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Efficient glycoengineering of GM3 on melanoma cell and monoclonal antibody-mediated selective killing of the glycoengineered cancer cell

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Abstract—To verify the principal of a new immunotherapeutic strategy for cancer, a monoclonal antibody 2H3 against *N*-phenylacetyl GM3, an unnatural form of the tumor-associated antigen GM3, was prepared and employed to demonstrate that murine melanoma cell B16F0 could be effectively glycoengineered by *N*-phenylacetyl-D-mannosamine to express *N*-phenylacetyl GM3 and that 2H3 was highly cytotoxic to the glycoengineered B16F0 cell in the presence of complements. It was further demonstrated that B16F0 cell could be glycoengineered 4–5 times more effectively than 3T3 A31 cell, a normal murine embryo fibroblast cell, and that the antibody and complement mediated cytotoxicity was at least 200 times more potent to the glycoengineered B16F0 cell than to the *N*-phenylacetyl-D-mannosamine-treated 3T3 A31 cell. These results show the promise for developing useful melanoma immunotherapies based on vaccination against *N*-phenylacetyl GM3 followed by treatment with *N*-phenylacetyl-D-mannosamine.

1. Introduction

Immunotherapy is an attractive direction for the development of caner therapies due to the potentially high potency and specificity of the activated immune system to eradicate tumors.[1] Therefore, the search for effective therapeutic cancer vaccines or cancer immunotherapies has been an active area in recent years.[2–5]

Since the early 1970's, many abnormal or excessively expressed glycans have been characterized on cancer cells.[6–9] These glycans, termed tumor-associated carbohydrate antigens (TACAs), play an important role not only in carcinogenic processes, such as cancer cell signaling and adhesion and cancer metastasis,[10–12] but also in the design of new cancer vaccines or cancer immunotherapies.[13–16] Some TACA-based cancer vaccines have been on clinical trials and shown promising clinical results.[4,5,17–19] However, among many TACAs identified, only a few can form functional vaccines via conjugation to a carrier protein. The majority of TACAs are essentially non-immunogenic due to the problem of immune tolerance, but since some tolerated TACAs are relatively abundant on cancer cells, they are useful immunological targets.

To overcome the problem of immune tolerance to TACAs for the design of effective cancer immunotherapies, we have recently explored a new strategy.[20–22] The strategy is based on joint application of synthetic vaccines made of unnatural derivatives of TACAs and glycoengineered expression of the unnatural TACA derivatives on cancer cells. Its basic concept is outlined as following. First, cancer animals are immunized with a conjugate vaccine made of an artificial derivative of a native TACA. Because the antigen is artificial, it is expected to induce a specific immune response easily. After the specific immune response is established, the animals are treated with an unnatural monosaccharide that can serve as a biosynthetic precursor of artificial oligosaccharides, which will initiate the expression of the artificial TACA derivative, in place of the native TACA, on tumor cell surfaces. Thereafter, the activated immune system will recognize and eradicate cancer cells that are marked by the artificial antigen. The modification of cell surface glycans via giving cells an unnatural

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monosaccharide precursor is broadly known as cell glycoengineering, pioneered by Reutter[23–25] and Bertozzi.[26,27] Since the glycoengineering of cell surface *N*-acetyl sialic acid (Neu5Ac) with unnatural derivatives of *N*-acetyl-D-mannosamine (ManNAc) as the bioengineering precursors has been proven easy and effective,[23,27–34] our study of the new strategy has been focused on sialylated TACAs.

Using GM3 (Fig. 1), a sialylated trisaccharide TACA abundantly expressed by malignant melanoma and other tumors,[35–38] as the target antigen, we have essentially verified the feasibility of the new strategy. For instance, after studying a series of *N*-acyl derivatives of GM3 in mouse, we have found that unnatural GM3 derivatives, especially *N*-phenylacetyl GM3 (GM3NPh Ac), were much more immunogenic than native GM3 and that GM3NPhAc could provoke a robust T-cell dependent immune response[21] which is critical for the antitumor activities of a cancer immunotherapy. We have also showed that the antisera obtained from GM3NPhAc-provoked animals could target tumor cells treated with *N*-phenylacetyl-D-mannosamine (ManNPhAc).[34] However, because polyclonal antisera were utilized in the latter research, the results were rather preliminary. In this work, an anti-GM3NPhAc monoclonal antibody (mAb) 2H3 was prepared and employed to analyze the expression of GM3NPhAc on tumor cells in detail.

