Tumorigenesis and Neoplastic Progression

Matrix Metalloproteinase-1 Associates with Intracellular Organelles and Confers Resistance to Lamin A/C Degradation during Apoptosis

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Since the first description of matrix metalloproteinase (MMP)-1 as an interstitial collagenase, great importance has been ascribed to this enzyme in extracellular matrix remodeling during tumoral, inflammatory, and angiogenic processes. As more evidence for the role of MMPs in targeting nonmatrix substrates emerges, casual observations that intracellular MMP-1 is found *in vitro* and *in vivo* prompt investigation of the role that MMP-1 may play on basic cell functions such as cell division and apoptosis. Here we show for the first time that MMP-1 not only has extracellular functions but that it is strongly associated with mitochondria and nuclei and accumulates within the cells during the mitotic phase of the cell cycle. On induction of apoptosis, MMP-1 co-localized with aggregated mitochondria and accumulated around fragmented nuclei. Inhibition of this enzyme by RNA interference or treatment with a broad MMP inhibitor caused faster degradation of lamin A, activation of caspases, and fragmentation of DNA when compared with untreated cells. These observations strongly suggest that intracellular association of MMP-1 to mitochondria and nuclei confers resistance to apoptosis and may explain the well-known association of this enzyme with tumor cell survival and spreading. (Am J Pathol 2005; 166:1555–1563)

Matrix metalloproteinase (MMP)-1, commonly known as collagenase-1, and able to cleave interstitial collagens,¹ is produced by various types of cells *in vitro* and *in vivo* and its expression has been associated to inflammation,

wound healing, and tumor invasion, growth, and metastasis.^{2–4} Although the main role ascribed to MMPs has been the degradation of extracellular matrix for facilitation of cell division, migration, and differentiation, more recent work has provided evidence for the role of these enzymes in targeting nonmatrix substrates, such as cell bound cytokines, enzymes, and cell surface receptors.^{5,6} It has been further suggested that there may still be more unknown substrates for these enzymes.⁶ Evidence for the role of various MMPs in mediating cell functions has been given by the demonstration that gelatinases A and B (MMP-2 and MMP-9) modulate cell proliferation and differentiation,^{7,8} and that inhibition of MMPs by natural inhibitors of matrix metalloproteinases (TIMPs) have diverse effects on proliferation and apoptosis in various cell types. For example, TIMPs-1 and -2 may exhibit growth factor-like activity,^{9,10} while TIMP-1 can also inhibit tumor cell growth.¹¹

Although most MMPs are secreted and activated extracellularly, some have been shown to be anchored to the cell surface by a transmembrane domain (MT1-, MT2-, MT3-, and MT5-MMPs) and to contain a short cytoplasmic tail at the C-terminal region of the protein that facilitates their interaction with intracellular proteins that regulate cell function.¹² Furthermore, intracellular MMP-1 in platelets has been shown to activate pathways that signal phosphorylation of intracellular proteins, distributes β_3 integrins to cell contact points, and primes these cells for aggregation. This provides new evidence that MMP-1 can regulate outside-in signaling events that control cell function and phenotype.¹³ In addition to platelets, intracellular localization of MMP-1 has been documented in various cells, including human vascular endothelium¹⁴ and rabbit synovial fibroblasts cultured in the presence of anti- $\alpha 5$ integrin antibodies.¹⁵ However, at present there is

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no indication of any potential role that MMP-1 may have on the mediation of cellular functions such as cell division and apoptosis. Given the functional importance of MMP-1 in the promotion of cell migration and matrix degradation, and based on preliminary observations in our laboratory, we have investigated the intracellular expression of this enzyme and its potential role in functions related to cell division and apoptosis.

Materials and Methods

Cell Culture

Cells used in this study included the human Muller glia cell line MIO-M1,¹⁶ the retinal pigment epithelial cell line ARPE-19,¹⁷ and primary cultured corneal and Tenon's fibroblasts. MIO-M1 cells and ARPE-19 were maintained in culture using Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies, Paisley, UK) containing L-glutamax I, 10% newborn calf serum, 2 mmol/L L-glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin (all obtained from Gibco). Human corneal and Tenon's fibroblasts were isolated from eyes donated for research from the eye bank at Moorfields Eye Hospital (London, UK) according to well established methods in our laboratory.^{18,19} After 3 hours of incubation with 2 mg/ml of dispase (neutral protease II; Boehringer-Mannheim, Lewes, UK) in phosphate-buffered saline (PBS) (pH 7.2), corneas were brushed to remove epithelium, and fibroblasts scraped into 3 ml of PBS. After two washes with PBS at 300 $\times$ g for 5 minutes, cells were cultured in DMEM containing 10% newborn calf serum, 2 mmol/L L-glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin (all obtained from Gibco).

Cell-Cycle Synchronization

Preparations enriched for cells in the M phase were obtained by synchronization of MIO-M1 cells with 0.1 μ g/ml of nocodazole (Sigma, UK) for 18 hours.²⁰ Cells were washed with PBS before addition of fresh culture medium to continue the cell cycle. Synchronized cells were examined for their expression of MMP-1 and the cell cycle proteins cdc2 and cyclin B by confocal microscopy and Western blot analysis. Levels of MMP-1 in the cell lysates were also examined by enzyme-linked immunosorbent assays (ELISAs) using commercially available kits (Amersham Pharmacia Biotech, UK).

