

Suppression of GD1 α ganglioside-mediated tumor metastasis by liposomalized WHW-peptide

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Abstract GD1 α ganglioside-replica peptides were recently isolated from a phage-displayed random pentadecapeptide library by assaying for inhibition of adhesion of RAW117-H10 lymphosarcoma cells to hepatic sinusoidal microvessel endothelial (HSE) cells. We show here that the Trp-His-Trp (WHW) peptide was identified as a minimal sequence of the GD1 α -replica peptide WHWRHRIPLQLAAGR. The addition of WHW peptide-attached liposomes displayed efficient inhibition of liver metastasis of RAW117-H10 cells as well as of GD1 α -mediated adhesion of RAW117-H10 cells to HSE cells *in vitro*. These results suggest that engineered liposomes for peptide delivery are applicable to treatment for metastasis.

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Key words: Cell adhesion; Experimental liver metastasis; Liposome; Phage-displayed random peptide library; GD1 α -replica peptide

1. Introduction

Modern drug delivery systems should have potential advantages, such as continuous maintenance of drug levels in a therapeutically desirable range, reduction of harmful side effects due to targeted delivery to a particular cell type or tissue, potentially decreased amount of drug needed, decreased number of dosages and possibly less invasive dosing, and facilitation of drug administration for pharmaceuticals with short *in vivo* half-lives [1]. Among various drug delivery systems, we have been focusing on liposome-based delivery systems for developing *in vivo* long circulation of drugs [2–7].

Metastasis is the most life-threatening event in patients with cancer. Blood-borne cancer cells frequently form metastasis mediated through various cell adhesion molecules. Better understanding of the interactions between metastatic cells and host cells in target organs would facilitate the development of new therapeutic approaches.

In an experimental metastasis, the RAW117-H10 cell clone, derived from parental RAW117-P cells that were originated from murine spleen cells, are known to form approximately 200-fold more gross liver tumor nodules than did the parental cell line [8], because of a strong correlation between high adhesiveness of RAW117-H10 cells to hepatic sinusoidal microvessel endothelial (HSE) cells and high expression of the ganglioside GD1 α on RAW117-H10 cells [9,10].

Recently, we found that adhesion of RAW117-H10 cells to HSE cells is inhibited by the GD1 α -replica peptides that we isolated from a phage-displayed random 15-mer peptide library [11]. In this study, we identified WHW tripeptide as a minimal functional domain located in the GD1 α -replica peptide, and found that WHW tripeptide-attached liposomes efficiently inhibited GD1 α -mediated liver metastasis of RAW117-H10 cells. Our findings suggest that appropriate liposomalization of WHW tripeptide is applicable to therapeutic treatment for GD1 α -mediated liver metastasis.

2. Materials and methods

2.1. Cell lines, antibodies, peptides, and reagents

The low-metastatic lymphosarcoma cell line RAW117-P was established from splenocytes of Balb/c mice by *in vitro* infection with Abelson leukemia virus [12]. The RAW117-H10 cell clone is a variant of RAW117-P cells with high metastatic potential for the liver [8,13]. RAW117-H10 and RAW117-P cells were maintained in suspension culture in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 5% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified CO₂ incubator [5]. Hepatic sinusoidal microvessel endothelial (HSE) cells were previously established from liver cells [14], and maintained in adherent culture in Dulbecco's modified Eagle's medium:Ham's F-12 (DMEM/F12 1:1 mixture) supplemented with 10% FBS, 10 units/ml heparin sodium, and 50 μ g/ml Endothelial mitogen (Biomedical Technologies, Stoughton, MA, USA) in a humidified CO₂ incubator, as described previously [15]. RAW117-H10 cells, RAW117-P cells, and HSE cells were kindly provided by Drs. G.L. Nicolson and T. Irimura.

The ganglioside GD1 α [16–18] purified from frog livers was generously provided by Dr. M. Ohashi [18]. The monoclonal antibody (mAb) directed against GD1 α (clone KA17) was kindly provided by Dr. Y. Hirabayashi [19]. Horseradish peroxidase (HRP)-conjugated F(ab')₂ fragment of goat anti-mouse IgG+IgM antibody was purchased from Jackson ImmunoResearch Laboratories, (West Grove, PA, USA).

