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Comparison of Metalloproteinase Protein and Activity Profiling

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Abstract

Proteolytic enzymes play fundamental roles in many biological processes. Members of the matrix metalloproteinase (MMP) family have been shown to take part in processes crucial in disease progression. The present study used the ExcelArray Human MMP/TIMP Array to quantify MMP and tissue inhibitor of metalloproteinase (TIMP) production in the lysates and media of 14 cancer and one normal cell line. The overall patterns were very similar in terms of which MMPs and TIMPs were secreted in the media versus associated with the cells in the individual samples. However, more MMP was found in the media, both in amount and in variety. TIMP-1 was produced in all cell lines. MMP activity assays with three different FRET substrates were then utilized to determine if protein production correlated with function for the WM-266-4 and BJ cell lines. Metalloproteinase activity was observed for both cell lines with a general MMP substrate (Knight SSP), consistent with protein production data. However, although both cell lines promoted the hydrolysis of a more selective MMP substrate (NFF-3), metalloproteinase activity was only confirmed in the BJ cell line. The use of inhibitors to confirm metalloproteinase activities pointed to the strengths and weaknesses of *in situ* FRET substrate assays.

Keywords

matrix metalloproteinase; FRET substrate assay; *in situ* MMP assay; protein array; MMP protein production

Introduction

Enzymes with proteolytic activity play fundamental roles in many biological processes. In normal physiology proteases help regulate cell growth, cell death, blood clotting, matrix remodeling, and immune defense. They have also been implicated in pathologies such as

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arthritis and tumor metastasis. There has been considerable effort to understand the role of proteases in disease and to identify therapeutic agents targeting specific protease activities. Members of the matrixin (matrix metalloproteinase; MMP) subfamily of metalloendopeptidases have been shown to take part in processes crucial in disease progression, such as the remodeling of the extracellular matrix (ECM), processing bioactive molecules, and ectodomain shedding [1;2;3].

The vast majority of studies that have correlated metalloproteinases with disease progression have focused on modulation of mRNA expression and/or protein production. The most commonly used high-throughput methods for determining protein abundance are protein microchips or planar antibody microarrays [4]. Unlike the genome, which contains a fixed number of genes, the levels of protein within the cell are highly dynamic. Proteins are continuously produced and undergo a wide range of post-translational modifications. In addition, protein families are extremely diverse and have considerable differences in their physical sizes, chemical and structural properties, affinity constants, and relative abundance within the cells. As a result, accurately characterizing protein production is extremely challenging [5].

Secreted MMPs are produced in inactive forms as zymogens and must be activated extracellularly. Once activated, their ability to process substrates is regulated by protease inhibitors [6;7] as well as protein localization. Thus, protease activity assays may be more representative of the potential of MMPs to participate in pathogenic states than protein production analysis. Protease activity assays are typically based on fluorescence resonance energy transfer (FRET) and utilize synthetic peptides [8;9]. Fluorescence can be detected with outstanding sensitivity, and these continuous assays allow for rapid, convenient kinetic evaluation of proteases and thus greatly aid mechanistic studies of these enzymes [8;10]. For screening compounds that might potentially modulate selective protease activities, there is a need for the study of substrates, which correspond, as closely as possible, to the native substrates of proteases. The hydrolysis of collagen (collagenolysis) is one of the committed steps in basement membrane turnover [11]. The triple-helical structure of collagen renders it resistant to most proteases. In vertebrates, the enzymes that cleave triple-helical structure include cathepsin K and collagenolytic MMP family members [12]. Synthetic triple-helical peptide (THP) substrates have been developed for continuous assays, with Mca/Dnp as the fluorophore/quencher pair [13]. These fluorogenic THP substrates are specific enough to be utilized to discriminate between certain MMP family members in kinetic assays *in vitro* [14], as well as in transfer FRET assays in cultured cells [15]. This latter method proved to be a straightforward and powerful method to assay secreted, active MMPs in cultured cells. However, the conditioned media needed to be centrifuged after withdrawal to remove any floating cells or cellular debris. With this step, this assay (a) become unsuitable to assay surface-bound proteolytic activity, (b) is an end-point method, and (c) is cumbersome to adapt for use with robotics and high-throughput formats.

In the present study, protein production levels of several MMPs and TIMPs have been assayed utilizing protein microarrays. Fifteen cell lines were analyzed: melanoma (M14 clones, WM115, WM-266-4, SK-MEL-2 and Mel53UM) and non-melanoma (RKO, Du145, IC4, MCF-7 and PC3) cancer cell lines, and a fibroblast cell line (BJ) (Table 1). Enzyme activity has been quantified for two of the cell lines using a novel *in situ* FRET cellular assay with three different substrates, and results compared with protein production. Finally, some of the challenges for developing *in situ* triple-helical peptidase FRET assays are addressed.