Confocal Microscopy

For immunocytochemistry, cells were cultured in fibronectin-coated (5 μ g/ml) Lab-Tek glass chamber slides (Nunc, Inc., Naperville, IL). After appropriate treatments, cells were fixed in 4% paraformaldehyde in PBS for 10 minutes, and incubated for 3 hours with primary antibodies diluted in 0.5% blocking reagent (Roche, UK) in PBS. These included two monoclonal anti-MMP-1 antibodies (clones 41-1E5 and V13; Calbiochem, UK), sheep polyclonal anti-MMP-1 antibodies (The Binding

Site, UK, and antibody prepared by G.M.); monoclonal anti- α tubulin (Zymed, South San Francisco, CA). Mouse IgG isotypes matching those of the test antibodies, and total sheep IgG (Sigma) were used as negative controls. After incubation with primary antibodies, specimens were washed in Tris-buffered saline, followed by incubation for 1 hour with either rabbit anti-mouse antibodies conjugated with fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology, Santa Cruz, CA) or donkey anti-sheep antibodies conjugated with FITC or Cy5 (Santa Cruz Biotechnology). Golgi apparatus was visualized by staining with mouse monoclonal anti-human Golgin-97 antibody (Molecular Probes, The Netherlands). After staining for MMP-1 with primary and secondary antibodies, cells were co-stained either for mitochondria by incubation with 200 nmol/L MitoTracker red (Molecular Probes) for 15 minutes at room temperature, or lysosomes with 200 nmol/L LysoTracker green (Molecular Probes) for 2 hours. Slides were then washed and counterstained with 2 μ g/ml of 4',6'-diamino-2-phenylindole (DAPI) for 1 minute and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Fluorescent images were recorded using a Zeiss LSM 510 confocal microscope operating in multitrack mode for FITC, DAPI, and Cy5 fluorochromes.

Analysis of MMP-1 and Cyclins by Western Blotting

Cell lysis and Western blot analysis were performed as previously described.²¹ Aliquots of cell lysates (100 μ g protein) were resolved on 10% NuPAGE Bis-Tris-sodium dodecyl sulfate gels (Invitrogen, UK) for 50 minutes at 200 V in MOPS running buffer [50 mmol/L MOPS, 50 mmol/L Tris base, 0.1% sodium dodecyl sulfate, 1 mmol/L ethylenediamine tetraacetic acid (EDTA), pH 7.7; Invitrogen]. Proteins were then transferred to nitrocellulose membranes, blocked with 2% blocking reagent (Roche) in PBS and immunodetection performed using various monoclonal and polyclonal antibodies to MMP-1 (as for confocal microscopy), cyclin B, and cdc2 (Santa Cruz Biotechnology). Immunocomplexes were detected by enhanced chemiluminescence (Amersham) after incubation with goat or donkey antiserum against rabbit, sheep, or mouse IgG coupled to horseradish peroxidase (Santa Cruz Biotechnology). Images were analyzed and processed using a Fuji image reader LAS-1000 Pro, version 2.1 (Fuji, UK).

Preparation of Mitochondria

Mitochondria were isolated from MIO-M1 cells grown to confluence for 3 to 4 days. Cells were detached from monolayers by treatment with trypsin-EDTA (5% trypsin, 2% EDTA; Gibco BRL) for 3 minutes at 37°C. Crude mitochondria were obtained by incubating 10⁷ MIO-M1 cells in 1 ml of 10 mmol/L Tris-HCl buffer, pH 7.4, containing 10 mmol/L NaCl and 1.5 mmol/L MgCl₂ for 10 minutes on ice. After addition of 8 ml of 2.5 $\times$ MS buffer (12.5 mmol/L Tris-Cl, 525 mmol/L mannitol, 175 mmol/L

sucrose, 2.5 mmol/L EDTA, pH 7.4), cells were homogenized in a Dounce homogenizer and the suspension taken to a volume of 30 ml with 1× MS buffer. The mixture was centrifuged at $600 \times g$ for 20 minutes and the supernatant containing the platelets further centrifuged at $13,500 \times g$ for 15 minutes.²² The platelet pellet was washed twice with 1× MS buffer and resuspended in RIPA buffer containing 0.5 mmol/L dithiothreitol, 0.2 mmol/L phenylmethyl sulfonyl fluoride, 1 mmol/L EDTA, and 1:100 protease cocktail inhibitor (Sigma).

Preparation of HA and VsV-Tagged MMP-1 Plasmids

A polymerase chain reaction fragment exhibiting the full length sequence of human MMP-1 was generated from a pcD-X plasmid containing the MMP-1 sequence (American Type Culture Collection, Rockville, MD) according to established techniques.²³ This fragment was then cloned into the 2.1-TOPO Vector (Invitrogen) using the manufacturer's instructions. The cDNA fragment containing the MMP-1 sequence was then excised from the TOPO vector, sequenced to confirm orientation, and analyzed for homology using the Blast search of the Entrez Nucleotides database of the human genome. The fragment was finally cloned into a pCB6 vector expressing a C-terminal domain tagged to the VsV epitope (pCB6-MMP-1-VsV) and a pCB6 vector expressing the N-terminal domain tagged to the HA epitope (pCB6-MMP-1-HA).²³

Preparation of siRNA

The mU6pro vector was used for expression of RNA duplexes.²⁴ Two regions of human MMP-1 were targeted that are part of sequences coding for different protein domains (region I, 5'AACACAAGAGCAAGATGTGG3' and region II, 5'AAGGTGGACAACAATTTTCAG3'). As a control, a random sequence was also used: 5'AATCTACAAGCGAAAACAACT3'. Oligonucleotides and their correspondent anti-sense sequences (100 nmol/L/ml) were annealed in a slowly cooling bath with a starting temperature of 90°C, followed by ligation into the Bbs1/XbaI-digested and gel-purified mU6pro vector. Ligation was confirmed by release of the insert with Bbs1 and XbaI. Specific silencing was confirmed by at least three independent experiments.

