FEBS Letters 586 (2012) 2366–2374

journal homepage: www.FEBSLetters.org

Alendronate promotes plasmin-mediated MMP-9 inactivation by exposing cryptic plasmin degradation sites within the MMP-9 catalytic domain

Antonietta R. Farina [a], Lucia Cappabianca [a], Natalia Di Ianni [a], Pierdomenico Ruggeri [a], Marzia Ragone [a], Stefania Merolle [a], Alberto Gulino [b,c], Andrew R. Mackay [a,*]

[a] Section of Molecular Pathology, Department of Experimental Medicine, University of L'Aquila, 67100 L'Aquila, Italy
[b] Department of Molecular Medicine, University of Rome "La Sapienza", 00161 Rome, Italy
[c] Neuromed Institute, 86077 Pozzilli, Italy

ARTICLE INFO

Article history:
Received 11 May 2012
Accepted 22 May 2012
Available online 4 June 2012

Edited by Veli-Pekka Lehto

Keywords:
Bisphosphonate Alendronate
MMP-9
Divalent cation chelation
Catalytic-domain
Hemopexin–domain
Irreversible inhibition

ABSTRACT

Irreversible MMP-9 inhibition is considered a significant therapeutic goal in inflammatory, vascular and tumour pathology. We report that divalent cation chelators Alendronate and EDTA not only directly inhibited MMP-9 but also promoted irreversible plasmin-mediated MMP-9 inactivation by exposing cryptic plasmin-degradation sites within the MMP-9 catalytic-domain and producing an inhibitory hemopexin–domain fragment. This effect was also observed using MDA-MB-231 breast cancer cells, which activated exogenous plasminogen to degrade endogenous proMMP-9 in the presence of Alendronate or EDTA. Degradation-mediated inactivation of proMMP-9 occurred in the absence of transient activation, attesting to the incapacity of plasmin to directly activate proMMP-9 and direct MMP-9 inhibition by Alendronate and EDTA. Our study provides a novel rational for therapeutic Alendronate use in MMP-9-dependent pathology characterised by plasminogen activation.

1. Introduction

Matrix metalloproteinases (MMPs) and the plasmin generating system form part of an interactive proteolytic cascade that is involved in inflammatory, vascular and tumour pathology [1–6]. Plasmin generated from plasminogen by the action of plasminogen activators has been shown to activate selective MMPs, which in turn activate other MMPs, to amplify and diversify proteolytic activity [7–9]. This interactive proteolytic cascade exhibits negative feedback regulation, characterised by autocatalytic MMP and plasmin inactivation and the capacity of MMPs such as MMP-3 and MMP-9 to degrade plasmin to inhibitory fragments, including angiostatin [7,10,11].

Amongst the MMP family, MMP-9 is considered to play a critical role in inflammatory, vascular and tumour pathology, has been directly implicated in aberrant extracellular matrix remodelling associated with diabetes, kidney and destructive bone disease, tumour associated vasculargenesis, angiogenesis, invasion and metastasis, and is involved in the down-regulation of immunological surveillance [12–14]. Originally identified as a promoter of metastatic behaviour in Ras-transfected murine fibroblasts [15], MMP-9 is expressed by malignant tumour cells, tumour-associated stroma and inflammatory leucocytes [1–4,16]. It is secreted as an inactive, O-glycosylated, 92 kDa zymogen comprised of a N-terminal pro-peptide, a zinc-binding catalytic site and a carboxyl terminal hemopexin-like domain, and is activated either by allosteric perturbation, oxidation or proteolytic processing by serine proteases such as chymotrypsin, trypsin, cathepsin G and urokinase or by other MMPs [7,8,12,17]. Upon activation MMP-9 is subject to autocatalytic processing, which optimises activity prior to inactivating the enzyme [7,8,18].

MMP-9 activity is also regulated by its own hemopexin domain, which is involved in MMP-9 dimerization, interaction with tissue inhibitor of metalloproteinase (TIMP)-1 and bioavailability by binding cell surface LRP1, LRP2 and Ku [19–22]. In purified recombinant form, the MMP-9 hemopexin domain acts as a MMP-9 antagonist [23]; inhibits tumour xenograft growth, invasion, angiogenesis and metastasis; and also inhibits metastatic bone disease and destructive bone pathology [24–27]. The hemopexin domain is, therefore, considered a novel therapeutic agent for potential future use in MMP-9 dependent pathology. A significant drawback of current MMP-9 inhibitors is, however, reversibility and capacity to

Abbreviations: MMP-9, matrix metalloproteinase-9; TIMP, tissue inhibitor of metalloproteinases; APMA, aminophenylmercuric acetate; EDTA, ethylenediaminetetraacetic acid

* Corresponding author. Fax: +39 0862433523.
E-mail address: andrewreay.mackay@univaq.it (A.R. Mackay).

0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.05.048

promote proMMP-9 accumulation by preventing autocatalytic elimination. This raises the possibility of MMP-9 re-activation upon inhibitor removal, making the irreversible inhibition of MMP-9 a preferable therapeutic goal.

Bisphosphonates are well tolerated divalent cation binding drugs that exhibit direct MMP inhibitory activity, reduce MMP-9 expression and are currently employed to treat Paget's bone disease, osteoporosis, myeloma and metastatic bone disease, primarily due to inhibitory effects upon osteoclast function and bone resorption [28–37]. In this study, we provide novel important information concerning the bisphosphonate sodium Alendronate that provides a novel rational for its potential use in MMP-9-dependent pathology. We report that, in addition to direct MMP-9 inhibition, Alendronate promotes rapid plasmin-mediated irreversible MMP-9 inactivation by exposing cryptic plasmin-degradation sites within the MMP-9 catalytic domain, the plasmin-mediated degradation of which inactivates the MMP-9 catalytic site, in addition to generating an inhibitory MMP-9 hemopexin domain fragment.

2. Materials and methods

2.1. Cells and reagents

Mammalian recombinant TIMP-1-free 92 kDa human pro-MMP-9 was purchased from Calbiochem (Cambridge MA) and exhibited >95% purity by silver stained SDS–PAGE and did not contain TIMP-1, as judged by Western blot. Recombinant human 28 kDa TIMP-1 was purchased from Calbiochem and exhibited >95% purity by silver-stained SDS–PAGE. Purified human 66 kDa plasmin (specific activity against D-Val-Leu-Lys-P-Nitroanilide of 2 U/mg) was purchased from Sigma–Aldrich (St Louis, MO). Human MMP-9/TIMP-1 complexes were purified by gelatin-Sepharose affinity chromatography from 72 h MDA-MB-231 breast carcinoma cell serum-free conditioned medium, as previously described [38,39]. MMP-9/TIMP-1 complexes exhibited an approximate 1:1 molecular stoichiometry, as judged by silver stained SDS–PAGE and Western blot. Purified bacterial recombinant human MMP-9 catalytic domain was purchased from Anaspec (Fremont, CA). Aminophenylmercuric acetate (APMA), ethylenediaminetetraacetic acid (EDTA), bovine type I gelatin and α2-macroglobulin were purchased from Sigma–Aldrich (St Louis, MO). ^{3}H-labelled rat-tail type I collagen was purchased from Amersham (Bedford UK). The anti-MMP-9 hemopexin domain antibody was produced by inoculating New Zealand White rabbits with purified recombinant human MMP-9 hemopexin domain (amino acids 536–704), as previously described [40]. The anti-human MMP-9 catalytic-site antibody, raised against recombinant MMP-9 catalytic domain, was purchased from Millipore Inc (Milan, IT). Sodium Alendronate was kindly provided by Merck, Sharp and Dohme (Rome, IT).

2.2. Gelatinase assays

Assays were performed using heat denatured (30 min 60 °C) rat-tail type I collagen, as previously described [41]. Briefly, reactions containing TIMP-free MMP-9, pre-incubated with either: plasmin alone; plasmin plus Alendronate, plasmin plus EDTA or APMA, at the concentrations and for the times indicated, were subsequently incubated with ^{3}H-gelatin (3000 cpm per assay) for 24 h at 37 °C in a buffer containing 0.1 M Tris, 0.2 M NaCl and 1 mM $CaCl_2$ [pH 8.0] and plasmin inhibitors leupeptin (0.5 μg/ml), aprotinin (2 μg/ml) and PMSF (1 mM). Following incubation, undegraded material was precipitated with 10%TCA/0.5%TA for 30 min at 4 °C, precipitates removed by centrifugation at 5000*g* and degraded gelatin in supernatants counted in a β-liquid scintillation counter (Beckman model LS 5000TD).

