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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

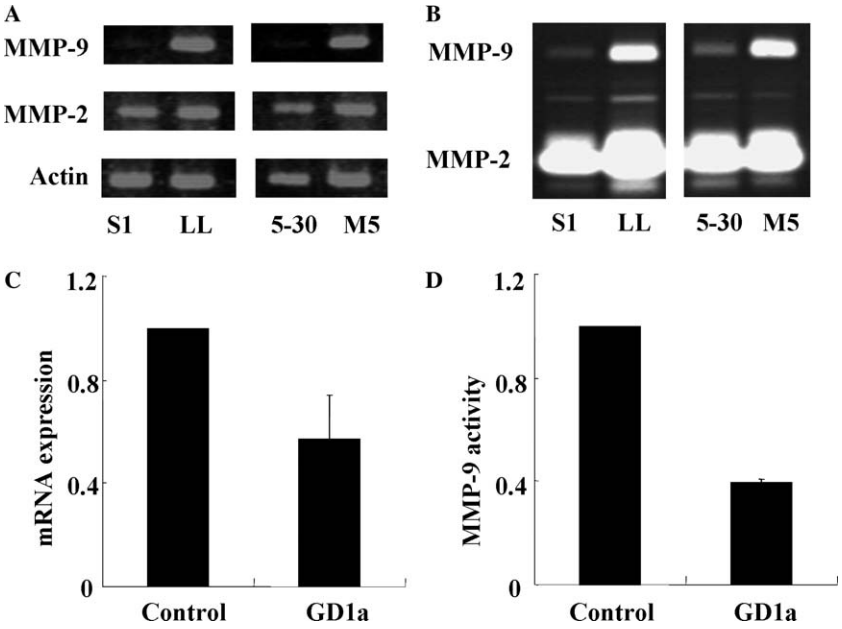

Fig. 1. Ganglioside GD1a negatively regulates metalloproteinase-9 (MMP-9) but not MMP-2. FBJ-S1, -LL, -M5, and -LA5-30 cells were cultured under standard conditions followed by determination of MMP-9 and -2 expression by RT-PCR (A). On aliquots of culture medium of FBJ-S1, -LL, -M5, and -LA5-30 cells 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 μM 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 β-actin in a way so that MMP-9 mRNA content divided by that of β-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 μM or 100 μM 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 meditate 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.

