Detection of endogenous matrix metalloprotease-12 active form with a novel broad-spectrum activity-based probe

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Running title: detection of MMP-12 active form

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Background: The detection of MMP active forms remains a challenge

Result: An endogenous active form of MMP-12 was detected in animal fluids with a new activity-based probe.

Conclusion: The presence of MMP active forms can be demonstrated only with a highly sensitive probe.

Significance: It should be possible to validate active forms of MMP as potential biomarkers in different physiopathological contexts.

SUMMARY

Matrix metalloproteases (MMPs) have attracted considerable attention as critical mediators of pathological tissue remodelling processes. However it remains an unresolved challenge to detect their active forms in biological samples. To prove the efficacy of a recently developed MMP activity-based probe, we examined the content in MMP active forms of bronchoalveolar lavage fluids, (BALf), from male C57BL/6 mice exposed to ultrafine carbon black nanoparticles, a model of chronic obstructive pulmonary disease. This probe was shown to label proteins, mostly expressed in BALf of mice exposed to nanoparticles. Using competition assays with a selective MMP-12 inhibitor, as well as MMP-12 knockout mice, one of these proteins was identified as the active form of the catalytic domain of MMP-12. This new probe can detect the active form of MMP-12 down to a threshold of 1 femtomole. Radioactive counting showed the concentration of the active form of MMP-12 to be around 1 femtomole/µL in BALf from nanoparticle treated mice. A less sensitive probe would therefore not have detected MMP-12. As the probe can detect other MMPs in the femtomolar range, it is a potentially powerful tool for monitoring the levels MMP active forms in various diseases.

Matrix metalloproteases (MMPs) belong to a family of structurally related extracellular/cell surface-anchored zinc-endoproteases (1-3). MMPs form a group of 23 members in human containing a catalytic domain belonging to the metzincin superfamily of proteases (4). MMPs cleave extracellular matrix proteins and are thus considered to be critical mediators of both normal and pathological tissue remodelling processes (5,6). Furthermore MMPs also act as key cell regulators of diverse processes by cleaving non-matrix extracellular proteins (7-9). This involvement in multiple pathways led to considerable efforts to detect MMPs overexpression in various pathological conditions, and to validate these proteins as biomarkers or therapeutic targets (6,10-12). Most MMPs are secreted aszymogens, which form active MMPs following the cleavage of a pro-peptide. Active forms of MMPs can be rapidly
inhibited by the natural tissue inhibitors of MMPs (TIMPs) (10,12). The misregulation of these processes may result in an excess of MMP active forms and uncontrolled protease activity. To detect such misregulation, a simple readout of MMP activity in biological fluids or tissue extracts can be thought by using quenched synthetic fluorogenic substrates. However, the presence of many proteinases in these complex media greatly limits the use of this type of approach, as non-specific cleavage may mask the contribution of MMP. Affinity capture methods based on the use of specific antibody have therefore been developed to increase the specificity of FRET assays (13,14). Alternatively, chemical probes able to label only protease active forms, termed activity-based probes (ABPs), have been successively developed for several families of proteinases (15-17). ABP development relies on the presence of a critical catalytic site residue essential for enzyme activity that is covalently modified by a warhead (18). In most cases, this warhead is an electrophilic group that covalently targets a nucleophilic active site residue. For MMPs with no such nucleophilic residues in their active site, ABP development has required the use of groups that can be photoactivated to achieve active site labelling through a non-specific covalent bond-forming event (19). The probe derives its specific affinity and selectivity from an inhibitor moiety interacting only with the free MMP active site (MMP active forms) (20-23). Following this approach, we previously reported an MMP probe incorporating an azide photolabile group. This group was placed on the tip of the P1′ side chain of a potent broad-spectrum phosphinic peptide inhibitor of MMPs (scheme 1, probe 1) (24). Probe 1 also incorporates a tritium atom (3H) to improve the detection by radioimaging of any covalent adduct formed after light activation (24). This probe was shown to be highly efficient for labelling the active site of human MMP-12 and MMP-3, but labelled other MMPs much less efficiently (25). Further studies demonstrated that the MMP crosslinking yield obtained with this probe depended on the presence a nucleophilic group on the side chain of the residue in position 241 of MMPs (MMP-12 numbering) (25,26). Indeed, the reactive intermediate generated by azide group in response to activation by light is known to react preferentially with nucleophilic groups, like that present on the side chain of Lys241 in MMP-12 and His241 in MMP-3. In addition to these probe-related difficulties with the labelling of different human MMPs (hMMPs), we also reported the poor modification of murine MMP-12 (mMMP-12), due to the weak nucleophilicity at neutral pH of the arginine residue in position 241 of this MMP (26). This precludes the use of this probe for the detection of active forms of mMMP-12 in samples from animal models. We overcame this problem, to facilitate testing for MMP in fluids or tissue extracts from animal models, by replacing the azide group in the P1′ position of probe 1 by a trifluoromethylaziridine one (probe 2, scheme 1), as the reactive intermediate generated by this photolabile group in response to light activation is known to react with various types of residues in close proximity (27). Probe 2 also includes radioactive tritium atoms to ensure the sensitive detection of protein covalent adducts by radioimaging and a short polyethylene linker to increase the solubility of the probe in water. This novel probe displays nanomolar affinities towards a large set of human MMPs and was shown to crosslink only active forms of MMPs with very high crosslinking yields, allowing their detection in the femtomolar range in vitro (28). These encouraging results led us to investigate, in the present study, whether probe 2 could be used to detect active form of MMPs in samples from mice. We used probe 2 to analyse the bronchoalveolar lavage fluid (BALF) of mice exposed to ultrafine carbon black nanoparticles, a model of chronic obstructive pulmonary disease (COPD). This animal model was chosen because the lung inflammation induced by these nanoparticles is thought to involve inflammatory cells overexpressing MMP-12 activity (29). In this study, casein zymography was used to infer the presence of the active form of MMP-12 (30). By contrast, the use of probe 2, which interacts only with the free MMP active site, should only result in the detection of active mMMP-12 if present in BALF. Our laboratory has developed a potent and selective inhibitor of
MMP-12 (RXP470.1) (31), which targets mMMP-12 in vivo, in a mouse model of atherosclerosis (32). Thus, MMP-12 selective inhibitor can be used in a competitive assay, to determine, indirectly, whether any of the potential MMP targets of probe 2, one can be identified as MMP-12. In addition to this competition assay, BALF from knockout MMP-12 mice were analysed in this study. To the best of our knowledge, this is the first attempt to detect active forms of MMP in animal fluids, without prior sample concentration. Indeed, in many previous studies, the detection of active forms of MMP in biological samples has been reported only after a step of concentration to increase the MMP quantity, which is a major limiting factor for the detection of active forms of MMP active forms in most samples (19,22,33-35).