Materials and Methods

All cell culture and molecular biology reagents were obtained from Invitrogen unless otherwise stated. All standard chemicals were of analytical or molecular biology grade and purchased from Fisher Scientific or VWR. 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, 1-hydroxybenzotriazole, and 9-fluorenylmethoxycarbonyl (Fmoc)-amino acid derivatives [including Fmoc-Lys(Mca) and Fmoc-Lys(Dnp)] were obtained from EMD Biosciences (San Diego, CA). Amino acids are of L-configuration (except for Gly).

General Cell Culture Conditions

Cells were maintained in OptiMEM I Reduced Serum Medium supplemented with 4% FBS, 25 units/ml penicillin, and 0.025 mg/ml streptomycin sulfate. Cells were cultured with complete medium at 37 °C in a humidified atmosphere of 5% CO₂ in air and harvested from sub-confluent (<80%) cultures using a trypsin-EDTA solution. All utilized cell lines are given in Table 1.

Quantification of Proteins

The cultured cells were directly lysed on the tissue culture plate using the following lysis buffer: 150 mM NaCl, 50 mM Tris HCl, and 1% Triton-X, pH 7.5. The lysis buffer also contained the following protease inhibitors: pepstatin A (1 µg/mL), AEBSF (1 mM), aprotinin (54,900 KIU/mL), and leupeptin (10 µg/mL). The cultured cells were rinsed once with PBS, then 300 µL lysis buffer was added to one standard size (100 mm diameter) tissue culture dish and collected with a tissue culture cell scraper. The lysates were kept on ice and stored at -20 °C. The protein content of the cell lysates was quantified by the Pierce BCA Protein Assay Kit (Thermo Scientific) carried out in a 96-well plate format in triplicate using a SpectraMAX Plus Absorbance Plate Reader (Molecular Devices).

Protein Microarray

The ExcelArray Human MMP/TIMP Array (Thermo Scientific) utilized here was a multiplex sandwich fluorescent immunoassay (FIA) for the simultaneous quantitative measurement of MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, MMP-13, TIMP-1, TIMP-2 and TIMP-4. Each ExcelArray Slide contained 16 wells (subarrays) of 12 target-specific antibodies and a negative and positive control, all spotted in triplicate on the PATH *plus* Protein Microarray Slide. The kit also contained lyophilized 12-plex Standards, for preparing the standard curve (10000, 3333, 1111, 370 and 123 pg/mL), diluted in Array Sample Diluent (a proprietary formulation containing Mg(NO₃)₂, 5-chloro-2-methyl-2,3-dihydroisothiazol-3-one, MgCl₂•6H₂O, and Cu(NO₃)₂•3H₂O, as well as other components not listed).

12,732 cells/cm² tissue culture dish surface was plated in full OptiMEM media 36 h before harvesting the conditioned media or lysing the cells. The array was washed once with the Wash Buffer (20–25% NaCl, 3–5% monobasic sodium phosphate, 1–3% Tween-20). 100 µL of the standards or sample (conditioned media neat or cell lysate diluted four-fold in Array Sample Diluent) was added to each well of the array. The covered slide was incubated at room temperature for 2 h with shaking at 200 rpm to allow for the spotted antibodies to capture MMPs or TIMPs in standards or samples added to the wells. The non-bound proteins were removed by washing the slide 3 times with Wash Buffer. 75 µL Biotinylated Detection Reagent was added to each well, and the covered slide was incubated at room temperature for 1 h with shaking at 200 rpm. After washing the slide 3 times to remove excess detecting antibody, 100 µL Streptavidin-DyLight 649 Reagent was added and the covered slide was incubated at room temperature for 30 min with shaking at 200 rpm. The

slide was finally washed 5 times with Wash Buffer and rinsed in Final Rinse Buffer. The array was centrifuged at $200 \times g$ for 1 min to remove excess liquid and sent in to the manufacturer for scanning and data analysis. The signal associated with each spot was measured by a fluorescence slide reader. The intensity of fluorescent signal is proportional to the amount of each MMP or TIMP in the standard or sample.

Visual Representation of the Protein Microarray Data

In the current study the protein production datasets are visualized with the aid of a heat map [16]. The data tables are represented graphically by coloring each cell on the basis of the measured production value. Cells with production values of 0 (under 0.1 ng/mL was undetectable) are colored black. Cells with production values above the detection limit were colored dark maroon (noted by “OL”) (Figure 1). The protein content of the cell lysates shows the total amount of protein in ng found associated with 1,000,000 cells seeded and the ECM components deposited onto the tissue culture dish (Figure 1A). The protein content of the media represents the amount of proteins secreted into the growth media during culturing in the span of 36 h (Figure 1B).