Cellular Transfections

For confocal analysis of MMP-1 localization, Muller cells were plated to a density of 2×10^4 cells per well in glass culture slides coated with fibronectin (10 µg/ml) on day 1. On day 2, 250 ng of pCB6-MMP-1-VsV or pCB6-MMP-1-HA plasmids were mixed into 25 µl of DMEM media (Invitrogen). In a separate reaction, 0.4 µl of Lipofectamine 2000 (Invitrogen) were suspended in 25 µl of DMEM and allowed to incubate at room temperature for 5 minutes. The mixtures were combined, incubated at room temperature for 20 minutes, and added directly into the

wells containing 100 µl of DMEM without antibiotics or fetal bovine serum. Slides were incubated for 6 hours at 37°C, followed by addition of 50 µl of DMEM containing 40% fetal bovine serum. On day 5 cells were stained with antibodies to VsV and HA as outlined above. Specificity of staining for VsV or HA was controlled by staining individually transfected cells with both antibodies. For RNA interference assays, Müller cells were plated to near confluence in 25-cm² tissue culture flasks on day 1. On day 2 cells were transfected as above using 12.5 µg of mU6pro siRNA plasmid per flask and corresponding proportions of medium and Lipofectamine 2000. On day 5 cells were lysed with RIPA buffer as described above and processed for Western blotting with anti-lamin A/C antibodies.

Induction of Apoptosis

Apoptosis was induced by addition of 1 µmol/L staurosporine to untreated (control) cells or cells undergoing MMP-1 down-regulation by transfection with mU6pro vector expressing siRNA for MMP-1, or 72 hours of incubation with 100 µmol/L of the metalloproteinase inhibitor Ilomastat (GM 6001) or Ilomastat control (GM 6001-negative control) (Calbiochem). Apoptotic cells were examined for either intracellular localization of MMP-1, integrity of lamin A/C by Western blotting analysis, or total caspase activation using the caspase-FITC-VAD-FMK marker (Promega) and DNA fragmentation using the ApoTag Fluorescein *in situ* apoptosis detection kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. Cells labeled with the caspase-FITC-VAD-FMK marker were washed three times before labeling with DAPI to remove nonspecific binding to inactive caspase.

Results

Intracellular Accumulation of MMP-1 during the Mitotic Phase of the Cell Cycle

To investigate whether there is a relationship between the intracellular levels of MMP-1 and cell cycle progression, we synchronized glial Müller cells (MIO-M1 cells) in the M phase of the cell cycle with nocodazole, and examined the relationship between the intracellular levels of this enzyme and the levels of expression of the cell-cycling proteins Cdc2 and cyclin B. Western blot analysis of cell lysates obtained at various times after nocodazole release showed that the highest intracellular levels of MMP-1 are present at the times when the highest levels of the mitotic proteins cyclin B and cdc2 are also observed (Figure 1A). Although the proenzyme form (57 kd) of MMP-1 was predominant, the mature form of this enzyme (45 kd) was also observed (Figure 1A). These observations correlated with the intracellular levels of MMP-1 detected by ELISA assays (Figure 1B). Cell monolayers were also investigated for their phenotypic expression of MMP-1 and α -tubulin as a marker of cell-cycling stage. A granular pattern of intracellular staining for MMP-1 was

Figure 1. Intracellular accumulation of MMP-1 during the mitotic phase of the cell cycle. **A:** Western blot analysis of Müller cell lysates obtained after nocodazole synchronization, showing increased intracellular levels of MMP-1 during the M phase of the cell cycle. Higher expression of MMP-1, predominantly the 57-kD proenzyme, was observed at 0, 3, and 6 hours culture after nocodazole release. This corresponded to the increased expression of the mitotic cell-cycle proteins cdc2 and cyclin B at the same times after nocodazole release. **B:** Levels of MMP-1 measured by ELISA in lysates of synchronized cells confirmed that MMP-1 accumulates within the cells at the times when cdc2 and cyclin B are increased, and that they decrease as the cell cycle progresses. **C:** Characteristic granular pattern of staining for MMP-1 observed within Müller cells at various stages of the cell cycle, as determined by visualization of microtubule organization with antibodies to α -tubulin. The greatest intracellular accumulation of MMP-1 was observed at the time in which the mitotic spindle was clearly present (anaphase/metaphase), in accordance with that seen by Western blot analysis and ELISA assays. Perinuclear staining was often observed during late telophase.

observed throughout the whole cell cycle, being more prominent during anaphase/metaphase and corresponding to the highest intracellular levels of mitotic proteins observed in lysates of synchronized cells (Figure 1A). Perinuclear staining was often seen during cytokinesis (Figure 1C).

Association of MMP-1 with Mitochondria and Nuclei

Because of the granular appearance of the intracellular staining for MMP-1, we investigated the association of MMP-1 to various intracellular organelles, including mitochondria, Golgi apparatus, and lysosomes (Figure 2). We observed that MMP-1 staining did not co-localize with Golgi apparatus (Figure 2A) or lysosomes (Figure 2B), but clearly co-localized with mitochondria and nucleus (Figure 2C). To determine whether MMP-1 was either bound to mitochondria and or whether it was present inside these organelles, we incubated enriched preparations of mitochondria with various concentrations of proteinase K in the presence or absence of 1% Triton X-100 and examined the supernatants for the levels of MMP-1 in relation to the total concentration of protein released. As observed in Figure 2D, by decreasing the concentrations of proteinase K there was an increase in the levels of MMP-1 released into the medium in relation to the total amount of protein freed from the mitochondria. In the absence of permeabilizing agents (such as Triton X-100), low levels of proteinase K are unlikely to dissociate intra-mitochondrial protein, for which these results suggest that this enzyme dissociates MMP-1 bound to the surface. Addition of Triton X-100 to increasing concentrations of proteinase K was aimed to dissociate surface and intramitochondrial protein, for which lower levels of MMP-1 in relation to the total concentration of protein released further, suggests that MMP-1 was associated to the mitochondria membrane.

