® Postepy Hig Med Dosw (online), 2018; 72: 1132-1137
e-ISSN 1732-2693

www.phmd.pl
Original Article

Received: 09.02.2018
Accepted: 21.08.2018
Published: 31.12.2018

Metalloproteinases secretion from cultured cells: Differentiation by detergents

Sekrecja metaloproteinaz macierzy w hodowlach komórkowych – różnicujące działanie detergentów

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Aim: Matrix metalloproteinases, particularly MMP-2 and MMP-9, are the active factors influencing the structure and properties of the extracellular matrix. This effect is enhanced in metastasis. Hence, it is necessary to enrich the knowledge of the relation between the accumulation and distribution of the enzymes in cells and the intensity of their secretion.

Material/Methods: The study was conducted on three cell lines of dermal origin: normal line of human dermal fibroblasts (NHDF) and two melanoma lines (BM and B16F10). The results were obtained with substrate zymography, immunofluorescence microscopy and detergent-complemented fluorimetric assay.

Results: All the studied cell lines revealed relatively rich content of MMP-2 (latent and active) with random intracellular localization. Nevertheless, the enzyme secretion was differentiated in various types of cells. The most intensive proMMP-2 secretion was obtained for NHDF, relatively lower for BM, and the lowest for B16F10 line. Zymographic detection of the active form of MMP-2 was restricted to NHDF and BM cell lines. On the other hand, differentiating usage of detergents Brij 35P and Triton X-100 evidenced an active fraction of MMP-2 present in cells of all studied lines. Triton X-100-generated lysis of cells of high MMP-2 secretion (NHDF, BM) revealed the presence of intracellular inhibitors.

Conclusions: From the obtained results it can be concluded that the active form of MMP-2 is localized pericellularly in studied cells, being a minor fraction of a total amount of cellular MMP-2. The rest of the enzyme content is located deeper in cell cytoplasm in a latent form. The activity of the pericellular fraction of the enzyme can be measured with detergent-complemented fluorimetric assay, constituting a potentially useful tool for evaluating the enzyme distribution.

Keywords: **matrix metalloproteinases • MMP-2 secretion • MMP-2 distribution • cultured cell lines**

GICID 01.3001.0012.8254
DOI: 10.5604/01.3001.0012.8254
Word count: 2148
Tables: 1
Figures: 3
References: 16

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Wykaz skrótów: **BM –** black melanoma (cell line); **MMP** – matrix metalloproteinase; **NHDF –** normal human dermal fibroblasts; **PBS** – phosphate buffered saline; **SFCM** – serum-free conditioned medium.

INTRODUCTION

Until now, it has been evidenced that matrix metalloproteinases (MMPs) are produced in healthy and cancerous cells and are secreted from them [10, 16]. A and B gelatinases (MMP-2 and MMP-9, respectively) are found to be essential for cancer progression and metastasis, mainly because of their broad substrate specificity (including hardly-degradable ECM components [11]) and multifunctional action [9, 12, 13]. These enzymes are factors contributing to the structure and properties of the intracellular matrix. The relation between expression of MMP-2 and the invasiveness of cancer cells has been shown [6, 7]. On the other hand, knowledge of the production and distribution of the ECM-degrading enzymes in the tumour microenvironment is surprisingly limited, even in the matter of their primary cellular source [8]. Hence, it is important to enrich the knowledge of the accumulation and distribution of the enzymes in cells and the intensity of their secretion.

In this study, three cultured cell lines of dermal tissue origin were used: normal human dermal fibroblasts (NHDF) and two cancerous – BM and B16F10. Immunofluorescence microscopy and substrate zymography were used to determine the activity and localization of MMP-2. Detergent-complemented fluorimetric assay was performed to distinguish between the activity focused on the cell surface and overall proteolytic potential. Such an assay may be considered as an alternative for the fluorescence-based *in situ* zymography, which, despite the simultaneous detection and localization of enzymatic activity, presents a rather qualitative approach and reveals some inconveniences in signal recognition and analysis [4, 15].

