FEBS Letters 441 (1998) 20–24 FEBS 21260

GD1α-replica peptides functionally mimic GD1α, an adhesion molecule of metastatic tumor cells, and suppress the tumor metastasis

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Received 4 November 1998

Abstract A novel peptide technology to produce mimicking peptides of carbohydrate moiety (which we propose to name glyco-replica peptides) is a useful tool to elucidate the functions of glycoconjugate. Carbohydrate moiety of ganglioside GD1α functions as a molecule involved in the adhesion between murine highly metastatic lymphoma RAW117-H10 cells and hepatic sinusoidal endothelial (HSE) cells. To prepare peptides which mimic the carbohydrate structure of GD1α, phage clones expressing peptides which bound to a monoclonal antibody against GD1α (KA17) were isolated from a phage-displayed random peptide library. Four phage clones having affinity to the monoclonal antibody KA17 were isolated, and these clones showed inhibitory effect on the binding of KA17 to GD1α. The amino acid sequences of the displayed pentadecamers were determined, and one of the phages displaying sequence WHWRHRIPLQLAAGR bound to HSE cells directly and showed the highest inhibitory effect on the adhesion between RAW117-H10 cells and HSE cells. The synthesized peptides having the same sequences to the displayed 15mers in the four isolated phage clones also showed the inhibitory effect on the adhesion of RAW117-H10 cells to HSE cells, and, again, the WHWRHRIPLQLAAGR peptide showed the highest inhibitory effect. Furthermore, intravenous injection of the peptide brought almost complete inhibition of the metastasis of RAW117-H10 cells to lung and spleen, and about 50% inhibition of the liver metastasis. These results indicate that GD1α plays an important role for metastasis of RAW117-H10 cells, and the peptides obtained by the present procedure are able to mimic the functional role of the glycoconjugate.

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Key words: GD1α-replica peptide; Phage-displayed random peptide library; Adhesion molecule

1. Introduction

Carbohydrate moiety of glycoconjugate on the cell surface changes during embryogenesis, cell differentiation, proliferation, and oncogenic transformation [1–4]. Their functional roles on cell behavior were elucidated to be cell recognition molecules such as ABH-blood group determinants, ligands of bacteria and virus, and adhesion molecules in inflammation and tumor metastasis [5–9]. Recently, we demonstrated that ganglioside GD1α on the cell surface of murine metastatic large cell lymphoma (RAW117-H10 cells) functions as an adhesion molecule against the endothelial cells of the target tissue [10]. More detailed experiments to elucidate the mechanism of GD1α-mediated adhesion to the endothelial cells required fairly large amounts of GD1α; however, there are several difficulties in large scale preparation of GD1α from limited materials. Difficulties in getting enough quantity of pure oligosaccharide moieties from either natural sources of synthetic procedure prohibit the progress of glycoconjugate research. To solve these difficulties, we attempted to create a new approach by means of peptide technology and glycobiology. In this approach, we used phage-displayed random peptide library and monoclonal antibody against carbohydrate structure to prepare peptides which mimic the carbohydrate antigen. In the present study, we proposed the selected phage clones displaying peptides which have affinity to anti-GD1α antibody and tested the effect of the selected GD1α-replica peptides displaying phage clones and peptides themselves on the adhesion between RAW117-H10 cells and hepatic sinusoidal endothelial (HSE) cells. Actually, replica peptides displaying phage clones and the peptides themselves inhibited the adhesion. Furthermore, the anti-metastatic effect of the synthetic peptide was observed in in vivo experiments.

2. Materials and methods

2.1. Phage-displayed random peptide library

A phage-displayed random peptide library expressing a pentadecamer (15mer) peptide at the N terminus of pIII proteins of filamentous phage (fd phage) affinity vector was kindly provided by Dr. Hideyuki Saya at Kumamoto University [11]. A library has approximately 2.5×10^8 recombinants.

2.2. Biotinylation of antibody

Four hundred micrograms of KA17 in 200 μl of 50 mM $NaHCO_3$, pH 8.8, were incubated with 210 μg of sulfo-NHS-biotin (Pierce) in dimethylsulfoxide (14 μl) overnight at 4°C. The reaction mixture was dialyzed at 4°C against TBS (0.14 M NaCl containing 20 mM Tris-HCl buffer, pH 7.2) and concentrated with a Centricon-30 concentrator (Amicon) by centrifugation at 3000 rpm for 30 min.

