II　研究成果に関する一覧表

研究成果の刊行に関する一覧表

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III　研究成果の刊行物・別冊

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ORIGINAL PAPER

Gene transfer by DNA/mannosylated chitosan complexes into mouse peritoneal macrophages

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Abstract Chitosan is a biodegradable and biocompatible polymer and is useful as a non-viral vector for gene delivery. In order to deliver pDNA/chitosan complex into macrophages expressing a mannose receptor, mannose-modified chitosan (man-chitosan) was employed. The cellular uptake of pDNA/man-chitosan complexes through mannose recognition was then observed. The pDNA/man-chitosan complexes showed no significant cytotoxicity in mouse peritoneal macrophages, while pDNA/man-PEI complexes showed strong cytotoxicity. The pDNA/man-chitosan complexes showed much higher transfection efficiency than pDNA/chitosan complexes in mouse peritoneal macrophages. Observation with a confocal laser microscope suggested differences in the cellular uptake mechanism between pDNA/chitosan complexes and pDNA/man-chitosan complexes. Mannose receptor-mediated gene transfer thus enhances the transfection efficiency of pDNA/chitosan complexes.

Keywords Mannose · Chitosan · Gene transfer · Receptor-mediated endocytosis · Macrophage · Plasmid DNA

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Introduction

Macrophages play an important role as effector cells in inflammation and antigen presentation. Macrophages accumulate at pathological sites, including tumors, atherosclerotic plaques, arthritic joints and sites of infection (Kelly et al. 1988). Activated macrophages can release cytokines such as interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α) and reactive oxygen intermediates to defend against microbial infection and lyse tumor cells (Higuchi et al. 1990). Therefore, gene transfer to macrophages can be applied to not only genetic disease, but also to DNA vaccination and cancer therapy (Griffiths et al. 2000).

Recently, several non-viral gene delivery systems have been developed. Chitosan is a naturally occurring polysaccharide consisting of D-glucosamine and *N*-acetyl-D-glucosamine linked by a $\beta(1 \rightarrow 4)$-glycosidic bond. It has the potential to condense anionic DNA to a compact structure

through electrostatic interaction. Since chitosan has been proved to be non-toxic, biodegradable and biocompatible (Hirano et al. 1988), chitosan is considered to be a promising DNA carrier. DNA/chitosan complexes have shown high transfection efficiency in several cell lines (Sato et al. 1996; Thanou et al. 2002; Corsi et al. 2003). However, they did not show uptake into white blood cells (Sato et al. 2001) and, to our knowledge, transfection of DNA/chitosan complexes to macrophages has not been reported yet.

Receptor-mediated endocytosis offers the potential to target specific cells and enhances their uptake. Active targeting using receptor-mediated interaction has been effective in gene delivery (Varga et al. 2000). For example, macrophages express a mannose receptor that is used for endocytosis and phagocytosis of a variety of antigens (Stahl et al. 1980). Mannose-mediated drug delivery systems have often been employed to target macrophages (Sato and Sunamoto 1992). For gene delivery systems, mannosylated PEI, PLL and liposomes have been employed to deliver genes into dendritic cells and macrophages (Ferkol et al. 1996; Kawakami et al. 2000). However, PEI has strong cytotoxicity (Moghimi et al. 2005), and PLL requires chloroquine to induce transfection (Midoux et al. 1993). In this study we have employed mannosylated chitosan as a gene carrier with high transfection efficiency and low cytotoxicity, and the uptake of pDNA/man-chitosan complexes and their transfection efficiency in mouse peritoneal macrophages was investigated.

Materials and methods

Preparation of mannose-modified (man-chitosan)

Chitosan (average molecular weight, 53 kDa; degree of deacetylation, 93%) was obtained from Koyo Chemical Co. Ltd. Mannosylated chitosan (man-chitosan) was synthesized according to the literature (Holme and Hall 1992). Briefly, after allyl mannoside was reductively ozonolyzed to provide formylmethyl mannoside, chitosan dissolved in 1% aqueous acetic acid was coupled with formylmethyl mannoside by reductive alkylation in the presence of sodium cyanoborohydride. The degree of substitution (DS) was calculated to be 5 and 21% from elementary analysis. Calc. for 5% man-chitosan ($[(C_8H_{13}NO_5)_{0.03}(C_6H_{11}NO_4)_{0.92}(C_{14}H_{25}NO_{10})_{0.05}]\,1\,H_2O$): C, 40.82; H, 7.25; N, 7.28. Found: C, 42.8; H, 7.597; N, 7.422. Calc. for 21% man-chitosan ($[(C_8H_{13}NO_5)_{0.03}(C_6H_{11}NO_4)_{0.76}(C_{14}H_{25}NO_{10})_{0.21}]\,1\,H_2O$): C, 41.67; H, 7.19; N, 6.21. Found: C, 43.98; H, 7.59; N, 6.024.

