



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

Dan Hu<sup>a</sup>, Xuan Tan<sup>a</sup>, Toshinori Sato<sup>b</sup>, Sadako Yamagata<sup>a</sup>, Tatsuya Yamagata<sup>a,\*</sup>

<sup>a</sup> *Laboratory of Tumor Biology and Glycobiology, Shenyang Pharmaceutical University, Shenyang 110016, People's Republic of China*

<sup>b</sup> *Department of Biosciences and Informatics, Keio University, Hiyoshi, Yokohama 223-8522, Japan*

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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.

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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  $\beta$ 1-4GalNAcT-1 (GM2/GD2-synthase) cDNA transfection [6], showed less MMP-9 production, compared to a mock-transfectant M5 cell. GD1a regulation was con-

firmed 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

\* Corresponding author. Fax: +86 24 23986433.

E-mail address: [tcyamagata@cool.odn.ne.jp](mailto:tcyamagata@cool.odn.ne.jp) (T. Yamagata).

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  $\beta$ 1-4GalNAcT-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  $\mu$ g/ml streptomycin, and incubated in a humidified (37 °C, 5% CO<sub>2</sub>, 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  $\mu$ M 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  $\mu$ M 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  $1 \times 10^6$  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  $\beta$ -actin, sense 5'-ACACTGTGTGCCCCATCTACGAGG-3' and antisense 5'-AGGGGCCGGACTCGTCGTCATACT-3'; for MMP-9, sense 5'-CTGACTACGATAAGGACGGCAA-3' and antisense 5'-ATACTGGATGCCGTCTATGTCG-3'; for MMP-2, sense 5'-ACCTGGATGCCGTCTGTCGAC-3' and antisense 5'-TGTGGCAGCACCAGGGCAGC-3'.

**Western blotting.** About  $1 \times 10^6$  cells were lysed in 1 ml sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerine, 5%  $\beta$ -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 Lamml'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  $\mu$ M 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 Lamml'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<sub>2</sub>, 50 mM Tris-HCl, pH 7.4, and 0.02% NaN<sub>3</sub>) 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, 1D 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  $\beta$ 1-4GalNAcT-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

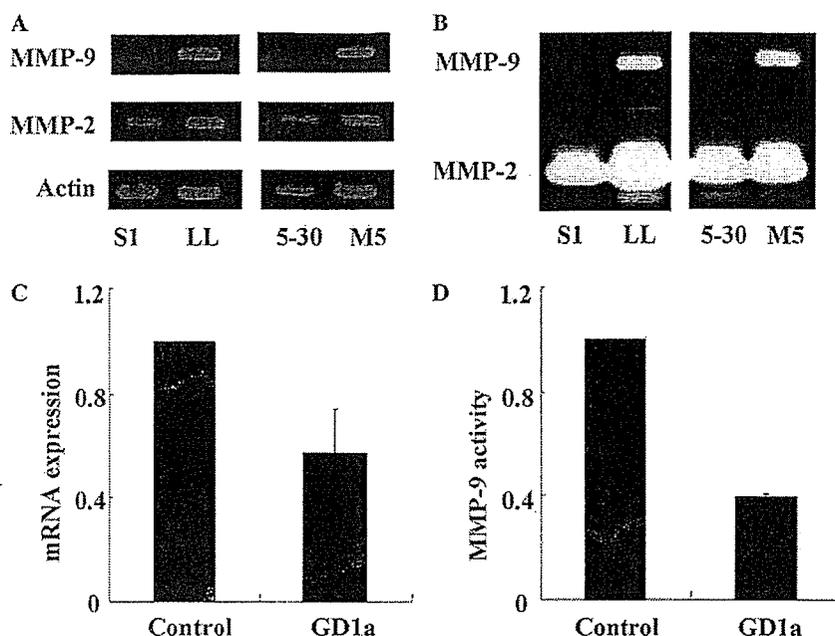


Fig. 1. Ganglioside GD1a negatively regulates metalloproteinase-9 (MMP-9) but not MMP-2. FBJ-S1, -LL, -M5, and -LA5-30 cells were cultured under standard conditions followed by determination of MMP-9 and -2 expression by RT-PCR (A). On aliquots of culture medium of FBJ-S1, -LL, -M5, and -LA5-30 cells gelatin zymography was carried out (B). For clarification of the effects of GD1a on MMP-9 expression, RNA was extracted from FBJ-M5 cells incubated with or without 50  $\mu$ M GD1a in serum-free medium for 12 h and examined for the expression of MMP-9 mRNA (C). Gelatin zymography was done using aliquots of culture medium (D). (C) RT-PCR measurement is normalized by the expression of  $\beta$ -actin in a way so that MMP-9 mRNA content divided by that of  $\beta$ -actin of the control is expressed as 1 with which mRNA expression of the cell treated with GD1a was compared. MMP-9 activity of the control is taken as 1 in (D). Independent experiments were conducted several times with results essentially in agreement. Typical results are shown here.

MMP-9 compared to the mock transfectant, FBJ-M5 cells. This situation was not observed for MMP-2.

GD1a regulation of MMP-9 was previously confirmed by treatment of FBJ-5-30 cells with D-PDMP, an inhibitor of glucosylceramide synthesis, or siRNA targeting St3gal2, which brought about increase in MMP-9 mRNA [5]. GD1a regulation of MMP-9 was further confirmed by exogenous addition of GD1a to M5 or LL cells, in both of which MMP-9 mRNA production was seen to decrease dose and time dependently. MMP-9 would thus appear negatively regulated by GD1a. Fig. 1C shows GD1a to suppress MMP-9 mRNA expression of FBJ-M5 cells and this suppression was reflected in MMP-9 activity as determined by gelatin zymography shown in Fig. 1D. The activity of MMP-9 (40%) of the cells incubated with GD1a was less than expected, judging from mRNA suppression (60%). But there was no effect of GD1a on MMP-2 mRNA or activity [5].

#### Possible degradation of MMP-9 by GD1a in conditioned medium

Examination was made as to whether GD1a suppresses not only MMP-9 gene transcription but also MMP-9 activity in culture medium. For this purpose, FBJ-M5 cells rich in MMP-9 were incubated in serum-free medium for 24 h to prepare conditioned medium containing MMP-9. Following centrifugation for cell removal, the conditioned

medium was incubated with 50  $\mu$ M or 100  $\mu$ M GD1a for 12 h. Each aliquot of incubation medium was assayed for MMP activity by gelatin zymography. As shown in Fig. 2A (FBJ-M5 cells) and B (FBJ-LL cells), MMP-9 activity decreased in conditioned medium with GD1a incubation, while MMP-9 activity of conditioned medium incubated without GD1a for 12 h was essentially the same as 0 time control (data not shown). MMP-2 activity remained unchanged throughout incubation. Fig. 2C shows MMP-9 activity in the medium incubated with GD1a to have decreased time dependently.

Decrease in MMP-9 activity may possibly have been due to its suppression or degradation during incubation in culture medium in the presence of GD1a. GD1a may bind to MMP-9 to suppress activity of the latter toward the gelatin substrate, to catalyze its degradation, or mediate some proteolytic enzyme for this degradation. To determine which of these is applicable, freshly obtained conditioned medium recovered from FBJ-M5 cells following 24 h in culture without GD1a was separated with a gelatin-containing gel and its SDS removed. The gel was then incubated in GD1a-contained reaction buffer. But as shown in Fig. 2D, enzyme activity on the gel was not affected by incubation with GD1a, thus ruling out any possibility that putative binding of GD1a to MMP-9 hinders the activity and that GD1a catalyzes MMP-9 degradation. Rather, GD1a would appear to assist and facilitate degradation of MMP-9 in conditioned medium.