2.3. Substrate gel electrophoresis

Regular gelatin and reverse zymograms were prepared, as previously described [38]. Briefly, samples were subjected to regular SDS–PAGE under non-reducing conditions in gels co-polymerised with either 0.1% gelatin for regular zymograms or 0.1% gelatin plus 100 ng/ml of MMP-9 for reverse zymograms. After electrophoresis, gels were washed in 2% Triton X-100, rinsed in water and incubated in 50 mM Tris, 0.2 M NaCl and 5 mM $CaCl_2$ containing 1 mM PMSF, 0.5 μg/ml leupeptin and 2 μg/ml of Aprotinin to inhibit plasmin within reactions. MMP-9 and MMP-9-inhibitor activities were visualised following staining and destaining in Coomassie blue.

2.4. Western blots

Samples separated by regular reducing SDS–PAGE were transferred electrophoretically to nitrocellulose (Hybond C-extra, Amersham, Bucks, UK). Non-specific protein binding sites on membranes were blocked by 5% non-fat milk in PBS. Membranes were then incubated with primary antibody diluted in blocking solution and subsequently with horseradish peroxidase-conjugated secondary antibody diluted in blocking solution. Immunoreactivity was demonstrated by chemiluminescence reaction (Amersham) and immunoreactive bands were visualised on XAR-5 film (Kodak, Rochester, NY). Molecular weights were approximated by comparison to pre-stained m.w. standards (Bio-Rad) using Molecular Analyst™/PC for the Bio-Rad Model GS-670 Imaging Densitometer. Antibody specificity was confirmed by comparison with pre-immune IgG preparations.

2.5. Statistical analysis

The Student's *t* test was used for statistical comparison of data. A comparison of means giving *t* values with associated probabilities of difference $\leqslant 0.05$ was considered to be statistically significant.

3. Results

3.1. In contrast to APMA, plasmin does not directly activate proMMP-9

The incubation of TIMP-free proMMP-9 (100 ng) with APMA (1 mM) resulted in MMP-9 activation and the degradation of 2800 ± 180 of the 3000 cpm ^{3}H-gelatin used in solution phase assay (Fig. 1A, the gelatinolytic activity of APMA activated MMP-9 is represented arbitrarily as 100%). The gelatinolytic activity of APMA-activated MMP-9 was completely inhibited by co-incubation with either recombinant TIMP-1 (10 μg/ml), EDTA (1 mM) or Sodium Alendronate, which at a concentration of 10 nM inhibited gelatinolytic activity by 12 ± 15% (NS, $n = 12$), by 55 ± 8.5% ($p < 0.05$; $n = 12$) at a concentration of 100 nM and by 98.5 ± 18.3% ($p < 0.001$; $n = 12$) at a concentration of 1 μM (Fig. 1A). Alendronate also inhibited the gelatinolytic activity of SDS-activated MMP-9 in a gelatin zymogram-strip assay, following incubation of renatured zymogram strips containing 100 ng of TIMP-free proMMP-9 for 16 h with increasing concentrations of Alendronate (Fig. 1B). Alendronate exhibited in an inhibitory IC_{50} concentration of approximately 100 nM in both solution phase and zymogram-strip assays.

Gelatin zymography revealed that APMA had induced the conversion of TIMP-free 92 kDa proMMP-9 to 83 kDa and 68 kDa species (Fig. 1C), confirming a previous report [18]. Both EDTA (1 mM) and Alendronate (1 mM) inhibited APMA-induced proMMP-9 processing to 83 kDa and 68 kDa species, consistent with

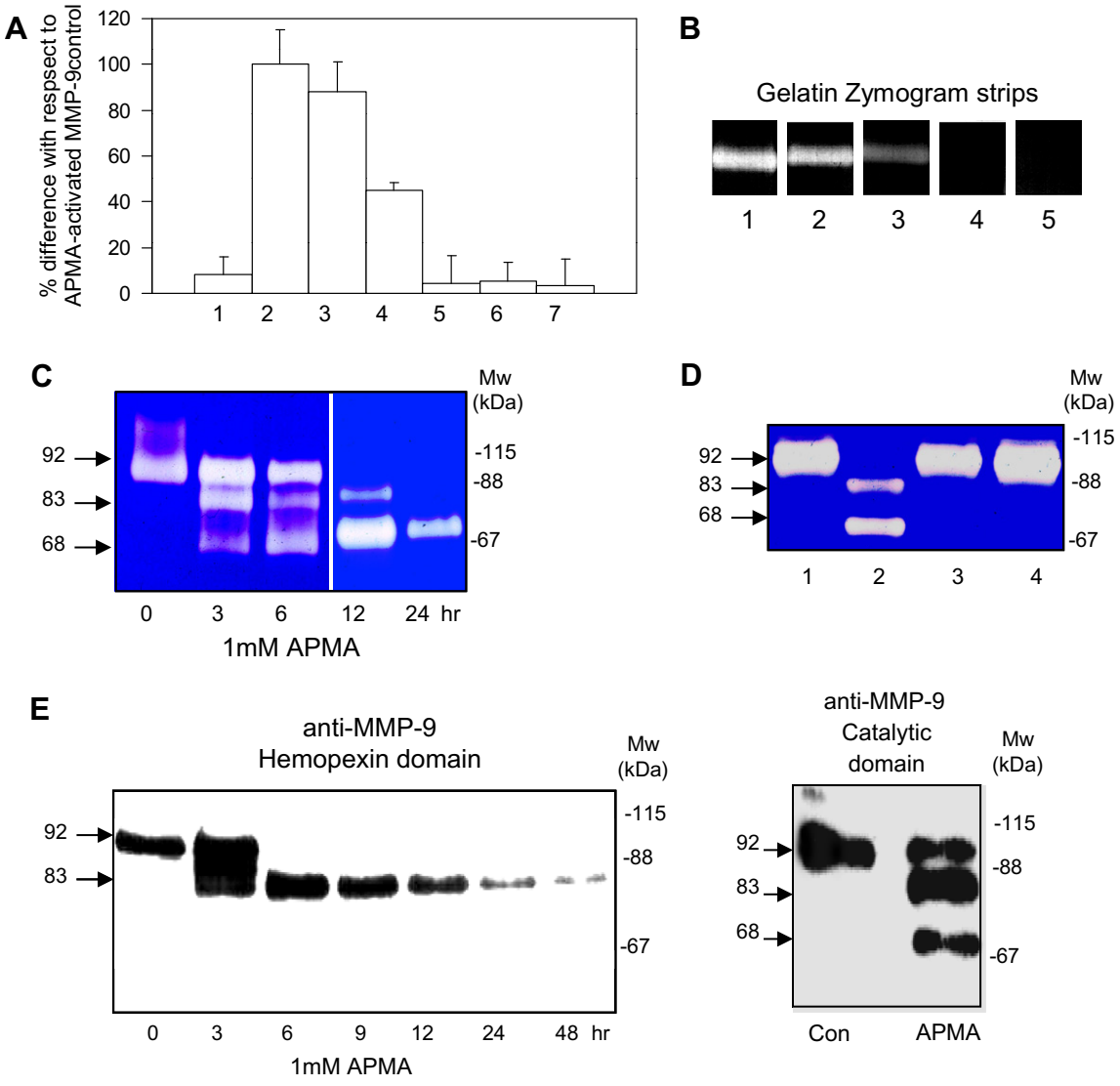

Fig. 1. (A) Histogram demonstrating the percentage of ^{3}H gelatin degraded by (1) 100 ng TIMP-1-free proMMP-9; (2) 100 ng APMA-activated (1 mM) TIMP-1-free MMP-9; (3) 100 ng APMA-activated (1 mM) TIMP-1-free MMP-9 plus 10 μM Alendronate; (4) 100 ng APMA-activated (1 mM) MMP-9 plus 100 μM Alendronate; (5) 100 ng APMA-activated (1 mM) TIMP-1-free MMP-9 plus 1 mM Alendronate; (6) 100 ng APMA-activated (1 mM) MMP-9 plus 10 mM Alendronate and (7) 100 ng APMA-activated (1 mM) TIMP-1-free MMP-9 plus 1 mM EDTA. Results are expressed as the mean ± s.d. percentage difference with respect to APMA-activated MMP-9 controls (100%) in duplicate assays each performed using six samples per group. (B) Representative gelatin strip assays demonstrating gelatinolytic activity of 100 ng of SDS-activated TIMP-1-free MMP-9 incubated for 16 h at 37 °C in (1) collagenase buffer alone; (2) collagenase buffer containing 10 μM Alendronate; (3) collagenase buffer containing 100 μM Alendronate; (4) collagenase buffer containing 1 mM Alendronate and (5) collagenase buffer containing 10 mM Alendronate. (C) Representative gelatin zymograms demonstrating the effect of incubating 100 ng TIMP-1-free proMMP-9 with 1 mM APMA for the times indicated. (D) Representative gelatin zymogram demonstrating the gelatinolytic activity of 100 ng TIMP-1-free proMMP-9: (1) alone; (2) following 6 h pre-incubation with 1 mM APMA; (3) following 6 h incubation with 1 mM APMA in the presence of 1 mM Alendronate; and (4) following 6 h incubation with 1 mM APMA in the presence of 1 mM EDTA. (E) Representative Western blots demonstrating anti-MMP-9 hemopexin domain-immunoreactivity of 100 ng TIMP-1-free proMMP-9 incubated for the times indicated with 1 mM APMA (left panel) and anti-MMP-9 catalytic domain immunoreactivity of 100 ng TIMP-1-free proMMP-9 incubated alone (con) or for 3 h with 1 mM APMA (right panel).