Another important question about the new strategy is how selective the glycoengineering and the immunotherapy can be to tumors. This issue is directly related to the in vivo efficacy and the side effects of an immunotherapy. To answer this question, mAb 2H3 was also employed to study the glycoengineered expression of GM3NPhAc on normal cells and the antibody-mediated complement-dependent cytotoxicity to ManNPhAc-treated tumor and normal cells. This work has for the first time revealed semi-quantitatively that the glycoengineering and the antibody and complement mediated cytotoxicity was highly selective to cancer cells.

2. Results

2.1. Preparation of the anti-GM3NPhAc mAb 2H3

An anti-GM3NPhAc immune response was first established in C57BL/6 mice by immunization of the animals with a synthetic vaccine made of GM3NPhAc and keyhole limpet hemocyanin (KLH), namely GM3NPhAc-KLH. The mouse splenocytes were then fused with SP2/0 myeloma cells according to standard hybridoma technology as described by Harlow and Lane.[39] The hybridoma cultures were analyzed by enzyme-linked immunosorbent assay (ELISA) with GM3NPhAc-HSA (human serum albumin) as the capture antigen. After several trials, one GM3NPhAc-specific antibody-secreting culture was obtained and then subjected to clone and sub-clone in the limiting dilution experiments. The established mAb, designated as 2H3, showed no cross-reactivity with natural GM3 in the ELISA experiments using GM3-HSA as the capture antigen. MAb 2H3 showed some cross-reactivity to *N*-phenylacetylated sTn-HSA conjugate, but this reaction was at least 60% less efficient than the reaction between GM3NPhAc and mAb 2H3. Therefore, mAb 2H3 was proved rather selective to GM3NPhAc. MAb 2H3 was identified as a murine IgM antibody by means of ELISA using class and sub-class specific goat anti-mouse immunoglobulins. The supernatants of cultures of mAb 2H3-secreting cell were used throughout the following experiments.

2.2. Glycoengineered expression of GM3NPhAc on tumor and normal cells

B16F0 cell, a murine melanoma cell line which expresses a high level of GM3,[35] was employed to examine the glycoengineered expression of GM3NPhAc on tumor cells resulting from ManNPhAc treatment. For this purpose, B16F0 cells were incubated in medium containing 0.02, 0.1, 0.5, or 2.0 mM of ManNPhAc, while B16F0 cell without ManNPhAc treatment was utilized as the control. After cells were incubated for 24, 48, and 72 h, respectively, they were collected and subjected to cellular ELISA using mAb 2H3 and alkaline phosphatase-linked goat anti-mouse IgM antibody as the primary and secondary antibodies. The expression levels of GM3NPhAc on cells were presented as the mean optical density (OD) obtained from triplicate ELISA experiments.

As shown in Figure 2, the ELISA results about B16F0 cell glycoengineering revealed that the OD value of B16F0 cell incubated with 0.02 mM of ManNPhAc was unchanged, while the OD value of B16F0 cell incubated with 0.1 mM of ManNPhAc showed a significant increase (ca 1.5- to 2-fold) compared to that of the control group. The OD values of cells incubated with 0.5 and 2 mM of ManNPhAc increased further to 5–5.5

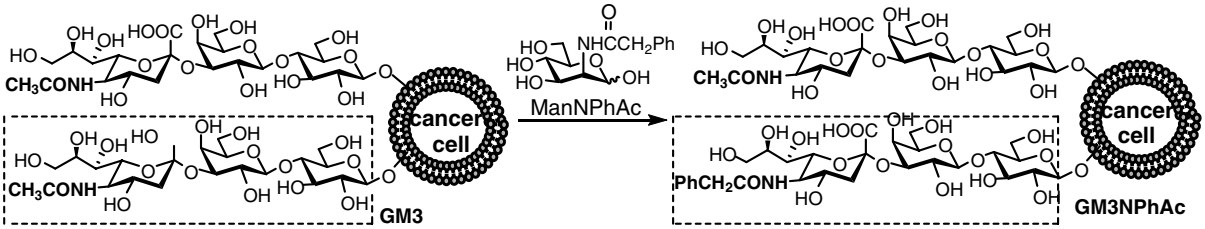

Figure 1. Glycoengineering of GM3 on cancer cell. Treatment of cancer with ManNPhAc induces GM3NPhAc expression in place of GM3 on cancer cell for immunological targeting.