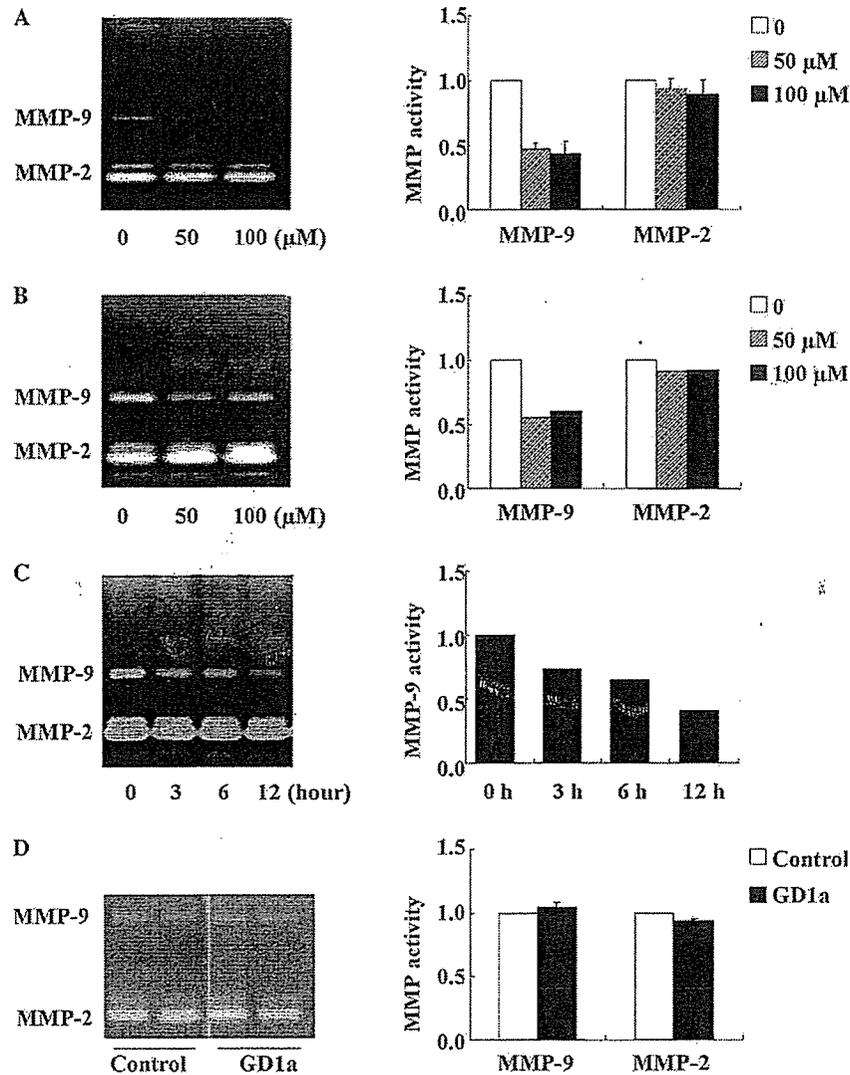


Fig. 2. GD1a in culture medium decreases MMP-9 but not MMP-2 activity. To examine the effects of GD1a on MMP-9, conditioned medium of FBJ-M5 (A) or FBJ-LL (B) cells incubated without GD1a and serum for 24 h was obtained and incubated with 50  $\mu\text{M}$  or 100  $\mu\text{M}$  GD1a for 12 h. Aliquots of the medium were subsequently assayed for MMPs. (C) Conditioned medium was incubated with 50  $\mu\text{M}$  GD1a for the time indicated and MMPs were determined by gelatin zymography. (D) Aliquots of conditioned medium were electrophoresed as in gelatin zymography and a portion of the gel containing MMPs was cut and rinsed in 2.5% Triton X-100 for 1 h at room temperature followed by incubation in reaction buffer containing 50  $\mu\text{M}$  GD1a for 16 h at 37  $^{\circ}\text{C}$ . The control gel was incubated with reaction buffer without GD1a. (A–C) Indicate experiments two times separately with similar results obtained. Typical zymograms appear on the left of the figure. In (D) the control and sample have been electrophoresed in duplicate.

#### Total MMP-9 not affected by GD1a despite apparently possible suppression

To find whether MMP-9 is degraded in conditioned medium with GD1a, MMP-9 protein was determined using an antibody. Gelatin zymography being much more sensitive than immunoblotting, culture medium of GD1a-treated and control cells was concentrated by about 20 times using an Amicon filtration tube (for MW 10,000). Aliquots of condensed medium (Fig. 3A) exhibited essentially the same activity as that of MMP-9 activity previously noted. Each aliquot was separated by Laemmli's electrophoresis (procedure including boiling for 5 min in the presence of 2-mercaptoethanol), electroblotted onto nitrocellulose membrane, and immunostained with anti-MMP-9 or anti-MMP-2 antibody. Fig. 3B shows Western blots of

MMP-9 and MMP-2 and, surprisingly, no differences in MMP-9 protein content for culture medium incubated with GD1a and the control. Any degradation of MMP-9 during MMP-9 incubation in culture medium appears not to have occurred through the action of GD1a. Gelatin zymography shown in Fig. 3A was performed under native conditions using SDS but not 2-mercaptoethanol, without boiling. Electrophoresis was thus conducted under the same (native) conditions as gelatin zymography followed by electroblotting (Fig. 3C). The density of MMP-9 immunoblotting with GD1a incubation was less than for the control, this being consistent with the results of gelatin zymography (Fig. 3A), indicating intact MMP-9 protein to be partially absent from its site. Careful examination of gelatin zymography of the GD1a-treated sample (Fig. 3A) disclosed faint activity bands, each with molecular mass of 120 kDa or

