection antigens are tumor-associated carbohydrate antigens (TACA) for which antibodies are the primary mediators of tissue destruction [1–3]. Carbohydrate targeting tissue rejection is best typified by the natural antibody response directed against the α-Gal antigen, a major barrier in porcine-to-human xenotransplantation [4]. α-Gal tissue rejection supports a mechanistic rationale for targeting TACA as tumor-induced antibody responses in general resemble autoimmune responses [5]. The potential impact of TACA vaccines is demonstrated in clinical trials where patient survival significantly correlates with TACA-reactive IgM levels [6–9].

Some TACAs are glycosphingolipids (GSL) [10]. Antibodies that recognize GSLs such as the gangliosides GD2, GM2 and the Lewis Y (LeY) antigen are demonstrated to mediate complement-dependent cytotoxicity (CDC) and have been suggested to be more cytotoxic to tumor cells than antibodies that recognize protein antigens or TACAs-linked to protein antigens [11] that kill tumor cells by antibody dependent cellular cytotoxicity (ADCC). Antibodies to GD2 [12], and GM2 [13] are also able to mediate apoptosis of tumor cells. GSL-induced responses could augment naturally occurring TACA-reactive IgM antibodies that trigger apoptosis of tumor cells [14]. Consequently, optimizing sustained immunity against TACAs might have a beneficial effect on the course of malignant disease.

A variety of approaches are being taken to generate responses to TACA. Carbohydrate mimetic peptides (CMPs) of TACA are one approach. The characterization of CMPs
is at present limited to preclinical studies. CMPs have been described for GD2 [15–17], GD3 [18], sialylated Lewis a/x [19], and LeY [15,20,21]. Importantly, in preclinical prophylactic and therapeutic vaccination studies, CMPs were efficacious in eliciting immune responses that reduced tumor burden and inhibited metastatic outgrowth [21–23]. CMPs can induce antibodies targeting common carbohydrate moieties on tumor cells of different origin and can be designed to induce antibodies with broad or specific TACA reactivities. Thus, CMPs represent a new and very promising tool to increase the efficiency of the immune response to glycan antigens. Here, we summarize some of the salient features associated with developing these novel immunogens for their translation into the clinic as breast cancer and melanoma vaccines.

2. Materials and methods

2.1. Mice and immunization

Animal studies have been reviewed and approved by the Institutional Care and Use Committee of the University of Arkansas for Medical Sciences. Peptides were synthesized as multiple antigen peptides (MAPs, Research Genetics, Huntsville, AL) made by Fmoc synthesis on poly-L-lysine groups resulting in the presentation of eight peptide clusters [21]. Six to eight week-old BALB/c or C57BL/6 female mice were purchased from The Jackson Laboratory (Bar Harbour, ME). Separate groups of mice were immunized three times at 2-week intervals via subcutaneous injection with 100 µg of the respective MAP and 20 µg QS-21 suspended in 100 µl of sterile PBS. Control animal groups received only 20 µg QS-21.

2.2. Flow cytometry

Staining, acquisition and analysis was performed as described earlier [24]. Briefly, cells were incubated with dilutions of mouse sera for 30 min and then stained with FITC-conjugated anti-mouse antibody for another 30 min on ice. Mean fluorescence intensity and percentage of positive cells were calculated from duplicates for each serum dilution.

2.3. ELISA assays

ELISA was performed as described before [21]. Briefly, plates were coated with gangliosides (3 µg/ml) dissolved in ethanol. After blocking of the surface, sera were added and incubated for 1 h at 37 °C. Mean absorbance was calculated from duplicates for each serum dilution and the dilution yielding half-maximal binding was determined.

2.4. Complement-dependent cytotoxicity

CDC was measured using GD2-expressing murine cell line B16F10 (ATCC) as previously described [21]. Briefly, 10 µl of cell suspension (4 × 10^6 cells/ml) were added to triplicate wells of a microtiter plate to which was added sera, followed by incubation on ice. Rabbit complement (Sigma) 1:4 was added and allowed to incubate for 4 h at 37 °C. Medium was discarded and cells were fixed using methanol. The number of viable cells was determined by Giemsa staining and the percentage of cytotoxicity was calculated. The assay was performed in duplicate with medium, sera, and complement controls.

2.5. Necropsy, histopathology, and in situ apoptosis detection

Fourteen days after the last vaccination, animals were euthanized via overdose of CO2. A complete necropsy was performed and organs were placed immediately into 10% neutral buffered formalin (NBF). Tissues were processed and embedded in paraffin and sectioned at 6 µm. Sections were stained with hematoxylin and eosin for routine histologic evaluation.