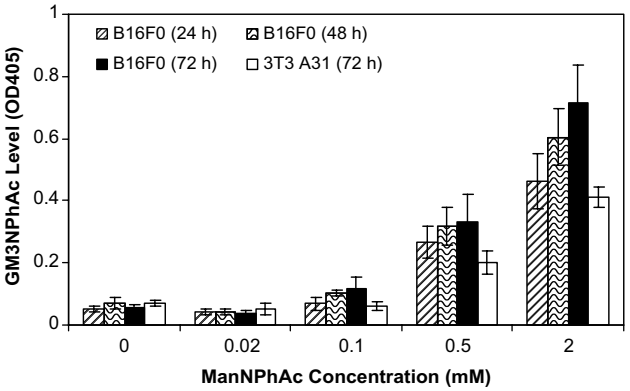

Figure 2. Levels of GM3NPhAc expression on B16F0 and 3T3 A31 cells treated with ManNPhAc. After B16F0 and 3T3 A31 cells were incubated with 0, 0.02, 0.1, 0.5, and 2.0 mM of ManNPhAc for indicated time (24, 48 or 72 h), they were analyzed by cellular ELISA using mAb 2H3 and goat anti-mouse IgM antibody as the primary and secondary antibodies, respectively. GM3NPhAc levels were presented as the mean OD values obtained from triplicate experiments.

and 9- to 12-fold higher, respectively, than that of the control group. These data suggest that B16F0 cell incubated with 0.1 mM of ManNPhAc, or may be with lower concentrations, started to express a significant level of GM3NPhAc and that the expression levels of GM3NPhAc on B16F0 cells were dependent on ManNPhAc concentrations. Moreover, the results also revealed that B16F0 cells treated by ManNPhAc for different lengths of time showed the same trend of increased GM3NPhAc expression as the result of ManNPhAc concentration increase, while the OD value of B16F0 cell treated by ManNPhAc for 24 h was constantly lower than that of cell treated for 48 h and both were lower than that of cell treated for 72 h. These data suggest that the glycoengineered expression of GM3NPhAc on B16F0 cell was also dependent on the time of ManNPhAc treatment.

The same methods and protocols were also employed to investigate the ManNPhAc-mediated glycoengineering of 3T3 A31 cell, a murine normal embryo fibroblast cell line which expresses a relatively high level of GM3.[40] The ELISA results concerning 3T3 A31 cell treated with different concentrations of ManNPhAc for 72 h are shown in Figure 2. The OD values of 3T3 A31 cells incubated with 0.02 and 0.1 mM of ManNPhAc were the same as that of the control group. Cells incubated with 0.5 and 2.0 mM of ManNPhAc showed significantly higher OD values (ca 3- and 6-fold higher, respectively) than cells of the control group. These results suggest that normal cell 3T3 A31 can also use ManNPhAc to biosynthesize GM3NPhAc, but less efficiently than cancer cell B16F0.

2.3. Anti-GM3NPhAc antibody-mediated complement-dependent cytotoxicities (CDCs) to ManNPhAc-treated cells

To study antibody-mediated CDCs to glycoengineered cells, B16F0 and 3T3 A31 cells were incubated first with 0.02, 0.1, 0.5, 2.0, and 4.0 mM of ManNPhAc for 72 h, respectively, and then with mAb 2H3 and complements. Cell lysis was finally analyzed by means of lactate dehydrogenase (LDH) assay,[41] and the cytotoxicity expressed as percentage of cell lysis was calculated by the following equation:

$$\%\text{lysis} = (\text{experimental A} - \text{low control A}) / (\text{high control A} - \text{low control A}) \times 100$$