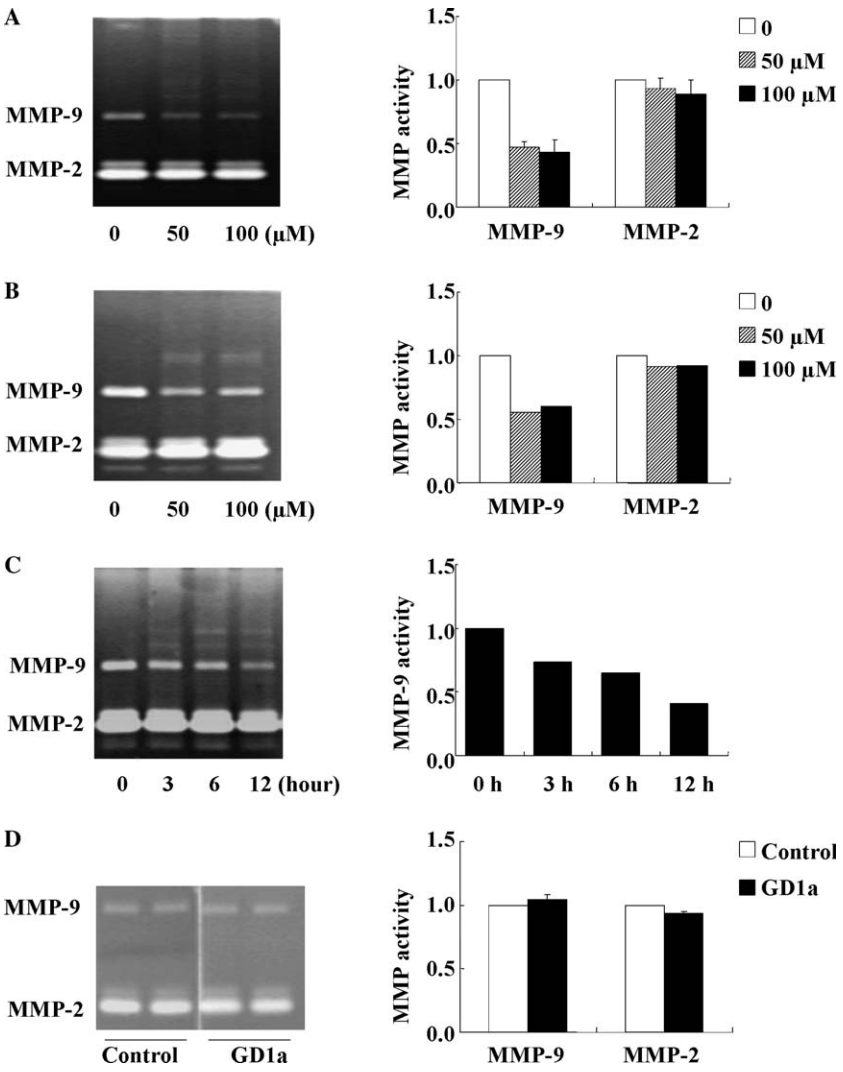

Fig. 2. GD1a in culture medium decreases MMP-9 but not MMP-2 activity. To examine the effects of GD1a on MMP-9, conditioned medium of FBJ-M5 (A) or FBJ-LL (B) cells incubated without GD1a and serum for 24 h was obtained and incubated with 50 μM or 100 μM GD1a for 12 h. Aliquots of the medium were subsequently assayed for MMPs. (C) Conditioned medium was incubated with 50 μM 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 μM GD1a for 16 h at 37 °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

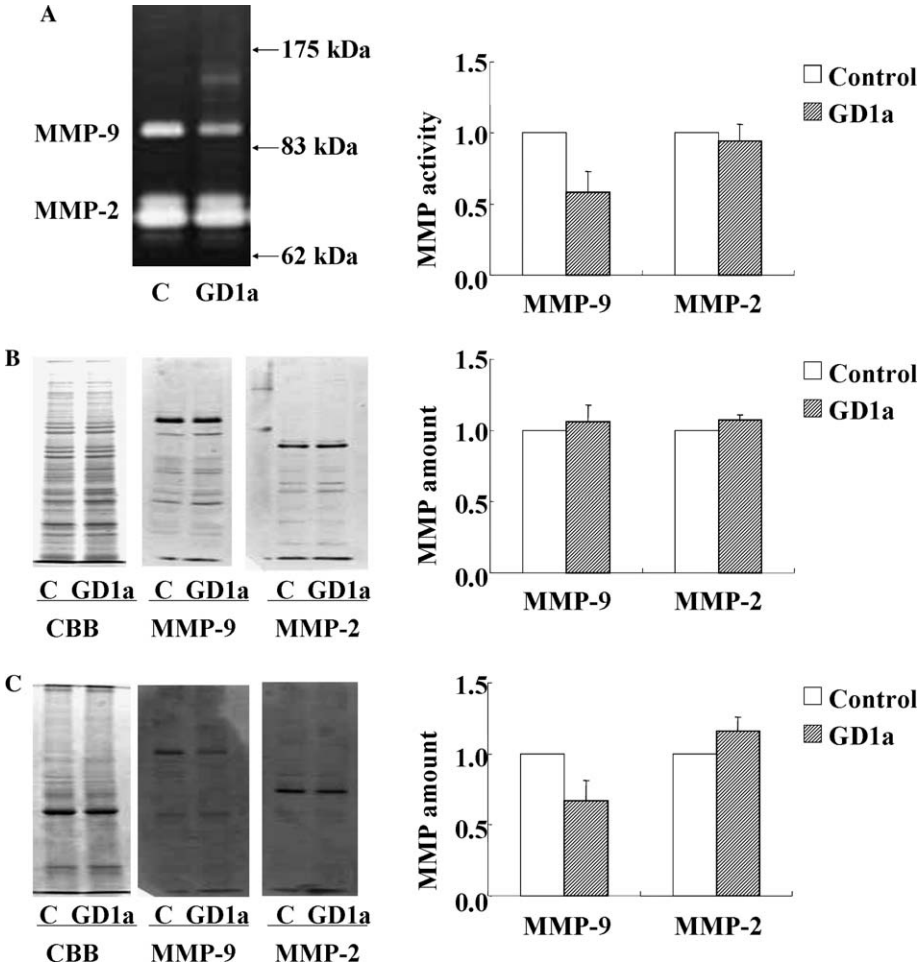

Fig. 3. MMP-9 activity is apparently suppressed by GD1a but total MMP-9 does not change. FBJ-M5 cells were incubated without GD1a to prepare conditioned medium which was subsequently incubated with or without 50 μM GD1a for additional 6 h and then concentrated by 20 times using an Amicon filtration tube (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.