In situ Enzyme Assay

The *in situ* enzyme assays were run in a 384-well format on a tissue-culture treated opaque microplate (Corning). The cells were seeded in OptiMEM I Reduced Serum Medium supplemented with 4% FBS at 4,000 cells/well density (approximately 30,000/cm² surface area) and then incubated for 3–5 h before starting the assay. The assays were carried out in serum-free OptiMEM I. The cells were incubated with the single-stranded or triple-helical fluorogenic substrates with or without inhibitors overnight (16 h). Proteolytic activity was determined by calculating the percentage increase in fluorescence compared to the background signal provided by the corresponding dilution of the substrates and/or inhibitors with no cells or exogenous enzymes using a multiwell plate fluorimeter (SpectraMAX Gemini EM, Molecular Devices) at $\lambda_{\text{excitation}} = 324 \text{ nm}$ and $\lambda_{\text{emission}} = 393 \text{ nm}$. Cell viability and proliferation was assessed using Alamar Blue. Viability was calculated as the percentage Alamar Blue reduction from multiwell plate fluorimeter at $\lambda_{\text{excitation}} = 540 \text{ nm}$ and $\lambda_{\text{emission}} = 590 \text{ nm}$.

All inhibitors were purchased from EMD Biosciences. All substrates and inhibitors were diluted in serum-free OptiMEM I medium. Type I collagen dilutions were prepared in 0.05 N acetic acid in 50% ethanol, and used to coat non tissue-culture treated surfaces at 6.25 $\mu\text{g}/\text{cm}^2$. ProMMP-8 was activated by incubating with 1 mM APMA for 1 h at 37 °C. Recombinant MMP-9 (catalytic domain without the fibronectin repeats), designated MMP-9CD-FN, was expressed in *E. coli* [17]. The syntheses, purification, and analyses of the linear and triple-helical fluorogenic substrates were performed as described [13;18].

Results

The production of MMPs and their inhibitors (TIMPs) was examined in lysates and media for 15 cell lines (Table 1). The overall patterns are very similar in terms of which proteins are secreted in the media versus associated with the cells in the individual samples (Figure 1). However, more protein was found in the media, both in amount and in variety. Looking at the cell lysate heat map (Figure 1A), the metastatic melanoma cell lines M14.11 and SK-Mel-2 and the non-melanoma IC4, Du145, and PC3 cells showed no detectable proteins. The cell lines M14.P, M14.FV, MCF-7, and RKO only produced low amounts (0.1 – 0.5 ng) of TIMP-1 or TIMP-2. M14.5 showed low production of MMP-8, TIMP-1, and TIMP-2 (<1.0 ng). There was no detectable production of MMP-9, MMP-12, MMP-13, or TIMP-4 in any of the assayed cell lines. The matched primary and metastatic melanoma cell lines

WM115 and WM-266-4 both showed moderate to high level production of a variety of MMP and TIMP proteins. The Mel53UM cell line and the BJ fibroblasts showed production of MMP-1, MMP-2, TIMP-1, and TIMP-2. MMP-3 was only produced in the WM115.b and BJ cell lines. MMP-10 was only produced in the WM115.b and Mel53UM cells.

Looking at the secreted protein production (Figure 1B), all cell lines produced at least moderate amounts (~25 ng) of TIMP-1. MMP-13 was the only protein that was undetectable across all samples. The four M14 clones showed some similarities. M14.11 and M14.FV produced low levels of MMP-1 and TIMP-2. The parental M14 cell line and M14.5 were nearly identical in that they both showed low levels of MMP-2 and TIMP-2. The parental cells produced some MMP-1 as well. The WM115.a and the WM-266-4 matched cells were nearly identical, producing moderate levels of MMP-1, MMP-2, MMP-8, and TIMP-2. The primary cell line WM115.a also had moderate levels of MMP-3. The WM115.b cells were again very similar to the original WM115.a cells, except that they produced low levels of MMP-7, MMP-9, MMP-10, MMP-12, and TIMP-4, as well as producing high levels of MMP-1 and TIMP-1. The SK-Mel-2 cells only produced low levels of MMP-8 and moderate levels of TIMP-2. IC4 (an MMP-9-overexpressing subclone of MDA-MB-231) showed low levels of MMP-9, TIMP-2, and TIMP-4. The Du145 cells produced low amounts of MMP-7.

