A Mimic of Tumor Rejection Antigen-Associated Carbohydrates Mediates an Antitumor Cellular Response

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ABSTRACT

Tumor-associated carbohydrate antigens are typically perceived as inadequate targets for generating tumor-specific cellular responses. Lectin profile reactivity and crystallographic studies demonstrate that MHC class I molecules can present to the immune system posttranslationally modified cytosolic peptides carrying O-β-linked N-acetylgalactosamine (GlcNAc). Here we report that a peptide surrogate of GlcNAc can facilitate an in vivo tumor-specific cellular response to established Meth A tumors that display native O-GlcNAc glycoproteins on the tumor cell surface. Peptide immunization of tumor-bearing mice had a moderate effect on tumor regression. Inclusion of interleukin 12 in the immunization regimen stimulated complete elimination of tumor cells in all of the mice tested, whereas interleukin 12 administration alone afforded no tumor growth inhibition. Adoptive transfer of immune T cells into tumor-bearing nude mice indicates a role for CD8+ T cells in tumor regression. This work postulates that peptide mimetics of glycosylated tumor rejection antigens might be further developed for immune therapy of cancer.

INTRODUCTION

The presence of carbohydrate antigens on the surface of common human malignant tumor cells has led to studies directed toward the development of synthetic carbohydrate-based anticancer vaccines (1). Although these vaccines elicit antibody responses, it would also be advantageous if T cells could be directed to tumor-associated carbohydrate antigens (2–6). Posttranslationally modified cytosolic peptides carrying O-β-linked N-acetylgalactosamine (GlcNAc) can be presented by class I MHC molecules to the immune system that activate CTLs, as resolved by wheat germ agglutinin (WGA)-binding profiles reacting with GlcNAc containing glycopeptides in the MHC Class I binding site (7, 8). Crystal structure analysis of T-cell receptor binding to model glycopeptides has indeed shown that T cells can recognize GlcNAc-linked glycopeptides bound by the MHC molecule (9, 10), T cells, therefore, have the potential to react with the GlcNAc moiety of glycopeptide antigens, suggesting that T cells can target to presented carbohydrate antigens on tumor cells.

In an effort to further define strategies to augment immune responses to tumor cells, we have been developing peptide mimics of tumor-associated carbohydrate antigens and have demonstrated that peptides synthesized as multivalent peptides can emulate or mimic the native clustering or presentation of tumor cell-displayed carbohydrate antigens (11). We have shown that prophylactic vaccination with a peptide surrogate, having the sequence GGIYWRYDIYWRYDIY-WRYD (and referred to as peptide 106), induces a tumor-specific cellular response inhibiting tumor growth of a methylcholanthrene-induced sarcoma (12). Here, we demonstrate the ability of this peptide to stimulate the regression of established Meth A tumor in a murine model via the activation of specific antitumor cellular responses. We demonstrate that peptide 106 is a mimic of O-GlcNAc, an antigen presented on Meth A surface-expressed glycoproteins as resolved by reactivity with WGA to which the peptide also binds. Immunohistochemistry demonstrates infiltrates of lymphocytes targeting Meth A tumor cells in peptide-immunized mice and adoptive transfer of peptide-specific T cells into tumor-bearing nude mice verifies a role for CD8+ T cells in mediating tumor regression. These studies highlight a new function for peptide mimotopes of carbohydrate-associated antigens by demonstrating that they possess in vivo antitumor activity with CD8+ T cells as the primary effector cells.

MATERIALS AND METHODS

Mice and Tumor Inoculation. BALB/c female mice, 6–8 weeks old, were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c nude mice (BALB/cAnNTac-Foxn1nu N9, nu/nu) were purchased from Taconic Farm Inc. (Germantown, NY). To establish tumor, each mouse was inoculated s.c. into the right flank with 5 × 103 Meth A cells (Methylcholanthrene-induced sarcoma of BALB/c origin; Ref. 11). Tumor growth was measured using a caliper and was recorded as the mean of two orthogonal diameters [a + b]/2.