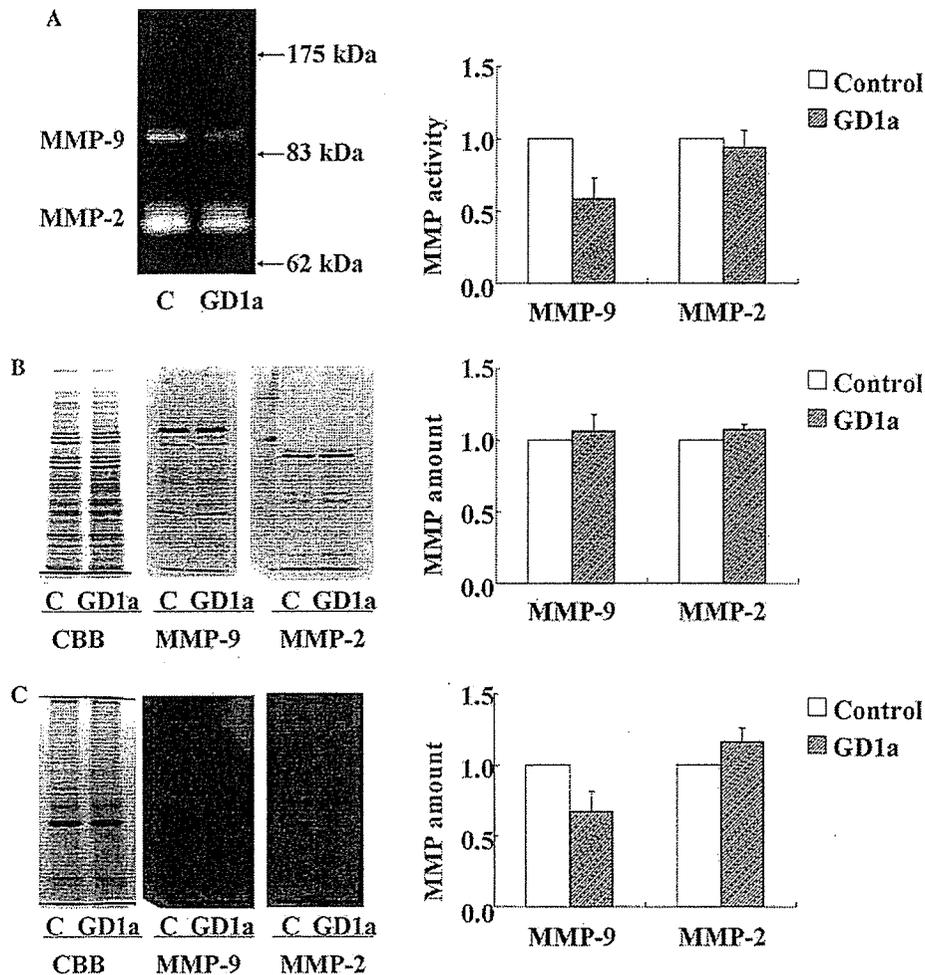


Fig. 3. MMP-9 activity is apparently suppressed by GD1a but total MMP-9 does not change. FBJ-M5 cells were incubated without GD1a to prepare conditioned medium which was subsequently incubated with or without 50  $\mu$ M GD1a for additional 6 h and then concentrated by 20 times using an Amicon filtration tube (for MW 10,000); its MMP activity was then determined (A). Molecular mass markers appear in the right lane of (A). Western blotting of MMP-9 and -2 was conducted subsequent to Laemmli's electrophoresis (procedure including boiling for 5 min in the presence of 2-mercaptoethanol) (B), or electrophoresis under native conditions as for gelatin zymography (C). Measurement of MMP activity and protein was each expressed as the mean value of two independent experiments. Typical results for gelatin zymography or Western blots appear on the left of the figures.

exceeding 190 kDa, possibly owing to the association of MMP-9 with some molecules present in culture medium in the presence of GD1a.

## Discussion

Due to its simplicity and sensitivity and lack of any better method, gelatin zymography is used for matrix metalloproteinase measurement in cells and tissue with or without drug application [8–10], and the term zymography often means gelatin zymography for assay of MMPs, though zymography has been used other than for the detection and measurement of MMPs, such as application to other enzymes [11–13].

MMP-9 and MMP-2 are secreted by cells and thus, for examination of its effects on MMPs, a drug is introduced into the culture medium. Aliquots to be assessed for effect on MMPs consequently contain the drug. Should MMP activity be shown suppressed on a gelatin

zymogram, the suppression may possibly be explained by the following: (1) The drug suppression of MMP mRNA expression with consequent reduction in enzyme protein content, (2) impairment of MMP protein synthesis, (3) inhibition of MMP secretion into the medium, (4) degradation of MMP secreted in the medium by GD1a itself or degradation mediated by GD1a, or (5) denaturing MMP in the medium via drug interaction during gelatin zymography. Any one or all these possibilities may be applicable.

GD1a administered to FBJ-M5 cells inhibited MMP-9 mRNA (Ref. [5] and Fig. 1C), reduction in enzyme activity (Fig. 1D), this possibly being a reflection of decrease in mRNA, shows at least possibility (1) to be applicable. MMP-9 activity (40%) of FBJ-M5 cells incubated with GD1a was less than expected, based on the degree of mRNA suppression (60%), so that GD1a would appear to suppress not only MMP-9 gene transcription but MMP-9 activity as well, in culture medium. Pure

MMP-9 being difficult to obtain, conditioned medium containing MMP-9 secreted by FBJ-M5 cells was used in this study to find whether additional incubation with GD1a would diminish enzyme activity in gelatin zymography.

Incubation of conditioned medium containing MMP-9 with GD1a was noted to decrease MMP-9 activity (Figs. 2A–C), suggesting (4) and/or (5) to be applicable. But incubation in GD1a-containing reaction buffer following electrophoresis of conditioned medium in the gel containing gelatin failed to diminish enzyme activity (Fig. 2D), indicating GD1a itself not to degrade MMP-9 or lessen MMP-9 activity. Still, GD1a may possibly mediate the degradation of MMP-9 by the enzyme in culture medium. Staining of MMP-9 with antibody following electrophoresis under reducing conditions (Fig. 3B) showed protein content not to change during conditioned medium incubation with GD1a. GD1a thus clearly does not mediate MMP-9 degradation in conditioned medium, and so (4) and (5) would not be applicable.

With electrophoresis of conditioned medium incubated with GD1a under native conditions, immunoblotting and staining with antibody, decrease in MMP-9 subsequent to GD1a treatment compared to the control (Fig. 3C) was essentially the same as that indicated on the gelatin zymogram (Fig. 3A), indicating MMP-9, once secreted into the culture medium, to be affected by GD1a in such a way as to cause MMP-9 to form a complex with GD1a or with other molecule(s) assisted by GD1a. The zymogram also showed several activity bands corresponding to positions of higher molecular mass (Fig. 3D). There thus should also be the possibility (6) that GD1a with micelles binds MMP-9 or GD1a assists MMP-9 to bind to some molecules in culture medium to form a higher molecular weight complex, thus decreasing apparent activity at the site where MMP-9 would normally appear. Human neutrophils have been shown to contain 25 kDa protein which, on binding to MMP-9, forms an activity band at 135 kDa in zymography [14]. Synovial fluid from patients with rheumatoid arthritis showed MMP-9 activity at 130 and 224 kDa as well as 92 kDa [15]. Analysis of mouse uterus indicated the presence of high molecular mass proteins that were immunoreactive toward the anti-MMP-9 antibody [16]. Pro-MMP-9 binds to TIMP-1 through a hemopexin domain and active MMP-9 binds to TIMP-1 and TIMP-2 [17,18], though either of which bound to proMMP-9 or MMP-9 is liberated in an electric field in gelatin zymography. MMP-9 has thus been shown to bind to other molecules, but in this study was noted to bind to certain molecules in culture medium in the presence of GD1a. The mechanism for this and nature of the bound molecules remain to be elucidated. The possibilities (2) and (3) have yet to be demonstrated, but it is beyond the scope of this work.

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# Hyaluronic acid and its derivative as a multi-functional gene expression enhancer: Protection from non-specific interactions, adhesion to targeted cells, and transcriptional activation

Tomoko Ito <sup>a</sup>, Naoko Iida-Tanaka <sup>b</sup>, Takuro Niidome <sup>c</sup>, Takahito Kawano <sup>c</sup>, Koji Kubo <sup>d</sup>, Kenichi Yoshikawa <sup>d</sup>, Toshinori Sato <sup>e</sup>, Zhihong Yang <sup>e</sup>, Yoshiyuki Koyama <sup>a,\*</sup>

<sup>a</sup> Department of Textile Science, Otsuma Women's University, Chiyoda-ku, Tokyo, Japan

<sup>b</sup> Department of Food Science, Otsuma Women's University, Tokyo, Japan

<sup>c</sup> Faculty of Engineering, Kyushu University, Fukuoka, Japan

<sup>d</sup> Department of Physics, Graduate School of Science, Kyoto University, Kyoto, Japan

<sup>e</sup> Faculty of Science and Technology, Keio University, Kanagawa, Japan

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## Abstract

Hyaluronic acid (HA), a natural anionic mucopolysaccharide, can be deposited onto the cationic surface of DNA/polyethyleneimine (PEI) complexes to recharge the surface potential and reduce nonspecific interactions with proteins. HA can also be used as a ligand to target specific cell receptors. Furthermore, HA-coating enhanced the transcriptional activity of the plasmid/PEI complexes, probably by loosening the tight binding between DNA and PEI, which facilitated the approach of transcription factors. Amphoteric HA derivative having spermine side chains (Spn-HA) with a structure similar to HMG protein showed higher transcription-enhancing activity than HA. Plasmid/PEI/Spn-HA ternary complex exhibited 29-fold higher transgene expression efficiency than naked plasmid/PEI complexes in CHO cells.

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**Keywords:** Transfection; Hyaluronic acid; Transcription; HMG

## 1. Introduction

A variety of cationic polymers or lipids, which can electrostatically bind to DNA, has been explored as synthetic non-viral vectors [1,2]. However, the surface of the DNA/polycation complex is usually positively charged. The activation of complements [3], coagulation of blood cells [4], and self-aggregation with serum proteins [5] have been major problems, especially for in vivo gene therapy. The positive charge on complexes should be shielded to obtain stable dispersion in blood. Ogris et al. reported that DNA complexes coated and shielded with a neutral polymer having active ester side chains showed enhanced systemic duration [6] and higher gene expression in the tumor [7]. We have developed a novel poly(ethylene glycol)

derivative with carboxylic acid side chains (PEG-C) as a self-assembling protective coat on DNA/polycation complexes [5], which can reduce nonspecific interactions with blood cells or serum proteins. Introduction of a ligand to PEG-C can also increase higher transgene expression in target cells [4,8].