Intracellular Expression of MMP-1 by Nonglial Cells

We further investigated whether the association of MMP-1 to mitochondria and nuclei is a unique characteristic of glial Müller cells or whether it is also a feature of cells of nonglial origin. For this purpose we cultured Tenon's capsule fibroblasts, corneal fibroblasts, and retinal pigment epithelial cells and stained their monolayers for both mitochondria and MMP-1. Immunohistochemical staining revealed that MMP-1 was also associated to the mitochondria of all of the cells investigated (Figure 3A), and to the nuclei of Tenon's fibroblasts (Figure 3A) and ARPE cells (not shown). We confirmed the presence of this intracellular enzyme by Western blotting analysis of mitochondrial lysates, and in accordance, both the proactive (57 kD) and active (45 kD) forms of MMP-1 were also observed by immunoblotting (Figure 3B).

Mitochondrial and Nuclear Targeting of MMP-1 Constructs

The specificity of intracellular localization of MMP-1 was confirmed by transient transfection of the MIO-M1 cells with MMP-1 constructs in a pCB6 vector expressing either the C-terminal domain tagged to the VsV epitope (pCB6-MMP-1-VsV), or the N-terminal domain tagged to the HA epitope

Figure 2. Intracellular localization of MMP-1 and nature of association to mitochondria and nucleus. **A:** Characteristic granular staining of Müller cells for MMP-1 (Cy5) did not co-localize with the Golgi apparatus (FITC), as shown in the merged image. **B:** Similarly, staining for MMP-1 did not co-localize with lysosomes (green). **C:** Co-localization of mitochondrial (red) and MMP-1 staining (FITC) is revealed by the orange staining in the merged image (**top**). Nuclear staining for MMP-1 was often observed in cultured cells as illustrated at the **bottom**. **D:** Enriched mitochondrial fractions were treated with various concentrations of proteinase K (1, 0.1, and 0.01 μ g/ml) either in the presence or absence of 1% Triton X-100. Supernatants examined for the levels of MMP-1 by ELISA assays showed that the highest levels of MMP-1 in relation to the concentration of protein released was increased by decreasing the concentrations of proteinase K alone. Increasing concentrations of proteinase K and addition of Triton X-100 to release total mitochondrial protein resulted in relatively lower levels of MMP-1 in relation to the concentration of protein.

(pCB6-MMP-1-HA).²³ Figure 4, A and B, shows that staining for VsV and HA co-localized with mitochondria and nuclei, thus confirming the association of this matrix-degrading enzyme with both cellular organelles.

Figure 3. Mitochondrial and nuclear expression of MMP-1 in nonglial cells. **A:** Tenon's fibroblasts, corneal fibroblasts, and ARPE cells were cultured in monolayers for 48 hours and co-stained for MMP-1 (FITC) and MitoTrack (red). Confocal images show co-localization of MMP-1 with mitochondria (first panel) and nucleus of Tenon's fibroblasts (second panel), and with mitochondria of corneal fibroblasts and ARPE cells. **B:** Western blot of mitochondrial lysates obtained after a 48-hour culture of nonglial cells show the presence of MMP-1. The proenzyme (57 kd) and mature (45 kd) forms of this enzyme were also observed.

Distribution of Intracellular MMP-1 during Staurosporine-Induced Apoptosis

To determine whether induction of apoptosis causes changes in the phenotypic expression of MMP-1, we incubated Muller cells with the apoptotic agent staurosporine (1 μ mol/L) and examined the intracellular distribution of this matrix metalloproteinase after various periods of time (Figure 5). We observed that the pattern of mitochondrial distribution of intracellular MMP-1 observed in untreated cells (Figure 5A) changed rapidly at 15 minutes after addition of staurosporine, and by 30 minutes there was clear association of MMP-1 to perinuclear mitochondrial clusters (Figure 5B). After 45 minutes, there was accumulation of MMP-1 in the grooves forming invaginations of the nuclear envelope, and after 3 hours,

Figure 4. Transfection of human Müller cells with MMP-1. **A** and **B**: Müller cells were transfected with MMP-1 constructs in a pCB6 vector expressing either the C-terminal domain tagged to the VsV epitope (pCB6-MMP-1-VsV) or the N-terminal domain tagged to the HA epitope (pCB6-MMP-1-HA). As a control, cells were transfected with the pcDNA 3.1 vector. After 72 hours of transfection, cells were co-stained for either VsV or HA (FITC) and mitochondria (red). **A**: Cells transfected with the pcDNA 3.1 vector did not stain with antibodies to VsV. Expression of MMP-1 localizing to mitochondria and nucleus was observed in cells transfected with the pCB6-MMP-1-VsV as shown by staining of both organelles for VsV (FITC). **B**: Control cells transfected with pcDNA 3.1 did not stain with antibodies for HA. Transfection of cells with the pCB6-MMP-1-HA construct also resulted in expression of MMP-1 localizing to mitochondria and nucleus as judged for their staining for HA (FITC).

accumulation of MMP-1 around nuclear fragments was characteristically observed. After 24 hours of incubation, apoptotic cells did not stain for MMP-1 (Figure 5B). These observations suggest that MMP-1 may be involved in intracellular processes leading to apoptosis.