EXPERIMENTAL PROCEDURES

Material: Enzymatic assays were performed with a Fluoroskan Ascent photon counter spectrophotometer (Thermo-Labsystems, Waltham, USA) equipped with a device controlling temperature and a plate shaker. For 1D-electrophoresis, the BioRad Mini-Protean III electrophoresis system (Bio-Rad Laboratories, Munich, Germany) was used with mini-gels (thickness 1 mm). The Polyvinylidene difluoride membranes (PVDF, Immobilon Transfer Membranes, pore size: 0.45 µm) used for protein gel-transfer were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Proteins were transferred using Trans-Blot SD Semi-Dry Transfer Cell from Bio-Rad (Bio-Rad Laboratories, Munich, Germany). Radioactivity imaging and counting on PVDF membranes were carried out with a beta-ImagerTM 2000 from Biospace (Paris, France).

Chemicals: Glycerol, Tris base (2-amino-2-(hydroxymethyl)-1,3-propanediol), CaCl2 (calcium chloride), SDS (sodium dodecyl sulfate), TEMED (N,N,N‘,N’-tetramethylethylenediamine), APS (ammonium persulfate), Na2CO3 (sodium carbonate), bromophenol blue, glycine, β-mercaptoethanol and mammalian protease inhibitor cocktail P8340 (targeting all proteases with the exception of metalloproteinases) were obtained from Sigma-Aldrich. Methanol, ethanol and acetic acid were obtained from de VWR Prolabo (Fontenay/sous Bois, France). Acrylamide 40%/bis solution, 37:5:1(2.6% C), molecular markers (Precision Plus Protein Standards, All Blue) and blotting paper (extra thick Blot paper) were obtained from Bio-Rad. Probe 2 was prepared as previously described (28).

Proteins—α-macroglobulin was obtained from R&D System. Catalytic domains of human and murine MMP-12 (hMMP-12 and mMMP-12 respectively) were produced in E. coli as previously described (25).