PEG-C can be deposited onto the surface of plasmid/polycation complexes to form ternary structures without destroying them, while common polyanions such as heparin and polyacrylic acid will disrupt DNA/polycation condensation [8–10]. We have explored various kinds of anionic polymers in the search for other polyanions which can form ternary complexes, and found that natural hyaluronic acid (HA) is one of the few polyanions which can coat DNA/polycation complexes without disrupting their structures.

HA is an acidic mucopolysaccharide distributed widely in the extracellular matrix and found in the liquid portion of mammalian joints. It is a relatively low toxic polymer approved

\* Corresponding author. Tel.: +81 3 5275 6017; fax: +81 3 5275 6932.

E-mail address: [koyama@otsuma.ac.jp](mailto:koyama@otsuma.ac.jp) (Y. Koyama).

by the FDA for injections. Moreover, a receptor for HA, CD44, is known to be overexpressed on various tumor cell surfaces [11,12]. HA would thus work both as a protecting coat against blood components and as a ligand for targeted cells.

For efficient *in vivo* gene transfection, non-viral vectors need to overcome many barriers such as internalization into cells, escape from endosomes and trafficking into the nucleus. Low efficiency in transcription of DNA/polycation complexes in the nucleus also represents an obstacle for high gene expression. The polycation may interfere with transcription, since access of transcription factors to a DNA molecule can be hindered when DNA tightly binds to the polymer. As mentioned above, addition of HA to the DNA/polycation complexes will not decompose them nor release free DNA molecule. However, HA might loosen the DNA-polycation binding to some extent by interacting with the polycation, and the approach of transcription-factors to the swollen complex would be facilitated. In this study, we examined the possibility of HA for loosening DNA/polycation complexes and its effect on enhancing transcriptional activity.

In the mammalian cell nucleus, tightly condensed chromosomal DNA is uncoiled by an HMG (High Mobility Group) protein before transcription begins. HMG protein is a non-histone DNA-binding protein having both cationic DNA-binding domains and an anionic C-tail. The acidic region has been reported to be essential for transcriptional activation [13]. A specific interaction between the acidic region of HMGB1 and histone was reported [14], but such ampholytes having both basic and acidic groups may also have non-specific physical properties for loosening the tight interaction between DNA and cationic polymers. In order to examine the transcriptional activation potential of polyampholytes, an amphoteric HA derivative, Spn-HA, was synthesized to mimic the structure of HMG protein and investigated for its transcription-enhancing activity potential.

## 2. Materials and method

### 2.1. Materials

Linear PEI (MW 25,000) was obtained from Polyscience, Inc. Hyaluronic acid sodium salt (from Microorganism), spermine, and bovine serum albumin (BSA) were purchased from Nacalai Tesque, Inc., Wako Pure Chemical Industries, Ltd., and SIGMA Chemical Co, respectively. YOYO-1 iodide and uridine 5'-triphosphate P<sup>3</sup>-(5-sulfo-1-naphthylamide) tetra (triethylammonium) salt ( $\gamma$ -AmNS-UTP) were purchased from Molecular Probes, Inc. Plasmid DNA containing firefly luciferase gene and cytomegavirus promoter was amplified in *Escherichia coli*, and purified with a QIAGEN Plasmid Mega Kit. The *E. coli* RNA polymerase holoenzyme was purchased from EPICENTRE Technologies. ATP, CTP and GTP were obtained as lithium salts from Roche Diagnostics, Co.

### 2.2. Synthesis of Spn-HA

HA sodium salt (10 mg) was dissolved in 4 mL of water and mixed with spermine (50.9 mg). Water-soluble carbodiimide (WSC) (77.1 mg) was then added to the solution, which was

adjusted to pH 4.9. After standing at RT for 5 h, the solution was dialyzed for 4 days against running water and 3 more days in distilled deionized water. The solution was freeze-dried and a white spongy Spn-HA was obtained (Fig. 1), with a yield of 10.7 mg. Spn-HA tested positive with the ninhydrin test, while a mixture of HA and spermine similarly dialyzed gave a negative result. Elemental analysis revealed that 27.4% of carboxyl groups had reacted with spermine. This was in good accordance with <sup>1</sup>H NMR of the product taken in D<sub>2</sub>O. A 27.3% value was estimated from the integration of the resonance peaks at 1.1 ppm (–N–CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–N– in spermine residue) and 1.9 ppm (–NCOCH<sub>3</sub>). IR spectrum of the product showed more absorption at 1650 cm<sup>–1</sup> arising from the amide linkage compared to the original HA.

### 2.3. Fluorescence microscopic observation

Fluorescence microscopic observation was performed using an IX70 microscope (Olympus) equipped with a 100× oil-immersion objective lens and a high-sensitivity Hamamatsu SIT TV camera. DNA was visualized with the fluorescent dye, 4',6-diamidino-2-phenyl-indole (DAPI). Mercaptoethanol was added as an antioxidant. Final concentrations of DNA, DAPI and mercaptoethanol were 3  $\mu$ M (in base), 3  $\mu$ M, and 3% (v/v), respectively. Bovine serum albumin (BSA) was added to the DNA complex suspension as a 20 mg/mL aqueous solution (final concentration of 10 mg/mL).

### 2.4. Distribution of DNA complex in the mouse peritoneal cavity

The fluorescent dye YOYO-1 was used instead of DAPI to visualize DNA. DNA/PEI/HA (or Spn-HA) complex was prepared at 1:1:9.7 (w/w/w) in saline. One hundred  $\mu$ L was injected into the peritoneal cavity of each mouse. The final concentration of YOYO-1 and DNA in the injected solution was 50 and 250  $\mu$ M, respectively. After 30 min, the mice were sacrificed by ether asphyxiation and the abdominal cavity was exposed through a midline incision. The internal organs were illuminated with a UV lamp to observe DNA distribution in tissues.

### 2.5. $\zeta$ -potential and size measurement

$\zeta$ -potential and size of the complexes were measured with a particle analyzer (MALVERN Zetasizer Nano ZS). Typically, to an aqueous solution of plasmid DNA (1.25  $\mu$ g in 12.5  $\mu$ L) was added an equal volume of linear PEI (1.25  $\mu$ g in 12.5  $\mu$ L in quadruple condensed PBS) followed by addition of HA (12.25  $\mu$ g in 25  $\mu$ L in H<sub>2</sub>O). The mixture was then diluted with 950  $\mu$ L of PBS and its  $\zeta$ -potential and diameter were analyzed.

### 2.6. Transcription procedure

The complex suspension was prepared by mixing the aqueous solutions of plasmid (0.33  $\mu$ g in 3.3  $\mu$ L), linear PEI



### 3.2. Protection effect of HA and Spn-HA against BSA-induced aggregation

Anionic coating of the particles with HA or Spn-HA appears to protect the DNA/PEI complexes from undesirable interactions with serum proteins. Dispersion stability of the complexes prepared at plasmid:PEI:HA=1:1:9.7 (w/w/w) in the presence of albumin was examined by fluorescence microscopic observation. DNA was visualized with DAPI and mixed with PEI. Globular DNA/PEI binary complexes (1:1, w/w) were observed as small fluctuating particles. Addition of HA to the solution did not cause visible changes in particle size or movement. When BSA was added to the plasmid/PEI binary complexes (final concentration of 10 mg/mL), the complexes aggregated and precipitated. On the other hand, the ternary complexes coated with HA or Spn-HA retained Brownian motion up to one-hour post-addition of BSA (Fig. 2).