Experimental A represents the optical absorption of analyzed cells at 490 nm; the low and high control A's represent the optical absorptions of untreated cells and completely lyzed cells at 490 nm, respectively. The results are shown in Figure 3. For B16F0 cell, the antibody-mediated cytotoxicity almost reached the maximum at 0.02 mM of ManNPhAc. With further increase of ManNPhAc concentration, the cytotoxicity was approaching a plateau (ca 85% cell lysis). For 3T3 A31 cell, however, no cell lysis was observed even at 0.1 mM of ManNPhAc. Cell lysis started to become evident when the concentration of ManNPhAc reached 0.5 mM, but only about 50% (after subtracting the background lysis) of 3T3 A31 cells were lyzed after incubation with 4 mM of ManNPhAc.

Since the cytotoxicity to B16F0 cell already reached the maximum at 0.1 mM of ManNPhAc, next, we evaluated the antibody-mediated cytotoxicity to B16F0 and 3T3 A31 cells at lower concentrations in great detail. The cells were incubated first with 0.01, 0.02, 0.04, 0.08, 0.16, and 0.32 mM of ManNPhAc, respectively, and then with mAb 2H3 and complements. Finally, the antibody-induced cell lyses were analyzed by LDH assay. As shown in Figure 4, mAb 2H3 started to exhibit obvious CDC to B16F0 cell incubated with a low concentration (0.01 mM) of ManNPhAc. The required ManNPhAc concentration to achieve 50% lysis of B16F0 cell was ca 0.016 mM. For 3T3 A31 cell, however, no CDC was observed at the ManNPhAc concentration as high as 0.16 mM. After incubation with 0.32 mM of ManNPhAc, 3T3 A31 cell exhibited a lysis rate similar to that

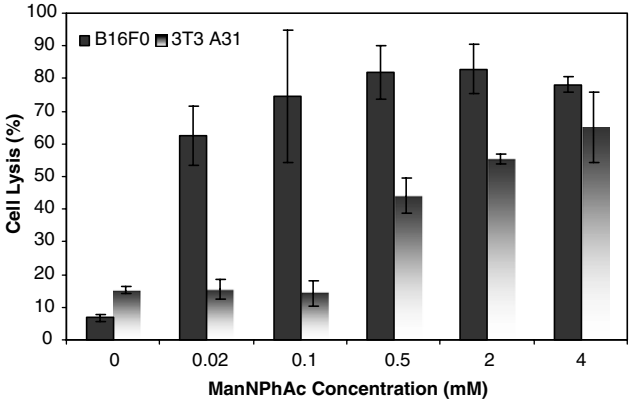

Figure 3. Anti-GM3NPhAc antibody-mediated cytotoxicity to B16F0 and 3T3 A31 cells treated with various concentrations (0, 0.02, 0.1, 0.5, 2.0, or 4.0 mM) of ManNPhAc. After cells were incubated with ManNPhAc for 72 h, cell lysis induced by mAb 2H3 and complements was assessed by the LDH assay.

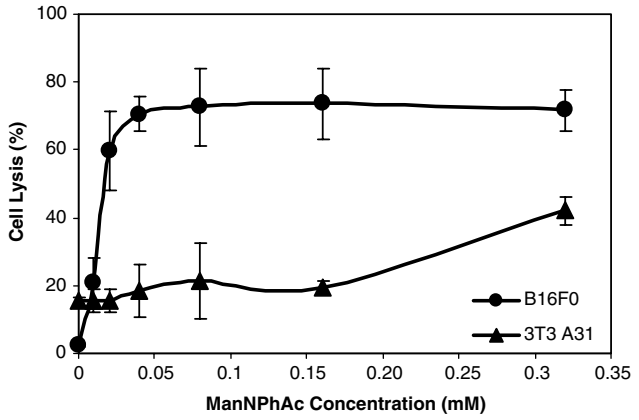

Figure 4. Comparison of the anti-GM3NPhAc antibody-mediated cytotoxicities to B16F0 and 3T3 A31 cells treated with different concentrations (0, 0.01, 0.02, 0.04, 0.08, 0.16, and 0.32 mM) of ManNPhAc. After cells were incubated with ManNPhAc for 72 h, cell lyses induced by mAb 2H3 and complements was assessed by the LDH assay.

of B16F0 cell incubated with 0.01 mM of ManNPhAc. A 50% lysis of 3T3 A31 cell was obtained only when the ManNPhAc concentration reached ca 4.0 mM. Thus, under the exactly same conditions, normal cell 3T3 A31 was at least 200 times less sensitive to antibody-mediated CDCs than cancer cell B16F0, though both cell lines were glycoengineered by ManNPhAc to express GM3NPhAc.