Enzyme assays: Enzyme inhibition experiments were carried out at 25 °C, in flat-bottomed 96-well nonbinding surface plates (Corning-Costar, Schiphol-Rijk, The Netherlands) in 100 µL of 50 mM Tris-HCl buffer, 10 mM CaCl2, pH 6.8, and the Mca-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg-NH2 (Mca-Mat) substrate. MMPs were incubated at a final concentration of 0.2 nM (hMMP-12) or 0.5 nM (mMMP-12) in the absence or the presence of various concentrations of probe 2. Following incubation in the dark for 45 min, Mca-Mat substrate was added to a final concentration of 9 µM. Fluorescence signals were monitored for 1 h. Ki values of probe 2 were determined towards the two MMPs, by the method proposed by Horovitz and Leviski (36). Titration experiments were carried out for hMMP-12 and mMMP-12 to determine enzyme concentration accurately for each MMP. For this purpose, a broad-spectrum potent MMP inhibitor LD500.1 (Ki (hMMP-2) = 0.02 nM; Ki (mMMP-12) = 1.5 nM) was used, with a final concentration of 10 nM for each MMP in the presence of 18 µM of Mca-Arg-Pro-Lys-Pro-Val-Glu-Nval-Trp-Arg-Lys(Dnp)-NH2. This substrate was selected because it is much less efficiently cleaved than Mca-Mat by these MMPs, thus less than 10% of substrate are degraded by high MMP concentration during the time of measurement.

Exposure by pharyngeal aspiration and bronchoalveolar lavage (BAL): Male C57BL/6 wild-type (WT) and MMP-12 knock-out mice (MMP-12-/-) were purchased from Charles Rivers (France). At the age of seven weeks, mice were anaesthetised with 2% isoflurane and treated with uICB particules (Printex 90, Orion Engineered Carbons, Germany) that had been ground and suspended in phosphate-buffered saline (PBS, pH 7.4). The suspension was dispersed by ultrasonication (20 min, 750 W, 28% of amplitude) (29). Exposure was achieved...
by pharyngeal aspiration with ufCB (300 µg/80 µL/mouse) as previously described (37). For controls, mice were exposed to sterile PBS (80 µL). Three days after exposure, the mice were euthanized by intraperitoneal injection of a mixture of ketamine (150 mg/kg) and xylazine (15 mg/kg). BAL were collected by exposing the lungs and cannulating the trachea for lavage of the bronchoalveolar space with 1 mL PBS. BAL were centrifuged at 300 x g for 10 min at 4°C to afford BAL fluids (BALf) as supernatant and the corresponding pellets. BALf was immediately diluted two-fold in 50 mM Tris-HCl, 10 mM CaCl₂, 0.01% Brij-35, pH 6.8 in the presence of 1X P8340 inhibitor cocktail (1 % v/v), which blocks all proteases other than metalloproteinases. Total amounts of protein were determined using a Bio-Rad Bradford Assay according to the manufacturer’s instructions.

Samples irradiation: Photo-irradiation experiments were performed after the incubation of probe 2 with samples. Samples were irradiated for 10 min at 10 °C with a 1000-Watt mercury lamp (Osram, France Lampes, Saint Cirq, France) at 353 nm at 30 mW.cm⁻², in a room provided with inactinic lighting (sodium light), as previously described in (24).

Buffered mMMP-12 and hMMP-12 labelling with Probe 2: MMP (final concentration 250 nM) and probe 2 (final concentration 1 µM) were incubated in 50 µL of 50 mM Tris-HCl, 10 mM CaCl₂, 0.01% Brij-35, pH 6.8 and then for 45 min in complete darkness, before photo-irradiation. For competition assays with RXP470.1, a sample containing a solution of mMMP-12 (final concentration 250 nM) was first incubated for 45 min with the selective MMP-12 inhibitor RXP470.1 (final concentration 10 µM) in 50 µL of 50 mM Tris-HCl, 10 mM CaCl₂, 0.01% Brij-35, pH 6.8, and then for 45 min incubation in the presence of probe 2 (final concentration 1 µM) before photo-irradiation. For the determination of the mMMP-12 detection threshold, various amounts of mMMP-12 (final concentrations of 0.1 nM, 0.2 nM, 0.5 nM, and 1 nM) and probe 2 (final concentration 100 nM) were incubated in 50 µL of 50 mM Tris-HCl, 10 mM CaCl₂, 0.01% Brij-35, pH 6.8 for 45 min, in complete darkness, and then photo-irradiated.