References

[1] S. Curran, G.I. Murray, Matrix metalloproteinases: molecular aspects of their roles in tumour invasion and metastasis, Eur. J. Cancer 36 (2000) 1621–1630.

[2] G. Klein, E. Vellenga, M.W. Fraaije, W.A. Kamps, E.S.J.M. de Bont, The possible role of matrix metalloproteinase (MMP)-2 and MMP-9 in cancer, e.g. acute leukemia, Crit. Rev. Oncol./Hematol. 50 (2004) 87–100.

[3] J.E. Rundhaug, Matrix metalloproteinases and angiogenesis, J. Cell. Mol. Med. 9 (2005) 267–285.

[4] L.A. Liotta, W.G. Stetler-Stevenson, Tumor invasion and metastasis: an importance of positive and negative regulation, Cancer Res. 51 (1991) 5054–5059.

[5] 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.

[6] S. Hyuga, S. Yamagata, Y. Takatsu, M. Hyuga, H. Nakanishi, K. Furukawa, T. Yamagata, Suppression of FBJ-LL cell adhesion to vitronectin by ganglioside GD1a and loss of metastatic capacity, International J. Cancer 83 (1999) 685–691.

[7] S. Yamagata, R. Tanaka, Y. Ito, S. Shimizu, Gelatinase of murine metastatic tumor cells, Biochem. Biophys. Res. Commun. 158 (1989) 228–234.

[8] S. Yamagata, Y. Yoshii, J.G. Suh, R. Tanaka, S. Shimizu, Occurrence of an active form of gelatinase in human gastric and colorectal carcinoma tissues, Cancer Lett. 59 (1991) 51–55.

[9] K. Tamakoshi, F. Kikkawa, A. Nawa, H. Ishikawa, K. Mizuno, A. Tamakoshi, S. Yamagata, N. Suganuma, Y. Tomoda, Characterization of extracellular matrix-degrading proteinase and its inhibitor in gynecologic cancer tissues with clinically different metastatic form, Cancer 76 (1995) 2565–2571.

[10] M. Hamaguchi, S. Yamagata, A.A. Thant, H. Xiao, H. Iwata, T. Mazaki, H. Hanafusa, Augmentation of metalloproteinase (gelatinase) activity secreted from Rous sarcoma virus-infected cells correlates with transforming activity of src, Oncogene 10 (1995) 1037–1043.

[11] R.O. Miura, S. Yamagata, Y. Miura, T. Harada, T. Yamagata, Analysis of glycosaminoglycan-degrading enzymes by substrate gel electrophoresis (zymography), Anal. Biochem. 225 (1995) 333–340.

[12] M. Cao, M. Sahmi, J.G. Lussier, C.A. Price, Plasminogen activator and serine protease inhibitor-E2 (protease nexin-1) expression by bovine granulosa cells in vitro, Biol. Reprod. 71 (2004) 887–893.

[13] S. Hattori, H. Fujisaki, T. Kiriyama, T. Yokoyama, S. Irie, Real-time zymography and reverse zymography: a method for detecting activities of matrix metalloproteinases and their inhibitors using FITC-labeled collagen and casein as substrates, Anal. Biochem. 301 (2002) 27–34.

[14] L. Kjeldsen, A.H. Johnsen, H. Sengeløv, N. Borregaard, Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase, J. Biol. Chem. 268 (1993) 10425–10432.

[15] G.S. Makowskia, M.L. Ramsby, Zymographic analysis of latent and activated forms of matrix metalloproteinase-2 and -9 in synovial fluid: correlation to polymorphonuclear leukocyte infiltration and in response to infection, Clin. Chim. Acta 329 (2003) 77–81.

[16] J. Hu, X. Zhang, W.B. Nothnick, T.E. Spencer, Matrix metalloproteinases and their tissue inhibitors in the developing neonatal mouse uterus, Biol. Reprod. 71 (2004) 1598–1604.

[17] I. Stamenkovic, Matrix metalloproteinases in tumor invasion and metastasis, Cancer Biol. 10 (2000) 415–433.

[18] K. Brew, D. Dinakarpandian, H. Nagase, Tissue inhibitors of metalloproteinases: evolution, structure and function, Biochim. Biophys. Acta 1477 (2000) 267–283.