Various peptides were synthesized from Rink amide resin (0.4–0.7 mmol/g) using a peptide synthesizer ACT357 (Advanced Chem-Tech), resulting in the form of amide at the carboxy termini. Alkylated peptides were synthesized using the DIPCI-HOBt coupling method and the formulas of the peptides were indicated as follows: NH₂-WHW-CO-NH-(CH₂)₁₁-CO-NH₂; NH₂-WHWRHRI-CO-NH-(CH₂)₇-CO-NH₂; CH₃(CH₂)₁₆-CO-NH-WHW-CO-NH₂. The former two were used in *in vitro* cell adhesion shown in Fig. 4 and the latter one was in *in vivo* experimental metastasis shown in Table 1. Integration of alkylated peptides into liposomes was confirmed by spectrophotometry.

2.2. Preparation of liposomes

Liposomes were prepared as described [2]. In brief, dipalmitoylphosphatidyl choline (DPPC) or distearoylphosphatidylcholine (DSPC), cholesterol, and alkylated WHW (10:5:1 or 4:2:1 as molar ratio) dissolved in chloroform were dried under low pressure to prepare thin lipid film, and stored *in vacuo* for at least 1 h. Liposomes

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were produced by hydration of thin lipid film with 0.3 M glucose, freeze-thawing for three times using liquid nitrogen, and subsequent sonication for 5 min with 50% duty cycles by using Braonson Sonifier (Model 250). Thus obtained liposomes were essentially small unilamellar vesicles. Initial concentrations of DPPC-containing liposomes and DSPC-containing liposomes were adjusted with 0.3 M glucose to 1.3 mM as DPPC and 1.25 mM as DSPC, respectively. DPPC and DSPC were kindly provided by Dr. Y. Namba (Nippon, Hyogo, Japan).

2.3. Preparation of BCECF-labeled cells

Cells were labeled with 2',7'-bis(2-carboxyethyl)-5(and 6)-carboxy-fluorescein, acetoxymethylester (BCECF-AM, Dojindo Laboratories, Kumamoto, Japan), as previously described [16]. In brief, RAW117 cells (1.2×10^6 cells/ml) were loaded with 3 μ M BCECF-AM (final 0.3% dimethylsulfoxide) in high-glucose DMEM containing 5% heat-inactivated FBS for 30 min at 37°C in a humidified CO₂ incubator. Cells were washed twice with phosphate-buffered saline (PBS), and suspended in high glucose DMEM containing 1% bovine serum albumin (BSA).

2.4. Cell adhesion assay

HSE cells (6.8×10^5 cells/ml) were seeded in 96-microwell plates precoated with 0.1% gelatin in PBS. Cells were cultured in DMEM/F12 1:1 mixture containing 10% FBS, heparin, and endothelial mitogen overnight at 37°C in a humidified CO₂ incubator. After the resulting monolayers were washed with PBS, 25 μ l of BCECF-labeled cells (4×10^5 cells/ml) and 25 μ l of various peptides or liposomes at the indicated concentrations were added onto HSE monolayer cells, and incubated for 20 min at 37°C in a humidified CO₂ incubator. Then, non-adherent cells were removed by washing with PBS. Adherent cells were solubilized in 100 μ l of 10% Triton X-100 at 4°C overnight, and were quantitated by fluorometry. BCECF was excited at 490 nm and emission signals were detected at 526 nm.

2.5. ELISA assay

50 μ l of GD1 α solution (64.34 μ g/ml methanol) was placed into each well in 96-microwell plates (polysop, NUNC) and methanol was evaporated. GD1 α -coated plates were washed three times with Tris-buffered saline, pH 7.4 (TBS) and then blocked with TBS containing 1% BSA (200 μ l/well) for 2 h at room temperature. After washing with TBS, 25 μ l of the anti-GD1 α mAb KA17 in TBS containing 0.1% BSA and 25 μ l of various peptides or buffer alone were added to each well, and incubated for 2 h at 4°C. After washing, bound mAb KA17 was detected by further incubation with HRP-conjugated goat anti-mouse IgG+IgM antibody for 2 h at 4°C. The bound antibodies were detected by peroxidase activities using *o*-phenylenediamine as substrate. The signals were measured with a microplate reader at 490 nm.