BALf labelling with probe 2: For samples stability purpose, immediately after collection and processing, equal volumes of BALf samples were incubated by probe 2, photo-irradiated and subjected to denaturation for electrophoresis, blotting and radioimaging analysis (see below). In parallel, protein concentration determination was determined to make it possible to take differences in protein concentration between samples into account. For BALf samples labelling, 50 µL of each samples were incubated for 45 min on ice in the presence of probe 2 (final concentration 100 nM), in complete darkness, before photo-irradiation. For competition experiments with RXP470.1, a similar protocol was applied, but 1 µL of RXP470.1 solutions was added first (yielding a final competitor concentration of either 10 nM, 100 nM, 1 µM or 10 µM), the samples were then incubated for 45 min on ice before the addition of probe 2 (final concentration 100 nM).

α-macroglobulin effect: α-macroglobulin was added (final concentration 760 nM) to 50 µL BALf samples or buffered mMMP-12 samples (10 nM). These samples were incubated for 1 h on ice, and then in the presence of probe 2 (final concentration 100 nM) for 45 min on ice, in complete darkness, before photoirradiation.

Buffered mouse serum albumin labelling by probe 2: 20 µL of mouse serum albumin (0.6 µg/µL, Sigma-Aldrich, A3139) in 50 mM Tris-HCl, 10 mM CaCl₂, 0.01% Brij-35, pH 6.8 were incubated with probe 2 (100 nM) for 45 min, in complete darkness, before photo-irradiation.

Electrophoresis and blotting: Immediately after photo-irradiation, BALf samples (50 µL) were diluted in 12.5 µL of 5X Laemmli loading buffer (0.25% (w/v) bromophenol blue, 10% (w/v) SDS, 60% (w/w) glycerol, 250 mM Tris-HCl pH 6.8 and 10 % β mercaptoethanol) and 20 µL of each samples (1.4 µg to 3.7 µg of total protein amount) were subjected to SDS-PAGE. For the characterization of covalent modification of hMMP-12 and mMMP-12 by probe 2, photo-irradiated samples were diluted 1:20 in 1X Laemmli buffer, to give solutions with a final concentration of 10 fmol,µL⁻¹ for use in 1D-electrophoresis (10 µL, 100 fmol). For the buffered mouse serum albumin experiments, 1X Laemmli buffer (5 µL) were added and 6.2 µL (3 µg) were processed for 1D-electrophoresis. For the mMMP-12 sensitivity experiment, 12.5 µL of each mMMP-12 solution diluted in Laemmli buffer (final concentration 0.08 nM, 0.16 nM, 0.4 nM, 0.8 nM or 1.6 nM, corresponding to 1, 2, 5, 10 and 20 fmol of mMMP-12, respectively) was subjected to 1D-electrophoresis. Samples
were boiled for 5 min at 95°C and immediately resolved by SDS-PAGE electrophoresis in a 1-mm thick 12% or 15% SDS-PAGE gel. Samples and molecular markers were loaded into the wells and a current of 15 mA for 15 min and 25 mA for 1 hour (for one gel) was applied. Proteins were transferred onto PVDF (polyvinylidene fluoride) membrane, using a semi-dry transfer blotting apparatus. Transfer was performed for 1 hour at 400 mA, with a maximum voltage of 50 V. After transfer, membranes were air-dried and radioactivity was counted. Last, lab-made radioactive ink was used to make visible to radioimager the defined positions of molecular markers on the PVDF membranes analyzed.