MATERIAL AND METHODS

Enzymes and chemicals

Matrix prometalloproteinase 2 (progelatinase A, human, recombinant; RHproMMP-2), matrix prometalloproteinase 9 (progelatinase B, human, recombinant; RHproMMP-9) and Mca-PLGL-Dpa-AR-NH_2 fluorogenic substrate ([7-Methoxycoumarin-4-yl]-acetyl-Pro-Leu-Gly-Leu-N3-[2,4-Dinitrophenyl]-L-2,3-Diaminopropionyl-Ala-Arg) were purchased from R&D Systems Inc. Non-ionic detergents: Brij 35P and Triton X-100 were purchased from Serva Inc. Pyruvate sodium salt and reduced β-nicotinamide adenine dinucleotide ($NADH_2$) sodium salt were obtained from Sigma-Aldrich Inc. All other reagents were of laboratory grade. For all experiments, distilled-deionised water was exclusively used.

Cell cultures

BM and B16F10 melanoma cells, as well as NHDF normal human dermal fibroblasts, were cultured in Dulbecco's modified Eagle's medium (DMEM). All the cell lines were obtained from the Institute of Immunology and Experimental Therapy, Polish Academy of Science, Wroclaw, Poland. Cell cultures were supplemented with 1% GlutaMAX and 10% fetal bovine serum (Gibco Inc.) and carried out under the standard conditions (37°C, 5% CO_2) until the confluence of about 90% was reached. Subsequently, the medium was replaced with its serum-free equivalent (SFCM, serum-free conditioned medium) and the culturing was continued for 24 hours. Cells were detached with non-enzymatic cell dissociation reagent (Gibco Inc.) and counted using the Countess cell counter (Invitrogen Inc.), according to the protocol provided by the manufacturer. SFCM were also collected.

Substrate zymography

Serum-free culture medium samples were analysed for the activities of secreted MMP-2 and MMP-9. Total protein concentration in SFCM samples was estimated with a commercial BCA protein assay kit (Sigma-Aldrich Inc.). Zymography was performed according to the protocol as established by Troeberg and Nagase [14]. SFCM aliquots were electrophoresed on the 8% polyacrylamide gels containing 0.1% porcine skin gelatine. About 0.5 ng of the RHproMMP-2 was used as a positive control. Electrophoresis gels were washed three times for 15 minutes in the renaturing buffer (Tris 50 mM, NaCl 150 mM, $CaCl_2$ 10 mM, Triton X-100 2.5% *v/v*, pH 7.5), and then incu-

bated overnight at 37°C in the developing buffer (Tris 50 mM, NaCl 150 mM, $CaCl_2$ 10 mM, pH 7.5). For the gel staining, 0.1% Coomassie Brilliant Blue R-250 solution in 50% methanol/20% acetic acid was used. Gels were subsequently destained in 30% methanol and documented with Gel-Doc XR system, equipped with ImageLab software (Bio-Rad Inc.). Proteolytic activity was identified as transparent bands on a blue background.

Immunofluorescence microscopy

For the microscopy experiments, cells were cultured directly on 3-well diagnostic slides (Menzel-Gläser/Thermo Fisher Scientific Inc.) under conditions described above and then were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes, washed with PBS and then treated with 0.3% Triton X-100 solution in PBS for 10 minutes. After the second washing step, cells were blocked with 3% solution of bovine serum albumin (BSA) in PBS for 30 minutes and incubated overnight at 4°C with monoclonal, mouse IgG_1 antibodies (concentration of 5 µg/mL; R&D Systems Inc.), detecting both zymogens and active forms of human MMP-2. Subsequently, cells were washed once with 0.1% Triton X-100 and twice with PBS and were incubated for 30 minutes with FITC-conjugated secondary antibodies (R&D Systems Inc.). Cells were washed with 0.1% Triton X-100 and with PBS and sealed with Mowiol 4-88 solution (Sigma-Aldrich Inc.). DAPI was used to stain nuclei. Images were obtained using Olympus FluoView 1000 confocal microscope and analysed with dedicated software.

Lactate dehydrogenase activity assay

To determine the cell membrane permeability in the presence of detergents, an assay of lactate dehydrogenase (LDH) activity in the cell suspension was employed (Bergmeyer's method [1]). In brief, the cells were washed twice in PBS and then suspended in the developing buffer to the final concentration of $5x10^5$ cells/ml. Samples were subsequently complemented with mild detergent Brij 35P or strong detergent Triton X-100 (to final concentration of 0.1%) or water (control). All samples were incubated at 37°C for 30 minutes and then centrifuged at 350 x g for 4 minutes for complete recovery of the supernatants, in which the activity of LDH was measured. In parallel, the effect of detergents on cell samples was evaluated by microscopic observation (data not shown).