2.6. Experimental metastasis

Experimental metastasis was performed as described [5,11]. In brief, 7-week-old female Balb/c mice (SLC, Shizuoka, Japan) were anesthetized with sodium pentobarbital (0.05 mg/g body weight). Mice were cared for, according to the animal facility guidelines of the University of Shizuoka. For injection of RAW117-H10 cells into the hepatic portal system, an incision was made along the midline of the abdomen to expose the mesentery. Cultured RAW117-H10 cells were washed with high-glucose DMEM to remove FBS. Immediately after cell

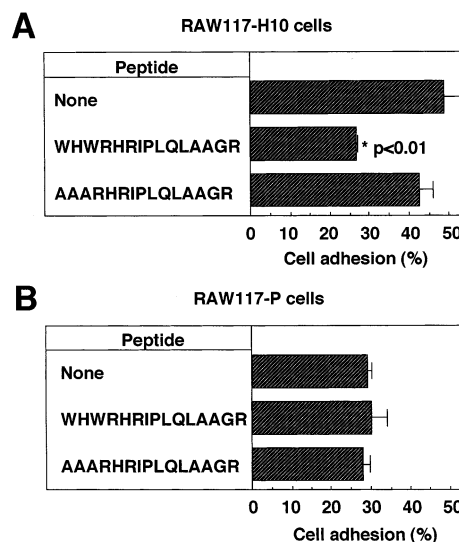


Fig. 1. Effect of the GD1 α -replica peptide on adhesion of RAW117 cells to HSE cells. BCECF-labeled RAW117-H10 (A) or RAW117-P cells (B) were added onto hepatic sinusoidal microvessel endothelial cells (HSE) in the presence or absence of peptides (660 μ M), and incubated for 20 min at 37°C. After non-adherent cells were washed out, adherent cells were quantitated by fluorometry. Peptides used in this study were WHWHRHRIPLQLAAGR and AAARHRIPLQLAAGR. The data represent the mean \pm S.D. ($n=4$). An asterisk indicates the significant difference (* $P<0.01$) calculated by Student's *t*-test.

suspensions (1×10^5 cells/0.1 ml) were mixed with either an equal volume of liposome solution or medium alone, 0.2 ml of the mixture was injected into mice via the portal vein. The animals (5 per group) were killed on day 7 after injection and the livers were removed. Establishment of metastasis was confirmed by pathological examination. The removed livers were weighted to determine the extent of metastasis because of difficult evaluation of RAW117 cell colonies inside the livers.

3. Results and discussion

Our recent study showed that, of 24 independent phage clones that we selected for inhibition of adhesion of RAW117-H10 cells to HSE cells, 13 clones possessed the WHW tripeptide domain in pentadecapeptides. Among them, the phage clone displaying the WHWHRHRIPLQLAAGR sequence showed the highest inhibitory activity [11].

To examine the significance of the WHW sequence in adhesion of RAW117-H10 cells to confluent monolayers of HSE cells, we synthesized mutated peptides for the ability to inhibit the adhesion. The GD1 α -replica peptide WHWHRHRIPLQ-

Table 1

Suppression of experimental metastasis of RAW117-H10 cells in the liver by liposomal GD1 α -replica peptide

Group	Liver weight (g \pm S.D.)	Increase in liver weight (g \pm S.D.)	Inhibition (%)
Sham ^a	0.861 \pm 0.014	—	—
RAW117-H10 cells	1.363 \pm 0.195	0.502 \pm 0.195	0
RAW117-H10 cells+control liposomes	1.305 \pm 0.110	0.444 \pm 0.110	11.6
RAW117-H10 cells+WHW-liposomes	1.137 \pm 0.040*	0.276 \pm 0.040*	45.0

RAW117-H10 cells (1×10^5 cells/0.2 ml/mouse) were injected into mice (7-week-old Balb/c female mice, $n=5$) via the portal vein with or without liposomes (1.25 mM as a peptide). After 7 days, liver weight was measured for evaluating experimental metastasis of RAW117-H10 cells in the liver. Components of liposomes were DSPC:cholesterol=2:1 for control liposomes and DSPC:cholesterol:peptide=4:2:1 for WHW peptide-modified liposomes.

^a0.2 ml of medium alone was injected into mice as a control.

*Significantly different from the liver weight of mice injected with RAW117-H10 cells alone ($P<0.05$), calculated by Student's *t*-test.