Change in complex size before and after BSA addition was measured with a particle analyzer. The size of DNA/PEI binary complexes in PBS was approximately 230 nm. HA addition increased the diameter to 350 nm, most likely due to cross-linking of the complexes by this large polysaccharide. Spn-HA addition did not affect complex size, perhaps due to the small exclusion volume of this polyampholyte. BSA was added (final concentration of 10 mg/mL) to the DNA/PEI binary complexes and the measured diameters increased up to 500 nm. Aggregation gradually proceeded and within 30 min reached sizes of 600–900 nm (Fig. 3). When BSA was added to ternary complexes coated with HA or Spn-HA, they did not show apparent increases in size under similar conditions. This confirms that particle coating with HA or Spn-HA can minimize interactions with serum proteins.

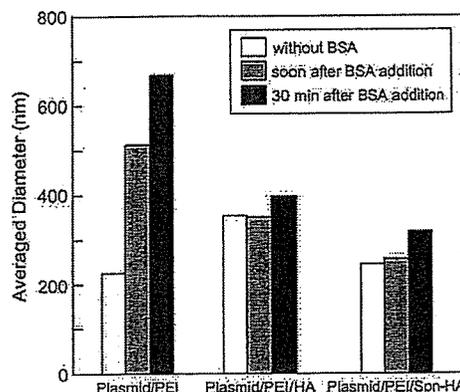


Fig. 3. Averaged diameter of the complexes.

### 3.3. Distribution in the peritoneal cavity

Since DNA/PEI complexes coated with HA did not interact with serum proteins, they were expected to diffuse to target organs without aggregating or precipitating in body fluids. Fluorescently labeled DNA/PEI/HA ternary complexes (1:1:9.7, w/w/w) were injected into the peritoneal cavity of mice (ddY, male, 6–7 w) and their diffusing behavior in the cavity was examined. Since the binding of DAPI to DNA is reversible, a stable intercalating fluorescent dye YOYO-1 was used to visualize the DNA complex. Large amounts of YOYO-1 can extend the persistent length of the DNA molecules. YOYO-1 was therefore added at a ratio of YOYO-1/DNA=1/5 or less (molar ratio based on nucleotides) to minimize effects on complex formation. DNA from salmon sperm was used for the experiment. The labeled DNA/PEI complexes with or without HA in 5% glucose (100  $\mu$ L) were injected into the peritoneal cavity of mice (8.25  $\mu$ g DNA/mouse). After 30 min, DNA/PEI/HA ternary complexes spread throughout the cavity whereas

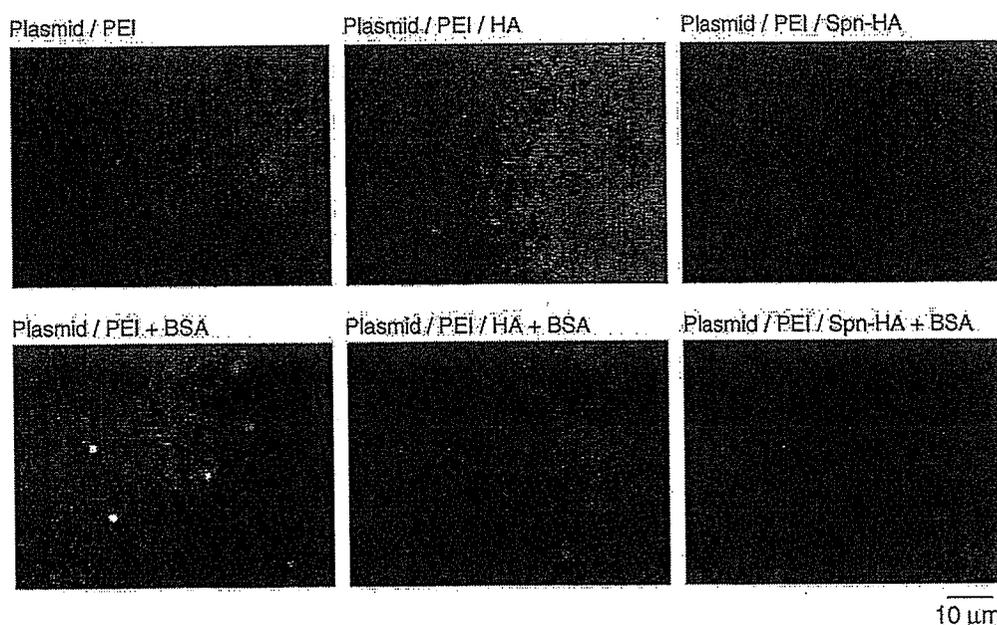


Fig. 2. Fluorescence images of the plasmid/PEI binary complex (1:1 in weight) and plasmid/PEI/HA (or SPN-HA) ternary complexes (1:1:9.7 in weight) before and after the addition of albumin. Final concentration of BSA=10 mg/mL.

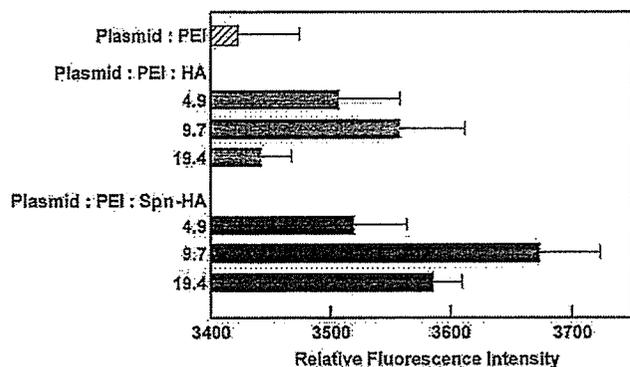


Fig. 4. Relative fluorescence intensity of the AmNS-UTP solutions after the incubation for 90 min with RNA polymerase and plasmid complexes ( $n=3$ ).

binary complexes without HA were retained in the injected area. Inertness of the ternary complexes coated with HA enhanced their diffusion and penetration through the intestines. This enhanced diffusion of HA-coated complexes could be favorable for the delivery of DNA complexes to target diseased organs in gene therapy.

### 3.4. Transcription study

HA was expected to loosen the plasmid/polycation complex through electrostatic interactions with the polymer, leading to an overall improvement in transcription factor access to the DNA. This was investigated with an *in vitro* system without using cells. Ternary complexes of plasmid:PEI:HA=1:1:4.9–19 (w/w/w) (or 1:8:4–16 in terms of charge) were prepared. Nucleotide triphosphates and RNA polymerase (from *E. coli*) were then added. Following incubation at 37 °C for 90 min, the transcription reaction was stopped by addition of EDTA and the fluorescence intensity was measured. As shown in Fig. 4, plasmid/PEI/HA induced higher production of AmNS-pyrophosphoric acid compared to plasmid/PEI binary complexes. This indicates that HA can enhance the transcription of the plasmid.

The behavior of HA resembles that of HMG proteins, which can uncoil tightly compacted chromosomal DNA to facilitate its association with transcription factors [13]. An amphoteric derivative of HA was thus synthesized to mimic the HMG protein structure by grafting spermine onto HA with a water-soluble carbodiimide. Excess spermine was used to prevent

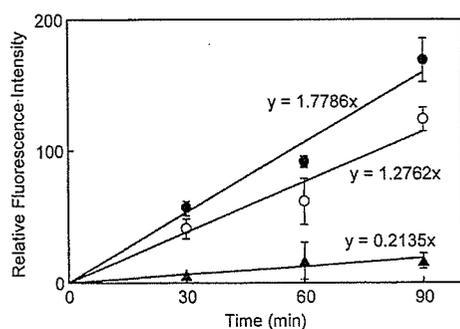


Fig. 5. Transcriptional activity of the plasmid complexes; ▲: Plasmid/PEI, ○: Plasmid/PEI/HA, ●: Plasmid/PEI/Spn-HA ( $n=3$ ).