Plasmid DNA

Plasmid DNA (pDNA), pGL3-Luc, encoding luciferase (Promega) was amplified in *E. coli* DH5 and isolated with a Qiagen Endotoxin-free Plasmid Giga Kit (Qiagen Inc.) according to the instruction manual provided. pGL-3Luc was labeled with YOYO-1 (Molecular Probes Inc.) for the evaluation of cellular uptake (Ogris et al. 2000). Briefly, 10 μl of pGL-3Luc (1 mg/ml) was mixed with 10 μl 10 μM YOYO-1 and incubated at room temperature for 1 h in the dark.

Mouse peritoneal macrophages

Female ICR mice were purchased from Japan SLC Co. The mice used in the study were 6–8 weeks old, and were housed under pathogen-free conditions in the animal facility of the Center for Disease Biology and Integrative Medicine, the University of Tokyo. Elicited macrophages were harvested from the peritoneal cavity of female ICR mice 3 days after intraperitoneal injection of 2 ml 3% (w/v) thioglycolate medium (Nissui Pharmaceutical). Cells were washed and then suspended in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, JRH). The cells were plated on either a 24- or 96-well culture plate at a density of 3×10^5 cells/cm^2. After incubation for 2 h at 37°C in 5% CO_2-95% air, non-adherent cells were washed away with PBS (−) and the residual adherent was cultured under the same conditions for another 24 h.

Preparation of pDNA/chitosan complexes

pDNA/chitosan complexes were prepared as in the method previously reported (Sato et al. 2001). pDNA solution (0.1 mg/ml water) was mixed with a given amount of man-chitosan solution (0.3 mg/ml) in PBS (−) adjusted to pH 6.5. The mixture of pDNA and man-chitosan was incubated at room temperature for 15 min. The YOYO-1-labeled pDNA described above was used for the evaluation of cellular uptake. The ratio of the plasmid phosphate anion to the chitosan amino groups (N/P) was 5.

Measurement of zeta potential and particle size

The concentration of pDNA was adjusted to 1.5 μg/ml by diluting with 20 mM HEPES buffer (pH 6.5). Zeta potentials and particle sizes of pDNA complexes were measured with DELSA 440 (Beckman Coulter) at 25°C.

Cellular uptake

Mouse peritoneal macrophages were seeded at 1×10^5 cells/well in 96-well plates 24 h prior to transfection. pDNA complexes containing 1 μg YOYO-1-labeled pDNA were incubated with cells in RPMI1640 adjusted to pH 6.5 containing 10% (v/v) FBS at 37°C under a 5% CO2 humidified atmosphere for 1 h. The culture medium was replaced with PBS (−) after rinsing with PBS (−) three times. The cells were then measured for their fluorescence intensity using a fluorescence microplate reader (Safire, TECAN) at excitation and emission wavelengths of 490 and 520 nm, respectively. For competition studies, the cells were treated with pDNA/man-chitosan complexes in the presence of 72 μg/ml mannan.

Luciferase assay

Mouse peritoneal macrophages were seeded at 4×10^5 cells/well in 24-well plates 24 h prior to transfection. pDNA complexes containing 5 μg of pDNA were incubated with cells in RPMI1640 (pH 6.5) containing 10% (v/v) FBS at 37°C under a 5% CO_2 humidified atmosphere for 4 h. The medium was replaced with fresh complete medium and the cells were further incubated for 20 h before assays. Transfection with Lipofectin® (Gibco BRL) and mannosylated PEI (in vivo-jet-PEI-Man, Polyplus transfection) was carried out according to the instruction manuals. The transfected cells were washed three times with PBS (−) and lysed with cell lysate buffer (Promega). Luciferase gene expression was measured using a luciferase assay kit (Promega). The protein concentrations were determined with a DC protein assay kit (BIO-RAD). The relative light units (RLU) were measured with a luminometer and were corrected for by the protein concentration.