the inhibition of MMP-9 autocatalytic processing (Fig. 1D, lanes 3 and 4). In Western blots, 92 kDa proMMP-9 and 83 kDa APMA-activated MMP-9 but not 68 kDa APMA-activated MMP-9 species were recognised by anti-MMP-9 hemopexin–domain antibody (Fig. 1E, right panel). Over a 48 h activation time course, >95% of the APMA-activated 83 kDa MMP-9 species was lost due to continuous autocatalytic processing (Fig. 1E). Both the APMA-activated 83 kDa and 68 kDa MMP-9 species were recognised by anti-catalytic domain MMP-9 antibody (Fig. 1E, left panel).

In contrast to the effect of APMA, incubation of proMMP-9 (100 ng) with purified human plasmin (5 μg/ml – 0.01 U) for times up to 24 h prior to ^{3}H-gelatinase assay under plasmin inhibitory conditions, did not activate MMP-9 to degrade gelatin (Fig. 2A). Plasmin did, however, induce the low-level conversion of 92 kDa proMMP-9 to a single 82 kDa species, detected by gelatin zymography (Fig. 2B). This species was recognised in Western blots by both anti-MMP-9 hemopexin–domain and catalytic-domain antibodies (Fig. 2C, data displayed for anti-hemopexin domain antibody only). Plasmin generation of the 82 kDa MMP-9 species was inhibited by leupeptin (0.5 μg/ml) but not by TIMP-1 (10 μg/ml) in gelatin zymograms (Fig. 2D). The incubation of reactions containing plasmin-generated 82 kDa MMP-9 with fourfold molar excess α2-macroglobulin for 1 h did not result in α2-macroglobulin binding of this species, as judged by gelatin zymography (Fig. 2E). In contrast to the 83 kDa APMA-activated MMP-9 species, the 82 kDa MMP-9 species generated by plasmin was stable, continued to accumulate

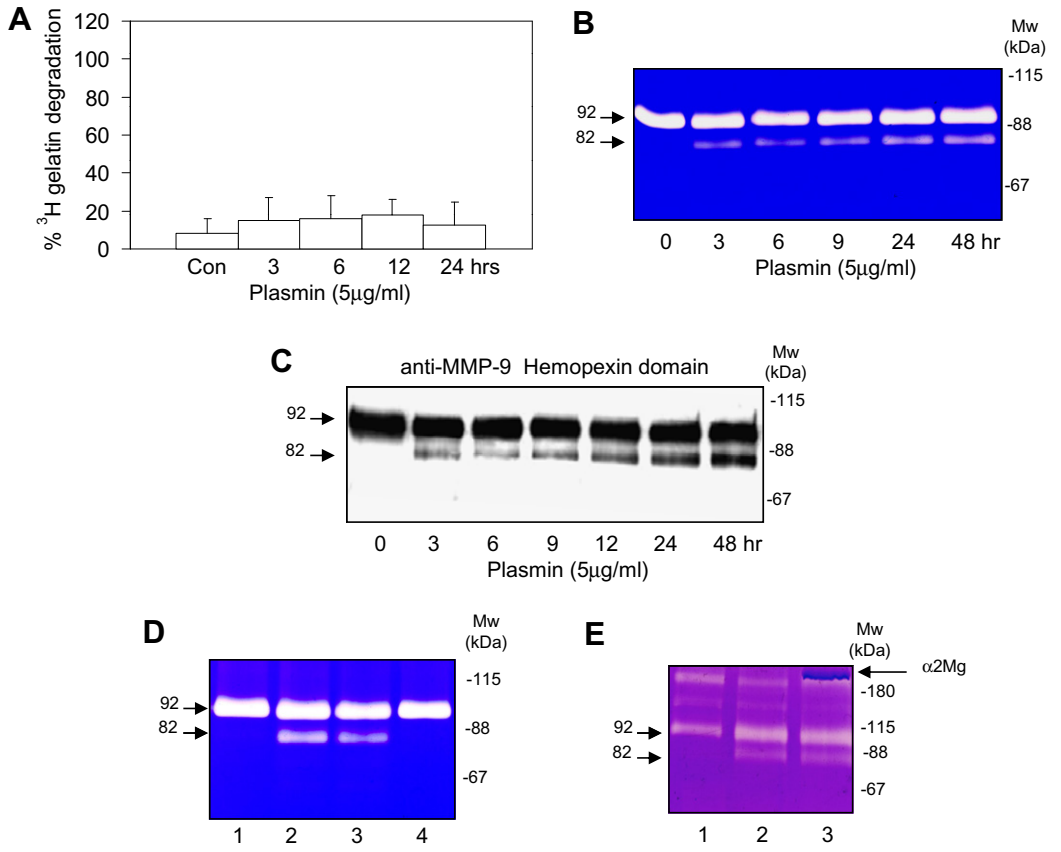

Fig. 2. (A) Histogram demonstrating the percentage of ^{3}H gelatin degraded by 100 ng of TIMP-1 free proMMP-9 alone (con) or following pre-incubation with plasmin (5 μg/ml) for the times indicated in hours, prior to ^{3}H gelatinase assay under plasmin inhibitory conditions. (B) Representative gelatin zymogram demonstrating the effect of incubating 100 ng of TIMP-1-free proMMP-9 with plasmin (5 μg/ml) for the times indicated. (C) Representative Western blot demonstrating anti-MMP-9 hemopexin domain immunoreactivity of 100 ng TIMP-1-free proMMP-9 incubated with plasmin (5 μg/ml) for the times indicated. (D) Representative gelatin zymogram demonstrating the gelatinolytic of 100 ng TIMP-1-free pro-MMP-9: (1) alone; (2) pre-incubated for 3 h with plasmin (5 μg/ml); (3) pre-incubated for 3 h with plasmin (5 μg/ml) plus TIMP-1 (10 μg/ml); and (4) pre-incubated for 3 h with plasmin (5 μg/ml) plus leupeptin (0.5 μg/ml). (E) Representative gelatin zymogram demonstrating the gelatinolytic activity of 100 ng TIMP-1-free pro-MMP-9: (1) alone; (2) pre-incubated for 4 h with plasmin (5 μg/ml); and (3) pre-incubated for 3 h with plasmin (5 μg/ml) followed by 1 h with fourfold molar excess α2-macroglobulin.

and did not associate other MMP-9 forms, assessed over a 48 h period (Fig. 2B and C).

3.2. Alendronate and EDTA promote plasmin-mediated degradation of pro-MMP-9 and APMA-activated MMP-9

Unexpectedly, the co-incubation of 100 ng proMMP-9 with plasmin (5 μg/ml) in the presence of either 1 mM EDTA or 1 mM Alendronate resulted in complete degradation of proMMP-9 within 3 h to a major 49 kDa fragment recognised by anti-MMP-9 hemopexin–domain but not catalytic-domain antibody (Fig. 3A right and left panels), and to 26 kDa and 8 kDa species recognised by anti-MMP-9 catalytic-domain but not hemopexin–domain antibody in Western blots (Fig. 3B). This degradation was dose-dependent, with 10 μM Alendronate promoting little, 100 μM Alendronate partial and 1 mM Alendronate complete plasmin-mediated degradation of 100 ng proMMP-9, within 3 h (Fig. 3A, lanes 5 and 6). Promotion of plasmin-mediated MMP-9 degradation by Alendronate and EDTA resulted in the complete loss of MMP-9 activity in zymograms (Fig. 3C, lanes 3 and 4). This effect was inhibited by leupeptin (0.5 μg/ml) but not by recombinant TIMP-1 (10 μg/ml) (Fig. 3D, lanes 4 and 5). MMP-9 pre-activated by 3 h incubation with 1 mM APMA was also completely degraded by plasmin (5 μg/ml) in the presence of 1 mM Alendronate or 1 mM EDTA to inactive 26 kDa and 8 kDa catalytic-domain species (Fig. 4A, lanes 3 and 4). Under these conditions, however, degradation did not generate the 49 kDa hemopexin domain MMP-9 fragment, which was detected using proMMP-9. The degradation of APMA-activated MMP-9 by plasmin in the presence of Alendronate or EDTA resulted in the complete loss of MMP-9 gelatinolytic activity in zymograms (Fig. 4B, lanes 6 and 7). In the absence of plasmin, both 1 mM Alendronate and 1 mM EDTA inhibited autocatalytic processing of MMP-9 pre-activated by APMA (Fig. 4B, lanes 2 and 4).