Effects of MMP-1 Silencing or Inhibition on Lamin A Degradation, Caspase Activation, and DNA Fragmentation

Nuclear envelope proteins, including lamin A/C undergo degradation during apoptosis.²⁵ We therefore investigated whether silencing of MMP-1 expression by transient transfection with the U6pro vector expressing short strands of duplex MMP-1 RNA (interfering RNA or siRNA) or inhibition by the wide spectrum MMP inhibitor Ilomastat (GM6001),²⁶ had any effect on the degradation of this protein. Transfection of Müller cells with two different constructs of siRNA (siRNA-1 and siRNA-2) and a control construct of siRNA made of scrambled oligonucleotides from the siRNA-1 and siRNA-2 sequences (siRNA-C), showed that only the siRNA-2 preparation had a signifi-

Figure 5. Distribution of intracellular MMP-1 after staurosporine-induced apoptosis. Müller cells were incubated for various periods of time with 1 μ mol/L staurosporine and stained for MMP-1 (FITC) and mitochondria (red). **A**: Untreated control cells. **B**: Within 15 minutes after addition of staurosporine, MMP-1 localizes to initial nuclear envelope invaginations and aggregated mitochondria (arrow). After 30 to 45 minutes of the addition of staurosporine, there was a predominance of large aggregates of MMP-1 co-localizing with mitochondrial aggregates. At these time points there was accumulation of MMP-1 in deep nuclear envelope invaginations where nuclear fragments appear to be emerging (arrows). As nuclear fragmentation occurs (3 hours), MMP-1 can be seen surrounding nuclear fragments (arrow), and after 24 hours there was no staining for MMP-1 within the apoptotic cells.

cant effect in reducing MMP-1 secretion as quantified by ELISA methods (Figure 6A). Partial down-regulation of MMP-1 (less than twofold) could be ascribed to the fact that only 30 to 40% of the cells could be transfected with the U6pro vector, as judged by the percentage of cells that are transfected with other plasmids, including HA-MMP-1 and VsV-MMP-1 (shown in this study), as well as EGFP vectors (not shown). Western blotting of Müller cell lysates using antibodies to lamins A/C showed that Müller cells express lamin A (72 kd) and lamin C (65 kd) (Figure 6B). A small band of ~60 kd was also observed and appears to be an early degradation product of lamin A/C. This is consistent with the results obtained on induction of apoptosis. Four hours after the addition of 1 μ mol/L staurosporine, we observed greater lamin A/C degradation in cells that had been transfected with the siRNA-2 construct than in cells transfected with the siRNA-1 and

Figure 6. Inhibition of MMP-1 results in rapid lamin A/C degradation, caspase activation, and DNA fragmentation on induction of apoptosis. **A:** Quantification of MMP-1 in Müller cell lysates by ELISA assay showed that the siRNA construct down-regulated production of MMP-1 while the siRNA-1 and siRNA-C (control) constructs did not modify MMP-1 production when compared with untreated cells. **B:** Western blot analysis of cell lysates shows that untreated Müller cells express lamin A/C (72 kd and 65 kd, respectively). Degradation of lamin A after 4 hours of induction of apoptosis with staurosporine was greater in cells previously transfected with the siRNA-2 construct than in untreated cells or cells transfected with the siRNA-1 or siRNA-C constructs. Inhibition of MMP-1 protein expression by Ilomastat before induction of apoptosis also caused faster lamin A/C degradation when compared with cells treated with Ilomastat control. **C:** After induction of apoptosis, there was a greater number of caspase-positive cells at 3 and 7 hours in preparations treated with Ilomastat than in preparations treated with Ilomastat control or medium alone. Similarly, DNA fragmentation, as judged by the number of TUNEL-positive cells, was greater at 3 and 7 hours in cells treated with Ilomastat than in cells treated with Ilomastat control or medium alone.

siRNA-C constructs or untreated cells (Figure 6B). Similarly, greater lamin A/C degradation was observed at 4 hours in cells that had been treated with Ilomastat (GM6001) than in untreated cells or cells treated with Ilomastat-negative control (Figure 6B). Because perturbation of mitochondrial membrane causes release of cytochrome C and subsequent activation of initiator or executor caspases that cleave nuclear envelope proteins, we further examined whether inhibition of MMP-1 by Ilomastat may cause *in vivo* changes on caspase activation, as judged by binding of the FITC-VAD-FMK marker, and DNA fragmentation as determined by terminal dUTP nick-end labeling (TUNEL) assay. We observed that the proportion of cells showing total caspase activation 3 and 7 hours after addition of staurosporine was significantly increased ($P = 0.024$ and 0.008 , respectively) in cells in which MMP-1 had been inhibited, when compared with cells treated with Ilomastat control. Similarly, the percentage of TUNEL-positive cells was significantly higher in cell preparations treated with Ilomastat ($P = 0.032$ and 0.023 , respectively) than in preparations treated with Ilomastat-negative control (Figure 6C).