2.3. Selection of GD1α-replica peptide displaying phage clones from the library (biopanning)

For isolation of GD1α-replica peptide displaying phage clones, a 96-well microtiter well (Falcon 3072) was coated with 10 μg of streptavidin in 100 μl of 100 mM $NaHCO_3$ overnight at 4°C and treated with blocking solution (100 mM $NaHCO_3$, 5 mg/ml BSA, 0.1 μg/ml streptavidin, and 0.02% NaN_3) for 1 h at room temperature. The

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Abbreviations: BSA, bovine serum albumin; ConA, concanavalin A; ELISA, enzyme-linked immunosolvent assay; HPTLC plate, high-performance thin-layer chromatography plate; HSE cell, hepatic sinusoidal endothelial cell; mAb, monoclonal antibody; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; TBS, trisaminomethane buffered saline; TLC, thin-layer chromatography; GD1α, NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAcβ1-4Galβ1-4Glcβ1-1Cer

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PII: S0014-5793(98)01511-7

biotinylated KA17 (1 μg) was adsorbed onto the streptavidin-coated well, and affinity-isolation of phages from the peptide epitope library by four cycles of biopanning was carried out as described by Scott and Smith [12].

2.4. DNA sequence

Phages from supernatants of positive colonies were precipitated with polyethyleneglycol (PEG 6000), and their single strand DNA was prepared by phenol extraction. DNA was sequenced by the protocol of Pharmacia Cy5 dye-primer system. A Cy5-conjugated oligonucleotide 5′-TAACACTGAGTTTCGTCACCAGTA was used as an anti-sense primer.

2.5. Binding of antibody to selected phages

Affinity of the isolated phage clones to the antibody KA17 was analyzed by ELISA.

Each well of microtiter plates (Falcon) was coated with isolated phage clones (10^9–10^{14} particles) in 100 μl of 100 mM $NaHCO_3$ by incubation overnight at 4°C. The coated plates were washed three times with TBS, blocked with TBS/1% BSA for 1 h at room temperature, washed three times with TBS, and KA17 in 100 μl of TBS/1% BSA was added to the wells, then incubated overnight at 4°C. After washing the wells with TBS, the bound KA17 was detected by incubation with peroxidase-conjugated anti-mouse IgM, diluted 1:1000, for 2 h at room temperature. The bound antibody was monitored by peroxidase activity, with orthophenyldiamine used as substrate, and the color developed was determined by a microtiter plate reader at 490 nm. An irrelevant peptide containing phage clone was used as negative clone (control) in both experiments.

2.6. TLC immunostaining

TLC immunostaining was performed as follow. GD1α (2 μg) was developed on a high-performance thin-layer chromatography plate (HPTLC plate, a silica 60 precoated thin layer plate, E. Merck, Darmstadt, Germany) with a solvent system of chloroform/methanol/0.2% $CaCl_2$ (55:45:10, by volume). After developing, the HPTLC plate was coated with 0.05% polyisobutylmethacrylate for 30 s. Coated HPTLC plate was air dried, and GD1α on the plate was incubated overnight at 4°C with 0.2 μg of KA17 in 200 μl of PBS/1% BSA. The HPTLC plate was washed with PBS, incubated with peroxidase-conjugated anti-mouse IgM (diluted 1:100) at room temperature for 4 h, and washed with PBS again. GD1α was made visible with Konica Immunostaining Kit (Konica, Japan).

2.7. Inhibition study of cell adhesion of RAW117-H10 cells to hepatic sinusoidal endothelial (HSE) cells

Adhesion of RAW117-H10 cells to HSE cells was done as described previously [10]. For inhibition experiments, the selected phage clones or synthesized peptides and the HSE cells were incubated for 30 min at 37°C in 50 μl of RPMI 1640 without serum in a 96-well micro-titer plate. [^{35}S]Methionine-labeled H10 cells (1×10^5 cells/50 μl) were added to the mixture and incubated for 30 min at 37°C, after which each well was washed with PBS twice and H10 cells adhered to the HSE cells were solubilized with 50 μl of 1 N NaOH containing 1% SDS. After neutralizing the solubilisate with 2 N HCl, the radioactivity of H10 cells was measured.