Immunization. As in our previous studies (11, 12), peptide 106, having the sequence GGIYWRYDIYWRYDIY-WRYD, was synthesized as a multiple-antigen peptide (Research Genetics, Huntsville, AL). Each mouse received 100 μg of 106 multiple-antigen peptide and 20 μg of QS-21 (Antigenics Inc., Framingham, MA) i.p., both resuspended in 100 μl of PBS three times at 5-day intervals. Recombinant murine interleukin (IL)-12 (Sigma, St. Louis, MO) was administered i.p. once daily for 5 days, starting on the day of the last peptide immunization.

Flow Cytometry. Acquisition and analysis were performed as described earlier (12). Cells were resuspended in a buffer containing, Dulbecco’s PBS, 1% BSA, and 0.1% sodium azide and incubated with biotinylated peanut agglutinin or WGA (10 μg/ml; Vector laboratories, Burlingame, CA) for 30 min on ice. Cells were then stained with FITC-conjugated streptavidin at 1:500 dilution for another 30 min on ice.

ELISA and Inhibition Assays. ELISA was performed as described previously (11). Briefly, plates were coated with 106 multiple-antigen peptide. Biotinylated WGA was added, and binding was visualized with streptavidin-horseradish peroxidase (Sigma, St. Louis, MO). Absorbance was read, using a Bio-Tek ELISA reader (Bio-Tek instruments, Inc, Highland Park, Vermont). For inhibition assay, GlcNAc and N-acetylgalactosamine, attached to a polyacrylamide polymer (GlycoTech Corporation, Rockville, MA), were used as carbohydrate competitor. Biotinylated WGA (2.5 μg/ml) combined with serial concentrations of carbohydrates and incubated overnight at +4°C. Lectin/carbohydrate mix was added to the peptide-coated plates, and lectin binding was visualized by streptavidin-horseradish peroxidase as above. Mean absorbance was calculated from duplicates for
each carbohydrate concentration, and percentage of inhibition was calculated as: \(1 \frac{(\text{mean of test wells/mean of control wells}) \times 100.}

**T-Cell Purification.** Splenocytes were harvested from spleens and prepared by lysis of erythrocytes and consequent washing several times with fresh medium (12). Splenocytes were first passed through nylon wool and then, using MiniMACS (Miltenyi Biotec, Auburn, CA), natural killer cells were depleted using anti-natural killer cell (DX5) microbeads. Finally, T cells were positively purified by Thy1.2-coated beads. Purified T cells were tested for purity as >97% positive for anti-CD3 antibody. For cell transfer experiments, after nylon wool passage, cells were enriched in CD4+ or CD8+ population using MiniMACs and depletion of unwanted cell populations.

**IFN-γ Production by Purified T Cells.** Purified T cells (1 × 10^6/ml) were cultured in 96-well or 24-well plates with various doses of recombinant IL-12. After 48 h of stimulation, supernatant was harvested and stored at −20 until use. Concentration of IFN-γ was measured using a quantitative ELISA kit (BioSource International Inc., Camarillo, CA) according to the manufacturer’s instructions.

**Adaptive Transfer of Cells.** Splenocytes were collected from cured mice after tumor eradication and were used in transfer experiments. Immune splenocytes (1.5 × 10^7) were transferred i.p. to syngeneic nude tumor-bearing mice 7 to 10 days after inoculation of 0.5 × 10^6 Meth A cells into the right flank. To in vitro deplete CD4+ and CD8+ cells, splenocytes (1.5 × 10^6/each sample) were first passed through nylon wool column, and then, using MACS, we depleted CD4+ and CD8+ cells.

**Histology.** Tumors with surrounding tissues were excised and fixed in 10% formalin. Fixed samples were embedded in paraffin, sectioned, and stained with H&E. Sections were analyzed histologically for lymphocyte infiltration.

**Statistical Analysis.** Statistical analyses were performed using Student’s t test and the χ² test; \(P < 0.05\) were regarded as statistically significant. EXCEL and Statistica softwares were used for analyses. All of the experiments were performed at least three times.