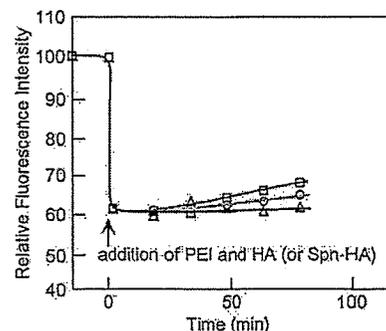


Fig. 6. Fluorescence recovery of the YOYO-1 in the plasmid/PEI complexes by □: HA, ○: Spn-HA, △: control.

cross-linking and the reaction was discontinued after 5 h to yield water-soluble amphoteric HA derivatives (Spn-HA) having both amino and carboxylic groups. The effect of Spn-HA on the transcription activity was then investigated simultaneously with HA and results are shown in Fig. 4. Spn-HA, as expected, had a superior enhancing effect to HA on the transcriptional activity. Both HA and Spn-HA displayed maximal transcriptional activation at a weight ratio of PEI:HA (or Spn-HA)=1:10. The transcriptional rate of these ternary complexes was then measured and compared with those of plasmid/PEI binary complex (Fig. 5). The complex with HA or Spn-HA prepared at plasmid:PEI:HA (or Spn-HA)=1:1:9.7 (w/w/w) showed 6.0- or 8.3-fold higher transcription rate, respectively, than those with plasmid/PEI binary complexes. Neither HA nor Spn-HA had any effect on the transcription efficiency of naked plasmid without PEI. These results suggest that HA and its amphoteric derivatives could possess HMG-like transcriptional enhancing properties.

### 3.5. Loosening behavior of the plasmid/PEI complex

DNA complex-loosening by HA and Spn-HA was evaluated by measuring the fluorescence intensity of the YOYO-1-intercalated DNA molecules. When YOYO-1 intercalates with DNA, it fluoresces with more intensity than when free. If the DNA is compacted in a globular state, its fluorescence intensity would be much lower than in the random coiled state. When the DNA complex becomes more loose due to presence of HA or

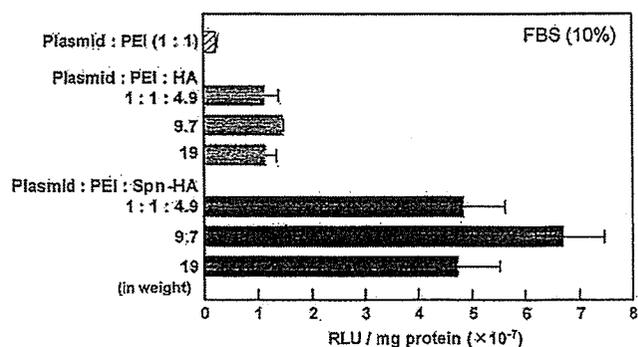


Fig. 7. Transgene expression efficiency of HA- or Spn-HA-coated plasmid/PEI complexes on CHO cells ( $n=3$ ).

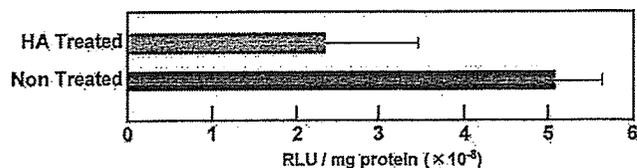


Fig. 8. Effect of HA-preaddition on the transgene expression on CHO cells mediated by plasmid/PEI/HA (1:0.71:4.9 in weight) ( $n=3$ ).

Spn-HA, the fluorescence intensity of any intercalated YOYO-1 in the complex should cause a recovery. Therefore, change in the fluorescence intensity of YOYO-1-labeled DNA complexes before and after the addition of HA or Spn-HA was measured. YOYO-1-labeled naked plasmid yielded high fluorescence. This value decreased down to 61% of its original value upon addition of PEI (1:1, w/w). Addition of HA recovered the fluorescence to 68%. Spn-HA also recovered the fluorescence but to a smaller degree (Fig. 6). These results reveal that both HA and Spn-HA can loosen the plasmid/PEI complex to some extent, although they could not completely cause the complexes to fall apart. Recovery of the fluorescence intensity by Spn-HA was not as high as expected based on the transcription results. The influence of Spn-HA itself on the fluorescence intensity was examined. Spn-HA without PEI reduced the fluorescence intensity of YOYO-1/DNA to 80%, whereas HA addition did not have any effects. Since Spn-HA has a quenching effect, this amphoteric polysaccharide seems to effectively loosen the DNA/PEI complex, leading to overall higher transcription efficiency.

### 3.6. Transfection mediated by the DNA/PEI/HA (or Spn-HA) ternary complexes

Ternary complexes coated with HA or Spn-HA can easily disperse in serum-containing medium, favorably bind to CD44 positive-cells and can loosen DNA/PEI interactions favorable for enhanced transcription. Transfection efficiency of the HA- or Spn-HA-coated DNA/PEI complexes on CD44-expressing CHO cells was examined. Fig. 7 shows the results for plasmid/PEI/HA and plasmid/PEI/Spn-HA ternary complexes. Based on the total protein content, cell toxicity was not observed in either case. HA enhanced the transgene expression with a 6.4-fold higher luciferase activity than plasmid/PEI binary complexes. As was expected from the transcription study, Spn-HA had higher transfection enhancing effect than HA, and the ternary complex prepared at plasmid/PEI/Spn-HA = 1:1:9.7 (w/w/w) (P:N:COOH=1:8:3.6), had 29-fold higher gene expression than the plasmid/PEI binary complex. This superior enhancing effect of Spn-HA could be due to its HMG-like transcriptional activation properties.

HA-receptors (CD44) present on CHO cells improve uptake of HA-coated ternary complexes and lead to higher gene expression. This was examined by preaddition of excess HA to cells prior to incubation with the plasmid complex. The complex was prepared with relatively small amount of HA (plasmid:PEI:HA = 1:0.71:4.9 (w/w/w), (P:N:COOH = 1:5:4)) in order to minimize un-complexed HA in the medium. Before

the DNA complex was added, 200  $\mu$ L of HA solution (2.4 mg/mL in PBS) was added to CHO cells to which 1 mL of F12 medium was added in advance. After incubation at 37  $^{\circ}$ C for 90 min, the DNA/PEI/HA complexes were added to the wells, and the cells were treated and assayed as previously mentioned in the transfection study. The CHO cells pretreated by HA had diminished gene expression (less than 1/2 of the control cells treated with PBS alone) (Fig. 8). This reveals that the highly enhanced luciferase expression of HA-coated ternary complexes was also mediated by particle uptake through HA-specific receptors present on the surface of CHO cells.

## 4. Conclusion

HA can be deposited onto the cationic surface of DNA/polyethyleneimine (PEI) complexes via electrostatic means, minimize nonspecific interactions with serum proteins, act as a ligand to specific cells and also behave like a transcriptional activator. An amphoteric HA derivative having spermine side chains (Spn-HA) exhibited higher transcription-enhancing activity and gene expression in cultured cells than HA. Water-soluble polyampholytes such as the one investigated having multifunctional properties can improve gene transfection and simultaneously also serve as artificial HMG protein models.