Recombinant 37 kDa human MMP-9 catalytic domain (100 ng) was completely degraded by plasmin (5 μg/ml) within 3 h to 26 kDa, 17 kDa and 8 kDa species recognised by anti-MMP-9 catalytic domain antibody, both in the presence and absence of 1 mM EDTA, in association with complete loss of activity in gelatin zymograms. EDTA alone did not alter recombinant MMP-9 catalytic domain (Fig. 4C and D).

Purified proMMP-9 in TIMP-1-complexed form (150 ng) (Fig. 5A) exhibited low level conversion from 92 kDa to 82 kDa when incubated with plasmin (5 μg/ml) for 3 h (Fig. 5B), and was degraded by plasmin (5 μg/ml) in the presence of 1 mM Alendronate or 1 mM EDTA to the 49 kDa hemopexin–domain fragment, in the absence of changes in 28 kDa TIMP-1 immunoreactivity in Western blots (Fig. 5C). This was associated with the complete loss of MMP-9 activity in gelatin zymograms (Fig. 5B, lanes 3 and 4).

MDA-MB-231 breast cancer cells, characterised by constitutive MMP-9 and plasminogen activator expression [38,39], were cultured for 72 h in serum-free conditions prior to being incubated

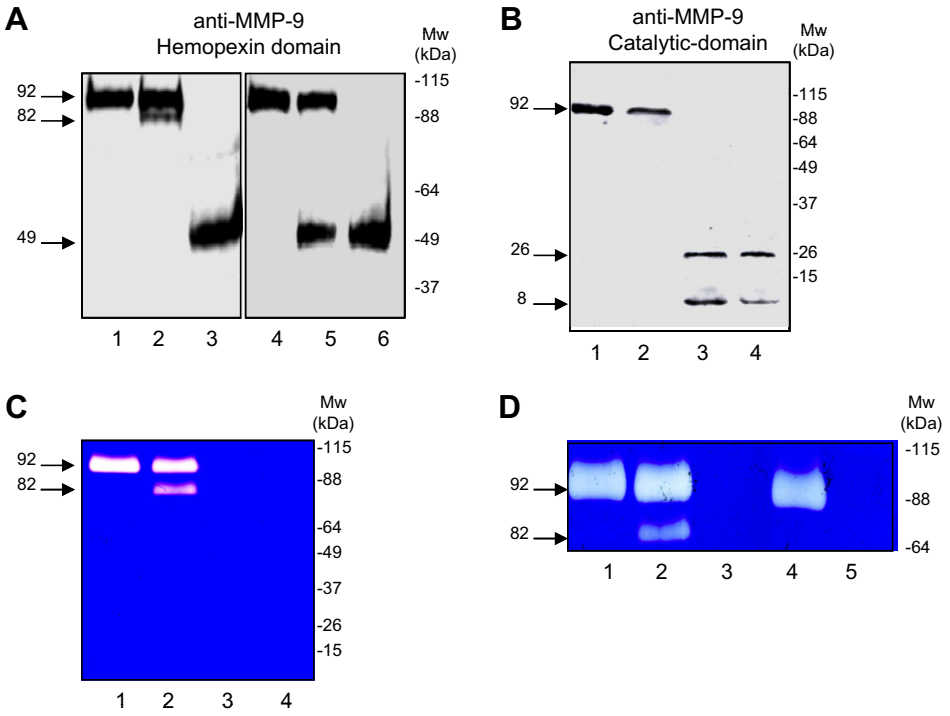

Fig. 3. (A) Representative Western blots demonstrating anti-MMP-9 hemopexin–domain immunoreactivity of 100 ng TIMP-1-free pro-MMP-9: (1) alone; (2) pre-incubated for 3 h with plasmin (5 μg/ml); (3) pre-incubated for 3 h with plasmin (5 μg/ml) plus 1 mM EDTA; (4) pre-incubated for 3 h with plasmin (5 μg/ml) plus 10 μM Alendronate; (5) pre-incubated for 3 h with plasmin (5 μg/ml) plus 10 μM Alendronate; and (6) pre-incubated for 3 h with plasmin (5 μg/ml) plus 1 mM Alendronate. (B) Representative Western blots demonstrating anti-MMP-9 catalytic-domain immunoreactivity of 100 ng TIMP-1-free pro-MMP-9: (1) alone; (2) pre-incubated for 3 h with plasmin (5 μg/ml); (3) pre-incubated for 3 h with plasmin (5 μg/ml) plus 1 mM EDTA; (4) pre-incubated for 3 h with plasmin (5 μg/ml) plus 1 mM Alendronate. (C) Representative gelatin zymogram demonstrating the gelatinolytic of 100 ng TIMP-1-free pro-MMP-9: (1) alone; (2) pre-incubated for 3 h with plasmin (5 μg/ml); (3) pre-incubated for 3 h with plasmin (5 μg/ml) plus 1 mM EDTA; (4) pre-incubated for 3 h with plasmin (5 μg/ml) plus 1 mM Alendronate. (D) Representative gelatin zymogram demonstrating the gelatinolytic of 100 ng TIMP-1-free pro-MMP-9: (1) alone; (2) pre-incubated for 3 h with plasmin (5 μg/ml); (3) pre-incubated for 3 h with plasmin (5 μg/ml) plus 1 mM Alendronate; (4) pre-incubated for 3 h with plasmin (5 μg/ml) plus 1 mM Alendronate plus leupeptin (0.5 μg/ml); (5) pre-incubated for 3 h with plasmin (5 μg/ml) plus 1 mM Alendronate plus TIMP-1 (10 μg/ml).

for 6 h with exogenous plasminogen (10 μg/ml) in the presence or absence of 1 mM Alendronate or 1 mM EDTA. Under these conditions, MDA-MB-231 cells activated exogenous plasminogen, which in the absence of Alendronate or EDTA resulted in plasmin-mediated endogenous proMMP-9 conversion from 92 kDa to 82 kDa, and in the presence of 1 mM Alendronate or 1 mM EDTA resulted in the complete elimination of MMP-9 activity, associated with complete degradation of proMMP-9 to the 49 kDa hemopexin–domain fragment (Fig. 5D and E, lanes 3 and 4). Neither EDTA nor Alendronate alone altered endogenous proMMP-9 secreted by MDA-MB-231 cells in gelatin zymograms or Western blots (data not shown).

Purified TIMP-1-free proMMP-9 was not processed by 6 h incubation with either purified human glu-plasminogen (10 μg/ml) alone; purified human uPA (1 μg/ml) alone; purified glu-plasminogen (10 μg/ml) plus 1 mM EDTA; purified glu-plasminogen (10 μg/ml) plus 1 mM Alendronate; purified uPA (1 μg/ml) plus EDTA or purified uPA (1 μg/ml) plus 1 mM Alendronate (data not shown).

3.3. Plasmin generated MMP-9 hemopexin–domain inhibits the activity of MMP-9

Reverse MMP-9 zymography detected MMP-9 inhibitory activity of approximately 45 kDa in reactions containing MMP-9 degradation products, resulting from 3 h incubation of proMMP-9 with plasmin (5 μg/ml; 0.01 U) in the presence of 1 mM EDTA or 1 mM Alendronate (Fig. 6, lanes 2 and 5) but not in reactions containing proMMP-9 alone or proMMP-9 incubated with plasmin in the absence of Alendronate or EDTA (Fig. 6, lanes 1 and 3). Inhibitory activity was confirmed by the complete absence of equivalent bands in the same samples in Coomassie blue-stained regular non-reducing SDS–PAGE gels (not shown).

4. Discussion

In this study, we report that the divalent cation-binding bisphosphonate Alendronate and the divalent cation-chelator EDTA not only directly inhibit MMP-9 but also promote rapid plasmin-mediated irreversible inactivation of pro-form and active MMP-9. This results from divalent cation chelation-mediated exposure of cryptic plasmin degradation sites within the MMP-9 catalytic domain to plasmin-mediated inactivation by degradation, and in the case of proMMP-9 also generation of an inhibitory MMP-9 hemopexin–domain fragment, defining a novel mechanism for irreversible MMP-9 inhibition.