2.6. Statistical analysis

Data were expressed as arithmetic means ± S.D. Means were compared using Student’s t test. Differences between groups were considered significant if the p was <0.05. Saturation binding curves (of the type: conc. × maximal level/(K + conc.) + background) were fitted to the experimental data with the help of the non-linear regression unit of the STATISTICA for Windows (STATSOFT, Tulsa, OK). The parameter K was used as a measure of the concentration yielding half-maximal binding.

3. Results

3.1. CMPs target shared carbohydrate residues on differing carbohydrate structures

CMP sequences were first described with a high prevalence of tryptophan and tyrosine residues that were identified to be associated with differing carbohydrate moieties [20,25–28]. The sequence similarities that define these peptides suggest that antibodies to homologous peptides might cross-react with similar subunits expressed on what are otherwise dissimilar carbohydrate structures. Based upon these motifs we constructed CMPs that antigenically mimic the LeY antigen expressed on human breast cancer cell lines and also a lipid associated, structurally related difucoganglioside (6B ganglioside) [29], expressed on murine Meth-A fibrosarcoma cells and on human tumor cells [21]. In particular CMP 106 (H-GGIYWRYDIYWRYDIYWRYD) 8-MAP and CMP 107 (H-GGIYWRYDIYWRYDIYWRYD) 8-MAP induce antibodies reactive with LeY expressed on breast cancer cell lines and with Meth-A fibrosarcoma cells expressing...
the 6B antigen [21]. The basis for this cross-reactivity is a shared Fucα1-3GlcNAcβ1-3Galβ1 epitope as suggested by conformational analysis of these peptides interacting with the antibody-combining site of the BR55-2 monoclonal [30]. CMP 106 is effective in vivo in prophylactic vaccination followed by challenge with Meth-A tumor cells [21].

The 6B antigen is also expressed on the murine B16F10 melanoma cell line. Both FH6 and serum antibodies induced by CMP 106 mediate CDC of this cell line (Fig. 1). We have also adopted a B16F10 lung metastasis model in which FH6 reactive B16F10 cells were used in challenge experiments after vaccination with CMP 106 (Fig. 2). As observed, the number of lung colonies were less in the CMP-immunized mice compared with naïve tumor bearing animals (p < 0.05). Collectively, these results, combined with our previous studies, indicate that CMP 106 induces serum antibodies that target TACA expressed on murine models of fibrosarcoma [21], breast cancer [22], and melanoma.

3.2. Defining CMPs that induce serum antibodies to multiple carbohydrate antigens

Gangliosides are typically considered expressed with neuroectodermal tumors (melanoma and neuroblastoma) but can also be expressed on breast cancer cells. The composition of cell surface gangliosides is largely dependent on the relative activities of Golgi resident glycosyltransferases. Transfer steps leading to synthesis of the gangliosides GM3, GD3, GM2 and GD2 from LacCer are functionally coupled in the Golgi membranes [31]. Individual carbohydrate-based vaccines are in clinical trials targeting these important TACAs. Another approach would be to synthesize several different tumor-associated carbohydrate antigens into a single molecule. Alternatively, antibodies can be induced by CMPs with broad specificity towards these gangliosides.

The monoclonal antibody ME36.1 whose crystal structure is known [32], displays reactivity with GD2 and GD3 [33]. Using the program LIGPLOT [34], primary hydrogen bonding partners are illustrated in the recognition of the GalNAc moiety by ME36.1 residues Thr H33, Asn H59 and Asp H50, the Gal moiety by ME36.1 residues Thr H33, His 35 and Ser 100H, Neu5Aca2-3 residue by Ser 100H and Neu5Aca2-8 residue by Tyr L93 (Fig. 3). This primary interaction scheme suggests that ME36.1 could react with GD2 and GD3, and perhaps with GM2, GM3, GD1b and GD1a. Such broad specificity for these important tumor-associated TACA in fact has been argued for using ME36.1 in the clinic [33] and emphasizes the importance of inducing multiple specificity towards tumor antigens, i.e., binding of an antibody to two or more TACA. Consequently, defining CMPs reactive with ME36.1 might in turn induce antibodies with broader ganglioside reactivity.

