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# Intracellular localization of matrix metalloproteinases and their inhibitors in cultured tumor cell lines: Flow cytometric analysis

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Received August 26, 2005; Accepted November 15, 2005

**Abstract.** Coordinate cell-surface expression of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) on cancer cells, tumor-infiltrating lymphocytes (TIL) and cancer-associated myofibroblasts has been shown to play an important role in tumor invasion and metastasis in organ tissues. However, precise localization of tumor cell lines *in vitro* has remained obscure. The aim of this study was to investigate the spatial localization of these proteolytic enzymes in 21 tumor cell lines consisting of 8 gastric, 4 pancreatic, 2 gallbladder, 1 extrahepatic bile duct cancer and 6 T- or B-cell tumors. The cell-surface and/or intracellular localization of MMP-2, -7, and -9, membrane type-1 (MT1)-MMP and TIMP-2 and -4 was quantitatively determined by flow cytometry in living (non-permeabilized) and permeabilized tumor cell lines. As a result, all of the tumor cell lines showed a high level of intracellular expression, but a low or undetectable level of cell-surface expression of MMPs and TIMPs. The findings revealed that intracellular MMPs and TIMPs in tumor cell lines were preserved as latent pro-enzymes.

## Introduction

The spatial localization or regulation of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) within the organ tissues are poorly understood. Recently, however, it has been shown that enhanced and coordinate cell-surface expression of MMPs and TIMPs on cancer cells, tumor-infiltrating lymphocytes (TIL) and cancer-associated myofibroblasts in organic tissues facilitates cancer invasion and metastasis, and the progression of chronic atrophic gastritis in *Helicobacter pylori*-infected gastric mucosa (1-3). However, data are limited on the localization of MMPs and TIMPs of tumor cell lines *in vitro*.

This study was designed to quantitatively analyze cell-surface and intracellular expression of MMP-2, -7 and -9, membrane type-1(MT1)-MMP, and TIMP-2 and -4 in 21 various tumor cell lines by the method of flow cytometry. MMPs and their inhibitors, TIMPs, were intracellularly expressed in cultured tumor cell lines, but rarely detected on the cell surface. The reason and biological significance of the results are discussed with special reference to the necessity of certain antigen stimulations and/or tumor-stromal relationships to have a crucial signal for the activation of the MMP proteolytic enzymes.

## Materials and methods

*Human cell lines*. Gastric carcinoma cell lines SC-1, SC-2, SC-3, SC-4 and GCIY; gallbladder carcinoma cell lines G-415 and G-930; extrahepatic bile duct carcinoma cell line BD-1; and pancreatic carcinoma cell lines P1119 and P1229 used in this study were established and cultured continuously in our laboratory (4-7). Other gastric carcinoma cell lines MKN-28, MKN-45 and MKN-74 were obtained from the Human Science Research Resources Bank (Osaka, Japan). Other tumor cell lines, Panc-1 (pancreatic carcinoma cell line), MIA-PaCa2 (pancreatic carcinoma cell line), CCRF-CEM (T cell leukemia cell line), Molt-3 (T cell leukemia cell line), Jurkat (T cell leukemia cell line), Raji (Burkitt's lymphoma cell line), K562 (chronic myelocytic leukemia cell line) and Wa (B-blastoid cell line) were obtained from the Laboratory Products Division of Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). These cell lines were maintained in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; M.A. Bioproducts, Walkerville, MD, USA) and 100 $\mu$g/ml kanamycin (Meiji Seika Co., Ltd, Tokyo) in 5% $CO_2$ at 37˚C.

*Monoclonal antibodies (mAbs) and flow cytometric analysis*. mAbs for MMP-2 (mouse IgG1/κ, clone 42-5D11), MMP-7 (mouse IgG1/κ, clone 141-7B2), MMP-9 (mouse IgG1/κ, clone 56-2A4), MT1-MMP (mouse IgG1/κ, clone 114-1F2), TIMP-2 (mouse IgG1/κ, clone 67-4H11) and TIMP-4

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*Key words:* matrix metalloproteinase, MMP-2, MT1-MMP, TIMP-2, tumor cell line