Detection of proteolytic activity in living cells' suspension

Cells were washed in PBS and suspended in developing buffer to the final concentration of 10^5 cells/ml. After preincubation, the reaction was initiated by the addition of Mca-PLGL-Dpa-AR-NH2 fluorogenic substrate (final concentration of 5 mM), hydrolysed preferentially by MMP-2 and MMP-9. The reaction was carried out at 37°C for 10 minutes. One of the detergents – Brij 35P or Triton X-100 – was added (to final concentration of 0.1%) and the reaction was continued for 10 minutes. The increase in fluorescence was measured at two-minute intervals (t0-t20) with excitation and emission wavelength values of 325 nm and 393 nm, respectively using Cary Eclipse fluorescence spectrophotometer (Varian Inc.). To confirm that matrix metalloproteinases are the only source of detected proteolytic activity, the following inhibitors: E-64 (10 µM), PMSF (100 µM) and EDTA (1 mM) were used. Direct effect of detergents for the used concentrations on the enzymatic activity was also excluded (not shown). Three independent experiments were performed, and the results were presented as the mean ± standard deviation (SD). Within each cell line, differences in fluorescence intensity between Brij 35P and Triton X-100 data sets were tested with the Student's t test for independent samples. The data was selected and analysed using Statistica 13 software (Statsoft Inc.). Data point t10 was excluded from the analyses due to the measurement aberrations, caused by the manual application of detergent.

RESULTS

Substrate zymography is a method appropriate for the detection of both active and latent forms of gelatinases [14]. SFCM of the studied cell lines were analysed by substrate zymography to determine whether the MMP-2 or proMMP-2 is secreted. As shown in Fig. 1., NHDF and cancerous cells of BM line extensively secreted proMMP-2, although the secretion of the mature form was considerably less efficient. B16F10 cell line revealed low activity of secreted proMMP-2 and the lack of detectable mature form of MMP-2. No secretion of MMP-9 from all studied cell lines was evidenced by the same method. A trace of proMMP-9 was found only for B16F10 line after 10-fold concentration of the medium. The activity of no other matrix metalloproteinases was obtained in the investigated samples.

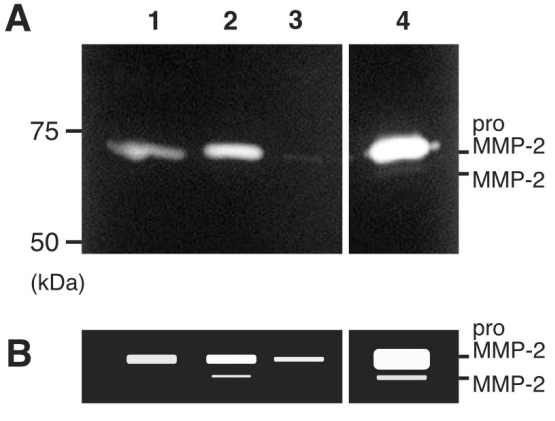

Fig. 1. (A) Proteolytic activity in serum-free conditioned media, evaluated with gelatine zymography; (1) recombinant human proMMP-2, used as positive control; (2) BM cell line; (3) B16F10 cell line; (4) NHDF cell line. (B) Schematic representation of detected activity

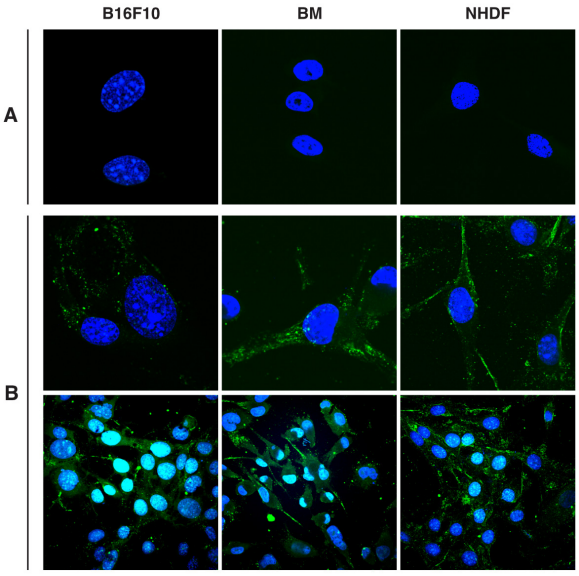

Fig. 2. Direct detection of MMP-2 in cell cultures with immunofluorescence microscopy; (A) cell samples treated exclusively with secondary antibodies (control); (B) cell samples treated with primary monoclonal antibodies, detecting both latent and active forms of MMP-2. Two different magnification levels were applied. FITC-conjugated anti-MMP-2 antibodies were applied (green); DAPI (blue) was used to stain nuclei