3. Discussion

GM3 is a TACA significantly overexpressed by a number of tumors, such as melanoma.[35] However, many normal cells also express a lower concentration of GM3.[40] Consequently, for cancer immunotherapy targeting unnatural GM3 derivative on tumor cells resulting from cell glycoengineering, in addition to the question whether GM3 on cancer cell can be effectively glycoengineered, another important question is how selective the cell glycoengineering and the provoked immune reactions are for cancer cell.

The results of cell glycoengineering (Fig. 2) obtained from ManNPhAc treatments have showed that cancer cell B16F0 could use ManNPhAc to express higher levels of GM3NPhAc than normal cell 3T3 A31. For example, by means of mAb 2H3, we have detected a significant expression of GM3NPhAc on the surfaces of B16F0 cell incubated with 0.1 mM of ManNPhAc, while 3T3 A31 cell incubated under the same condition did not show any detectable amount of GM3NPhAc. The level of GM3NPhAc expressed on 3T3 A31 cell incubated with 0.5 mM of ManNPhAc was significant, but it was only comparable to that on B16F0 cell incubated with 0.1 mM of ManNPhAc. Thus, it is estimated that the level of GM3NPhAc expressed on B16F0 cell was ca 4–5 times higher than that on 3T3 A31 cell. However, we cannot rule out the possibility that the actual difference in GM3NPhAc expression on these cell lines was higher or lower than 4- to 5-fold, because mAb 2H3 might have some cross-reactions with other *N*-phenylactylated sialo glycans on cells. The higher expression of GM3NPhAc on cancer cell than on normal cell can be attributed to the increased GM3 biosynthetic activities in the former.[40,42] We further hypothesize that in competitive environments the difference between normal and cancer cells in GM3NPhAc expression could be more significant, because the faster proliferation of cancer cell will make it more competitive for uptaking the glycoengineering precursor.

When ManNPhAc concentration was lower than 0.10 mM, no GM3NPhAc was detected on B16F0 cell by ELISA. However, our previous research,[34] which employed flow cytometry assay (FACS) to detect GM3NPhAc, has disclosed a significant expression of GM3NPhAc on B16F0 cell incubated with as low as 0.02 mM of ManNPhAc. The discrepancy in conclusion from these studies is probably owing to the different sensitivities of analytic methods used. ELISA detects the absorption of an enzymatic reaction product at 405 nm caused by the bound antibody, which should be less sensitive than FACS that detects the florescence of florescent tags attached to cells via the bound antibody. Therefore, the concentration of ManNPhAc required for functional cell glycoengineering may be lower than that required for ELISA detection of GM3NPhAc on cells, as demonstrated by the cytotoxicity experiments.

The anti-GM3NPhAc mAb 2H3 showed significant cytotoxicity to B16F0 cells treated with as low as 0.01 mM of ManNPhAc, and the cytotoxicity reached the maximum at the ManNPhAc concentration of ca 0.04 mM (Fig. 4). This is a concentration that agrees with that required for detection of GM3NPhAc on cancer cells by FACS in our previous study[34] but significantly lower than that required in the ELISA experiments above. Higher concentrations of ManNPhAc resulted in the expression of more GM3NPhAc on cells as revealed by the cell glycoengineering experiments (Fig. 2), but when the ManNPhAc concentration was over 0.04 mM, GM3NPhAc expression would exceed the level necessary for cell killing and the cytotoxicity would approach the plateau. This means that so long as the cells express certain levels of GM3NPhAc they would be recognized and killed by antibody and higher level of GM3NPhAc would give the same result, because each cell can only be killed once. In these experiments, the calculated maximum of cell lysis was ca 75–85%. Close inspection of the cells with a microscope proved that essentially all cells incubated with 0.04 mM of ManNPhAc or above were dead after antibody and complement treatments, indicating a cell lysis rate close to 100%. This discrepancy may be attributed to the method used to obtain the high control in LDH assay. Treating cells with a high concentration of detergent destroys cells completely and thus releases all cell contents to give a high absorption. For antibody-induced cell lysis, it is possible that some killed cells were partially destroyed to leak only a fraction of the contents to produce an