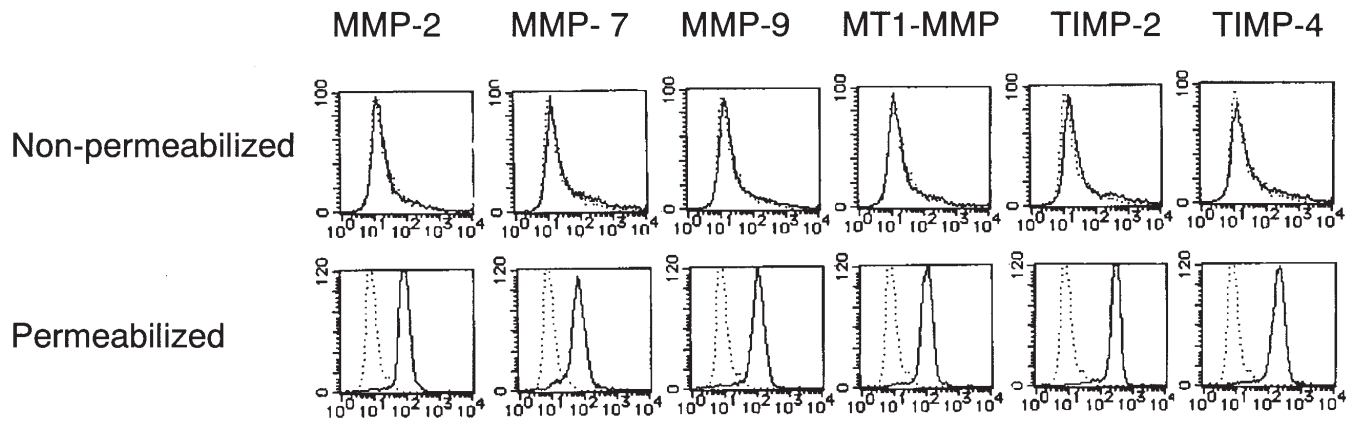


Figure 1. Cell-surface and intracellular expression of MMP-2, -7, and -9, MT1-MMP, and TIMP-2 and -4 in non-permeabilized and permeabilized gastric cancer cell line, MKN-45. The cells were stained with each mAb and FITC-conjugated goat anti-mouse IgG antibody (solid lines). Dotted lines indicate background staining with isotype control IgG and FITC-conjugated secondary antibody.

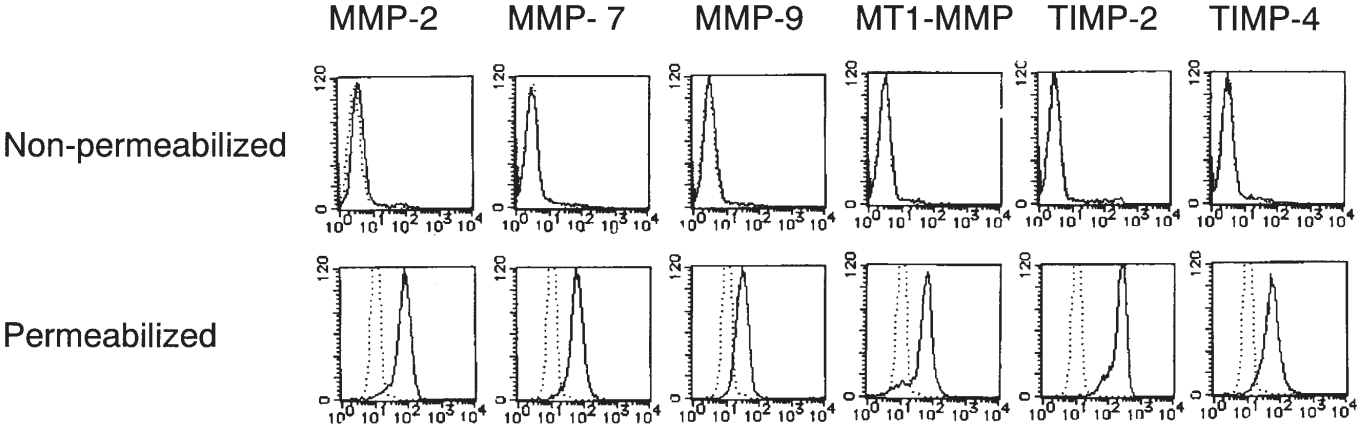


Figure 2. Cell-surface and intracellular expression of MMP-2, -7, and -9, MT1-MMP, and TIMP-2 and -4 in non-permeabilized and permeabilized T cell leukemia cell line, CCRF-CEM. The cells were stained with each mAb and FITC-conjugated goat anti-mouse IgG antibody (solid lines). Dotted lines indicate background staining with isotype control IgG and FITC-conjugated secondary antibody.