Confocal laser scanning microscopy

Cells were seeded at 2×10^5 cells/well in glass-bottomed dishes (diam. 35 mm), and incubated with pDNA/chitosan complexes for 4 h. The medium was replaced followed by washing with PBS (−). The subcellular distribution of YOYO-1-labeled pDNA/chitosan complexes was analyzed with a confocal laser-scanning microscope (TCS-NT, Leica) equipped with a heating stage (Temp-Control 37-2, Leica) and a Kr/Ar laser.

Cell viability

Mouse peritoneal macrophages adhered to a 96-well microplate were incubated with pDNA complexes for 24 h. Ten μl of WST-1 dye solution (10 mM WST-1 and 0.2 mM 1-methoxy PMS (Dojindo, Laboratories) per well was added to the culture medium. After 2 h, absorbance at 450 nm with a reference wavelength of 690 nm was measured using a microplate reader (Multiskan, Labsystem).

Results

The particle size and zeta potential of pDNA/chitosan, pDNA/5% man-chitosan and pDNA/21% man-chitosan complexes was about 300 nm and +15 mV, respectively (Table 1). The modification of mannose to chitosan with substitution degrees of 5% and 21% did not affect the size and zeta potential of pDNA/man-chitosan complexes.

Table 1 Particle size and zeta potential of pDNA/chitosan complexes

pDNA complex	Size	Zeta potential
Chitosan	316 ± 33	+17.4 ± 1.6
5% man-chitosan	205 ± 50	+14.1 ± 1.5
21% man-chitosan	334 ± 50	+15.5 ± 0.9

The carobohydrate recognition of pDNA/man-chitosan complexes was determined by agglutination induced by concanavalin A (ConA). With the addition of ConA, the turbidity of the pDNA/man-chitosan complexes increased significantly at 350 nm, while the pDNA/chitosan complex showed no change in turbidity (Fig. 1). Recognition of the pDNA/man-chitosan complexes depended on the substitution degree of mannose residues in chitosan. The increase in turbidity of the pDNA/21% man-chitosan complex was twice that of the pDNA/5% man-chitosan complex. Agglutination of the pDNA/man-chitosan complexes was reversible by adding 10 mM mannose. These results indicated that the mannose moieties of the pDNA/man-chitosan complexes were specifically recognized by ConA.

The interaction of pDNA/man-chitosan complexes with mouse peritoneal macrophages was investigated using YOYO-1-labeled pDNA. The amount of pDNA/man-chitosan complexes taken up into macrophages was almost the same as pDNA/chitosan complexes (Fig. 2). In the presence of mannan as an inhibitor, the amount of cellular uptake of pDNA/man-chitosan complexes was significantly decreased, while that of pDNA/chitosan complexes was not.

The transfection efficiency of pDNA/man-chitosan complexes was investigated by luciferase assay in macrophages. The transfection efficiency of both pDNA/5% man-chitosan and pDNA/21% man-chitosan complexes was significantly higher than that of pDNA/chitosan complexes, and was comparable to pDNA complexed with mannosylated PEI, a commercially available transfection reagent (Fig. 3A). The transfection activity of pDNA/man-chitosan complexes was also investigated in COS7 cells (Fig. 3B). The transfection efficiency of pDNA/chitosan complexes was almost the same as pDNA/5% man-chitosan complexes, and that of pDNA/21% man-chitosan complexes was about a quarter of pDNA/chitosan complexes.

The subcellular distribution of pDNA/man-chitosan complexes in mouse peritoneal macrophages was visualized by a confocal laser scanning

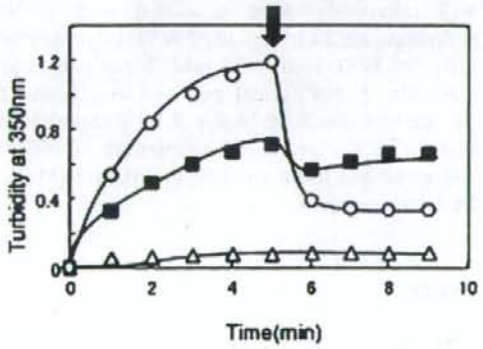

Fig. 1 Time courses of turbidity changes of pDNA complexes at 350 nm by the addition of ConA. pDNA = 25 μg/ml, ConA = 0.5 mM. Mannose (final concentration 10 mM) was added at the time marked by the arrow. Open triangle: pDNA/chitosan complex, filled square: pDNA/5% man-chitosan complex, open circle: pDNA/21% man-chitosan complex