**Casein zymography:** Casein zymography was performed with precast 12.5 % zymogram (Bio-Rad, 345-0084). BALf from nanoparticle-exposed mice (60 µL, 10.7 µg of proteins) were lyophilized and 15 µL of zymography loading buffer (2 % SDS, 10 % glycerol, 50 mM Tris-HCl, 1‰ bromophenol blue) were added. Samples were heated 15 min at 37 °C before 1D-electrophoresis. After migration, the gel was washed twice 30 min in a 2.5 % Triton solution, then washed with distilled water (4 x 15 min) and incubated for 48 h at 37 °C in 50 mM Tris-HCl, 10 mM CaCl2, pH 7.4. The gel was stained with Coomassie Blue (Coomassie Blue/EtOH/H2O/acetic acid 0.5/250/250/5 w/v/v/v/v) for 30 min and unstained (water/MeOH/acetic acid 60/30/30 v/v/v). Radioimaging: Imaging and radioactivity counting of PVDF membranes were carried out with a beta-ImagerTM 2000 from Biospace (Paris, France), using beta-acquisition and beta-vision+ softwares. This apparatus yields images consisting of a graduated colour projection of radioactivity emitted from the surface of the membrane. Absolute counts of the tritium beta particles on any chosen surface can also be obtained. Direct images analysis provides a preliminary view of “qualitative information”, whereas quantitative information requires precise counting on selected areas of the images, with beta-vision+ software. Counts are obtained in cpm (counts per minute) for a given membrane. Using an internal calibration (involving the spotting of known amounts of radioactivity on the acquisition plate: triplicates of 1, 2, 10 and 50 pCi) and background subtraction, each selected area can be associated with a value expressed in pCi. For the labelling experiment with buffered recombinant MMP-12, a comparison of the cpm obtained for labelled hMMP-12 and for labelled mMMP-12 was sufficient for the assessment of the percentage mMMP-12 crosslinking, as this percentage is know for hMMP-12 (28). In the case of BALf labelling experiment analysis, we took differences in protein concentration between samples into account by carrying out a second normalisation, in all cases, to provide counts in pCi per µg of total protein, based on the protein concentration determined for each sample.

**RESULTS**

**Labelling of catalytic domains of human and murine MMP-12 with probe 2**

The full-length MMP-12 is a 54 kDa pro-enzyme with three domains: an amino-terminal pro-domain and a catalytic domain attached through a hinge-peptide to a hemopexin-like carboxy-terminal domain. Upon activation by proteolytic cleavage of the pro-domain, the hemopexin-like domain is commonly shed from MMP-12 (38-40), which is consistent with the capacity of the MMP-12 catalytic domain to cleave various substrates, including elastin (41). Thus, we first characterised probe 2 with the catalytic domain of human and murine MMP-12. Probe 2 had a similar affinity for these two MMP-12 forms, with a Ki value of 1 nM and 2.4 nM for hMMP-12 and mMMP-12. The covalent modification of MMP-12 by probe 2 (probe labelling) involved the following steps: enzyme and probe incubation, photo-irradiation of the enzyme/probe complex, SDS gel electrophoresis, protein transfer to the membrane and radioimaging. The detection of a radioactive signal for both hMMP-12 and mMMP-12 samples demonstrates that probe 2 covalently modifies both these two proteins (Fig. 1A). The relative intensity of these bands can be used to calculate the crosslinking yield of the covalent modification. We previously reported a crosslinking yield of about 11% for hMMP-12 modification (28). The more intense signal observed with mMMP-12 indicates that probe 2 modifies mMMP-12 with a higher crosslinking yield (23% based on radioactivity counting). When mMMP-12 is first incubated with RXP470.1 (10 µM), a potent selective inhibitor of murine MMP-12 (Ki 4 nM), before the addition of probe 2 and photo-irradiation, the
labelling of murine MMP-12 was abolished (Fig. 1B). Thus, as expected, probe 2 labelled mMMP-12 by covalently modifying only its free active site, demonstrating that probe 2 can be used as an ABP. By targeting only the free MMP active site, probe 2 will not label either pro-MMP or the TIMP/MMP complex, as previously shown (24,28). In buffer, a detection threshold for mMMP-12 with probe 2 of 1 femtomole was obtained (Fig. 1C).

**Labelling of BALf proteins from control and nanoparticles-exposed mice with probe 2.**

Three days after exposure to nanoparticles or PBS as a control, BAL were recovered from mice and centrifuged to discard the cell fraction and obtain a clear fluid (BALf) that was treated with probe 2 for proteins labelling. In control mice, an intense band was observed at about 75 kDa, while faint bands were detected at 25 kDa and 80 kDa (Fig. 2A). In contrast, when probe 2 was incubated with BALf from mice exposed to nanoparticles, the signal around 75 kDa was of similar intensity, but the bands at 25 kDa and 80 kDa were more clearly detected (Fig. 2B), with a 3-fold intensity increase as compared to control (Fig. 2D). Exposure of the animals to nanoparticles thus increased the secretion of proteins in the BALf, which were covalently modified by probe 2. The strong signal at about 75 kDa can mostly be assigned to serum albumin labelling, as a band with a similar molecular weight was observed when a solution of serum albumin was treated with probe 2 and photo-irradiated (Fig. 2C). On casein zymography analysis, BALf from exposed mice yielded a band at 25 kDa suggesting that this proteolytic activity is the one targeted by probe 2. Labelling experiments performed on cell membranes isolated from exposed mice BAL revealed no radioactive bands at 25 and 80 kDa (supplemental Fig. S1). Moreover, the disappearance of the 75 kDa band in these samples is consistent with serum albumin labelling by probe 2 in BALf. In subsequent experiments, only BALf samples were further investigated.