Immunofluorescence microscopy assay was continued only for MMP-2 as MMP-9 was not detected. The antibodies applied could not distinguish between MMP-2 pro – and mature forms; hence, the results represent an overall amount of MMP-2 and its cellular localization. As shown in Fig. 2 B, in all lines the enzyme is present abundantly and rather evenly distributed in cell interior. No pericellular aggregation can be seen here.

The possible presence of the enzyme in the pericellular zone (even in small concentration) can be recognized with a fluorimetric assay complemented with detergents Brij 35P and Triton X-100. In the LDH test, lysis of the cells was observed exclusively upon treatment with Triton X-100 (Table 1). Cell suspensions of all lines before the addition of the detergent revealed low metalloproteinase activity in fluorimetric assay (Fig. 3). This might be due to the presence of active form of the enzyme on the cell surface and/or damage of some cells in rinsing procedure. After addition of Brij 35P (measurement between the10th and 20th minute) the assay reaction rate increased for all lines, specifically: 100% for NHDF cell line, 250% for BM cell line and over 50% for B16F10 cell line. We interpret this increase in the enzyme activity as a result of probable weakening of its binding to the membrane and the release of its pericellular fraction. A quite different effect was observed after the addition of Triton X-100 (Fig.3). For suspensions of NHDF and BM cells, we also obtained an increase in the reaction rate, however, markedly less than for Brij 35P (125% and 60%, respectively). For B16F10, no marked differences in the effect of two applied detergents could be observed (Fig. 3). Undoubtedly, these results were caused by lysis of the cells releasing their inner content, intracellular fraction of MMP-2 and its inhibitors included.

DISCUSSION

Our zymography experiments showed that cells of the studied cultured lines secrete detectable levels of MMP-2, mainly in a latent form. The secretion seems to be independent on the intracellular enzyme supply. Noncancerous line NHDF revealed the highest intensity of the secretion despite a relatively even total amount of MMP-2 (active plus latent) in all lines (Fig.2). The secretion extent from BM cells was several times lower and it was the lowest from B16F10 (Fig.1). Immunofluorescence microscopy investigation showed no noticeable separate pericellular fraction for all lines. However, this kind of aggregation of MMP-2 observed in CS-1 hamster melanoma cells may suggest that a kind of pericellular localization of the enzyme, not detectable with this method, cannot be excluded [3]. The experiment with Brij 35P-complemented assay showed the increase in MMP-2 activity (Fig. 3). The detergent affects the structure of membrane without causing lysis, which might make the cells leaky and weaken the association of protein with membranes as well. The authors interpret this marked increase in the activity as a release of pericellular fraction of MMP-2 [9]. Logically, completely destroying the cell membranes and lysis would result in an increase in the activity of MMP-2 due to the exposure of intracellular supplies. However, this is not the case here. As shown in Fig. 3 and described in *Results,* lysis induced by Triton X-100 caused the decrease of the activity for NHDF and BM and no

Table 1. Changes of the cell membrane permeability under the influence of non-ionic detergents, estimated by spectrophotometric measurement of L-lactate dehydrogenase (LDH) leakage. In control, distilled water was used instead of the detergent

Cell line	Control (△Abs/min ± SD)	Brij 35P (△Abs/min ± SD)	Triton X-100 (△Abs/min ± SD)
B16F10	0.032 ± 0.008	0.131 ± 0.008	0.386 ± 0.010
BM	0.053 ± 0.003	0.092 ± 0.011	0.334 ± 0.010
NHDF	0.063 ± 0.020	0.101 ± 0.022	0.372 ± 0.015