Discussion

The present work further support suggestions that the role of MMPs goes far beyond that of digesting extracellular matrix molecules alone, and that these enzymes may have unknown substrates that are not extracellular matrix components.^{5,6} For more than 4 decades after the first description of MMP-1 as an interstitial collagenase,²⁷ many reports have shown that this matrix-degrading en-

zyme may be found highly expressed in inflammatory and tumoral tissues,²⁻⁴ but very little or no importance has been given to observations that this MMP may be found intracellularly. Recent reports that MMP-1 binds to $\alpha 2 \beta 1$ integrins on the cell membrane of human keratinocytes and monocytes,^{28,29} causing regulation of migratory activities, highlights the biological importance of this cell-associated molecule in inflammatory and proliferative cell processes.

The present results indicate that MMP-1 localizes to mitochondria and nuclei of various cell types and suggest that intracellular localization of MMP-1 is not confined to cells of glial origin and that it might be a common feature in many cells (Figures 2 and 3). They also indicate that intracellular levels of this enzyme vary with cell-cycle progression, being at their highest during the M phase (Figure 1). We observed both the proenzyme and the mature forms of MMP-1 in all cell types examined. This contrasts with the predominant expression of the proenzyme form of MMP-1 after nocodazole arrest of the cell cycle at mitosis. This might be attributed to the inability of the cell to mobilize intracellular MMP-1 for activation and functionality due to microtubule de-polymerization. That both, the proenzyme and mature forms of MMP-1 may be found in cell lysates of untreated cells, may indicate an active role of MMP-1 in intrinsic cell functions, in which proenzyme stored on mitochondria or nucleus could be readily activated by unknown mechanisms, possibly by binding to organelle surfaces to perform essential intracellular functions. Association of MMP-1 to intracellular organelles was further confirmed by transfection of cells with the MMP-1 constructs pCB6-MMP-1-VsV and pCB6-

MMP-1-HA. Immunostaining for VsV- and HA-tagged proteins indicated that these were localized on the mitochondria and nuclei of the transfected cells. No staining for these proteins could be identified in control plasmids lacking the MMP-1 transcript (Figure 5). It is of interest that as early as 1969, it had been reported that a collagenolytic activity was present in the mitochondrial fraction of rat liver.³⁰ However, until the present investigation, no further reference to this observation or follow-up of the study has been made.

Because the nuclear envelope is a dynamic structure that breaks down at mitosis and reforms in an ordered manner as a result of reversible phosphorylations of membrane, lamina, and chromatin proteins,^{31,32} it could be suggested that intracellular accumulation of MMP-1 during metaphase may aid in the temporary dissociation of nuclear membrane proteins during cell division and therefore may promote cell growth. This could explain reported observations that treatment of cells with MMP-1 inhibitors acting at the mitochondrial level such as doxycycline, causes inhibition of cell growth,³³ and that increased production and mRNA expression of MMP-1 by tumor cells is associated to tumor growth and metastasis.³⁴

Cytosolic presence of tissue inhibitor of metalloproteinase 1 (TIMP-1) in bovine pulmonary artery smooth muscle³⁵ and of TIMP-1 and -2 in hepatic fibroblasts,³⁶ as well as nuclear accumulation of TIMP-1 in the nuclei of human gingival fibroblasts³⁷ have been recently identified. In addition, TIMP3 has been shown to accumulate during the G₁ phase of the cell cycle, at the time when cell division ceases.³⁸ These observations suggest that intracellular TIMPs, as natural inhibitors of MMPs, may bind to these enzymes to physiologically control their activity during various intracellular processes and merits further investigation. That intracellular MMP-1 accumulates during the mitotic phase of the cell cycle, further supports our suggestion that MMP-1 may have a role in cell growth. However, we could not demonstrate that inhibition of MMP-1 by broad-spectrum inhibitors of MMPs or specific silencing of MMP-1 by siRNA had any effect on Müller cell growth (data not shown), yet, the possibility that MMP-1 may promote proliferation of other cell types may not be excluded and deserves further investigation.

Due to the association of MMP-1 with mitochondria and the important role that these organelles play during apoptosis,³⁵ we further examined a possible role of MMP-1 in this process of programmed cell death. Examination of the intracellular distribution of MMP-1 after exposure to staurosporine showed that this matrix-degrading enzyme co-localizes with mitochondria clustered around the nucleus shortly after the induction of apoptosis. At later stages, it accumulates around the nuclei and nuclear fragments (Figure 5), suggesting a possible role in the breakdown of the nuclear envelope. Important proteins of the nuclear envelope are the lamins, which are type V intermediate filament proteins arranged into a meshwork that maintains nuclear envelope integrity and organization of the interface chromatin.³⁶ Although it has been shown that during apo-

ptosis the lamin network is degraded by proteolytic enzymes known as caspases, very little is known about the sequence of events that take place during lamin proteolysis.³¹ Due to the intracellular localization of MMP-1 on the mitochondrial surface, we investigated whether this enzyme may potentially be involved in the degradation of lamins. Western blotting of cell lysates in which MMP-1 had been ablated by siRNA or inhibited by Ilomastat (a broad spectrum MMP inhibitor), showed that breakdown of lamin A/C occurs faster after staurosporine-induced apoptosis when compared with untreated cells (Figure 6). These results strongly suggest that MMP-1 is not directly involved in the *in vivo* degradation of lamin A/C, but that it may have a regulatory effect on other factors responsible for its degradation. Because perturbation of mitochondrial membrane causes release of cytochrome C and subsequent activation of initiator or executor caspases that cleave nuclear envelope proteins,³⁷ we further examined whether broad spectrum inhibition of MMP may cause *in vivo* changes on caspase activation and DNA fragmentation. We observed that the proportion of cells binding the caspase-FITC-VAD-FMK marker 3 and 7 hours after induction of apoptosis was significantly higher in cells in which MMP had been inhibited by Ilomastat, when compared with control cells. Similarly, the number of TUNEL-positive cells was significantly increased at 3 and 7 hours after induction of apoptosis in cells treated with Ilomastat than in control cells (Figure 6C). Because Müller cells are known to produce MMP-2 and MMP-9,²¹ it would be possible to speculate that the Ilomastat effects on caspase activation and TUNEL formation may have been due to inhibition of these two MMPs. However, specific ablation of MMP-1 with siRNA resulted in faster degradation of lamin A/C, which indirectly correlates with the effects observed with Ilomastat. These findings therefore suggest that intracellular MMP-1 confers resistance to apoptosis, and have important implications for the control of cell growth and matrix remodeling during physiological and pathological conditions. That MMP-1 inhibition increases the rate of apoptosis may explain previous reports that blocking of MMP-1 activity by TIMP-1 inhibits apoptosis,³⁸ that increased expression of this enzyme leads to tumor progression and metastasis,³⁴ and that low levels of MMP-1 are favorable markers for tumor spreading.³⁹