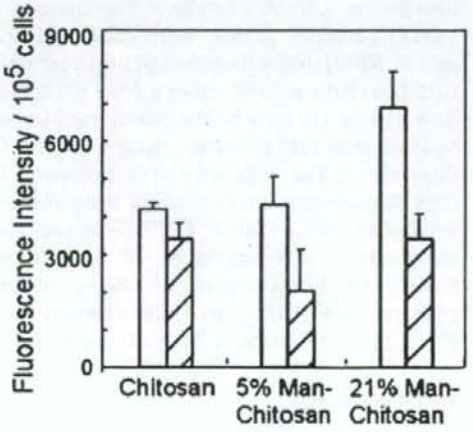

Fig. 2 Cellular uptake of pDNA complexes in mouse peritoneal macrophages. Macrophages (10^5 cells/well) were incubated with pDNA complexes (DNA = 10 μg/ml) in the absence (white bar) or presence (hatched bar) of mannan. After incubation for 1 h, the cells were washed and analyzed for fluorescence intensity. The values represent the mean ± SD, $n = 4$

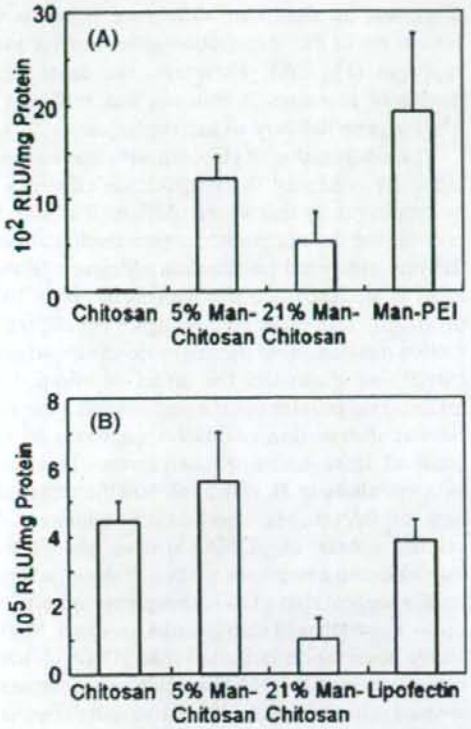

Fig. 3 Transfection efficiency of pDNA complexes for (**A**) mouse peritoneal macrophages and (**B**) COS7 cells. The concentrations of pDNA were 5 μg/ml for COS7 cells and 10 μg/ml for macrophages. The cells were incubated for 4 h with the pDNA complexes. The cells were lysed and their luciferase activity measured. pDNA complexes were prepared at N/P = 5

microscope. pDNA/chitosan complexes were mainly distributed near cell surface after 4 h incubation (Fig. 4A). On the other hand, pDNA/man-chitosan complexes were largely taken up into macrophages (Fig. 4B, C).

The influence of pDNA/chitosan and pDNA/man-chitosan complexes on the cell viability of mouse peritoneal macrophages was investigated by MTT assay. As shown in Fig. 5, cell viability was reduced by increasing the concentration of pDNA/chitosan complexes, while pDNA/man-chitosan complexes showed no cytotoxicity below 10 μg/ml of DNA concentration. The commercially available mannosylated PEI showed strong cytotoxicity depending on the concentration.

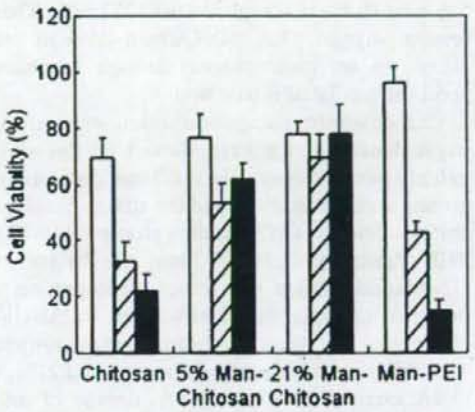

Fig. 5 Cytotoxicity of pDNA/chitosan complexes and pDNA/man-chitosan complexes in mouse peritoneal macrophages. The concentrations of pDNA were 1 μg/ml (white bar), 5 μg/ml (hatched bar), and 10 μg/ml (black bar). The cells were incubated with pDNA complexes for 24 h