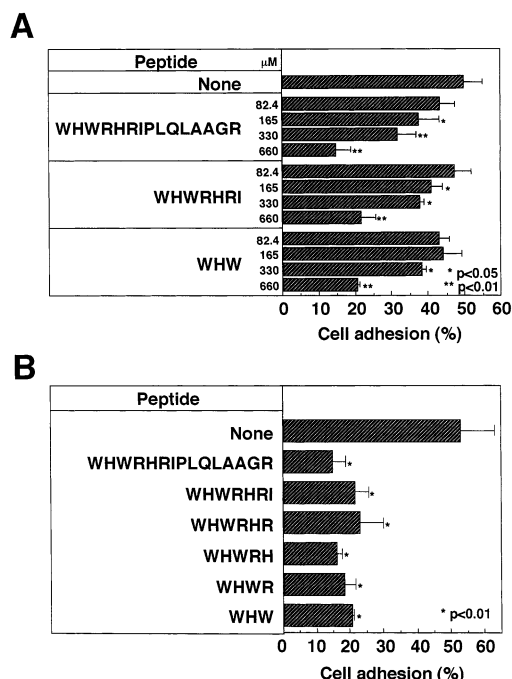


Fig. 2. Identification of a minimal sequence of the GD1 α -replica peptide for inhibition of cell adhesion. Adhesion of RAW117-H10 cells to HSE cells was analyzed in the presence or absence of the indicated concentrations of peptides (μ M) (A), as described in Fig. 1. Various peptides were used at a concentration of 660 μ M (B). The data represent the mean \pm S.D. ($n=4$). Asterisks indicate significant differences (A, * $P<0.05$, ** $P<0.01$; B, * $P<0.01$) calculated by Student's t test.

LAAGR and its alanine-substituted peptide AAARHRIPLQLAAGR were compared for inhibitory effects on cell adhesion. Fig. 1A shows that addition of WHWRHRIPLQLAAGR inhibited adhesion of RAW117-H10 cells to HSE cells, consistent with our recent observations [11]. In contrast, the addition of AAARHRIPLQLAAGR revealed no effect on adhesion of RAW117-H10 cells to HSE cells. Although the extent of adhesion of parental RAW117-P cells to HSE cells was less than 30%, no inhibition of the adhesion was observed by the addition of WHWRHRIPLQLAAGR or AAARHRIPLQLAAGR (Fig. 1B). These results suggest that GD1 α -mediated adhesion of RAW117-H10 cells to HSE cells is specifically blocked by WHWRHRIPLQLAAGR, and that the WHW domain plays a critical role in the cell adhesion.

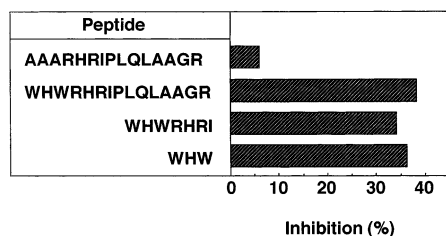


Fig. 3. Effect of WHW peptides on the binding of anti-GD1 α mAb to GD1 α . Anti-GD1 α mAb was added to ELISA plates coated with GD1 α in the presence or absence of various peptides (1.32 mM), and incubated for 2 h at 4°C. The binding of anti-GD1 α mAb to GD1 α was measured by ELISA using HRP-conjugated secondary Ab, and expressed as the values (means (%)) ($n=2$) relative to the reactivity in the absence of peptides.