2.8. Binding of the selected phage clone to HSE cell surface

Binding of the isolated phage clone by panning with KA17 was performed as described by Barry et al. [13]. HSE cells were cultured (10^3 cells) in 10% FBS containing RPMI 1640 in an 8-well chamber slide (Nunc) at 30°C for 3 days. Cells were washed with PBS once and incubated at 37°C for 1 h in 100 μl of serum free medium. The medium was removed and approximately 10^{12} titer of the phage clone was added to the chamber in 100 μl of serum free medium and incubated at 37°C for 30 min. The cells were washed six times with PBS and then fixed in 200 μl of 1% formaldehyde in PBS for 10 min at room temperature. The fixed cells were washed three times with PBS and incubated with 200 μl of peroxidase-conjugated anti-M13 rabbit polyclonal antibody (Pharmacia), diluted 1:100, in PBS/1% BSA for 2 h at room temperature. The chambers were washed six times with PBS and incubated with Coumarin Tyramide (TSA-Direct (Blue) kit, DuPont NEN, Boston, MA, USA) for 10 min. The chambers were washed three times with PBS, and the binding of phage clones was observed with a fluorescein microscope.

2.9. Animal experiment

The inhibitory effect of peptides on experimental metastasis was determined as follows. Seven-week-old female BALB/c mice used were cared for according to our institute's animal facility guidelines. The mice were anesthetized with sodium pentobarbital (0.05 mg/g mouse body weight). For the injection of lymphoma cells into the hepatic portal system, an incision was made along the midline of the abdomen to expose a large vein located in the mesentery. RAW117-H10 mouse lymphoma cells were cultured with high glucose DMEM (hG-DMEM) supplemented with 5% fetal bovine serum at 37°C in a CO_2 incubator, and washed with hG-DMEM three times to remove the serum. Cell suspension (1×10^5 cells/0.1 ml) were mixed with the same volume of 200 mg/0.1 ml peptide solution or that of medium and, immediately after the mixing, 0.2 ml of mixture was injected into 7-week-old BALB/c mice via the portal vein. The animals (8 per group) were sacrificed at day 5 after injection, and the removed organs, i.e. liver, spleen and lung, were weighed to determine the extent of metastasis.

3. Results

3.1. Selection of GD1α-replica peptide displaying phage clones from the phage library by mAb KA17

To select peptides which mimic GD1α-function as an adhesion molecule, we screened the phage-displayed random 15mer peptide library with biotinylated mAb KA17. After four cycles of biopanning, 24 individually isolated bacterial colonies were randomly picked up, and thus obtained phage clones from the host bacterium were subjected to DNA sequence analysis. The deduced peptide sequences shown in Table 1 classified the obtained phage into four clones (Ø1, Ø2, Ø3, and Ø4), and each peptide appeared in 11, 9, 2, and 2 clones, respectively. Proline and arginine appeared in all clones, and tryptophan (Ø1, Ø3, and Ø4), tyrosine and phenylalanine (Ø1, Ø2, and Ø4) appeared in three clones. A unique sequence WHW occurred in Ø1 and Ø3.

Fig. 1 shows that all isolated clones had higher affinity to KA17 than that of irrelevant phage clone used as a negative clone. In ELISA, KA17 binding to the individually isolated phage increased in association with the titer of each phage clone fixed on the wells, whereas KA17 weakly bound to the negative phage (below 0.1 at OD_{490}).

3.2. Inhibition of the binding of KA17 to GD1α by the selected phage clones

Fig. 2 shows the inhibitory effect of the isolated phage clones on the KA17 binding to GD1α on an HPTLC plate. Detection of GD1α by KA17 was completely inhibited by the addition of phage clones Ø3 (10^{13} and 10^{14} titer/ml) and Ø2 (10^{13} titer/ml), whereas the negative phage (ØN) had no effect as control. Two phage clones Ø1 and Ø4 inhibited the binding of KA17 to GD1α, but activities were less than that of Ø3 (data not shown).