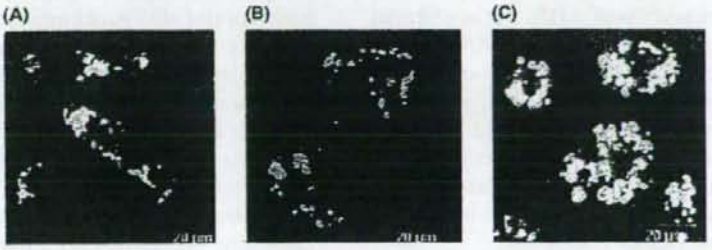

Fig. 4 The subcellular distribution of pDNA/chitosan complexes and pDNA/man-chitosan complexes in mouse peritoneal macrophages. (**A**) pDNA/chitosan complex, (**B**) pDNA/5% man-chitosan complex, and (**C**) pDNA/21% man-chitosan complex. The cells were incubated with pDNA complexes for 4 h

Discussion

Macrophages expressing a mannose receptor has been utilized for several target-specific drug delivery systems. In this study, we showed that mannosylated chitosan is a good gene carrier with high transfection efficiency and low cytotoxicity in macrophages. The substitution degree of mannose in chitosan was sufficient at 5% to enhance the transfection efficiency to macrophages. The cellular uptake of pDNA/man-chitosan complexes by macrophages was inhibited by mannan, while the transfection efficiency of pDNA/chitosan complexes was almost the same as the pDNA/5% man-chitosan complexes in COS7 cells. These results suggest that pDNA/man-chitosan was taken up by macrophages through mannose–receptor mediated interaction.

Carbohydrate recognition often depends on sugar density and is strengthened by the multivalent effect; however, the modification of amino groups in chitosan decreased the charge density of chitosan and the DNA-binding strength as well as PEI (Zanta et al. 1997). Thus, the balance of DNA condensation and receptor-recognition by modified chitosan would affect cell transfection efficiency. We then synthesized man-chitosans with different substitution degrees, 5 and 21%, as DNA carriers. The substitution degree of mannose in chitosan did not affect the size and zeta potential of pDNA complexes (Table 1). When pDNA complexes were incubated with DNase I under the condition that naked pDNA was digested completely, pDNAs complexed with chitosan, 5% man-chitosan and 21% man-chitosan showed resistance to DNase I (data not shown). We also examined DNase digestion of pDNA complexed with 47% man-chitosan. Although the formation of the pDNA/47% man-chitosan complex was confirmed by agarose gel electrophoresis, pDNA complexed with 47% man-chitosan was completely digested by DNase I (data not shown). Therefore, it was confirmed that low substitution degrees (5 and 21%) of mannose in chitosan were preferable for the formation of stable pDNA complexes.

The pDNA/21% man-chitosan complex was taken up in macrophages with the same efficiency as the pDNA/5% man-chitosan complex, and there was no significant difference between the complexes in the transfection efficiency for macrophages (Fig. 3A). Therefore, the substitution degree of mannose in chitosan was sufficient at 5% for gene delivery to macrophages.

The modification of chitosan with mannose significantly enhanced the transfection efficiency in macrophages in this study. Although it was reported that the mannose receptor-mediated gene delivery enhanced transfection efficiency (Kawakami et al. 2000), the mechanism has been little investigated. In order to investigate the cell transfection mechanism of the mannose-mediated gene carrier, we evaluated the effect of endocytosis inhibitor on cellular uptake and observed the subcellular distribution of pDNA comlexes with a confocal laser scanning microscope. Treatment with cytochalasin B, which inhibits the accumulation of microtubes, significantly inhibited the cellular uptake of pDNA/chitosan and pDNA/man-chitosan complexes (data not shown). These results suggest that pDNA complexes were taken up by macrophages through phagocytosis. Microscopic observation indicated that pDNA/chitosan complexes and pDNA/man-chitosan complexes showed different intracellular transport in macrophages (Fig. 4); while pDNA/chitosan complexes were localized in early phogosomes near the plasma membrane, pDNA/man-chitosan complexes were delivered inside the cells.