The rate of apoptosis in a tumor determines its growth, invasion, and metastasis and in nearly all instances a significant correlation between expression of MMP-1 and cancer survival has been documented.^{34,39–41} Because control of cell survival may be achieved through inhibition of apoptosis, it is possible that this is another mechanism by which MMP-1 promotes cell invasion and growth in inflammatory, angiogenic, and tumoral processes. Elucidation of the intracellular pathways that MMP-1 utilizes to confer resistance to apoptosis may further our understanding of the molecular mechanisms by which this matrix-degrading enzyme exert a wide variety of functions.

References

- Lovejoy B, Welch AR, Carr S, Luong C, Broka C, Hendricks RT, Campbell JA, Walker KA, Martin R, Van Wart H, Browner MF: Crystal structures of MMP-1 and -13 reveal the structural basis for selectivity of collagenase inhibitors. *Nat Struct Biol* 1999, 6:217–221
- Stamenkovic I: Extracellular matrix remodelling: the role of matrix metalloproteinases. *J Pathol* 2003, 200:448–464
- Pilcher BK, Sudbeck BD, Dumin JA, Welgus HG, Parks WC: Collagenase-1 and collagen in epidermal repair. *Arch Dermatol Res Suppl* 1998, 290(Suppl):S37–S46
- Brinckerhoff CE, Rutter JL, Benbow U: Interstitial collagenases as markers of tumor progression. *Clin Cancer Res* 2000, 6:4823–4830
- Brickerhoff CE, Matrisian LM: Matrix metalloproteinases: a tail of a frog that became a prince. *Nat Rev Mol Cell Biol* 2002, 3:207–214
- McCawley LJ, Matrisian LM: Matrix metalloproteinases: they're not just for matrix anymore! *Curr Opin Cell Biol* 2001, 13:534–540
- Johnson S, Knox A: Autocrine production of matrix metalloproteinase-2 is required for human airway smooth muscle proliferation. *Am J Physiol* 1999, 277:L1109–L1117
- Aoudjit F, Masure S, Opdenakker G, Potworowski EF, St.-Pierre Y: Gelatinase B (MMP-9) but not its inhibitor (TIMP-1) dictates the growth rate of experimental thymic lymphoma. *Int J Cancer* 1999, 82:743–747
- Hayakawa T, Yamashita K, Tanzawa K, Uchijima E, Iwata K: Growth-promoting activity of tissue inhibitors of metalloproteinase-1 for a wide range of cells. *FEBS Lett* 1994, 298:29–32
- Hayakawa T, Yamashita K, Ohuchi E, Shinagawa A: Cell growth-promoting activity of tissue inhibitor of metalloproteinase-2 (TIMP-2). *J Cell Sci* 1994, 107:2372–2379
- Forough R, Koyama N, Hasenstab D, Lea H, Clowes M, Nikkari ST, Clowes AW: Overexpression of tissue inhibitor of matrix metalloproteinases-1 inhibits vascular smooth muscle functions in vitro and in vivo. *Circ Res* 1996, 79:812–820
- Murphy G, Knäuper V, Atkinson S, Gavrilovic J, Edwards D: Cellular mechanisms for focal proteolysis and the regulation of the microenvironment. *Fibrinol Proteol* 2000, 14:165–174
- Galt SW, Lindemann S, Allen L, Medd DJ, Falk JM, McIntyre TM, Prescott SM, Kraiss LW, Zimmerman GA, Weyrich AS: Outside-in signals delivered by matrix metalloproteinase-1 regulate platelet function. *Circ Res* 2002, 90:1093–1099
- Naito S, Shimizu S, Matsuo M, Nakashima M, Nakayama T, Yamashita S, Sekine I: Ets-1 upregulates matrix metalloproteinase-1 expression through extracellular matrix adhesion in vascular endothelial cells. *Biochem Biophys Res Commun* 2002, 291:130–138
- Kheradmand F, Werner E, Tremblon P, Symons M, Werb Z: Role of Rac1 and oxygen radicals in collagenase-1 expression induced by cell shape change. *Science* 1998, 280:898–902
- Limb GA, Salt TE, Munro PM, Moss SE, Khaw PT: In vitro characterization of a spontaneously immortalized human Müller cell line (MIO-M1). *Invest Ophthalmol Vis Sci* 2002, 43:864–869
- Dunn K, Aotaki-Keen AE, Putkey FR, Hjelmeland LM: ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. *Exp Eye Res* 1996, 62:155–169
- Daniels JT, Limb GA, Saarialho-Kere U, Murphy G, Khaw PT: Human corneal epithelial cells require MMP-1 for HGF-mediated migration on collagen I. *Invest Ophthalmol Vis Sci* 2003, 44:1048–1055
- Daniels JT, Cambrey AD, Ocleston NL, Garrett Q, Tarnuzzer RW, Schultz GS, Khaw PT: Matrix metalloproteinase inhibition modulates fibroblast-mediated matrix contraction and collagen production in vitro. *Invest Ophthalmol Vis Sci* 2003, 44:1104–1110