APMA induced autocatalytic activation of TIMP-1-free proMMP-9 to 82 kDa and 68 kDa species, confirming a previous report [18]. In contrast, purified human plasmin did not activate TIMP-1 free proMMP-9, despite low level 92 kDa proMMP-9 conversion to a single 82 kDa MMP-9 species. This species exhibited activity in gelatin zymograms consistent with an intact catalytic domain but did not bind α2-macroglobulin, which binds activated MMP-9 forms [42,43], indicating that this species is inactive. In support of this and in contrast to 83 kDa APMA-activated MMP-9 that exhibits time-dependent autocatalytic elimination associated with the generation of additional species [this study and 18], the 82kda MMP-9 species generated by plasmin was stable, accumulated over 48 h and did not precede the generation of additional autocatalytic

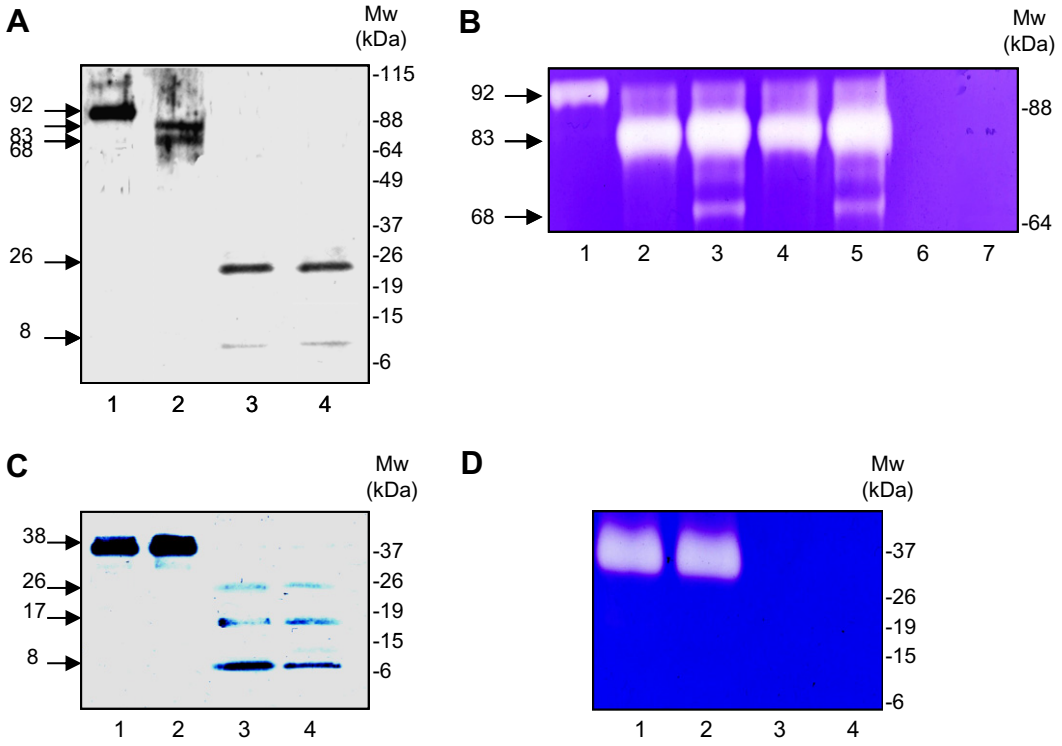

Fig. 4. (A) Representative Western blots demonstrating anti-MMP-9 catalytic-domain immunoreactivity of 100 ng TIMP-1-free pro-MMP-9: (1) alone; (2) pre-incubated for 3 h with 1 mM APMA; (3) pre-incubated for 3 h with 1 mM APMA followed by 3 h with plasmin (5 μg/ml) plus 1 mM EDTA; (4) pre-incubated for 3 h with 1 mM APMA followed by 3 h with plasmin (5 μg/ml) plus 1 mM Alendronate. (B) Representative gelatin zymogram, demonstrating gelatinolytic activity of 100 ng TIMP-1-free proMMP-9: (1) alone; (2) pre-incubated for 3 h with 1 mM APMA then for 3 h with 1 mM EDTA; (3) pre-incubated for 6 h with 1 mM APMA; (4) pre-incubated for 3 h with 1 mM APMA then for 3 h with 1 mM Alendronate; (5) pre-incubated for 3 h with 1 mM APMA then for 3 h with plasmin (5 μg/ml); (6) pre-incubated for 3 h with 1 mM APMA then for 3 h with plasmin (5 μg/ml) plus 1 mM EDTA; and (7) pre-incubated for 3 h with 1 mM APMA then for 3 h with plasmin (5 μg/ml) and 1 mM Alendronate. (C) Representative Western Blot, demonstrating anti-MMP-9 catalytic-domain immunoreactivity of 100 ng purified recombinant MMP-9 catalytic domain; (1) alone; (2) pre-incubated for 3 h with 1 mM EDTA; (3) pre-incubated for 3 h with plasmin (5 μg/ml); and (4) pre-incubated for 3 h with plasmin (5 μg/ml) plus 1 mM EDTA. (D) Representative Gelatin zymogram, demonstrating the gelatinolytic activity of 100 ng purified bacterial recombinant human MMP-9 catalytic domain; (1) alone; (2) pre-incubated for 3 h with 1 mM EDTA; (3) pre-incubated for 3 h with plasmin (5 μg/ml); and (4) pre-incubated for 3 h with plasmin (5 μg/ml) plus 1 mM EDTA.

MMP-9 species. Furthermore, EDTA and Alendronate but not leupeptin inhibited generation of the 83 kDa MMP-9 by APMA and vice versa for the 82 kDa MMP-9 species generated by plasmin, confirming that the former depends upon autocatalytic processing and the latter depends upon plasmin activity and not autocatalysis. These data support some [44,45] but not all reports [46], that human plasmin does not directly activate human proMMP-9 and suggests that the inactive 82 kDa MMP-9 species generated by plasmin may represent a novel MMP-9 pro-form similar to that reported to be expressed by human leukaemia cells [47]. This presumably indicates that 82 kDa proMMP-9 generated by plasmin must contain the cysteine-switch sequence of the pro-peptide domain and must, therefore, result either from carboxyl terminal degradation or amino terminal degradation upstream of the cysteine switch [9].

Consistent with previous reports that bisphosphonates directly inhibit MMP activity, Alendronate directly inhibited the activity of both APMA-activated and SDS-activated MMP-9 in a dose-dependent manner, with an approximate inhibitory IC_{50} of 100 μM. This adds MMP-9 to other MMPs directly inhibited by bisphosphonates and adds Alendronate to other MMP-inhibitory bisphosphonates [28,30–32]. Consistent with this, Alendronate like EDTA, also inhibited APMA-induced autocatalytic MMP-9 processing.

Unexpectedly, plasmin in the presence of Alendronate or EDTA rapidly degraded both latent and APMA-activated MMP-9 to inactive 26 kDa and 8 kDa catalytic-domain fragments and, in the case of proMMP-9 generated a 49 kDa hemopexin–domain fragment, indicating that these divalent cation chelators expose cryptic plasmin-degradation sites within native MMP-9. Under these conditions, MMP-9 degradation resulted in the eventual complete and irreversible loss of MMP-9 activity, in the absence of even transient MMP-9 activation. Under these conditions, plasmin did not degrade APMA-activated MMP-9 to the 49 kDa hemopexin domain fragment, since the hemopexin domain of MMP-9 is eliminated by autocatalysis upon APMA-activation [18]. In contrast to pro-form and APMA activated-MMP-9, plasmin degraded and inactivated purified bacterially expressed recombinant human MMP-9 catalytic-domain both in the presence and absence of divalent cation chelators to 26 kDa, 17 kDa and 8 kDa fragments. This confirms the presence of at least two plasmin degradation sites within the MMP-9 catalytic domain. The fact that these sites do not appear to be cryptic in purified bacterial recombinant human MMP-9 catalytic domain may be the result either of the bacterial expression and purification process or may indicate that these sites are rendered cryptic in the full length native protein by divalent cation regulated structures that lie outside of the catalytic domain, rather than catalytic domain divalent cations [48,49]. In any case, these degradation sites lie within the catalytic-domain amino acid sequence 107–425, downstream of the pro-peptide sequence removed from AMPA-activated MMP-9 by cleavage at amino acids A^{74} and M^{75} [9] and not present in recombinant MMP-9 catalytic domain. Furthermore, it is clear that degradation of these sites must separate the catalytic active site from the catalytic zinc-binding site, since the presence of both is required for MMP-9 catalytic activity and the deletion of the catalytic Zn^{2+} binding site inactivates recombinant MMP-9 catalytic-domain. Within this sequence,