underestimated absorption. Nevertheless, the results of this research indicated that mAb 2H3 started to show evident cytotoxicity to B16F0 cell incubated with 0.01 mM of ManNPhAc and that complete cell killing was achieved with B16F0 cell incubated with 0.04 mM of ManNPhAc.

On the other hand, mAb 2H3 did not show any toxicity to 3T3 A31 cell incubated with up to 0.16 mM of ManNPhAc. The higher background with 3T3 A31 cell than with B16F0 cell was probably due to the death of 3T3 A31 cell induced by complements alone. It is well established that culturing normal cells is more difficult than culturing cancer cells as the former die easily even under normal culturing conditions. Cytotoxicity started to become observable with 3T3 A31 cell incubated with 0.32 mM of ManNPhAc, but the lysis rate was similar to that of B16F0 cell incubated with 0.01 mM of ManNPhAc. Therefore, under the same conditions, the cytotoxicity of mAb 2H3 was at least 32 times more potent to cancer cell than to normal cell. Moreover, the change of cell lysis rate as a result of ManNPhAc concentration increase was significantly slower for 3T3 A31 cell than for B16F0 cell. For example, the necessary concentration of ManNPhAc to achieve 50% lysis of 3T3 A31 cell was ca 4.0 mM, while it was only ca 16 μM for B16F0 cell. Based on these results, we estimated that mAb 2H3 should be over 200 times more cytotoxic to melanoma cell than to normal cell.

Obviously, the selectivity of antibody-mediated cytotoxicity to glycoengineered cancer cell was significantly higher than the difference in GM3NPhAc expression levels on normal and cancer cells. One hypothetical explanation for the results is that the difference in GM3NPhAc expression on the two cell lines was significantly underestimated because of the cross-reactions between mAb 2H3 and *N*-phenylacetylated sialo glycans on normal cell. If this is the case, it is also necessary to assume that the weak and nonspecific cross-reactions did not provoke obvious cytotoxicity. To verify such hypotheses, quantitative analysis of GM3NPhAc levels on both cell lines and detailed studies of CDC mechanism are required. Another hypothesis is that the higher selectivity of antibody-mediated cytotoxicity was due to certain special TACA microdomains formed on cancer cell.[13] Hakomori and coworkers[13,41,43] observed that TACA-raised antibodies could selectively bind to cancer cell in the presence of normal cell, even under the condition that the latter also expressed a low level of the same antigen. Their hypothesis for this selectivity was that the immune reaction was antigen density-dependent[40] and that the higher concentration of TACA on cancer cell might create some special microdomains to facilitate the discrimination of cancer cell from normal cell.[13] This observation is the cornerstone for the development of cancer vaccines or immunotherapies based on natural TACAs. In our case, both cell glycoengineering and the density of GM3NPhAc will distinguish cancer cell for its selective targeting, thus cancer immunotherapies based on our strategy can be more specific than traditional immunotherapies targeting natural TACAs.