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## Construction of an oligosaccharide library by cultured cells for use in glyco-biotechnology

Hideki Nakajima<sup>1</sup> and Toshinori Sato<sup>2</sup>

<sup>1</sup>Glycomedics Inc., <sup>2</sup>Keio University, Department of Bioscience and Informatics  
3-14-1 Hiyoshi, Kouhoku-ku, Yokohama 223-8522, Japan

### Abstract

*The preparation of cell surface glycans by chemical synthesis techniques or purification from natural products has proved to be extremely difficult. Therefore, we developed the "saccharide primer method", which uses cultured cells to obtain large quantities of cell surface glycans. The "saccharide primer" is an artificial precursor of glycans mimicking the structure of the intermediate form for oligosaccharide synthesis in the cell. Saccharide primers introduced in the media are internalized into*

*the cell, are glycosylated by glycosyltransferase expressed in the cell, and are secreted to the media as oligosaccharide. It is relatively easy to recover the glycosylated saccharide primer from the media by ODS column (solid phase extraction), because the saccharide primer has a hydrophobic region in the molecule. The advantages of this method are that large quantities of oligosaccharide can be acquired with less contamination, resulting in a library representing the same composition as the cell. By devising aglycone of the saccharide primer, it was possible to increase by around 10-fold the oligosaccharide production secreted into the medium. An aglycone with a dodecyl group was the best structure for the saccharide primer to obtain effective amounts of glycans from the cultured media. We have succeeded not only in synthesizing various glycolipid glycans but also O-linked glycans by devising the structure of the saccharide primer. It is possible to put a reactive group, like azido, in the aglycone of the saccharide primer without affecting glycosylation efficiency in cultured cells. Thus, glycosylated saccharide primers can be combined to another molecule. These glycans can also immobilize on solid phase, and utilized as a "glyco-array".*

## **Introduction**

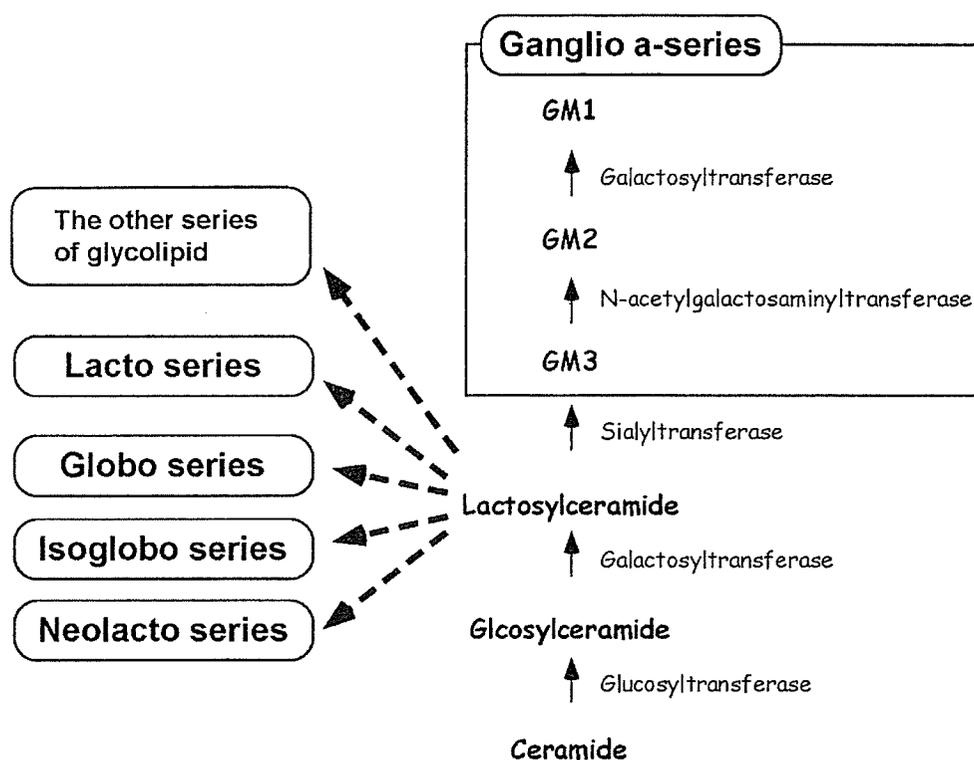
Biological roles of glycans including the oligosaccharide, glycolipid, glycoprotein or proteoglycans are quite varied. They are crucial for the development, growth, function, and survival of an organism. Furthermore, several human disease states are characterized by changes in glycan biosynthesis that can be of diagnostic and therapeutic significance. Thus, glycans are becoming increasingly important in modern biotechnology.

However, it is necessary to rapidly advance current technologies for the analysis and synthesis of oligosaccharides if we are to effectively put to use the therapeutic and diagnostic potential of these molecules. The fields of genetics and proteomics has significantly advanced due to the use of libraries, sequencers, synthesizers, amplification techniques using *Escherichia coli*, etc. To date, technologies for the analysis of oligosaccharides are not as developed.

Complicating this issue are the complex structures of oligosaccharides. In the galactose dimer, there are 15 types of structures, whereas in nucleic acid and amino acid dimers there is only one structure. Further, it has proven difficult to study glycans, given the complexity of the biosynthetic pathways cells use to produce them (Fig. 1).

To study the therapeutic potential of glycans, it is necessary to develop the means to produce stable supplies of them. Currently, glycans are obtained by the following means: Extraction from the natural product, organic synthesis, or enzymatic synthesis.

Polysaccharides like starch, chitin, cellulose, etc. can be produced in large quantities by extraction and purification from natural products. However,



**Figure 1.** Glycolipid glycan synthesis pathway.

oligosaccharides and glycans are hard to obtain because of the small abundance and difficulty of purification. Hence, current production of oligosaccharides and glycans utilizes organic synthesis. However, this synthesis technique becomes more difficult as the sugar unit increases in the glycan, leading to a great decrease in yield. In addition, this procedure is not very cost-effective.

Oligosaccharides can also be produced using glycosyltransferase in cells. Although many different glycosyltransferases have been cloned, available varieties of glycosyltransferase are limited at present, and it is necessary to prepare several expensive sugar nucleotides as sugar donors.

Due to these difficulties, we have conducted research for the development of a novel technique that uses cells to produce oligosaccharide in culture media. We call this technique the “saccharide primer method”, which has advantages in that it is able to generate a representative library of cellular glycans without using organic synthesis, glycosyltransferase, or expensive sugar nucleotides.

Glycans incorporated into lipids and proteins are synthesized in the endoplasmic reticulum and Golgi. We utilized this glycan synthesis system in the cell using the “saccharide primer method” for producing large amounts of oligosaccharide. There is a saccharide precursor to glycan synthesis in the cell. In case of the glycolipid, the glycan is synthesized by connecting sugar molecules

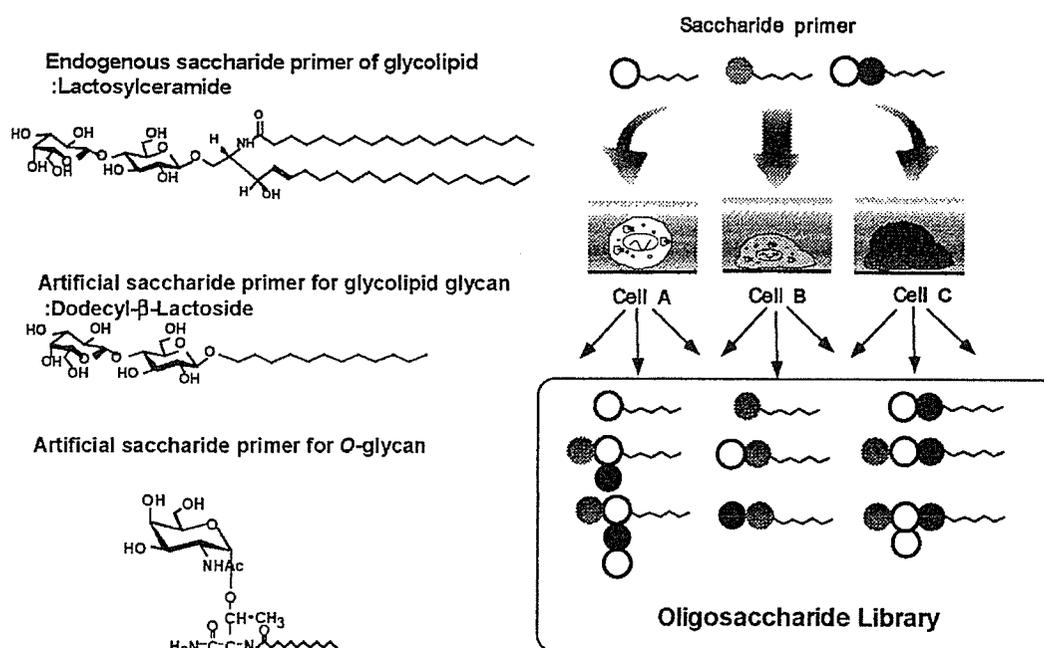
one by one to ceramide (Fig. 1). Lactosylceramide is synthesized from the ceramide by combining the glucose and galactose, respectively. This lactosylceramide (LacCer) is a common precursor of many series of glycolipids, including the ganglio series (NeuAc-Lac), globo series (-Gal $\alpha$ 1-4Lac), lacto series (-GlcNAc $\beta$ 1-3Lac), and neo lacto series (-GlcNAc $\beta$ 1-3Lac). The biosynthetic pathway of the ganglio glycolipid series is shown in Fig. 1 as one example. In the case of the glycosaminoglycan in proteoglycan or *O*-glycan glycoproteins, xylose or galactosamine combines to serine or threonine residue of peptides, respectively, as a first step of glycan synthesis. Then, glycan synthesis begins on the glycoside residue of these molecules. These glycosides act as an endogenous primer for the glycan.