**RESULTS**

**Peptide Mimic of GlcNAc Moiety.** It has become evident that both CD4+ and CD8+ T cells can recognize glycopeptides carrying mono- and disaccharides in a MHC-restricted manner provided the glycan group is attached to the peptide at suitable positions (13). Reactivity patterns of lectins with Meth A cells indicate that GlcNAc glycyl epitopes are more highly expressed on Meth A tumor cells than the T antigen Galβ1–3 N-acetylgalactosamine epitope, because WGA displays greater reactivity with Meth A cells than peanut agglutinin (Fig. 1, A and B). WGA binds to the peptide 106 mimotope in a concentration-dependent manner as assessed by ELISA (Fig. 1C). This binding is selectively and significantly inhibitable by WGA-reactive GlcNAc in a concentration-dependent manner (Fig. 1D), further indicating that the peptide mimotope is reactive with the GlcNAc-binding site of WGA, and, therefore, peptide 106 is an effective antigenic mimic of GlcNAc.

**Therapeutic Peptide Immunization Induces Tumor Regression.** To study the outcome of peptide immunization on the growth of solid tumors in vivo, we evaluated the antitumor effect of the peptide 106 on established Meth A tumors. BALB/c females were inoculated s.c. with Meth A cells, and 7 days later, treatment was started with the peptide. As shown in Fig. 2A, immunization moderately affected the growth of Meth A sarcoma, because 6 mice of 11 immunized were cured (χ² test, \(P = 0.01\), as compared with animals that were given IL-12 only). Fig. 2B demonstrates that treatment of animals with IL-12 after peptide immunization tended to enhance the immune response and was successful in mediating complete eradication of established tumors (χ² test, \(P = 0.008\), as compared with peptide-immunized only). Treatment of tumor-bearing mice with only IL-12 did not affect tumor growth (Fig. 2C) in keeping with other such studies (14).

We further determined that peptide/IL-12 combination therapy is highly effective even at lower doses of IL-12, because 100 ng of daily IL-12 treatment in the combined therapy, but not alone (χ² test, \(P = 0.001\)), eradicated tumors in five mice of five challenged (Fig. 3, A and B). The time of the beginning of immunization and the size of tumor at the time of immunization affect the efficacy of immunization. When immunizations were started at day 14 or later or when treating tumors with a mean diameter larger than 7 mm, the efficacy of immunization dropped (Fig. 3C), ruling out the possible effect of hyperimmunization. To further rule out nonspecific effects of hyperimmunization, we observed that cell-based vaccination using 10^6 mitomycin-C-inactivated Meth A cells, followed by IL-12 administration, also failed to induce tumor regression (Fig. 3D). This latter result confirmed a previous study in which Meth A immunization along with IL-12 failed to induce tumor regression (14). Our results are in agreement with other therapeutic vaccine studies on Meth A cells, in which enhancement of antitumor T-cell responses led to quick eradication of established tumors (15, 16).
Adoptive Transfer of Splenocytes Stimulates Eradication of Tumors in Nude Mice. To further assess whether the antitumor activity mediated by peptide/IL-12 therapy is T-cell dependent, we evaluated our therapeutic strategy in nude mice. BALB/c-nu/nu mice bearing Meth A tumors were immunized with the peptide followed by IL-12 treatment. Combined peptide/IL-12 therapy had no effect on tumor growth of nude mice, indicating the dependence of mediated tumor regression on T cells (data not shown). Next, nude mice were transplanted with Meth A cells and were given injections i.p. of fresh splenocytes, isolated from cured mice, 10 days later (Fig. 4). Immune cells transferred had a dramatic effect on tumor size because by day 15 after transfer, tumor was eradicated completely in all four mice tested ($\chi^2$ test, $P = 0.005$).

In a follow-up study, splenocytes were depleted of B cells and enriched for CD4+ or CD8+ cells, in vitro, and then were transferred to tumor-bearing nude mice. Our data indicate that CD8+ cells are required for efficient eradication of tumor; however, the process seems dependent on both CD4+ and CD8+ cells (Fig. 5). Histological sections of tumor sites and surrounding tissues were prepared (Fig. 6). Contrary to nonimmunized tumor-bearing mice, we detected lymphocytes around the periphery and infiltrating into tumor mass of immunized mice (Fig. 6, A and B). Staining of sections obtained from the tumor site of a cured mouse shows the presence of lymphocytes, although no tumor is detectable microscopically (Fig. 6C).