4. Conclusion

In short, an anti-GM3NPhAc mAb 2H3, which does not cross-react with natural GM3, was established by the hybridoma technology. MAb 2H3 was proved to be a useful tool for studying cell glycoengineering and for in vitro studies of new cancer immunotherapies. Results about the glycoengineering of GM3 on B16F0 cell and 3T3 A31 cell have verified that B16F0 cell can incorporate ManNPhAc in the biosynthesis of GM3NPhAc more effectively than 3T3 A31 cell. This has provided the necessary molecular basis for selective targeting and killing of cancer cell by means of anti-GM3NPhAc antibodies or immune reactions. Moreover, glycoengineering and cytotoxicity studies have confirmed that B16F0 cell cultured in 20–40 μM of ManNPhAc could be recognized and effectively killed by the GM3NPhAc-specific antibody in the presence of complements. This concentration of ManNPhAc is equivalent to an acceptable dosage of ca 100–200 mg per person, indicating the practicability of potential cancer immunotherapies based on treatment with ManNPhAc. Most importantly, CDC studies have demonstrated that mAb 2H3 is much more cytotoxic to ManNPhAc-treated cancer cell than to ManNPhAc-treated normal cell. We have revealed here for the first time that ManNPhAc can selectively glycoengineer tumor cells for their specific targeting and killing. These results suggest the feasibility to selectively eradicate cancer in vivo by the new immunotherapy without affecting normal cells. In agreement with this conclusion, we have observed that animals treated with a high dose of ManNPhAc, for example, 2 mg/mouse, for months did not show any obvious side effect or abnormal behavior. The results of this work also support that the new cancer immunotherapy combining vaccination against GM3NPhAc and ManNPhAc treatment is promising for further development. We propose that this immunotherapy may be applicable to other tumors so long as they express GM3. Presently, we are focused on studying the efficacy of this immunotherapy to cure and prevent metastatic melanoma in animals.

5. Experimental

5.1. Materials

ManNPhAc and GM3NPhAc-KLH/HSA conjugates were prepared by methods reported previously.[21] The LDH Assay Kit was purchased from Takara Bio Inc. MPL+TDM adjuvant (Rabi) and rabbit complement sera were purchased from Sigma-Aldrich. PEG 4000 (50%, w/v) was purchased from Hampton Research. Hypoxanthine–Thymidine (HT, 50X) and Hypoxanthine–Aminopterin–Thymidine (HAT, 50X) were purchased from Mediatech, Inc. Cell culture media RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM) were purchased from Mediatech, Inc. and American Type Culture Collection (ATCC, Manassas, VA), respectively. Bovine calf serum (CBS) and bovine fetal serum (FBS) were purchased from ATCC. Penicillin–streptomycin (pen-strep) and trypsin–EDTA were

purchased from Invitrogen. The alkaline phosphatase linked goat anti-mouse kappa, IgM, IgG1, IgG2a, and IgG3 antibodies were purchased from Southern Biotechnology, Buckingham, AL.

5.2. Animals

Female C57BL/6 mice of 6–8 weeks of age were used, and they were purchased from Jackson Laboratory. Mice were maintained in Department of Laboratory Animal Research (DLAR) at Wayne State University and allowed free access to food and water.

5.3. Cell Lines and culture conditions

Murine myeloma cell SP2/0-Ag 14, murine melanoma cell B16F0 and murine normal fibroblast cell3T3 A31 were purchased from ATCC. SP2/0-Ag 14 and B16F0 cells were cultured at 37 °C in RPMI-1640 and DMEM, respectively, supplemented with 10% heat-inactivated FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin. 3T3 A31 cell was cultured as B16F0 except the displacement of FBS with 10% CBS. Cells were detached from cell culture flasks with trypsin–EDTA.

5.4. Enzyme-linked immunosorbent assay (ELISA)

ELISA plates were first treated by 100 μL of a GM3NPhAc-HSA solution (1 μg/mL) in the coating buffer (0.1 M bicarbonate, pH 9.6) overnight at 4 °C, followed by washing 3 times with PBS containing 0.05% Tween 20 (PBST). Then, hybridoma culture supernatants or optimal dilutions of sera from immunized mice were added in the coated ELISA plates (100 μL/well) and incubated at 37 °C for 2 h. The plates were washed and incubated with 1:1000 dilution of alkaline phosphatase linked goat anti-mouse kappa, IgM or IgG2a antibody or with a 1:2000 dilution of alkaline phosphatase linked goat anti-mouse IgG1 or IgG3 antibody for 1 h at room temperature. Finally, plates were washed and developed with 100 μL of PNPP solution (1.67 mg/mL in PNPP buffer) for 30 min at room temperature for colorimetric readout using a Bio-Rad 550 plate reader at 405 nm wavelength.