Considering both cell lysates and media, MMP-13 was the only MMP not to be produced. MMP-7, MMP-9, MMP-10, and MMP-12 had low and sporadic production. MMP-1 was found in high amounts (as much as 10 ng) in cell lysates, as well as in secreted form in the media, except for the BJ fibroblasts, where it was predominantly detected in media. MMP-2 was evenly distributed between the cell associated and the secreted form. The in-house derived Mel53UM cell line proved to be the exception, as it had high levels of MMP-2 in the cell lysate. MMP-3 was only expressed in some of the cell lines and mainly in the secreted form. Only two samples showed low, but detectable levels of cell-associated MMP-3 protein. MMP-8 was mainly found secreted in media. However, with the exception of one cell line, all samples that expressed the protein in the media showed low, but detectable levels of MMP-8 in the cell lysates. The exception was the SK-Mel-2 metastatic melanoma cell line that did not have any cell-associated MMP-8.

In order to perform MMP activity profiling, several substrates were considered (Table 2). The Knight SSP is an excellent substrate for a wide variety of MMPs [19;20]. It is also readily hydrolyzed by ADAM10 and ADAM17 [21] and cathepsins D and E [22]. NFF-3 is selectively hydrolyzed by MMP-3 and MMP-10 within the MMP family [18], and also cleaved by trypsin, hepatocyte growth factor activator, and factor Xa [23]. fTHP-15 is based on a consensus sequence derived from the collagenolytic MMP cleavage sites in human types I-III collagen [12;24] and is hydrolyzed by MMP-1, MMP-2, MMP-8, MMP-9, MMP-13, MMP-14, and MMP-15 [24;25;26].

The WM-266-4 cell line was chosen for activity profiling based on its appreciable levels of cell associated and soluble MMP-1, MMP-2, and MMP-8, as well as the presence of TIMP-1 and TIMP-2 (Figure 1). The BJ cell line was chosen for comparison as it produced a similar, but not identical, profile as WM-266-4. For example, both cell lines produced MMP-1, MMP-2, TIMP-1, and TIMP-2, but only WM-266-4 produced MMP-8, while BJ cells produced MMP-3 (Figure 1). Using a constant number of WM-266-4 cells, overnight treatment with an increasing Knight SSP concentration resulted in increasing fluorescence emission which plateaued at ~17 μ M substrate (Figure 2). After overnight reaction the cells were found to be viable. Thus, the increase in fluorescence signaled proteolytic activity by live cells. The saturation curve was still in the linear range at 5 μ M substrate concentration

and thus this was chosen as the working concentration for the Knight SSP for all subsequent experiments.

Profiling experiments were repeated using NFF-3, to profile potential activities of stromelysins (MMP-3, MMP-10). Using a constant WM-266-4 cell number, overnight treatment with an increasing NFF-3 concentration resulted in increasing fluorescence emission which plateaued at ~20 μ M substrate (Figure 2). The concentration range used was well within the previously determined range (2.5 – 75 μ M) where self-quenching did not occur [18]. After overnight treatment with substrate, the cells were found to be viable. Thus, the increase in fluorescence signaled NFF-3 hydrolysis by live cells. The saturation curve was still in the linear range at 7.5 μ M substrate concentration and thus this was chosen as the working concentration for NFF-3 for all subsequent experiments.

NFF-3 can be considered more specific than the Knight SSP, as it is only hydrolyzed by two MMPs, and has not been described to have an affinity for other metalloenzymes such as ADAM family members. However, both the Knight SSP and NFF-3 can be hydrolyzed by non-metalloenzymes. Therefore, we attempted to verify the extent of metalloenzyme activity towards Knight SSP and NFF-3 hydrolysis. The homophenylalanine-hydroxamic acid broad-spectrum, cell-permeable, reversible inhibitor of metalloenzymes (designated “MMP Inhibitor III” by the manufacturer) was used to evaluate metalloproteinase activity. The inhibitor IC₅₀ is in the nM range, and it proved to be non-cytotoxic in the μ M range (data not shown). The Knight SSP was added to WM-266-4 cells or to MMP-9 in the presence or absence of inhibitor. The presence of the purified enzyme or the cells resulted in very similar results, with an increase of ~600 RFU overnight (Figure 3). In case of MMP-9, this activity was completely inhibited by the addition of MMP Inhibitor III (Figure 3). In case of the live cells, the same concentration of MMP Inhibitor III resulted in a 50–75% decrease in proteolytic activity (Figure 3). When MMP-9 was spiked in with the cells, the activity doubled. After the addition of the inhibitor, the measured activity decreased ~8-fold (Figure 3). The WM-266-4 metastatic melanoma cell line did not show detectable levels of MMP-3 or MMP-10 (Figure 1). When NFF-3 was added to cells in the presence or absence of the MMP Inhibitor III, there was no appreciable change in the observed proteolytic activity (Figure 3), indicating non-specific proteolysis.