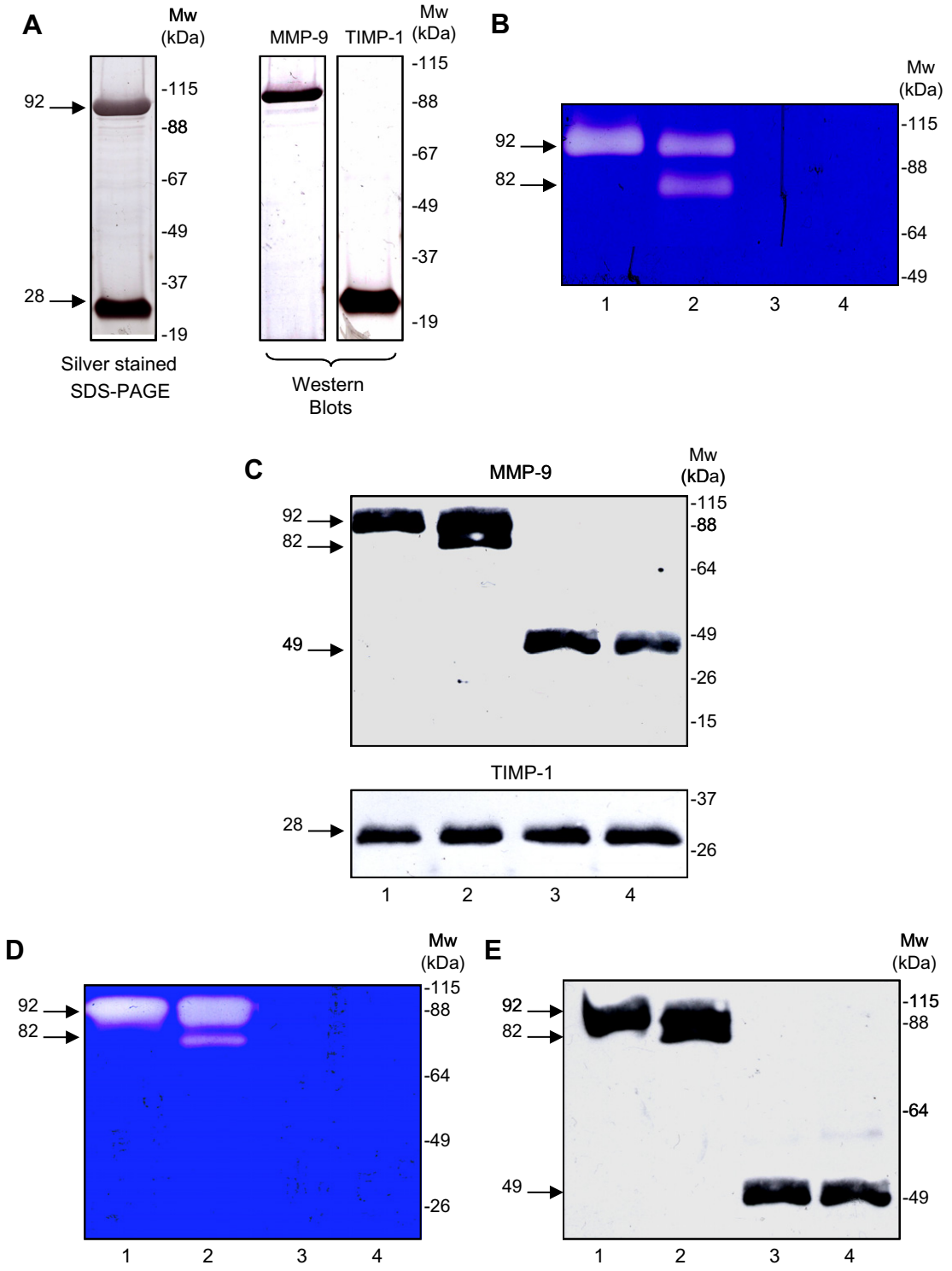

Fig. 5. (A) Silver stained SDS–PAGE (left panel) plus Western blots (middle and right panels) demonstrating relative MMP-9 and TIMP-1 levels in 150 ng MMP-9/TIMP-1 complexes purified from human MDA-MB-231 breast cancer cells; (B) Representative gelatin zymogram, demonstrating gelatinolytic activity of 150 ng purified MMP-9/TIMP-1 complexes: (1) alone; (2) pre-incubated for 3 h with plasmin (5 μg/ml); (3) pre-incubated for 3 h with plasmin (5 μg/ml) plus 1 mM EDTA; (4) pre-incubated for 3 h with plasmin (5 μg/ml) plus 1 mM Alendronate. (C) Representative Western blots demonstrating anti-MMP-9 hemopexin–domain (upper panel) and TIMP-1 (lower panel) immunoreactivity of 150 ng purified MMP-9/TIMP-1 complexes: (1) alone; (2) pre-incubated for 3 h with plasmin (5 μg/ml); (3) pre-incubated for 3 h with plasmin (5 μg/ml) plus 1 mM EDTA; and (4) pre-incubated for 3 h with plasmin (5 μg/ml) plus 1 mM Alendronate. (D) Representative gelatin zymogram, demonstrating gelatinolytic activity of endogenous proMMP-9 in unconcentrated serum-free 72 h MDA-MB-231 conditioned medium: (1) alone; (2) pre-incubated for 6 h with exogenous plasminogen (10 μg/ml); (3) pre-incubated for 6 h with exogenous plasminogen (10 μg/ml) plus 1 mM EDTA; (4) pre-incubated for 6 h with exogenous plasminogen (10 μg/ml) plus 1 mM Alendronate. (E) Representative Western blot demonstrating anti-MMP-9 hemopexin–domain immunoreactivity of endogenous proMMP-9 in unconcentrated serum-free 72 h MDA-MB-231 conditioned medium: (1) alone; (2) from cultures pre-incubated for 6 h with exogenous plasminogen (10 μg/ml); (3) cultures pre-incubated for 6 h with exogenous plasminogen (10 μg/ml) plus 1 mM EDTA; and (4) cultures pre-incubated for 6 h with exogenous plasminogen (10 μg/ml) plus 1 mM Alendronate.

the FN2 repeats separate the active site and catalytic Zn1 binding site and do not depend upon divalent cations for their conformation. This suggests that plasmin degradation site(s) exposed within the catalytic domain by divalent cation chelators may lie outside

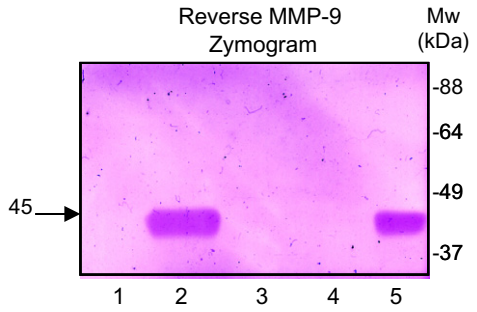

Fig. 6. Reverse MMP-9 zymogram demonstrating (1) no MMP-9 inhibitory activity in a reaction containing 100 ng TIMP-1-free proMMP-9 pre-incubated for 3 h with 1 mM EDTA alone; (2) 45 kDa MMP-9 inhibitory activity in a reaction containing TIMP-1-free pro-MMP-9 pre-incubated for 3 h with plasmin (5 μg/ml) plus 1 mM EDTA; (3) No MMP-9 inhibitory activity in a reaction containing TIMP-1-free pro-MMP-9 pre-incubated for 3 h with plasmin (5 μg/ml) alone; (4) No MMP-9 inhibitory activity in a reaction containing 100 ng TIMP-1-free proMMP-9 pre-incubated for 3 h with 1 mM Alendronate alone; and (5) 45 kDa MMP-9 inhibitory activity in a reaction containing 100 ng TIMP-1-free proMMP-9 pre-incubated for 3 h with plasmin (5 μg/ml) plus 1 mM Alendronate.

the FN2 repeats and either separates the active site from the FN2 repeats plus the functional Zn1 binding site; the active site plus FN2 repeats from the functional Zn1 binding site, or both [48–50].

The effects observed with MMP-9 may also extend to MMP-2, which exhibits close catalytic and hemopexin–domain structural and functional similarity to MMP-9 [48], and is degraded by plasmin in osteoblast cultures in the presence of bisphosphonates [51].