Table 1
Epitope sequence of phage clones selected by biopanning with biotinylated mAb KA17

Phage	Epitope sequence[a]	Clones
Ø1	FRSDVRFWHWSTPFM	11
Ø2	VRVYFGFGPPPYFGG	9
Ø3	WHWRHRIPLQLAAGR	2
Ø4	RYWLYGDPASFPVNH	2

[a]Epitope sequences were deduced from DNA of the inserted pentadecapeptide. The amino acid sequence is expressed by single-letter abbreviation; aromatic amino acids are in bold type, positive charged amino acids are underlined.

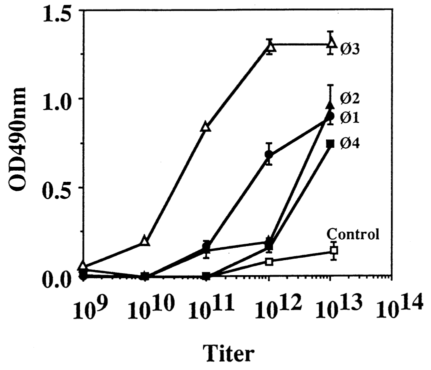

Fig. 1. Affinity of the selected phage clones against a CD1α MAb KA17 (ELISA). This experiment was done in triplicate.

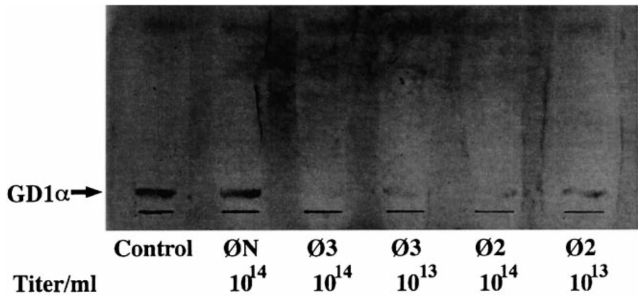

Fig. 2. Inhibition of the selected phage clones on the binding of KA17 to GD1α on an HPTLC plate. Isolated phage clones (final 10^{13} or 10^{14} titer/ml) were added to the KA17 solution, and the mixture was incubated with GD1α on the HPTLC plate overnight at 4°C. The complex of GD1α-KA17 was made visible with horseradish peroxidase-conjugated anti-mouse IgM and Konica Immunostaining Kit.

3.3. *Binding of selected phage clone to the HSE cells*

Binding of phage Ø3 to HSE cells was examined (Fig. 3). Fig. 3d shows that a larger amount of Ø3 bound to the HSE cells, compared with the negative phage shown in Fig. 3b, indicating that the binding of Ø3 is due to the specific recognition of HSE cell surface.

3.4. *Inhibition by the selected phage clones and synthetic peptides on the adhesion of RAW117-H10 cells to HSE cells*

We previously revealed KA17 inhibited the adhesion of RAW117-H10 cells to HSE cells [10]. Thus, the effect of the phage clones on the adhesion of RAW117-H10 cells to HSE cells was investigated (Fig. 4). The adhesion between RAW117-H10 cells and HSE cells was inhibited by the addition of a phage clone Ø3 (5.5×10^{14} titer/ml) and the inhibitory effect was 60% (Fig. 4A and B). Selected phage clones inhibited the adhesion in a dose dependent manner and the inhibition by phage clone Ø3 was stronger than the others. This inhibitory effect shown in Fig. 4B was well correlated with the affinity of phage clones to KA17 shown in Fig. 1. An inhibition study of the adhesion of RAW117-H10 cells to HSE cells was similarly performed by using synthetic 15mer peptides (Fig. 4C). A peptide Ø3P, which derived from a phage clone Ø3, had strongest inhibitory effect on the adhesion of RAW117-H10 cells and the adhesion of RAW117-H10 cells was suppressed to approximately 50% in the presence of 2.5 mg/ml of peptide Ø3P. The order of the inhibitory activity of these peptides was Ø3P > Ø4P > Ø1P > Ø2P. This order obtained by the peptides is the same as that obtained by using phage clones at a dose of 5.5×10^{4}/ml (Fig. 4B).