The Knight SSP and NFF-3 was then added to the BJ cells in the presence or absence of inhibitor. The proteolytic activity was inhibited ~80% in the hydrolysis of the Knight SSP and ~60% in the hydrolysis of NFF-3 (data not shown). Thus, the proteolytic activity in BJ cells for both substrates was metalloproteinase-based. This is not unexpected considering BJ cells produced MMP-3, whereas WM266-4 cells produced undetectable levels of this enzyme (Figure 1).

In situ collagenolytic behavior was examined using a triple-helical peptide model of the collagenase cleavage sites in types I-III collagen (fTHP-15). fTHP-15 was added to WM-266-4 cells and the fluorescence measured after 16 h (Figure 2). The observed increase in fluorescence was never greater than 100 RFU above background over the concentration range tested. The highest change in fluorescence detected, 74 RFU above the background, was observed at 8 μ M substrate concentration. This extremely low level of observed activity led us to believe that (a) MMPs with triple-helical peptidase activity were not being produced or activated under these conditions, or (b) the assay conditions required further optimization.

In order to induce collagenolytic activity, WM-266-4 cells were seeded on a surface that previously had been coated with collagen type I. Prior studies had shown that type I collagen induces expression of MMP-1 via the α 2 β 1 integrin [27]. In another approach, cells were

grown to over 100% confluency. The focal points that formed in the “over-confluent” cultures were meant to model the three-dimensional surroundings of the cells under physiological conditions. However, there was no appreciable change in proteolytic activity overnight after the addition of fTHP-15 in either case (data not shown). Phorbol myristate acetate (PMA) treatment upregulates and turns on expression of gelatinases, collagenases, and stromelysin [28]. Thus, the cells were treated with PMA in order to induce MMPs. Increasing concentrations of PMA and/or varying the length of treatment did not increase triple-helical peptidase activity. However, PMA did induce proliferation, and the increased Knight SSP and NFF-3 proteolysis observed correlated with the increased cell numbers (data not shown).

MMPs are produced as inactive zymogens and thus need to be proteolytically processed in order to be activated. *p*-Aminophenyl-mercuric acetate (APMA) or trypsin is routinely employed to activate latent MMPs *in vitro*. We thus examined the role of endogenous activators on triple-helical peptidase activity. APMA was found to be highly cytotoxic over a wide range of concentrations, and thus could not be used. Trypsin, however, was found to have a proliferative effect on the cultured cells. Still, there was no increase in proteolytic activity after employing trypsin treatment compared to pre-treating the cells with the trypsin inhibitor, aprotinin (data not shown).

Detergents such as Brij-35 (polyoxyethyleneglycol-dodecyl-ether) or Tween-20 are routinely employed in enzyme assay buffers. In many reports on refolding MMPs, a low level (0.5%) of Brij-35 was used to improve the refolding rate and activity [13]. As Brij-35 is a detergent, however, it disrupts cell membranes, conferring significant cytotoxicity even at extremely low concentrations (data not shown). In order to evaluate the necessity of Brij-35 and to explore the use of other, nontoxic detergents in place of Brij-35 in the enzyme assay, 1% Pluronic F-68 (polyoxyethylene-polyoxypropylene block copolymer) was tested in the WM-266-4 and BJ cell lines. Pluronic F-68 is a nonionic detergent that is commonly used in cell cultures as a nontoxic cytoprotectant, to prevent shear induced cell damage [29].

For WM-266-4 induced hydrolysis of 5 μ M Knight SSP or 10 μ M fTHP-15, activity levels were similar in OptiMEM and Pluronic F-68 (Figure 4A). When the same number of BJ cells compared with WM-266-4 cells were utilized, 5 μ M Knight SSP or 7.5 μ M NFF-3 was processed overnight at a much higher rate, ~3,200 RFU and ~2,200 RFU above the baseline, respectively (Figure 4B). Pluronic F-68 supported similar levels of activity towards the Knight SSP and fTHP-15 as Brij-35 for the BJ cells (Figure 4B). This result indicates that some of the BJ cell activity was generated by MMPs that were less sensitive to Brij, possibly MMP-1, MMP-2, and/or MMP-3.

The difference between WM-266-4 and BJ cell activities may be attributable to MMP-8 (Figure 1). To examine this possibility, full-length MMP-8, freshly activated with APMA, was added at four different concentrations to 5 μ M Knight SSP in serum-free OptiMEM supplemented with 0.5% Brij-35 (Figure 5A). When comparing the proteolytic activities observed in the presence of Brij-35 to the signal observed in the absence of the detergent, there was an 80–90% drop in activity without Brij-35. The lowest enzyme concentration used was 0.5 nM. Without the detergent, the activity of this amount of enzyme was below detection limits. An in-house derived MMP-8 overexpressing cell line expressed MMP-8 in the 0.1 nM range (data not shown). Therefore, even cell lines that overexpress MMPs do not express enough to be in the detectable range of the fTHP-15 assay without Brij-35. Thus, Brij-35 is absolutely necessary to achieve the sensitivity to be able to detect specific enzyme activity in this concentration range. However, with its level of cytotoxicity, this detergent cannot be used for the *in situ* fluorogenic assay. The addition of Pluronic F-68 did not

induce the same level of MMP-8 activity as Brij-35 with cell medium as the reaction buffer (Figure 5B).