Phagosome movement in macrophages is modulated by several receptors associated with cell uptake (Aderem and Underhill 1999). Therefore, it is considered that the phagocytosis of pDNA/man-chitosan complexes is distinct from that of pDNA/chitosan complexes. Since pDNA/chitosan complexes stayed near the plasma membrane, interaction of the pDNA/chitosan complexes with cell surface receptor may induce signal transduction to arrest phagosome movement. On the other hand, bacteria such as *M. tuberculosis* with lipoarabinomannan on the surface, were efficiently internalized via the mannose receptor (Kag and Schlesinger 1998). Likewise, it is considered that phagosome movement of pDNA/man-chitosan complexes occurred in macrophages. pDNA/man-chitosan complexes showed little cytotoxicity against mouse peritoneal macrophages, while pDNA/chitosan complexes exerted a toxic effect

on the proliferation of macrophage cells in a dose-dependent manner as well as pDNA/man-PEI complexes (Fig. 5). The cytotoxicity of pDNA/PEI complexes has been reported in several papers (Köping-Höggård et al. 2001; Moghimi et al. 2005), while that of pDNA/chitosan complexes is known to be low compared to other complexes (Thanou et al. 2002; Corsi et al. 2003). pDNA/chitosan complexes also showed little cytotoxicity for COS7 cells employed in this study (data not shown). Thus, their cytotoxicity may be confined to macrophages.

The mannosylation of chitosan decreased the cytotoxicity of pDNA/chitosan complexes. The hydrophilic surface of pDNA/man-chitosan complexes suppresses the formation of aggregation followed by non-specific adhesion to the cell surface. The low cytotoxicity of pDNA/man-chitosan complexes may be caused by the hydrophilicity of its surface.

Conclusion

In conclusion, we found that mannose-modified chitosan is a good gene carrier for macrophages. The modification of mannose to chitosan significantly enhanced the transfection efficiency of the pDNA/chitosan complex and reduced its cytotoxicity in macrophages.

Acknowledgements This work was partly supported by the Special Coordination of Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government (T.S.).

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Apparent suppression of MMP-9 activity by GD1a as determined by gelatin zymography

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Abstract

Gelatin zymography is widely used to detect and evaluate matrix metalloproteinase-9 (MMP-9) activity. MMP-9 transcription was previously shown to be negatively regulated by ganglioside GD1a. [D. Hu, Z. Man, T. Xuan, P. Wang, T. Takaku, S. Hyuga, X.S. Yao, T. Sato, S. Yamagata, T. Yamagata, Ganglioside GD1a regulation of matrix metalloproteinase-9 (MMP-9) expression in mouse FBJ cell Lines: GD1a suppression of MMP-9 expression stimulated by PI3K-Akt and p38 though not by the Erk signaling pathway, 2006, submitted for publication.] Zymography of MMP-9 of FBJ-M5 cells preincubated with GD1a indicated a greater decrease in activity than expected from mRNA suppression. Incubation of conditioned medium containing MMP-9 with GD1a caused MMP-9 activity to decrease. Examination was thus made to confirm that MMP-9 activity is actually suppressed and/or MMP-9 protein undergoes degradation by GD1a. GD1a was found to have no effect on MMP-9 activity and Western blots indicated GD1a not to diminish MMP-9 during electrophoresis under reducing conditions. GD1a appeared to mediate the binding of a portion of MMP-9 with certain molecules, with consequently greater molecular mass on the gel, to cause decrease in the activity of MMP-9 at the site where it would normally appear. Caution should be used in doing gelatin zymography since molecules other than GD1a may similarly work, causing decrease in MMP-9 activity in zymography.