Screening a random peptide phage display library with the anti-GD2 ganglioside monoclonal antibody ME36.1 defined another WRY containing peptide with the sequence GVWWRYTA PVHLGDG and referred to as P10 [15]. ELISA analyses of serum antibody induced by the P10 CMP, also synthesized as an 8-mer MAP, suggest that immunization might enhance preexisting ganglioside reactive IgM serum antibodies (Fig. 4A, empty bars). Comparing the ratios of the dilutions yielding half-maximal binding for naïve and immune sera, we observe about a three-fold increase in GD1a and GD1b reactivity (GD1b is related to the GD3/GD2 synthetic pathway while GD1a is related to GM3/GM2/GM1 pathway). This is equivalent to increasing the titer to the same fold degree. We found a correlation between the IgM titer thus measured before and after immunization implying that the reactivity induced probably depended on the one already existing or that the CMP stimulated preexisting carbohydrate-reactive B cells (Fig. 4B, crosses). These data suggest that B cells capable of responding to GD1a and GD1b exist in an innate pool that can be polyclonally activated and/or in a B cell pool that can be specifically amplified by CMP. On the other hand, peptide immunization
Fig. 3. LIGPLOT of GD2 recognition by ME36.1. Docking was performed using FlexX followed by energy optimization (Tripos). The network of hydrogen bonds and hydrophobic forces involved in stabilizing GD2 binding to ME36.1 are shown by two-dimensional plot using LIGPLOT [34]. This structure was of lower energy with more extensive hydrogen bonding than that originally reported [32].

The ELISA results suggest that P10 might induce serum IgM antibodies reactive with multiple gangliosides. To further test this hypothesis we examined the binding of serum antibodies induced by P10 to the human breast cancer cell line MDA-231 and MCF7 (Fig. 5) as MDA-231 cells are purported to differentially express a variety of gangliosides relative to MCF7 cells [35]. As observed by flow cytometry, serum IgM antibodies bound considerably stronger to MDA-231 cells compared to MCF7 cells (Fig. 5).
3.3. Structural basis for CMP mimicry

Understanding the origin and structural basis for antibody responses arising in response to TACA through molecular mimicry is of critical importance to understanding anti-tumor response and the mechanisms by which tolerance operates. To understand how CMP P10 might functionally mimic GD1a, GD1b and GD2 we used conformational and energy analysis to define potential binding modes of this peptide in the crystallographically defined ME36.1 binding pocket (Table 1) [36].

Molecular modeling of the P10 CMP in the ME36.1 binding site indicates that this CMP only has two hydrogen bonds in common with the GD2 antigen in binding to ME36.1 (Table 1). This would suggest that serum raised to this peptide might be skewed to cross-react with gangliosides emphasizing Neu5Aca2-3 Gal residues shared with GD1a as Thr33 and Ser 100H are observed to interact with these two carbohydrate moieties. Terminal saccharides are typically viewed as dominant epitopes. Consequently, reactivity with the terminal Neu5Ac-α2-3Gal-β1 epitope might be a basis for reactivity with GD1a in that it has two potential epitopes. The modeling also suggests the GalNAc1-4Gal epitope might also be mimicked by P10 as Thr33 and SerH100 are contacting the Gal and GalNAc moieties on GD2 as well. However, it is not clear if orientation matters, which would explain the GD1b reactivity as there are potentially two residues that interact with Gal and only one interacting with GalNAc. We are exploring this possibility.

To test the hypothesis that CMPs can be redesigned to increase the level of mimicry, we developed a peptide with the sequence WRYTAPVHLGD (referred to as P10 short or P10s) with an increased number of hydrogen bonds relative to its parent peptide GVVWRYTAPVHLGDG. The redesigned P10s shares five hydrogen bonds with GD2 in binding to ME36.1 (Table 1). The increased number of hydrogen bonds would suggest that the antibody population induced by P10s might be redirected towards GD2 reactivity. We observe that P10s immunization changes the preexisting anti-ganglioside reactivity of serum from that of immunization with P10 (Fig. 4A), augmenting the response to GD2, GD3 and GM2. Serum IgM induced by P10s is observed to selectively bind to GD2 expressing melanoma WM793 cells (Fig. 6).

3.4. CMPs induce anti-tumor responses with absence of normal tissue damage

It is generally recognized that the pathology observed from tumor-reactive antibodies can mirror autoimmune-mediated tissue damage and antibody-inducing antigens can serve as rejection antigens if they are recognized as foreign [5]. We observe that CMPs 106, 107, P10, or P10s induce antibodies that mediate tumor growth inhibition without tissue damage to normal murine tissues that express the model antigens including brain and kidney (Fig. 7). The relative expression density of carbohydrate antigen on the surface of a tumor cell is generally suggested as a mechanism that diminishes normal tissue damage. In keeping with these general concepts our histopathology studies suggest that CMPs do not induce antibodies that mediate normal tissue destruction.