Discussion

The present study has profiled MMPs on the levels of protein production and protein activity. In several cases, distinct differences have been observed regarding the localization of the proteins examined. The protein content of the cell lysates and conditioned media from 15 cell lines were tested. The proteins found in the cell lysates were presumably associated with the ECM components deposited by the cell or bound on the cell surface. Cell-directed focal proteolysis by plasma membrane-associated proteinases is critical to a number of physiological and pathological processes including tissue remodeling, angiogenesis, and leukocyte and tumor cell migration [6]. Therefore, the association of secreted proteases to the cell surface increases the proteolytic potential of a cell and results in high local concentrations of the proteases.

Possible cell association was observed for MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, and MMP-10 (Figure 1). The cell surface proteoglycan CD44 has been previously shown to bind to MMP-7, MMP-9, and MT1-MMP [30;31;32;33;34;35]. The overexpression of CD44 is found on a variety of tumor cells [36], and elevated CD44 expression is associated with tumor growth and metastasis [37]. MMP-2 binds to MT1-MMP and the $\alpha\text{v}\beta 3$ integrin [38;39], while MMP-1 binds to the $\alpha 2\beta 1$ integrin [40;41]. At present, it is not known what cell surface molecules MMP-3, MMP-8, and MMP-10 may bind to, or if their presence in cell lysates is due to intracellular release or release from ECM components.

A continuous assay method utilizing an increase in fluorescence upon hydrolysis allows for rapid and convenient kinetic evaluation of proteolytic activity. Ideally, protease function should be assayed under conditions representative of physiological conditions such as cell culture. The foundations of an *in situ* FRET assay have been established here for a better understanding of the behavior of metalloproteinases in a cellular context. Using linear substrates (Knight SSP and NFF-3) an increase in fluorescence was verified to signal proteolytic activity by live cells and in part due metalloenzyme activity. The Knight SSP is processed by MMP-1, MMP-2, MMP-3, and MMP-8, amongst others [19;21]. As demonstrated here, the WM-266-4 and BJ cells each produced three of these enzymes (Figure 1 and Table 3). Utilizing the *in situ* FRET assay with a hydroxamate-based metalloenzyme inhibitor, the observed proteolytic activity was verified to signal (for the most part) the presence of active metalloproteinases (Figure 3 and Table 3), presumably the presence of MMP-1, MMP-2, MMP-3, and/or MMP-8.

NFF-3 is cleaved by MMP-3 and MMP-10 and other non-metalloenzymes. When utilizing the *in situ* FRET assay, NFF-3 proteolysis was detected (Figure 2). This was, in the case of WM-266-4 cells, not inhibited by the metalloenzyme inhibitor (Figure 3), clearly signaling the absence of detectable levels of active MMP-3 or MMP-10 produced by these cultured melanoma cells. Consistent with this, the WM-266-4 cells did not produce these enzymes on the protein level (Figure 1 and Table 3). Therefore, the absence of MMP-3 and MMP-10 was verified on two levels: protein production and activity. In case of the BJ cells, MMP-3 was produced (Figure 1) and the processing of NFF-3 inhibited by the MMP Inhibitor III (Figure 4B). Therefore, MMP-3 in the BJ cells was observed as protein production and consistent with enzyme activity (Table 3).

Protease function was also assayed using the physiologically more relevant and narrower specificity triple-helical fluorogenic substrate fTHP-15. The hydrolysis of the triple-helical substrate fTHP-15 only resulted in 74 RFU above the fluorescence baseline (Figure 2). The