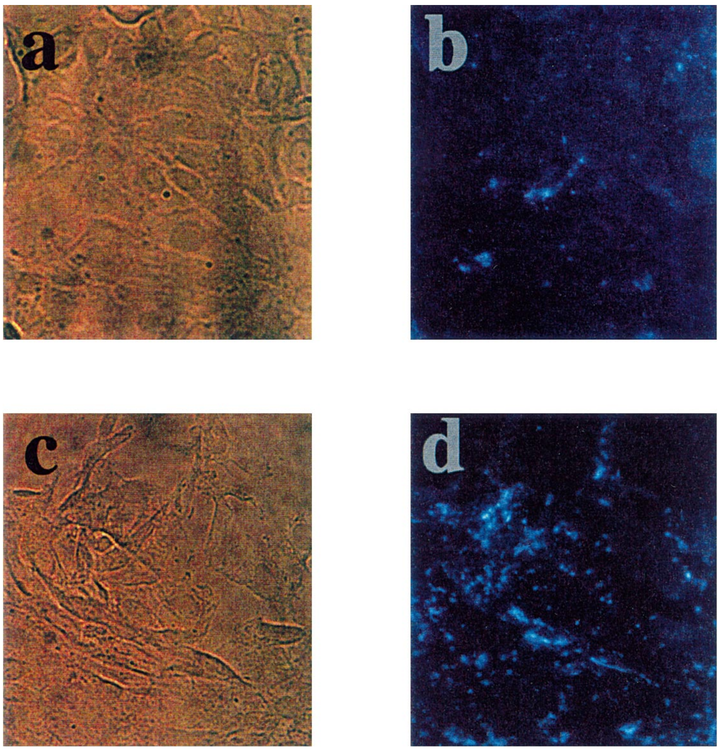

Fig. 3. Binding of Ø3 clone to HSE cell surface. HSE cells were incubated with (c,d) or without (a,b) Ø3 clone which has highest affinity to KA17. After this the Ø3 clone on the HSE cell surface was observed on a microscope with (b,d) or without fluorescein filters (a,c).