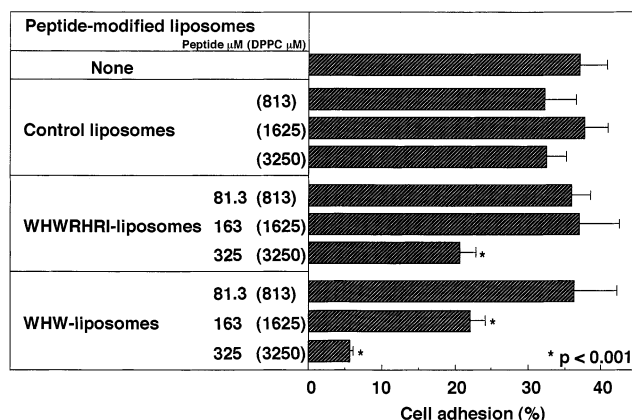


Fig. 4. Effect of GD1 α -replica peptide-attached liposomes on adhesion of RAW117-H10 cells to HSE cells. Adhesion of RAW117-H10 cells to HSE cells was analyzed in the presence or absence of peptide-attached liposomes, as described in Fig. 1. Peptide-attached liposomes (DPPC:cholesterol:peptide=10:5:1) were used at the indicated concentrations of peptides present in liposomes (μ M). Control liposomes (DPPC:cholesterol=2:1) were prepared without peptides. Concentrations of liposomes expressed as DPPC concentrations (μ M) were given in parentheses. The data represent the mean \pm S.D. ($n=4$). Asterisks indicate significant difference (* $P<0.001$) calculated by Student's t -test.

To dissect the role of the WHW domain in GD1 α -mediated cell adhesion, we created various deletion mutants of WHWRHRIPLQLAAGR. As shown in Fig. 2, WHW peptide per se as a minimal sequence exerted strong inhibitory activity toward adhesion of RAW117-H10 cells to HSE cells. To substantiate the binding of the WHW domain to mAb KA17 directed against GD1 α , we performed a competitive binding assay by measuring the binding of mAb KA17 to GD1 α -coated plates in the presence of various peptides. Fig. 3 shows that WHW peptide as well as WHW-containing peptides inhibited the binding of mAb KA17 to GD1 α , suggesting that the WHW domain is a minimal sequence mimicking the GD1 α epitope recognized by mAb KA17.

Liposomes coated with high densities of peptides are useful for drug delivery to target organs that express receptors for the peptides. We therefore tested whether WHW-containing peptides covalently attached to liposomes could efficiently inhibit adhesion of RAW117-H10 cells to HSE cells that express hypothetical GD1 α receptors. As shown in Fig. 4, the specific activity of WHW-containing peptides for inhibition increased approximately two-fold in case of liposomalization of the peptides (compare with the results using soluble peptides in Fig. 2). The inhibitory activity of liposomes modified with trimer peptides was stronger than those modified with 7-mer peptides, since the trimer peptide derivative was incorporated in a lipid bilayer more stable due to the hydrophilic-lipophilic balance than the 7-mer one. Thus the following experiment was performed by using palmitoyl-WHW, which was stably incorporated into the lipid bilayer of liposomes.

To ascertain whether WHW-attached liposomes were effective for the inhibition of adhesion of RAW117-H10 cells to HSE cells in vivo, we performed experimental metastasis of RAW117-H10 cells in the liver. Injection of RAW117-H10 cells into mice via the portal vein brought about increases in liver weight (approximately 158%) after 7 days (Table 1). Metastasis of RAW117-H10 cells to the liver was confirmed by pathological examination and the extent of the metastasis

was evaluated by an increase in liver weight. Intriguingly, the experimental liver metastasis was drastically inhibited by the addition of WHW peptide-attached liposomes. In contrast, the addition of soluble WHW peptide did not affect the experimental liver metastasis, presumably because of short in vivo half-life of this tripeptide (data not shown). These results suggest that WHW peptide-attached liposomes competitively block the binding of GD1 α expressed on RAW117-H10 cells to hypothetical GD1 α receptors present on hepatic sinusoidal endothelial cells. This event could lead to the inhibition of extravasation of RAW117-H10 cells from liver blood vessels.

Attachment of peptides to the surface of liposomes can enhance their stability in vivo and confer high availability for binding to their receptors, because proper orientation and well-controlled density of peptides on the liposome surface are easily achieved. In the present study, we identified WHW peptide as a minimal sequence of the GD1 α -replica peptide and showed that the addition of WHW peptide-attached liposomes suppressed GD1 α -mediated experimental metastasis of RAW117-H10 cells in the liver. Given that the size of WHW peptide is small, we imagine that GD1 α receptors can recognize WHW peptide as a haptenic molecule.

Use of phage-displayed random peptide libraries enables us to search for new peptide sequences targeting selective organs, tissues and cells in vivo [20,21]. Identification of minimal peptide sequences that actually function can prevent side effects which might stem from full-length peptides. Liposome-based drug delivery systems utilizing ligand-mimicking haptenic peptides will be useful for efficient inhibition of ligand-receptor interactions in cell adhesion such as tumor metastasis.

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