DISCUSSION

Carbohydrates are abundantly expressed on the surface of malignant cells, and induction and enhancement of a cell-mediated immune response toward these antigens has outstanding implications in vaccination for and treatment of cancer. T-cell recognition of nonpeptidic and modified peptide antigens is, however, still poorly understood. Peptide mimetics of carbohydrate antigens can activate peptide-specific cellular responses, but they have also been shown to activate cellular responses that might be cross-reactive with carbohydrate moieties (12, 17). The induction of carbohydrate-reactive T-lymphocytes with peptide mimics is based on a functional definition of T-cell mimotopes. One possible explanation is that the peptide mimotope activates cross-reactive CTLs that recognize a processed O-linked glycopeptide associated with MHC class I. It is also possible to generate carbohydrate-specific unrestricted CTL responses with MHC class I-binding carrier peptides (18). However, we previously showed that anti-MHC Class I antibody blocks CTL killing of Meth A cells in vitro by T cells derived from peptide 106 immunized mice (12). Immunization with cells in combination with IL-12 had no obvious enhancement of antitumor immune effects. Our data propose that replacing cell immunization with peptide 106 enhanced a potential immune responses resulting in a significant but moderate tumor eradication. Further treatment of peptide-immunized mice with IL-12 helped significantly in stimulating eradication of established tumor in all of the animals tested. Lack of tumor shrinkage on cell-based immunization rules out the possibility that hyperimmunization had a bearing on tumor regression. Taken together, these results indicate that peptide immunization enables an effective antitumor immune response, the potential of which can be significantly enhanced with IL-12 administration. Other groups have performed therapeutic immunization on Meth A cells by immunization with p53 mutant epitope, starting the immunizations 7 days after tumor inoculations, and have demonstrated an efficient enhancement of antitumor cellular immune responses leading to eradication of tumor mass in the majority of animals within 2 weeks after the first immunization (15, 16). Our findings are in concert with the results of these studies.
Because resting T cells do not express the IL-12 receptor (19) and IL-12 responsiveness is only activated after T-cell receptor stimulation (20), we observed that purified peptide-specific T cells were stimulated with IL-12 \textit{in vitro} (data not shown). IL-12 treatment is ineffective in the Meth A tumor model (14) as further observed in our studies. Because IL-12 responsiveness of T cells is induced after T-cell receptor stimulation, the lack of IL-12 responsiveness suggests that T cells in Meth A-bearing mice are not sensitized to Meth A tumor antigen on immunization with Meth A cells. In contrast, our data suggest that peptide immunization can sensitize tumor-reactive T cells that are responsive to IL-12. It is possible that peptide immunization further expands B and T cells that have been primed via shed glycoprotein(s) processing. We propose that peptide immunization activated a population of Th1 and CTLs with production of IFN-\(\gamma\) (12), and \textit{in vivo} IL-12 treatment further helps to expand the T-cell population and IFN-\(\gamma\) production. In previous studies, the failure of IL-12 treatment to induce tumor regression was also considered to be associated with the lack of T-cell migration to tumor sites (14). It was argued that sensitization of T cells to tumor antigens and generation of IL-12 responsiveness are insufficient to induce tumor regression when sensitized T cells are not allowed to migrate to tumor sites. In our studies, we observe lymphocyte migration to tumor sites.

In summary, this work further postulates the occurrence of saccharide epitopes for T cells linked to peptides with anchoring motifs for MHC Class I (6, 13). Although analogous to the hapten trinitrophe- nyl and O-\(\beta\)-linked acetyl-glucosamine, the potential implications of natural carbohydrates as antigenic epitopes for CTL in biology are considerable and are understudied. Consequently, it might be possible for peptide mimetics to activate T cells that recognize carbohydrate moieties on native glycopeptides (21). Peptides that mimic carbohydrate structures attached to class I or class II anchoring peptides would
extend our notion of vaccine design for cancer immunotherapy in the adjuvant setting.

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REFERENCES

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