In contrast to the MMP-9 catalytic domain, hemopexin–domain structure does not depend upon divalent cations [48,49], helping to explain why plasmin in the presence of Alendronate or EDTA generated a 49 kDa hemopexin–domain fragment from proMMP-9. MMP-3 also degrades MMP-9 to a 50 kDa hemopexin–domain fragment by cleaving amino acid 429 at the sequence EPE within the linker region that divides the proline-rich O-glycosylation-domain upstream of the hemopexin domain from the catalytic Zn^{2+} binding site [18]. The 49 kDa hemopexin–domain fragment generated by plasmin is likely, therefore, to result from the exposure of a cryptic plasmin degradation site close to this region.

Reverse MMP-9 zymography detected 45 kDa MMP-9 inhibitory activity in reactions containing MMP-9, plasmin and either Alendronate or EDTA, confirming the presence of MMP-9 inhibitory activity, following Alendronate and EDTA removal. This supports and extends a previous report, that recombinant human MMP-9 hemopexin–domain acts as an MMP-9 antagonist [23] and characterises a biological function for the MMP-9 fragment generated by plasmin in this study. Recombinant MMP-9 hemopexin domain has also been reported to inhibit tumour cell invasion, angiogenesis, xenograft growth, metastasis, metastatic bone disease and destructive bone pathology [24–28].

In addition to its effects upon purified pro-MMP-9, APMA-activated MMP-9 and recombinant MMP-9 catalytic domain, plasmin in the presence of Alendronate or EDTA also inactivated proMMP-9 in the presence of molar TIMP-1 excess and in TIMP-1-complexed form indicating that TIMP-1 interaction with the hemopexin–domain [20] does not protect proMMP-9 from plasmin-mediated inactivation by degradation upon cryptic degradation site exposure. Furthermore, in a MDA-MB-231 breast cancer cell model characterised by constitutive proMMP-9 and plasminogen activator expression [38,39], activation of exogenous plasminogen only in the presence of Alendronate or EDTA resulted in proMMP-9 degradation to the 49 kDa hemopexin–domain fragment, associated with complete loss of MMP-9 gelatinolytic activity without evidence of TIMP-1 degradation. The eventual irreversible plasmin-mediated inactivation of MMP-9 by degradation in the presence of divalent cation chelators occurred in the absence of even transient MMP-9 activation, attesting to the incapacity of plasmin to activate proMMP-9 and the direct MMP-9 inhibitory activity of Alendronate and EDTA, which is exemplified by the complete direct inhibition, associated with complete plasmin-mediated degradation of 100 ng MMP-9 by 1 mM Alendronate.

In conclusion, our data provide a novel rational for therapeutic use of Alendronate in MMP-9-dependent pathologies characterised by plasmin activation, both as a direct MMP-9 inhibitor and promoter of irreversible plasmin-mediated inactivation of MMP-9 in zymogen, activated, TIMP-free and TIMP-complexed forms, in addition to providing a novel method for the irreversible inactivation of MMP-9 and production of biologically active MMP-9 hemopexin–domain.

Acknowledgements

The first two authors contributed equally to this manuscript. This work was supported by AIRC, PRIN Cofin and the "Maugeri" Foundation.

References

[1] DeClerck, Y.A. and Laug, W.E. (1996) Cooperation between matrix metalloproteinases and the plasminogen activator-plasmin system in tumour progression. Enzyme Protein 49, 72–84.
[2] Lo, E.H., Wang, X. and Cuzner, M.L. (2002) Extracellular proteolysis in brain injury and inflammation: role for plasminogen activation and matrix metalloproteinases. J. Neurosci. Res. 69, 1–9.
[3] Egeblad, M. and Werb, Z. (2002) New functions for the matrix metalloproteinases in cancer progression. Nat. Rev. Cancer 2, 161–174.
[4] Zucker, S. and Cao, J. (2009) Selective matrix metalloproteinase (MMP) inhibitors in cancer therapy: ready for prime time? Cancer Biol. Ther. 8, 2371–2373.
[5] Chung, A.W., Yang, H.H., Sigrist, M.K., Brin, G., Chum, E., Gourlay, W.A. and Levin, A. (2009) Matrix metalloproteinase-2 and -9 exacerbate arterial stiffening and angiogenesis in diabetes and chronic kidney disease. Cardiovasc. Res. 84, 494–504.
[6] Pepper, M.S. (2001) Role of matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. Aterioscler. Thromb. Vasc. Biol. 21, 1104–1117.
[7] Ra, H.J. and Park, W.C. (2007) Control of matrix metalloproteinase catalytic activity. Matrix Biol. 26, 587–596.
[8] Ramos-DeSimone, N., Hahn-Dantona, E., Sipley, J., Nagase, H., French, D.L. and Quigley, J.P. (1999) Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin cascade enhances tumor invasion. J. Biol. Chem. 274, 13066–13076.
[9] Sang, Q.X., Birkedal-Hansen, H. and Van Wart, H.E. (1995) Proteolytic and non proteolytic activation of human neutrophil progelatinase B. Biochim. Biophys. Acta 1251, 99–108.
[10] Cornelius, L.A., Nehring, L.C., Harding, E., Bolanowski, M., Welgus, H.G., Kobayashi, D.K., Pierce, R.A. and Shapiro, S.D. (1998) Matrix metalloproteinases generate angiostatin: effects on neo-vascularization. J. Immunol. 161, 6845–6852.
[11] O'Reilly, M.S., Wiederschain, D., Stetler-Stevenson, W.G., Folkman, J. and Moses, M.A. (1999) Regulation of angiostatin production by matrix metalloproteinase-2, in a model of concomitant resistance. J. Biol. Chem. 274, 29568–29571.
[12] Zhao, Y., Lyons, C.E., Xiao, A., Templeton, D.J., Sang, Q.A., Brew, K. and Hussaini, I.M. (2008) Urokinase directly activates matrix metalloproteinase 9: a potential role in glioblastoma invasion. Biochem. Biophys. Res. Commun. 369, 1215–1220.
[13] Fiore, E., Fusco, C., Romero, P. and Stamenkovic, I. (2002) Matrix metalloproteinase 9 (MMP-9/gelatinase B) proteolytically cleaves ICAM-1 and participates in tumor cell resistance to natural killer cell-mediated cytotoxicity. Oncogene 21, 5213–5223.
[14] Murphy, G. and Nagase, H. (2008) Progress in matrix metalloproteinase research. Mol. Aspects Med. 29, 290–308.
[15] Ballin, M., Gomez, D.E., Sinha, C.C. and Thorgeirsson, U.P. (1988) Ras oncogene mediated induction of a 92 kDa metalloproteinase: strong correlation with the malignant phenotype. Biochem. Biophys. Res. Commun. 154, 832–838.
[16] Ardi, V.C., Kupriyanova, T.A., Deryugina, E.I. and Quigley, J.P. (2007) Human neutrophils uniquely release TIMP-free MMP-9 to provide a potent catalytic stimulator of angiogenesis. Proc. Natl. Acad. Sci. U.S.A. 104, 20262–20267.
[17] Nagase, H. and Woessner Jr., J.F. (1999) Matrix metalloproteinases. J. Biol. Chem. 274, 21491–21494.
[18] Shapiro, S.D., Fliszar, C.J., Broekelmann, T.J., Mecham, R.P., Senior, R.M. and Welgus, H.G. (1995) Activation of 92-kDa gelatinase by Stromelysin and 4-aminophenylmercuric acetate. Differential processing and stabilization of the