**Identification of the active form of mMMP-12 in BALf from nanoparticles-exposed mice.**

It has been suggested that mMMP-12 expression was induced in this animal model (29). This MMP is known to shed its hemopexin domain to produce acatalytic domain that migrates at 22 kDa (39). Labelling experiments were therefore repeated with the addition of various concentrations of RXP470.1 before the addition probe 2, to provide an indirect demonstration of the presence of the active form of mMMP-12 in BALf. We determined the optimal concentration of RXP470.1 for full competition between RXP470.1 and probe 2 in a complex proteome, by carrying out, preliminary competition assays, in BALf from controls, complemented with recombinant mMMP-12 (1 nM). By identifying the band corresponding to the recombinant catalytic domain of mMMP-12 (18 kDa), these experiments revealed that a full blockade of probe 2 labelling in this complex medium required the use of RXP470.1 concentration of up to 10 µM (Fig. 3A). These competition assays yielded an IC₅₀ of 22 nM for RXP470.1 towards recombinant mMMP-12 in BALf from controls. This difference in IC₅₀ values between buffer and BALf may be due to non-specific binding of RXP470.1 to serum albumin or other protein components present at high concentration in BALf, decreasing the free concentration of RXP470.1 available for mMMP-12 inhibition.

When BALf from nanoparticle-exposed mice were treated with probe 2 in the presence of RXP470.1, a clear dose competition effect was also observed for the 25 kDa band. Indeed, the increase in RXP470.1 concentration resulted in a decrease in the intensity of the band at 25 kDa (Fig. 3B). In figure 3B, the mean intensity of the band at 25 kDa was determined in absence and presence of various concentration of RXP470.1 in six independent animal experiments. These results strongly suggest that the labelled band at 25 kDa corresponds to endogeneous mMMP-12. From this competition assay, an IC₅₀ of 57 nM was determined for RXP170.1 towards endogeneous mMMP-12 in BALf, a value close to that determined for recombinant mMMP-12 (22 nM) under the same conditions. This shift in the RXP470.1 IC₅₀ value may be due not only to binding to serum albumin, as suggested above, but also to differences between recombinant and endogenous mMMP-12. The difference in molecular weight between these two proteins suggests the presence of a C-terminal extension in the endogenous mMMP-12. Indeed, a rather long hinge-peptide has been shown to connect the catalytic domain to the hemopexin domain in
MMP-12.

Probe 2 labelled 80 kDa proteins. However, despite that partial blockade of probe 2 labelling was observed, no significant dose-effect of RXP470.1 was detected, ruling out that this band corresponds to a higher molecular weight of mMMP-12 (supplemental Fig. S2, grey columns). The active form of murine MMP-9 is known to migrate at MW of 95 kDa, whereas the active form of murine MMP-2 has a predicted molecular weight of 62 kDa. However, it should be pointed out that the analysis of BALf by gelatin zymography, which focuses on detection of gelatinase activity, revealed no MMP-2 or MMP-9 activities in these samples (data not shown).

Further support for the conclusion drawn above was obtained by exposing MMP-12 knockout mice (MMP-12-/-) to particles and treating their BALf as for wild-type animals (WT). The intensity of the radioactive labelling of the band at 25kDa was much lower in these animals that in the wild-type mice (Fig. 3C). Competition experiments with RXP470.1 on BALf from four nanoparticle exposed MMP-12 knockout mice (MMP-12-/-) suggested a competition effect. However, due to inter-assay imprecision, these differences were not statistically significant (Fig. 3C). The residual faint labelling at 25kDa may correspond to the catalytic domain of another MMP, in its active form. A faint radioactive signal at 80 kDa for MMP12-/- sample, slightly weaker than that for WT mice samples, was observed but no blockade or dose-effect of RXP470.1 was detected for the labelling extinction of this radioactive band (supplemental Fig. S2, white columns).

Labelling of α-macroglobulin/MMP-12 complex by probe 2.