production of the collagenase MMP-1 was observed on the protein level in both WM-266-4 and BJ cell lines, while MMP-8 was only produced in the WM-266-4 cells (Figure 1). Using this substrate, the corresponding triple-helical peptidase activity was not confirmed. The same substrate has been successfully used earlier in a transfer FRET assay to detect MMP-activity in conditioned growth medium [15]. In that work, however, the growth media containing the secreted MMPs were added to the fluorescent substrates containing Brij-35. Brij-35 presumably stabilized the active forms of the MMPs, leading to an enhanced signal. Therefore, there are two major challenges still to be considered for an *in situ* triple-helical peptidase assay. First, a non-cytotoxic Brij-35 replacement capable of stabilizing MMP activity must be identified. The present study showed some potential for Pluronic F-68 in this regard. Second, the sensitivity of detection needs to be improved, especially considering the low levels of MMPs expressed by cultured cells. Compounding this problem is the lower k_{cat}/K_M value for MMP hydrolysis of fTHP-15 compared with the single-stranded Knight SSP and NFF-3 substrates [18;42;43]. One possible solution would be incorporating alternative fluorophores into the existing FRET substrates. Such fluorophores could have higher quantum yields [such as 5-carboxyfluorescein (Fam), with $\Phi_F = 0.92$, compared with $\Phi_F = 0.49$ for Mca] or be less susceptible to fluorescence quenching, as exemplified by the prior labeling of NFF-3 with a CyDye pair, Cy3/Cy5Q [10;44].

TIMPs were detected in all cell lines (Figure 1), leading one to question why any MMP activity was observed. The primary function of TIMPs is often ascribed to controlling the activity of MMPs and other metalloproteinases. It has also been suggested that TIMPs could have a function in the cell nucleus, possibly playing a role in regulating gene transcription [45]. TIMPs affect cell growth and survival through their cell growth-promoting and anti-apoptotic activity [46;47;48]. There is a growing body of evidence that TIMP-1 and TIMP-2 transduce intracellular signaling [47;49;50;51;52]. Therefore, one possibility is that the TIMPs are present but not fully available as MMP inhibitors because they are associated in alternative protein-protein complexes. Unfortunately, it is not known if the ExcelArray Protein Chip can detect the target proteins in complexed forms.

While all profiling methods suffer some drawbacks, activity-based profiling presents the most relevant picture for the potential role of an enzyme in biological processes. Activity-based profiling of MMPs has been reported previously utilizing small molecule MMP probes [53;54;55;56;57;58] or MMP FRET substrates [15;59;60;61]. The present study presents encouraging results for cell-based screening of MMP activity. Of particular note is that the present approach may ultimately be fine-tuned to accommodate selective MMP substrates, both in linear and triple-helical form [14;62;63;64;65;66;67].

A recent comparison of MMP-9 ELISA and activity assays showed an excellent correlation for APMA-activated plasma samples that allowed for determination of enzyme specific activity [68]. In non-activated plasma samples the ELISA and activity assays were used to demonstrate the natural amount of active MMP-9 present [68]. It was ultimately determined that activity assays alone, using APMA activation and without activation, were sufficient to evaluate total and active MMP-9 [68]. However, these studies did not include an inhibitor to determine if all of the activity was due to a metalloproteinase.

Other approaches beyond FRET substrate assays have been described for profiling MMP activity from biological fluids and tissues. Several are based on mass spectrometry, including the aforementioned use of small molecule MMP probes. The small molecule probes are inhibitors that are used to isolate MMPs, with subsequent identification of enzymes by liquid chromatography-electrospray tandem MS [53;58]. This approach allows one to determine concentrations of active proteases, but not enzyme kinetics. MALDI-TOF MS has been utilized to monitor type I collagen fragments produced by different

collagenases [69]. Drawbacks of this method are that collagenases that produce the same fragments (such as MMP-1, MMP-8, and MT1-MMP) cannot be differentiated, and the kinetics of reactions are difficult to quantify. *In vivo* analysis of MMP activity has been achieved by infusion of MMP substrates via implanted microdialysis probes and LC-MS/MS analysis of products that diffuse back into the probe [70]. This method relies on selective substrates for individual MMP identification and mostly likely could not be utilized for kinetic analysis due to the non-quantitative nature of back diffusion of substrate products. Second ion mass spectrometry has been used to monitor MMP processing of surface-bound peptide-conjugated gold nanoparticles [71]. This method also relies on selective substrates and does not offer analysis of kinetics. Additional activity monitoring approaches require that the MMP be bound to surfaces [72;73;74], and thus are not applicable for screening MMPs in solution.

Conclusions

The present study has examined the use of the ExcelArray for profiling MMP protein production, compared MMP protein production with protease activity, and identified strengths and weaknesses in *in situ* MMP FRET substrate assays. The ExcelArray provided a rapid and convenient method for profiling soluble and membrane-bound MMP production by a variety of cell lines. The technology utilized for ExcelArray could be readily duplicated in multi-well formats with MMP-specific antibodies. The limitation in protein production analysis is that no indication is given as to the amount of active protein. Even if antibodies are utilized that recognize activated MMPs (as opposed to zymogens), MMPs inhibited by TIMPs may not be differentiated from inhibitor-free MMPs. Comparison of protein production to activity analysis showed consistency between the two (Table 3). However, activity analysis required confirmation with inhibitors to rule out non-specific proteolysis. Finally, monitoring *in situ* activity in the presence of detergents is problematic, but for some MMPs Pluronic F-68 may be used instead of Brij-35.