5.5. Preparation of an Anti-GM3NPhAc monoclonal antibody

Three C57BL/6 female mice were immunized intraperitoneally with GM3NPhAc-KLH (containing 3 μg of carbohydrate) mixed with MPL+TMD (Ribi) adjuvant on day 0, 14 and 21. Their blood samples were taken to measure the antibody responses by ELISA using GM3NPhAc-HSA as the capture antigen. The mouse with the highest serum antibody titer was selected as the spleen donor for hybridoma production and thus received the final intraperitoneal injection of GM3NPhAc-KLH 4 days before the cell fusion experiment. Spleen cells (2×10^7) of the immunized mouse were prepared aseptically and fused with SP2/0 myeloma cells (2×10^6) by using 50% (w/v) of PEG4000 as a fusing agent. After fusion, the resulting cells were resuspended gently with regular medium (RPMI1640 supplemented with 20% of heat-inactivated FBS) and distributed on 96-well culture plates with a feeder layer of C57BL/6 mouse spleenocytes. After 24 h of culture, the regular medium was replaced with the selection medium containing 1 × HAT. Half of the medium in the wells was replaced by fresh HAT medium every 3 days, and two weeks later, it was switched to 1 × HT medium and cultured for another 2 weeks. Cell-free culture supernatants were screened with GM3NPhAc-HSA as a coating antigen for the presence of GM3NPhAc-specific antibodies by ELISA about 1 week after cell fusion. Hybridoma cells from ELISA-positive wells were cloned by limiting dilution method, and the positive clones from single cell were recovered, expanded, and cryopreserved according to standard procedures. Typing of mAbs was done by ELISA using class and sub-class specific goat anti-mouse immunoglobulins from Southern Biotechnology Associates, following the manufacturer's recommendations.

5.6. Cell glycoengineering analyzed by cellular ELISA

Cells were first incubated with various concentration of ManNPhAc for different periods, and then trypsinized, counted, and seeded onto 96-well microplates (1.5×10^4 cells/well in 100 μL medium). After overnight incubation at 37 °C, cells were washed with DMEM and incubated at 37 °C for 2 h with 100 μL of anti-GM3NPhAc mAb 2H3 (cell culture supernatant) diluted 1:10 in DMEM. Cells were washed again and incubated with 100 μL of goat anti-mouse kappa antibody (1:1000 in DMEM) at 37 °C for 1 h. After washing and addition of PNPP substrate, cells were incubated at rt for 30 min for colorimetric readout using a Bio-Rad 550 plate reader at 405 nm wavelength. Data were presented as the mean of OD of triplicate experiments after subtracting that of the control group, that is, cells incubated without primary antibody.

5.7. Antibody-mediated complement-dependent cytotoxicity (CDC) assays

CDCs were determined using LDH cytotoxicity Detection Kit according to manufacture's protocol with some modifications. Briefly, after cells were incubated with different concentrations of ManNPhAc for 72 h, they were trypsinized, counted, seeded onto 96-well microplates (1.5×10^4 cells/well in 100 μL of medium) and then incubated at 37 °C overnight. After washing and incubation with 100 μL of anti-GM3NPhAc mAb 2H3 supernatant (1:10 dilution in DMEM) at 37 °C for 2 h, cells were washed and then incubated with 100 μL of rabbit complement sera (1:10 in DMEM) at 37 °C for 1 h. The low control (spontaneous LDH release) and high control (maximum LDH release) were obtained by adding 100 μL of DMEM or 100 μL of 1% tritone-100 in PBS to wells, respectively. After incubation, the microtiter plate was centrifugated, and then 20 μL of cell-free supernatants were carefully removed and transferred into corresponding wells of an optically clear 96-well plate, which contained 80 μL PBS and 100 μL LDH cytotoxicity Detection Kit reagent in each well. The mixtures were incubated at room temperature for 30 min.

The absorptions (A) were read at 490 nm wavelength using an ELISA reader. The percentage of cell lysis is calculated by the equation:

$$\%\text{lysis} = (\text{experimental A} - \text{low control A}) / (\text{high control A} - \text{low control A}) \times 100.$$

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