In the biosynthesis of glycolipids, lactose (Gal $\beta$ 1-4Glc) is the primer region for glycan elongation. In fact, it is possible to use the lactoside, which combines a mono alkyl group in aglycone, to imitate the lactosylceramide as a saccharide primer of glycolipid glycan synthesis (Fig. 2). The ability of artificial glycolipid primers to act as a precursor of glycolipid glycans was shown by Yamagata *et al.* using cultured cells [1, 2]. This artificial saccharide primer was incorporated to the cell from the culture media, glycosylated in Golgi, and secreted back to the media.

Ceramide is the hydrophobic region of endogenous glycolipids. In the case of the artificial saccharide primer, the amount of glycan recovered from the medium was greatest when dodecyl (C12) was used as hydrophobic region of the primer. This primer resulted in about a 10-fold increase in production relative to endogenously produced glycolipid glycans. The glycosylation of the saccharide primer did not occur when the test saccharide primer carried a shorter hydrophobic aglycone (ie. Octyl- $\beta$ -D lactoside). Also, although primer glycosylation occurred when using saccharide primers carrying a longer alkyl chain or two hydrophobic chains of aglycone, like ceramide, it remained in the cell, and was not secreted into media at high concentrations [2].

The oligosaccharide primer containing dodecyl group for glycolipid glycan is easy to synthesize at high yields. It is also possible to obtain the glycoprotein *O*-glycan when using GalNAc $\alpha$ -Thr-C12 as a saccharide primer as shown in Fig. 2. By utilizing the living cell, it is not necessary to prepare expensive sugar donors or glycosyltransferase, which is difficult to obtain. Glycan production by saccharide primer is may also be applied to large cultures. Thus, it is possible to achieve large yields of oligosaccharides by scaling up the culture.

In the production of glycans by the saccharide primer method, it is possible to obtain the oligosaccharides one by one via an ordered extension. Generally, the product is obtained using this method within 1 or 2 days. Because most of the produced oligosaccharide can be extracted easily with less contaminant from the media by ODS resin (solid phase extraction), structure



**Figure 2.** Saccharide primer.

analysis is greatly simplified compared with endogenous glycan analysis. The sequence of the oligosaccharide can also be determined using MS/MS. The synthetic pathway and expression pattern of glycans in the cell can also be determined by analyzing the glycan derived from saccharide primer. More detailed structural determination is possible by fragment analysis of the MS<sup>n</sup> spectrum, substrate specificity of the glycosidase degradation product, binding specificity with the carbohydrate recognition proteins such as lectin or antibody, NMR analysis, etc.

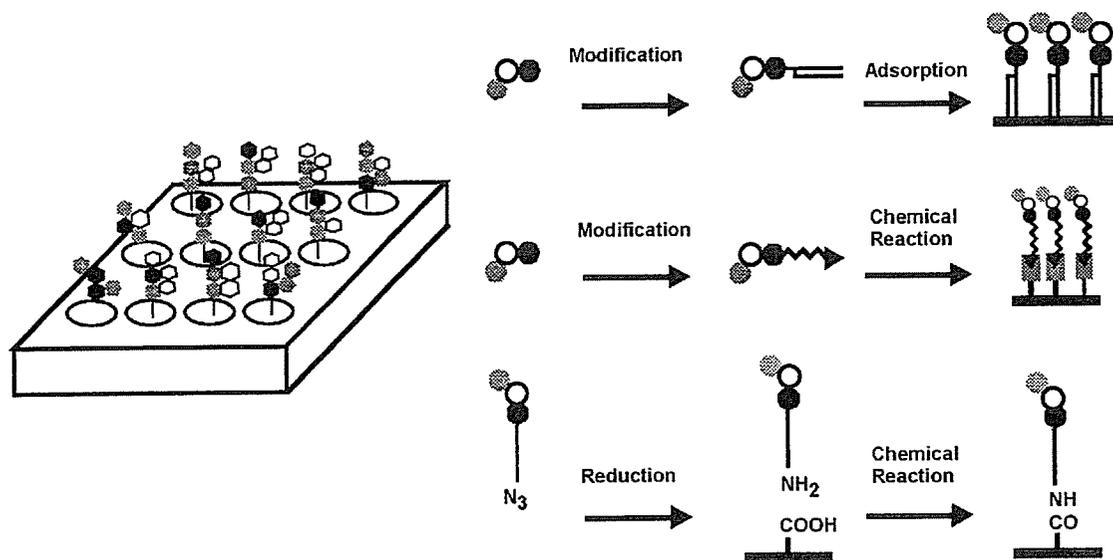
Combinatorial chemistry for producing libraries of chemical compounds has become standard in various fields. Useful compound are selected from the library by various screening assays. Similarly, genes of interest are also selected from cDNA libraries and cloned in the field of genetic engineering. To find useful oligosaccharides, it is necessary to construct oligosaccharide libraries. The inherent biocombinatorial chemistry mechanisms used by cells in culture will be utilized to generate this library. It is possible to construct the oligosaccharide library by the combination of several kinds of cells and saccharide primers (Fig. 2).

Each cell has specific oligosaccharide synthesis pathways. For example, mouse melanoma B16, Africa green monkey kidney cell COS7, and human leukemia HL60 express GM3 ganglioside, ganglio series glycolipids, and neolacto series glycolipids including sialyl and lewis X, respectively. We can obtain the same oligosaccharides present in these cells when oligosaccharide primers are administered. If different types of saccharide primers were used,

the primer would be used for another glycan synthesis pathway, leading to the acquisition of different types of glycans from only one type of cell. By the different combination of saccharide primers and cells, it is possible to increase the glycan copy number of the library.

Oligosaccharides with reactive groups are very useful for the research of glycobiology and glycomics. When an oligosaccharide is immobilized on a sensor chip, the interaction between glycan and ligand (e.g. lectin) can be detected. It is possible to modify the aglycone of saccharide primers using reactive groups that bind other molecules (Fig. 3). For example, saccharide primers with an azido group ( $N_3$ ) in aglycone can be glycosylated in the cell [3]. The azido group is converted to an amino group by reduction. Amino groups are covalently bound to carboxyl groups by an amide bond (Fig.3). Azido groups are also able to form covalent bonds by the Staudinger reaction with a linker molecule [4]. Interaction between a lectin and a saccharide primer molecule was detected by a surface plasmon resonance biosensor using this immobilization strategy [4]. As with the DNA and protein microarrays, the microarray of the glycan "glyco-array" will prove to be an important advancement in our ability to study the biological functions of glycans. The development of the "saccharide primer method" is an important step forward for the construction of a "glyco-array".

We have described a novel technology that utilizes cells as a factory of glycan production. Though manufacturing efficiency still needs to be improved, the trial to produce oligosaccharides using the cell engineering methodology has begun. Improvement of this technology will aid in the advancement of the glycan industry.



**Figure 3.** Application of Glycan derived from saccharide primer for "Glyco-array".

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