Keywords: Ganglioside; GD1a; Zymography; Metastasis; MMP-9; MMP-2

During the course of our study on ganglioside GD1a regulation of metastasis of mouse osteosarcoma-derived FBJ-LL cells, a matrix metalloproteinase-9 (MMP-9) but not MMP-2 mRNA expression was found to be down-regulated by GD1a [5]. MMP-9 and MMP-2 are implicated in metastatic potential of tumor cells [1–4]. MMP-9 was noted to be high in FBJ-LL with less GD1a, but suppressed in FBJ-S1 cells rich in GD1a [5]. The FBJ-LA5-30 cell, a GD1a-reexpressing FBJ-LL cell variant through β1-4GalNAcT-1 (GM2/GD2-synthase) cDNA transfection [6], showed less MMP-9 production, compared to a mock-transfectant M5 cell. GD1a regulation was confirmed by exogenous addition of GD1a to FBJ-M5 or FBJ-LL cells which displayed decreased production of MMP-9 in GD1a-dose- and time-dependent manners. Depletion of GD1a from cells such as FBJ-S1 and FBJ-5-30 by an inhibitor of glucosylceramide synthase D-PDMP or inhibition of sialyltransferase by siRNA targeting St3gal2 brought about increase in MMP-9 mRNA production. Assessment was subsequently made of MMP-9 activity by gelatin zymography using aliquots of culture medium subsequent to cell incubation with GD1a. mRNA production was suppressed by GD1a [5] and accordingly enzyme activity as determined by gelatin zymography should also be suppressed GD1a-dose and incubation time dependently. But the decrease in activity was actually more than expected. GD1a in the culture medium was kept in the aliquot to be assayed by gelatin zymography. There is thus

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the possibility that MMP-9 activity in the culture medium may have been suppressed by GD1a.

Though MMP-9 activity decreased when conditioned medium containing MMP-9 was pre-incubated with GD1a in the absence of cells, incubation of the gel with GD1a following electrophoresis of MMP-9 indicated GD1a to have no effect on MMP-9 activity in the gel, suggesting GD1a not to inhibit MMP-9 activity. From immunoblot analysis, following gel electrophoresis under native conditions, MMP-9 of the GD1a-treated conditioned medium was shown less compared to the control, this being consistent with zymographical results. Following the reducing PAGE, MMP-9 in the GD1a-treated conditioned medium was exactly as much as that in the control. Thorough scanning of the zymogram disclosed partial electrophoresis of MMP-9 as bands any of which had a molecular mass of more than 92 kDa. GD1a would thus appear to assist MMP-9 in its association with certain molecules in conditioned medium, so as to decrease the amount of MMP-9 at the site where it would normally appear. This was not seen to be the case with MMP-2. Caution should be used in doing gelatin zymography, since certain molecules other than GD1a may also function to decrease apparent MMP-9 activity in zymography.

Materials and methods

Cell lines and culture. The highly metastatic mouse osteosarcoma cell line, FBJ-LL, and poorly metastatic cell line, FBJ-S1, were produced from a FBJ virus-induced osteosarcoma of the BALB/c mouse [7]. FBJ-S1 cells expressed GM3 and GD1a, whereas FBJ-LL cells expressed GM3 and slightly expressed GD1a. LA5-30 cells were obtained by transfection of FBJ-LL cells with β1-4GalNAcT-1 (GM2/GD2-synthase), and mock-transfectant M5 cells, as control [6]. GD1a expression in LA5-30 cells was 5-fold that of M5. Migration capacity of LA5-30 cells was about one-tenth that of M5, comparable to the capacity of FBJ-S1 cells. When M5 cells were inoculated into mice, metastatic nodules were observed in liver, lung, kidney, and adrenal glands within 4–5 weeks, while LA5-22 cell transplantation did not show any sign of metastasis [6]. The cells were maintained in medium containing RPMI-1640 (Gibico, Invitrogen Corporation, NY, USA) supplemented with 10% fetal bovine serum (TBD-TianJin HaoYang Biological Company, TianJin, China), 100 U/ml penicillin, and 100 μg/ml streptomycin, and incubated in a humidified (37 °C, 5% CO_2, and 95% air) incubator (Sanyo, Tokyo, Japan). The cells were usually grown in a 60 mm culture dish (BD Falcon, CA, USA) and passaged on reaching 75% confluency. To see the effects of GD1a on cells, the cells were seeded, washed with serum-free RPMI-1640 at 24 h, and incubated with or without 50 μM GD1a in the absence of serum for the period of time indicated. In order to study the effects of GD1a on MMPs, cells were incubated in the absence of serum for 24 h, and the medium was collected and further incubated with or without 50 μM GD1a for the time indicated.

Chemicals and antibodies. Ganglioside GD1a from bovine brain was purchased from Wako (Osaka, Japan). The primers used in this study were designed by Primer3 software and synthesized by Invitrogen Shanghai. Rabbit anti-MMP-9 and anti-MMP-2 polyclonal antibodies were purchased from Chemicon. Horseradish peroxidase (HRP)-linked anti-rabbit IgG secondary antibody was from Cell Signaling (MA, USA).