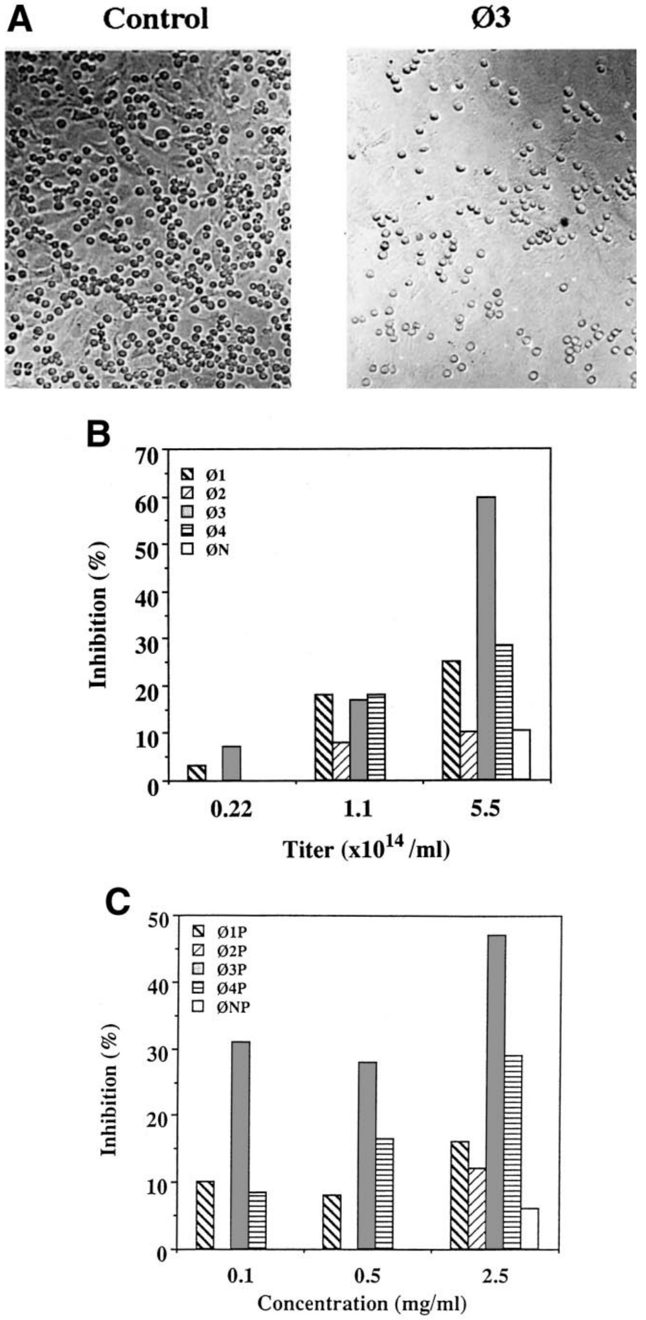

Fig. 4. Effect of the selected phage clones and synthetic peptides on adhesion between RAW117-H10 cells and HSE cells. Various titers of each phage clone were added to the medium when ^{35}S-labeled H10 cells and HSE cells were co-cultured. After 30 min incubation, H10 cells adhering to the HSE cells were observed with microscopy and counted with a scintillation counter. A: A phage clone Ø3 (5.5×10^{14} titer/ml) inhibited the H10 cell adhesion. B: Effect of dose of phage on the adhesion of H10 cells to HSE cells. When the H10 cells were co-cultured with HSE cells in the absence of phage clones, the radioactivity of the adhering H10 cells was estimated as 100% adhesion (0% inhibition). This experiment was done twice. C: Effect of synthetic 15mer peptides on the adhesion. Each peptide (Ø1P, Ø2P, Ø3P, and Ø4P) is respectively corresponding to the amino acid sequence shown in Table 1 (Ø1, Ø2, Ø3, and Ø4). Sequence of a peptide ØNP is corresponding to a negative phage clone ØN in B. This experiment was done twice.
←

3.5. Anti-metastatic effect of the synthetic peptide in vivo

Since the adhesion of RAW117-H10 cells to liver endothelia may be a prerequisite to the metastasis of the cells, the effect of the synthetic 15mer peptide Ø3P on the metastatic potential of RAW117-H10 cells in vivo was examined. Table 2 shows that the portal vein injection of RAW117-H10 cells resulted in increase of the tissue weights to 200%, 180%, and 164% in lung, liver, and spleen, respectively, at day 5 after injection. This increase in tissue weight was confirmed to be due to metastasis of RAW117-H10 cells by pathological examination (data not shown). On the other hand, the increase in weight of these tissues was suppressed when peptide Ø3P was injected with RAW117-H10 cells into mice. The inhibitory effect was observed to be 100%, 48.8%, and 91% in lung, liver, and spleen, respectively, at day 5 after injection.

4. Discussion

The phage-displayed random peptide library originally established by Scott and Smith [12] was reported to be useful for epitope mapping of monoclonal antibodies against a specific protein. Their report suggested that certain peptide displaying phage clones, which have high affinity to monoclonal antibody against carbohydrate and absorb the activity of the antibody, are possible to mimic both carbohydrate structure and their function. Concanavalin A (ConA)-binding peptides inhibited precipitation of α-glucan dextran by ConA [14,15]. These results seem to be that the selected peptides mimic carbohydrate moieties of ligands (α-mannose) for ConA. Furthermore, we selected lactotetraosylceramide (Lc_4Cer)-replica peptides, which mimic Galβ1-4GlcNAc structure, by a biopanning procedure and observed that the Lc_4Cer-replica peptides regulated Jack bean β-galactosidase activity [16]. On the basis of these findings and the idea of glyco-replica peptides, we isolated the peptide displaying phage clones which bind to a monoclonal antibody against GD1α ganglioside, which, we demonstrated, functions as an adhesion molecule on murine metastatic lymphosarcoma cell line RAW117-H10 [10]. By this procedure, four phage clones were selected. They bound to HSE cell surfaces and inhibited the adhesion between RAW117-H10 cells and HSE cells, indicating that the selected peptides mimic not only the three dimensional structure but also the function of GD1α as an adhesion molecule related to the metastatic process. From the present data, we would like to propose the terminology 'glyco-replica peptides' to the peptides which mimic functional roles of glycoconjugates. This procedure has some advantages: (i) glyco-replica peptide displaying phage clones are amplified easily by infecting to host bacterial cells; (ii) phage clones are water-soluble, compared with natural glycosphingolipids; (iii) synthetic peptides are prepared easily; (iv) a mutant peptide is easy to prepare; (v) searching of receptors for carbohydrate could be developed by using a glyco-replica peptide and two hybrid system; (vi) unknown functional role of the carbohydrate will be able to be suspected.