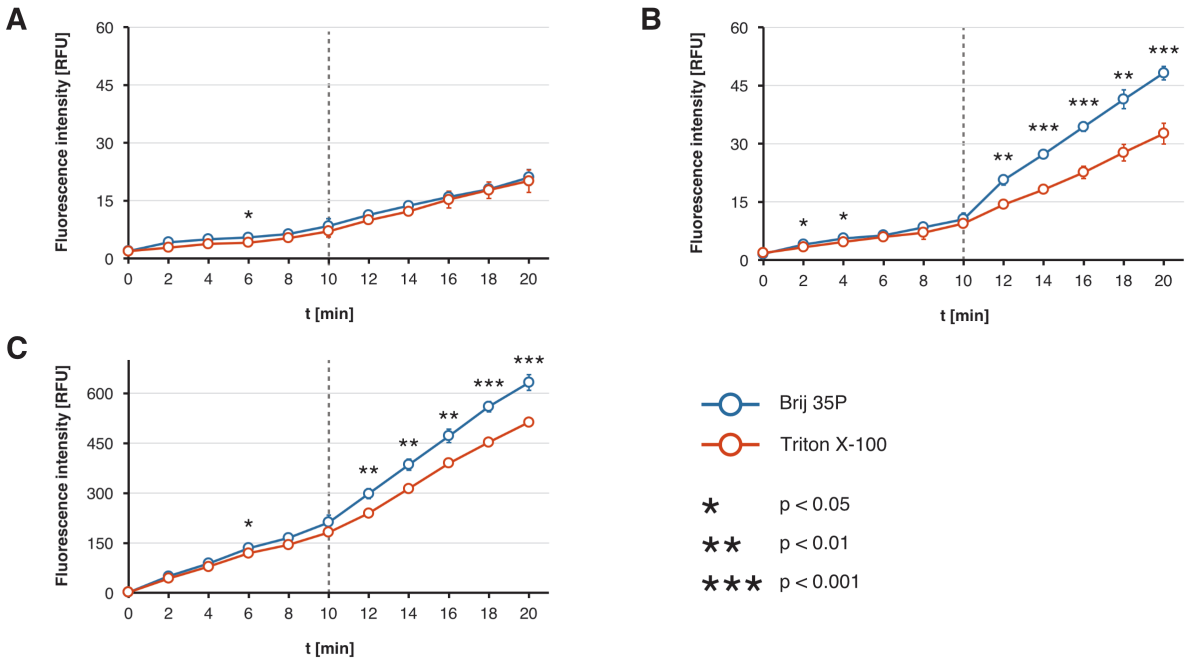

Fig. 3. Matrix metalloproteinase activity, detected in the living cells' suspension; (A) B16F10 cell line; (B) BM cell line; (C) NHDF cell line. The reaction rate before and after application of mild detergent (Brij 35P; blue line) or strong detergent (Triton X-100; red line) was evaluated with fluorimetric assay (RFU, relative fluorescence units). Vertical dashed lines indicate the point of detergent application (t10)

changes for B16F10. The authors interpret the result on the basis of a marked difference between cell lines in intensity of secretion of MMP-2 (both MMP-2 and proMMP-2), which was evidenced by zymography (Fig.1). A valid assumption is that the intensive secretion forces a cell to produce and keep a large supply of inhibitors of the enzyme that may be needed in case of perturbation of cell processes, e.g., secretion [2]. The inhibitors are likely located in a deeper part of the cytoplasm. Such a location of the inhibitors is evidenced elsewhere [5]. In cells with poor secretion of MMP-2 the amount of inner inhibitors would be low. Thus, destroying the cell membranes by Triton X-100 and lysis of B16F10 line cell (poorly secreting MMP-2) did not release enough inhibitors to affect the measured activity of MMP-2. The results observed after the addition of Triton X-100 suggest that an active form of MMP-2 is located only pericellularly and is a relatively small part of the overall amount of the enzyme, which is mainly located in the deep interior of the cytoplasm as a latent form (proMMP-2). In the other case, we would not observe the decrease of the protease activity for NHDF and BM (Fig. 3); for B16F10 rather an increase in the activity would be observed. In addition, such a large disproportion between the amounts of secreted pro-MMP-2 (inactive) and MMP-2 (active) could not be expected (Fig. 1 A, B).

The obtained results allow us to construct the short scheme of the relation between the intensity of cell secretion of MMP-2 and the effect of the used detergents as follows:

High MMP2 secretion → effect of Triton X-100 < effect of Brij 35P;

Low MMP2 secretion → effect of Triton X-100 = effect of Brij 35P.

Complementation of the fluorimetric assay with detergents of a different effect on the cell membrane leads to more efficient detection of MMPs activity in living cells' suspension. This simple tool might be useful for the investigation of MMPs production and secretion from cultured cells.

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The authors have no potential conflicts of interest to declare.

1137