We did observe mild liver inflammation with immunization with CMPs P10 and P10s (Fig. 8). Minimal to mild lymphocytic portal hepatitis and mild lobular lymphoplasmacytic hepatitis with rare intralesional apoptotic hepatocytes were present in all groups. The mild degree of inflammation is consistent with the lack of clinical signs and normal weight gain observed in these mice. Further tests including evaluation of serum chemistries for levels of hepatic enzymes to detect sub-clinical hepatocellular damage are planned. Importantly, no inflammatory or demyelinating lesions were
Table 1  
Modification of flanking residues enhance GD2 mimicy for ME36.1 binding

<table>
<thead>
<tr>
<th>Ligands</th>
<th>ΔG (free binding energy) (kJ/mol)</th>
<th>Residues on ME36.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>GD2</td>
<td>−10.06</td>
<td>H/Thr33 H/His35</td>
</tr>
<tr>
<td>GV VRYTAPVHLGDG</td>
<td>−17.60</td>
<td>H/Thr33 H/Asp50 H/Asn52</td>
</tr>
<tr>
<td>WRYTAPVHLGDG</td>
<td>−50.00</td>
<td>H/Thr33</td>
</tr>
</tbody>
</table>

* The mimotope residues forming hydrogen bonds with ME36.1/GD2 contact residues are underlined.

Fig. 5. Binding of serum IgM antibodies to MCF-7 (right column) and to MDA-MB-231 (left column) cells. Groups of mice (C57BL/6) were prebled and then immunized with P10 and P10s peptides. Serum was collected 7 days after the fourth immunization and pooled for each group. Cells were harvested using enzyme-free buffer and binding of serum IgM antibodies to cells was detected by flow cytometry. Percentage of positive cells (upper number) and MFI of whole population (lower number) for each histogram is shown. The reactivity of 1:200 dilution of serum is shown. X and y axes show relative fluorescence intensity and cell number, respectively.
Fig. 6. Comparison of serum binding to GD2 expressing cells. Mice (BALB/c) were prebled and then immunized with P10 short (P10s) peptide. Serum was collected 7 days after the third immunization and pooled for each group. Cells were harvested using enzyme-free buffer and binding of serum IgM antibodies (1:50 dilution) to cells was detected by flow cytometry. Percentage of positive cells for each histogram is shown. X and y axes show relative fluorescence intensity and cell number, respectively.

noted in nervous tissue, which generally has the highest tissue expression of gangliosides (data not shown). Normal mice appear to be resistant to neural injury during anti-GD1a antibody exposure, demonstrating the central role of membrane antigen density in modulating both immune tolerance to GD1a and axonal susceptibility to anti-GD1a antibody mediated injury [37]. In keeping with these general concepts our histopathology studies suggest that CMPs do not induce antibodies that mediate normal tissue destruction targeting gangliosides due to their low density of expression.

4. Discussion

We have made several striking discoveries in developing CMPs. (1) CMP-induced serum antibodies can be functionally related to the natural antibodies that represent an innate immune surveillance system; (2) the identification of pre-existing antibodies to CMP might also indicate preceding encounter of cross-reactive antigens and a possibility to elicit a swift recall response to TACA that suppresses the metastatic process; (3) immunization with CMPs is effective in eradicating cancer cells and impacting on tumor metastases without inducing significant immunopathology.

Circulating carbohydrate-reactive IgM antibodies are proposed as a mechanism of immune surveillance of the innate immune system [14]. Such antibodies might be designated as natural antibodies because of their ubiquitous occurrence with no basis for assignment of immunogenic induction. The fact that survival rates of cancer patients are correlated with low avidity and low titer carbohydrate-reactive antibodies [6–9] argues that having more robust IgG responses may not be of absolute necessity.

The same or similar TACAs are expressed on both breast cancer and melanoma cells. CMPs can induce IgM antibodies that target TACAs on breast cancer and melanoma
Fig. 7. Immunization with CMPs does not induce immunopathology. CMP 106 (A and B); CMP 107 (C and D); P10 (E and F); P10s (G and H); naïve control (I and J). Left column: cerebrum, 400× magnification, bar equals 40 μm. Right column: kidney, 200× magnification, bar equals 80 μm. H&E stain.
IgM antibodies to gangliosides are attributed as a contributory factor to multifocal motor neuropathy in some cancer patients and therefore must be monitored in clinical trials.

It is anticipated that CMPs can sustain TACA reactive responses in breast cancer and melanoma patients, facilitating long-term immunosurveillance through recall of carbohydrate immune responses that should contribute to patient survival. Preclinical studies support the hypothesis that vaccine-induced responses against TACA might have their greatest impact in the adjuvant setting as such responses inhibit tumor outgrowth in metastatic models [22,38]. The induction of long-lived responses capable of eradicating cancer metastases suggests that vaccines could be effective against tumor recurrence. However, unlike pathogen infections that provide “danger signals” to the immune system, cancer cells are rather a source of tolerance signals and therefore constant boosting of the immune surveillance and possibly suppression of Treg function may be warranted.

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References