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Figure 1. ExcelArray Human MMP/TIMP Array analysis of MMP and TIMP protein content for (A) cell lysates and (B) cell culture media in 15 cell lines. OL = over the detection limit.

Figure 2. WM-266-4 cell activity towards FRET substrates Knight SSP (closed, inverted triangles), NFF-3 (closed circles), and fTHP-15 (open circles) as a function of substrate concentration. 4000 WM-266-4 cells/well were utilized. RFU = relative fluorescence units.

Figure 3.

Inhibition of *in situ* peptidase activity by MMP Inhibitor III. Conditions were 4000 WM-266-4 cells/well, 2 nM MMP-9 catalytic domain (without the fibronectin motifs), 5 μ M Knight SSP (blue bars), 7.5 μ M NFF-3 (red bars), 10 μ M fTHP-15 (green bars), and 20 μ M MMP Inhibitor III. RFU = relative fluorescence units.

A **B** **Figure 4.**

Effect of detergents on cellular peptidase activity. Conditions were (A) 4000 WM-266-4 cells/well, 5 μ M Knight SSP (blue bars), 10 μ M fTHP-15 (green bars), 20 μ M MMP Inhibitor III, and no detergent (OptiMEM bars) or OptiMEM + 1% Pluronic F-68 (P F68 bars); (B) 4000 BJ cells/well, 5 μ M Knight SSP (blue bars), 7.5 μ M NFF-3 (red bars), 10 μ M fTHP-15 (green bars), 20 μ M MMP Inhibitor III, and no detergent (OptiMEM bars), OptiMEM + 1% Pluronic F-68 (P F68 bars), or OptiMEM + 0.5% Brij-35 (Brij-35 bars). RFU = relative fluorescence units.

A

B

Figure 5.

Effect of detergents on MMP-8 peptidase activity. Conditions were (A) 0.5, 1.0, 2.5, or 5.0 nM MMP-8, 5 μ M Knight SSP, and 0.5% Brij-35 (closed circles) or no Brij-35 (open circles); (B) 5 nM MMP-8, 5 μ M Knight SSP, and OptiMEM (left bar), OptiMEM + 1% Pluronic F-68 (middle bar), or OptiMEM + 0.5% Brij-35 (right bar). RFU = relative fluorescence units.

Table 1

Cell lines used in the protein array experiments.

Cell line	Type	Origin
M14.P	metastatic melanoma	*
M14.5	metastatic melanoma	*
M14.11	metastatic melanoma	*
M14.FV	metastatic melanoma	*
SK-Mel-2	metastatic melanoma	ATCC
WM115.a	primary melanoma	ATCC
WM115.b	primary melanoma	ATCC
WM-266-4	metastatic melanoma	ATCC
Mel53UM	metastatic melanoma, CHTN	CHTN, unknown tissue
BJ	foreskin fibroblast	ATCC
MCF-7	breast adenocarcinoma	†
IC4	breast carcinoma	‡
PC3	prostate adenocarcinoma	†
Du145	prostate carcinoma	†
RKO	human colorectal cancer	†

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† obtained as a generous gift from Dr. Ramaswamy Narayanan (Florida Atlantic University, Boca Raton FL);

‡ obtained as a generous gift from Dr. Sarah Dallas (University of Missouri - Kansas City, Kansas City, MO); ATCC, obtained from American Type Tissue Collection; CHTN, in-house derived cell line (tissue obtained from the Cooperative Human Tissue Network).

Table 2

FRET substrates utilized.

Name	Sequence
Knight SSP	Mca-Lys-Pro-Leu-Gly~Leu-Lys(Dnp)-Ala-Arg-NH ₂
NFF-3	Mca-Arg-Pro-Lys-Pro-Val-Glu~Nva-Trp-Arg-Lys-Lys(Dnp)-NH ₂ (Gly-Pro-Hyp) ₅ -Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-
fTHP-15	Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp) ₅ -NH ₂

“~” denotes the sites of hydrolysis.

Table 3

Comparison of MMP protein and activity detection.

Cell line	Protein Array (MMPs found in lysate)	Protein Array (MMPs found in media)	Activity Assay (Substrate hydrolyzed; class of protease)
WM-266-4	MMP-1, MMP-2, MMP-8	MMP-1, MMP-2, MMP-8	Knight SSP; metalloproteinase NFF-3; non-metalloproteinase
BJ	MMP-1, MMP-2, MMP-3	MMP-1, MMP-2, MMP-3	Knight SSP; metalloproteinase NFF-3; metalloproteinase