(IgG2a/κ, clone 216-1G2) were obtained from Daiichi Fine Chemical Ltd. (Takaoka City, Toyama, Japan) to screen for their usefulness in flow cytometry. To determine the cell-surface molecules of MMPs and TIMPs, the prepared cultured cells were incubated with heat-treated human aggregate IgG (GII 2388, Cohn fraction II and III; Sigma, St. Louis, MO, USA) for 15 min in PBS containing 0.1% BSA and 0.1% sodium azide to prevent antibody binding to FcR. The cells were then stained with a saturating concentration of primary mAb, followed by secondary antibody, fluorescein-isothiocyanate (FITC)-conjugated affinity purified goat anti-mouse (Jackson Immunoresearch Laboratories, Inc. West Grove, PA) for 30 min on ice and washed twice, as described previously (1,2). As a negative control, an aliquot of cells from each cell line was stained with an irrelevant mAb of the same phenotype and a secondary antibody. To determine the intracellular MMPs and TIMPs, the prepared tumor cells were first fixed with 1% formalin in PBS supplemented with 1% FCS for 5 min on ice, followed by permeabilization with 0.1% Triton X-100 in PBS with 1% FCS for 5 min at 0˚C. The staining method for each mAb was the same, as described above. Flow cytometric analysis was performed on FACScan (Becton-Dickinson, Mountain View, CA, USA), and data were processed using the CellQuest program (Becton-Dickinson). To analyze tumor cells, a gated area was selectively created around the tumor cells using forward angle vs. light scatter parameters to exclude cell debris as described previously (1-3,6,7). The data were collected on at least 10,000 cells per sample. The percentage of fluorescence-positive events was determined by setting gates to exclude ≥99% of positive cells (fluorescent) in the isotype control from each patient.

*Statistical analysis*. The results were expressed as the mean ± SEM percentage of cells having a given antigen expression. The data were compared using the Student's t-test and considered significant at a p-value of $<0.05$.

## Results

Representative FACS overlay histograms for the expression of MMP-2, -7, and -9, MTI-MMP, and TIMP-2 and -4 in the epithelial adenocarcinoma cell line (MKN-45) of the stomach are shown in Fig. 1. The upper part of Fig. 1 revealed low or undetectable levels of cell-surface expression of MMPs and TIMPs on non-permeabilized MKN-45 tumor cells. On the contrary, high levels of MMP and TIMP expression are

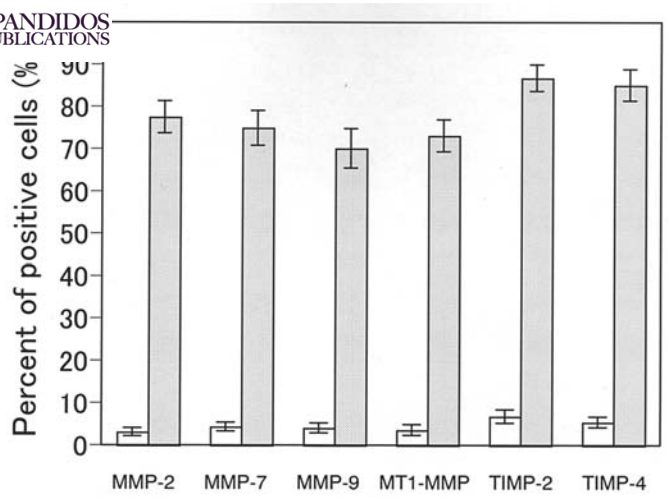


Figure 3. Summary results of the cell-surface and intracellular expression of MMP-2, -7, and -9, MT1-MMP, and TIMP-2 and -4 in 21 tumor cell lines. Open bar, non-permeabilized cells for cell-surface expression; hatched bar, permeabilized cells for intracellular expression. Values are the mean percentage of positive cells (mean ± SEM). There are significant differences in expression of MMPs and TIMPs between non-permeabilized and permeabilized cells ($p<0.001$).

