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