- Klein S, Kaszkin M, Barth H, Kinzel V: Signal transduction through epidermal growth factor receptor is altered in HeLa monolayer cells during mitosis. *Biochem J* 1997, 322:937–946
- Limb GA, Daniels JT, Pleass R, Charteris DG, Luthert PJ, Khaw PT: Differential expression of matrix metalloproteinases 2 and 9 by glial Müller cells: response to soluble and extracellular matrix bound TNF- α . *Am J Pathol* 2002, 160:1847–1855
- Spector DL, Goldman RD, Leinwand LA: Isolation of mitochondria. *Cells—A Laboratory Manual*, vol 1. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 1997, pp 41.2–41.4
- Balda MS, Whitney JA, Flores C, Gonzalez S, Cereijido M, Matter K: Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. *J Cell Biol* 1996, 134:1031–1049
- Yu JY, DeRuiter SL, Turner DL: RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci USA* 2002, 99:6047–6052
- Broers JLV, Bronnenberg NMHJ, Kuijpers HJH, Schutte B, Hutchison CJ, Ramaekers FCS: Partial cleavage of A-type lamins concurs with their total disintegration from the nuclear lamina during apoptosis. *Eur J Cell Biol* 2002, 81:677–691
- Grobely D, Poncz L, Galaray RE: Inhibition of human skin fibroblast collagenase, thermolysin, and *Pseudomonas aeruginosa* elastase by peptide hydroxamic acids. *Biochemistry* 1992, 31:7152–7154
- Gross J, Lapiere CM: Collagenolytic activity in amphibian tissues: a tissue culture assay. *Proc Natl Acad Sci USA* 1962, 48:1014–1022
- Dumin JA, Dickeson SK, Stricker TP, Bhattacharya-Pakrasi M, Roby JD, Santoro SA, Parks WC: Pro-collagenase-1 (matrix metalloproteinase-1) binds the $\alpha(2)\beta(1)$ integrin upon release from keratinocytes migrating on type I collagen. *J Biol Chem* 2001, 276:29368–29374
- Conant K, Haughey N, Nath A, St. Hilaire C, Gary DS, Pardo CA, Wahl LM, Bilak M, Milward E, Mattson MP: Matrix metalloproteinase-1 activates a pertussis toxin-sensitive signaling pathway that stimulates the release of matrix metalloproteinase-9. *J Neurochem* 2002, 82:885–893
- Heidrich H-G, Prokopova D, Hannig K: The use of synthetic substrates for the determination of mammalian collagenases. Is collagenolytic activity present in mitochondria? *Physiol Chem* 1969, 350:1430–1436
- Collas P, Courvalin JC: Sorting of nuclear membrane proteins at mitosis. *Trends Cell Biol* 2000, 10:5–8
- Goldman RD, Gruenbaum Y, Moir RD, Shumaker DL, Spann TP: Nuclear lamins: building blocks of nuclear architecture. *Genes Dev* 2002, 16:533–547
- Rubins JB, Charboneau D, Alter MD, Bitterman PB, Kratzke RA: Inhibition of mesothelioma cell growth in vitro by doxycycline. *J Lab Clin Med* 2001, 138:101–106
- Baschmeier BE, Nerlich AG, Lichtinghagen R, Sommerhoff CP: Matrix metalloproteinases (MMPs) in breast cancer cell lines of different tumorigenicity. *Anticancer Res* 2001, 21:3821–3828
- Zamzami N, Susin SA, Marchetti P, Hirsch T, Gomez-Monterrey I, Castedo M, Kroemer GJ: Mitochondrial control of apoptosis. *J Exp Med* 1996, 183:1533–1544
- Cohen M, Olson JF, Koshland Jr DE: Transcriptional repression, apoptosis, human disease and the functional evolution of the nuclear lamina. *Trends Biochem Sci* 2001, 26:41–47
- Fumarola C, Guidotti GG: Stress-induced apoptosis: toward a symmetry with receptor-mediated cell death. *Apoptosis* 2004, 9:77–82
- Murphy FR, Issa R, Zhou X, Ratnarajah S, Nagase H, Arthur MJ, Benyon C, Iredale JP: Inhibition of apoptosis of activated hepatic stellate cells by tissue inhibitor of metalloproteinase-1 is mediated via effects on matrix metalloproteinase inhibition. *J Biol Chem* 2002, 277:11069–11076
- Bendardaf R, Lamlum H, Vihinen P, Ristamäki R, Laine J, Pyrhönen S: Low collagenase-1 (MMP-1) and MT1-MMP expression levels are favourable survival markers in advanced colorectal carcinoma. *Oncology* 2003, 65:337–346
- Ito T, Iho M, Shiozawa J, Naito S, Kanematsu T, Sekine I: Expression of the MMP-1 in human pancreatic carcinoma: relationship with prognostic factor. *Mol Pathol* 1999, 12:669–674
- Murray GI, Duncan ME, O'Neil P, Melvin WT, Fothergill JE: Matrix metalloproteinase-1 is associated with poor prognosis in colorectal cancer. *Nat Med* 1996, 2:461–462