Hoess et al. showed that the tetramer peptide PWLY was critical for binding to mAb B3, which reacts with Lewis^Y (Le^Y) [17]. An isolated phage (Ø4) in the present study contained the tripeptide sequence WLY, which was found in the tetramer. Two selected phage clones (Ø1 and Ø3) had a tripeptide sequence WHW, which was reported to be in one clone (H11-1) of neolactotetraosylceramide (nLc_4Cer)-replica

Table 2
Effect of a synthetic peptide (Ø3P) on the metastasis of RAW117-H10 cells in mice

	Tissue weight (g ± S.D.)		
	Lung	Liver	Spleen
Control (medium) (0.1 ml)	0.138 ± 0.024	0.837 ± 0.067	0.119 ± 0.021
RAW117-H10+medium (1×10^5 cells) (0.1 ml)	0.277 ± 0.059	1.506 ± 0.044	0.196 ± 0.021
RAW117-H10+Ø3P (1×10^5 cells) (200 mg/0.1 ml)	0.136 ± 0.013*	1.185 ± 0.061	0.130 ± 0.036
Inhibition	100	48.8	91.0

RAW117-H10 cells (1×10^5 cells/0.1 ml) were mixed with the same volume of 200 mg/0.1 ml peptide solution or that of medium and 0.2 ml of suspension was injected into 7-week-old BALB/c mice via the portal vein. The animals (8 per group) were sacrificed at day 5 after injection and the individual tissue weight was determined to evaluate the extent of metastasis. *$P < 0.001$ against RAW117-H10 cells injected into mice.

peptides [16]. Therefore the three residues (WLY or WHW) seem to be critical for binding to mAb KA17. The four selected peptides were relatively enriched in aromatic amino acids, suggesting that the hydrophobic property relates to KA17 binding.

Adey et al. prepared peptides that have plastic-binding properties by this procedure and reported that plastic-binding peptides were enriched in tyrosine and tryptophan [18]. A phage clone Ø2 isolated in the present study had lower affinity to KA17 and inhibitory effect on the adhesion between RAW117-H10 cells and HSE cells compared with three other clones. Thus, it is possible that a phage clone Ø2 was isolated non-specifically.

Pasqualini and Rouslahti reported that phage-displayed randon peptide libraries might be useful for organ-selective targeting in vivo [19]. The synthetic peptide Ø3P was observed to inhibit actual tumor metastasis in vivo. Thus, it is possible that Ø3P peptide blocks the adhesion of RAW117-H10 cells to the target organ via occupying receptor(s) or certain molecule(s) which bind to GD1α on the endothelia of the target organ. Metastasis suppressing activity was complete in lung and spleen and 50% in liver, suggesting the presence of such GD1α receptors in these tissues. Alternatively, inhibition of liver metastasis by GD1α mimicking peptide caused suppression of further metastasis to lung and spleen, since liver is the most preferential organ of these metastatic tumor cells.

Recently, Pasqualini and co-workers isolated peptides having high affinity to integrin αvβ3 from a phage-displayed peptide library. Most of the peptides had the RGD motif and may be useful for targeting to the newly developed vasculature in the tumor site [20]. In this case peptides may mimic peptidic epitopes of the ligands for this integrin. Here, we prepared peptides which mimic GD1α, a carbohydrate epitope, from a phage-displayed peptide library, and observed the adhesion inhibitory function between the cells with hepatic endothelial cells in vivo. Furthermore, the peptide, WHWRHRIPLQLAAGR, actually suppressed metastasis of RAW117-H10 lymphoma cells in vivo. These results indicate that GD1α is involved in the metastatic process of the cells, and the mimicking peptide is useful for elucidating the function of the glycoconjugates. The peptide could also be useful for organ targeting and vaccine [21–24], although the actual receptor for GD1α remains to be identified.

Acknowledgements: This study was supported in part by the Special Coordination Fund from the Science and Technology Agency of the Japanese Government.

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