RNA extraction and RT-PCR. About 1×10^6 cells were harvested and total RNA was extracted using the Qiagen RNeasy Kit according to instructions of manufacturer. One microgram of RNA, taken as indicated by absorption, was subjected to RT-PCR using the TaKaRa RT-PCR kit (AMV) Ver. 3.0 with a PC707 Program Temp Cont System (ASTEC, Japan). The product obtained was analyzed by 2% agarose electrophoresis. Following ethidium bromide staining (0.05% ethidium bromide in TAE buffer) for 30 min, the intensity of the stained band was assessed with a Bio-profile Bio 1D image analyzer (Vilber Lourmat, Marne-la-Vallee, France) at 312 nm. Primers were synthesized at the Invitrogen (Shanghai, China) and primer sequences used for PCR in this study were as follows: for β-actin, sense 5′-ACACTGTGTGCCCATCTACGAGG-3′ and antisense 5′-AGGGGCCGGACTCGTCGTCATACT-3′; for MMP-9, sense 5′-CTGACTACGATAAGGACGGCAA-3′ and antisense 5′-ATACTGG ATGCCGTCTATGTCG-3′; for MMP-2, sense 5′-ACCTGGATGCCGT CGTGGAC-3′ and antisense 5′-TGTGGCAGCACCAGGGCAGC -3′.

Western blotting. About 1×10^6 cells were lysed in 1 ml sample buffer (62.5 mM Tris–HCl, 2% SDS, 10% glycerine, 5% β-mercaptoethanol, and 0.03% bromophenol blue) and boiled at 100 °C for 5 min. An aliquot of the lysate was loaded onto a 10% SDS–polyacrylamide gel. Following electrophoresis, the gel was blotted and subjected to Western blotting. The blotted membrane was reacted with primary antibody at 1/2000 dilution, followed by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (1/3000 dilution). Western blots were visualized by ECL Western blotting detection reagents (Amersham Biosciences) so as to enhance chemiluminescence subsequently to be exposed to Fuji XR film. Lanes were scanned and the optical density was determined by the Bio-profile Bio 1D image analyzer.

Gelatin zymography. Gelatinase activity was determined according to the method previously described [7]. In brief, 8% polyacrylamide gel of 1 mm thickness containing 0.3 mg/ml gelatin was used and the proteins were separated by Lammli's buffer system. Cells were inoculated into 60 mm culture dish with 4 ml culture medium. After overnight culture medium was discarded, and cells were washed with RPMI-1640 containing no serum and further incubated with 50 μM GD1a for the time indicated in the medium without serum. At the indicated time, conditioned medium was obtained to use for the measurement of gelatinase activity. An aliquot of conditioned medium was mixed with the equal amount of Lammli's sample buffer with no reducing reagent and without heating applied to electrophoresis. The gel following electrophoresis was rinsed with 2.5% Triton X-100 for 1 h at room temperature followed by incubation in the reaction buffer (10 mM $CaCl_2$, 50 mM Tris–HCl, pH 7.4, and 0.02% NaN_3) for 16 h at 37 °C, fixed with 50% methanol–10% acetic acid for 30 min, stained with 0.02% CBB in 50% methanol–10% acetic acid for 1 h, and destained with 20% methanol–10% acetic acid until clear white bands appeared on the blue background. Gelatin zymography depicts MMPs as negatively stained bands that were scanned, and the optical density was determined using the Bio-profile Bio, ID image analyzer.

Results

Inverse regulation of MMP-9 by GD1a

MMP-9 transcription was found inversely regulated by GD1a content; MMP-9 (95 kDa activity) was low in FBJ-S1 cells rich in GD1a, while several times more in FBJ-LL having less GD1a (Fig. 1A). MMP-9 but not MMP-2 mRNA expression was seen to be down-regulated by GD1a. Fig. 1A shows the FBJ-LA5-30 cell, a GD1a-reexpressing FBJ-LL cell variant produced through β1-4GalNAcT-1 (GM2/GD2-synthase) cDNA transfection [6], to have caused decrease in MMP-9 production, compared to mock-transfectant M5. Gelatin zymography indicated MMP-9 activity to be inversely related to GD1a. MMP-9 activity was low in GD1a-rich FBJ-S1 cells, though by one order of magnitude higher in GD1a-less FBJ-LL cells (Fig. 1B). FBJ-LA5-30 cells expressing GD1a to the same extent as FBJ-S1 cells had far less