shown in permeabilized MKN-45 cells in the lower part of Fig. 1. Similar findings were observed in the T-cell leukemia cell line, CCRF-CEM, which belonged to one of the stromal tumor cells (Fig. 2). The expression of MMP-2, -7, and -9, MTI-MMP, and TIMP-2 and -4 in non-permeabilized and permeabilized tumor cell lines is summarized in Fig. 3. Living or non-permeabilized tumor cell lines showed very low levels of cell-surface expression of MMPs and TIMPs. However, the mean percentages of MMP-$2^+$, -$7^+$, and -$9^+$, MTI-$MMP^+$, and TIMP-$2^+$ and -$4^+$ permeabilized tumor cell lines were significantly higher than the corresponding values of non-permeabilized tumor cell lines ($p<0.001$).

## Discussion

Quantitative flow cytometric analysis has clearly shown that the expression of MMP-2, -7, and -9, MTI-MMP, and their inhibitors TIMP-2 and -4 in 21 tumor cell-lines is largely intracellular and rarely on the cell surface. Previous flow cytometric analyses, however, have revealed that soluble or secreted MMPs and TIMPs as well as membrane type 1-MMP are mainly localized on the cell surface of tumor cells, TIL and myofibroblasts, particularly in metastatic carcinoma nests derived from malignant ascites to become an active form (1-3). On the contrary, data are limited on the localization of MMPs and TIMPs of tumor cell lines *in vitro*.

Tumor cell lines (SC-1, -2, -3, and -4, GCIY, G-415, G-930, BD-1, P1119 and P1229 cells) examined in the current study were established from carcinoma cells with malignant ascites in our laboratories. Based on previous studies, MMPs and TIMPs were expected to appear on the cell surface of tumor cells from these patients (1-3). However, the established tumor cell lines did not express MMPs and TIMPs on the cell-surface. It is important to explain the reason of their disappearance from the cell-surface after the long cultivation *in vitro*. Because, an elucidation of disappeared mechanism of the MMPs and TIMPs on the cell-surface makes clear the cellular or/and molecular activation mechanism of these proteolytic enzymes.

It has been reported that coculture with *H. pylori* and the MKN-45 human gastric cancer cell line or HT29 colonic adenocarcinoma cell line causes to significantly enhance steady state of protein or/and messenger RNA (mRNAs) of MMP-9 and MMP-7, compared with tumor cell lines alone (8,9). Similar findings have shown increased MMP-1, MMP-3, MMP-7, and TIMP-3 release or secretion by *H. pylori*-infected cultured human gastric fibroblast and adenocarcinoma cell lines *in vitro* (10-12). Furthermore, studies have shown the mechanism by which tumor-stromal cell contact influences the increased production and activation of MMPs in various tumor cell lines (13-15). Thus, the cell-surface expression of MMPs and TIMPs might be largely influenced by physical direct cell-cell contact among the different cell lineages. Indeed, evidence has shown that highly expressed intracellular MMPs and TIMPs in the cells rapidly appear on the cell surface after stimulation with tumor-associated antigen(s) or *H. pylori*-antigen(s), and then probably form the trimolecular complex, MT1-MMP/TIMPs/MMPs or bind to integrin, CD44, ICAM-1 and cell-surface heparin sulphate proteoglycan to become fully active (1-3,16-19). Intracellular expression of soluble MMPs and TIMPs in the tumor and TIL did not elucidate the actual function of proteolytic enzymes, since these molecules were secreted as latent pro-enzymes (1-3). The *in vitro* culture system presented here consists of tumor cells alone, and the MMPs and TIMPs do not express on the cell surface because the tumor cell alone *in vitro* is merely a microenvironment or society without stimulation, which is distinctly different from a real and complicated tissue-cell society *in vivo* with various stimulations. Therefore, the antigen-specific stimulation and/or paracrine tumor-stromal interactions within the local microenvironment *in vitro* and/or *in vivo* seem to be invariably needed to express and/or activate MMPs on the cell surface.

In conclusion, the MMPs and TIMPs are intracellularly localized at high levels, but rarely expressed on the cell surface in tumor cell lines alone. Hereafter, biological significance of the expression of secreted MMPs, TIMPs, and MT1-MMP in cultured tumor cell lines without evidence of cell-surface or/and intracellular expression should be evaluated with caution.

## Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan. Ms. Y. Jinsenji is greatly acknowledged for her technical help with flow cytometric analysis.

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