carboxyl-terminal domain by tissue inhibitor of matelloparoteinases (TIMP). J. Biol. Chem. 270, 6351–6356.
[19] O'Connell, J.P., Willenbrock, F., Docherty, A.J., Eaton, D. and Murpphy, G. (1994) Analysis of the role of the COOH terminal domain in the activation, proteolytic activity and tissue inhibitor of metalloproteinase interactions with gelatinase B. J. Biol. Chem. 269, 14967–14973.
[20] Cha, H., Kopetzki, E., Huber, R., Lanzendorfer, M. and Brandstetter, H. (2002) Structural basis of the adaptive molecular recognition by MMP-9. J. Mol. Biol. 320, 1065–1079.
[21] Van den Steen, P.E., Van Aelst, I., Hvidberg, V., Piccard, H., Fiten, P., Jacobsen, C., Moestrup, S.K., Fry, S., Royle, L., Wormald, M.R., Wallis, R., Rudd, P.M., Dwek, R.A. and Opdenakker, G. (2006) The hemopexin and O glycosylated domains tune gelatinase B/MMP-9 bioavailability via inhibition and binding to cargo receptors. J. Biol. Chem. 281, 18626–18637.
[22] Piccard, H., Van den Steen, P.E. and Opdenakker, G. (2007) Hemopexin domains as multifunctional ligand modules in matrix metalloproteinases and other proteins. J. Leukoc. Biol. 81, 870–892.
[23] Reob, E., Schleinkofer, K., Kernebeck, T., Potsch, S., Jansen, B., Behrman, I., Matern, S. and Grotzinger, J. (2002) The matrix metalloproteinase 9 (MMP-9) hemopexin domain is a novel gelatin binding domain and acts as an antagonist. J. Biol. Chem. 277, 50326–50332.
[24] Bjorklund, M., Heikila, P. and Koivunen, E. (2004) Peptide inhibition of catalytic and non catalytic activities of matrix metalloproteinase-9 blocks tumor cell migration and invasion. J. Biol. Chem. 279, 29589–29597.
[25] Monferran, S., Paupert, J., Dauvillier, S., Salles, B. and Muller, C. (2004) The membrane form of the DNA repair protein Ku interacts at the cell surface with metalloproteinase 9. EMBO J. 23, 3758–3768.
[26] Jadhav, U., Chigurupati, S., Lakka, S.S. and Mohanam, S. (2004) Inhibition of matrix metalloproteinase-9 reduces in vitro invasion and angiogenesis in human microvascular endothelial cells. Int. J. Oncol. 25, 1407–1414.
[27] Ezhilarasan, R., Jadhav, U., Mohanam, I., Rao, J.S., Gujrati, M. and Mohanam, S. (2009) The hemopexin domain of MMP-9 inhibits angiogenesis and retards the growth of intracranial glioblastoma xenograft in nude mice. Int. J. Cancer 124, 306–315.
[28] Melani, C., Sangaletti, S., Barazzetta, F.M., Werb, Z. and Colombo, M.P. (2007) Amino- bisphosphonate-mediated MMP-9 inhibition breaks the tumor bone marrow axis responsible for myeloid-derived suppressor cell expansion and macrophage infiltration in tumor stroma. Cancer Res. 67, 11438–11446.
[29] Gumienna-Kontecka, E., Jezierska, J., Lecouvey, M., Leroux, Y. and Kozlowski, H. (2002) Bisphosphonate chelating agents. Coordination ability of 1-phenyl-1hydroxymethylene bisphosphonate towards Cu(2+) ions. J. Inorg. Biochem. 89, 13–17.
[30] Teronen, O., Konttinen, Y.T., Lindqvist, C., Salo, T., Ingman, T., Lauhio, A., Ding, Y., Santavirta, S., Valleala, H. and Sorsa, T. (1997) Inhibition of matrix metalloproteinase-1 by dichloromethylene bisphosphonate (Clodronate). Calcif. Tissue Int. 61, 59–61.
[31] Teronen, O., Konttinen, Y.T., Lindqvist, C., Salo, T., Ingman, T., Lauhio, A., Ding, Y., Santavirta, S. and Sorsa, T. (1997) Human neutrophil collagenase MMP-8 in peri-implant sulcus fluid and its inhibition by clodronate. J. Dent. Res. 76, 1529–1537.
[32] Heikkila, P., Teronen, O., Moilanen, M., Konttinen, Y.T., Hanemaaijer, R., Leitinen, M., Maisi, P., van der Pluijm, G., Bartlett, J.D., Salo, T. and Sorsa, T. (2002) Bisphosphonates inhibit stromelysin-1 (MMP-3), matrix metalloelastase (MMP-12), collagenase-3 (MMP-13) and enamelysin (MMP-20), but not urokinase-type plasminogen activator, and diminish invasion and migration of human malignant and endothelial cell lines. Anticancer Drugs 13, 245–254.
[33] Rosen, C.J. and Kessenich, C.R. (1996) Comparative clinical pharmacology and therapeutic use of bisphosphonates in metabolic bone diseases. Drugs 51, 537–551.
[34] Finley, R.S. (2002) Bisphosphonates in the treatment of bone metastases. Semin. Oncol. 29, 132–138.
[35] Sato, M., Grasser, W., Endo, N., Akins, R., Simmons, H., Thompson, D.D., Golub, E. and Rodan, G.A. (1991) Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. J. Clin. Invest. 88, 2095–2105.
[36] Fleisch, H. (1994) Bisphosphonates: pharmacology. Semin. Arthritis Rheum. 23, 261–262.
[37] Shipman, C.M., Rogers, M.J., Apperley, J.F., Graham, R., Russel, G. and Croucher, P.I. (1998) Anti-tumor activity of bisphosphonates in human myeloma cells. Leuk. Lymphoma 32, 129–138.
[38] Mackay, A.R., Ballin, M., Pelina, M.D., Farina, A.R., Nason, A.M., Hartzler, J.L. and Thorgeirsson, U.P. (1992) Effect of phorbol ester and cytokines on matrix metalloproteinase and tissue inhibitor of metalloproteinase expression in tumor and normal cell lines. Invasion Metastasis 12, 168–184.
[39] Teti, A., Farina, A.R., Villanova, I., Tiberio, A., Tacconelli, A., Scortino, G., Chambers, A., Gulino, A. and Mackay, A.R. (1998) Activation of MMP-2 by human GCT23 giant cell tumour cells induced by osteopontin, bone sialoprotein and GRGDSP peptides is RGD and cell shape dependent. Int. J. Cancer 77, 82–93.
[40] Festuccia, C., Bologna, M., Vicentini, C., Tacconelli, A., Miano, R., Violini, S. and Mackay, A.R. (1996) Increased matrix metalloproteinase-9 secretion in short term tissue culture of prostatic tumor cells. Int. J. Cancer (Pred. Oncol.) 69, 386–393.
[41] Farina, A.R., Coppa, A., Tiberio, A., Tacconelli, A., Turco, A., Colletta, G., Gulino, A. and Mackay, A.R. (1998) Transforming growth factor-β1 enhances the invasiveness of human MDA-MB-231 breast cancer cells by up-regulating urokinase activity. Int. J. Cancer 75, 721–730.
[42] Morodomi, T., Ogata, Y., Sasaguri, Y., Morimatsu, M. and Nagase, H. (1992) Purification and characterisation of matrix metalloproteinase 9 from U937 monocytic leukaemia and HT-1080 fibrosarcoma cells. Biochem. J. 285, 603–611.
[43] Ogata, U., Itoh, Y. and Nagase, H. (1995) Steps involved in the activation of the pro-matrix metalloproteinase 9 (progelatinase B)-tissue inhibitor of metalloproteinase-1 complex by 4-aminophenylmercuric acetate and proteinases. J. Biol. Chem. 270, 18506–18511.
[44] Okada, Y., Gonoji, Y., Naka, K., Tomita, K., Nakanishi, I., Iwata, K., Yamashita, K. and Hayakawa, T. (1992) Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) from HT-1080 human fibrosarcoma cells. Purification and activation of the precursor and enzymic properties. J. Biol. Chem. 267, 21712–21719.
[45] Itoh, Y. and Nagase, H. (1995) Preferential inactivation of tissue inhibitor of metalloproteinases-1 that is bound to the precursor of matrix metalloproteinase 9 (progelatinase B) by human neutrophil elastase. J. Biol. Chem. 270, 16518–16521.
[46] Baramova, E.N., Bajou, K., Remacle, A., L'Hoir, C., Krell, H.W., Weidle, U.H., Noel, A. and Foidart, J.M. (1997) Involvement of PA/plasmin system in the processing of pro-MMP-9 and in the second step of pro-MMP-2 activation. FEBS Lett. 405, 157–162.
[47] Ries, C., Pitsch, T., Mentele, R., Zahler, S., Egea, V., Nagase, H. and Jochum, M. (2007) Identification of a novel 82 kDa proMMP-9 species associated with the surface of leukaemic cells: (auto-) catalytic activation and resistance to inhibition by TIMP-1. Biochem. J. 405, 547–558.
[48] Maskos, K. (2005) Crystal structure of MMPs in complex with physiological and pharmacological inhibitors. Biochimie 87, 249–263.
[49] Diaz, N. and Suarez, D. (2007) Molecular dynamics simulations of matrix metalloproteinase 2: role of the structural metal ions. Biochemistry 46, 8943–8952.
[50] Xia, Y., Garcia, G., Chen, S., Wilson, C.B. and Feng, L. (1996) Cloning of rat 92-kDa type IV collagenase and expression of an active recombinant catalytic domain. FEBS Lett. 382, 285–288.
[51] Ichinose, Y., Migita, K., Nakashima, T., Kawakami, A., Aoyagi, T. and Eguchi, K. (2000) Effects of bisphosphonate on the release of MMP-2 from cultured human osteoblasts. Tohuko J. Exp. Med. 192, 111–118.