α-macroglobulin is an abundant 720 kDa homotetrameric blood-plasma general protease inhibitor with a broad specificity, the main function of which is the covalent capture of medium-sized protease active forms in a large cavity, each of its subunit being a glycosylated multidomain protein of 1451 residues (42). Proteases covalently trapped in the large cavity of α-macroglobulin still have their active site that can cleave small synthetic substrates and be blocked by small inhibitors. The presence of the catalytic domain of the active mMMP-12 in the BALf of mice exposed to particles, as shown above, suggests that this fluid does not contain α-macroglobulin, because a radioactive band at high MW would otherwise have been detected. We have tested this hypothesis by determining whether a) α-macroglobulin can covalently trapped the catalytic domain of MMP-12 and b) if this complex can be labelled by probe 2. When BALf from control mice were complemented with recombinant mMMP-12, treated with probe 2, and photo-irradiated, a radioactive signal was detected at the expected molecular weight (18 kDa) (Fig. 4A). However, the prior addition of α-macroglobulin to such samples resulted in a new radioactive band at the expected MW for an α-macroglobulin subunit (150 kDa, Fig. 4A) with concomitant extinction of the 18kDa signal. Thus α-macroglobulin is able to capture active form of mMMP-12 and the corresponding complex can be labelled with probe 2. Similar results were obtained in experiments on BALf from nanoparticles exposed mice, without recombinant mMMP-12 complementation, with a band at 150 kDa and a decrease in the signal at 25 kDa (Fig. 4B). This suggests that the 25 kDa band corresponds to an active protease, with a free active that can be targeted by an activity-based probe. The same decrease in signal intensity was observed for the band at 80 kDa, suggesting that this band also corresponds to a protease including a free active site labelled with probe 2. By contrast, the presence of α-macroglobulin did not affect the signal corresponding to the labelling of serum albumin, as expected.

**DISCUSSION**

Activity-based protein profiling, a method based on the use of ABP to evaluate enzyme function in native biological systems, has led to remarkable applications for many classes of proteases, but also for other hydrolases and kinases (17,18). ABP optimisation is essential, as the integration of these tools into chemical proteomic platforms could accelerate the early stage of drugs discovery (43). Various ABP for MMPs have been described (20-24), but attempts to use them to detect active forms of MMPs in native biological systems was unsuccessful. The need for a photoactivatable group for the
covalent modification of active-site residues in MMPs imposes several constraints not encountered in the development of ABP for targets containing nucleophilic residues in their active sites. First, the covalent modification of the targets requires light activation. True in situ analyses of enzyme function in native biological systems are thus impossible with this type of ABP. Furthermore, given the considerable variability between MMPs in residue content within the active sites, the probe developed might not label all MMPs efficiently (24,25).

The tight regulation of MMP activity through proenzyme activation and natural TIMP inhibitors, together with the low abundance of active forms of MMP and the possible loss of inhibitors, are major considerations in the development of ABP. Furthermore, given the considerable variability between MMPs in residue content within the active sites, the probe developed might not label all MMPs efficiently (24,25). The tight regulation of MMP activity through proenzyme activation and natural TIMP inhibitors, together with the low abundance of active forms of MMP and the possible loss of inhibitors, are major considerations in the development of ABP. Furthermore, given the considerable variability between MMPs in residue content within the active sites, the probe developed might not label all MMPs efficiently (24,25).

We report here the detection, by a novel ABP, of an active form of mMMP-12 in BALf from mice exposed to carbon black nanoparticles. This conclusion was supported by competition assays between probe 2 and the selective RXP470.1 inhibitor and the analysis of experiments in MMP-12-knockout animals. It was possible to distinguish between BALf from controls and treated animals with probe 2, which showed the levels of active forms of MMP-12 in treated animals to increase by 3-fold as compared to controls, thus suggesting that this labelling can be used to monitor pathological progression. Using calibration experiments based on the relationships between the signal intensity observed on radioimaging and amount of recombinant mMMP-12, the actual concentration of active MMP-12 in BALf samples was estimated from a combination of observations to be about 1 nM (1 femtomole/µL). The detection threshold of probe 2 for the recombinant catalytic domain of mMMP-12 is about 1 femtomole. This threshold depends on the crosslinking efficiency of the probe, and the use of radioactivity as a means of detection. We used 8 µL of native BALf samples, in this study, so probe 2 has detected about 8 femtomoles of active endogenous mMMP-12, per experiments. Thus, without the current detection threshold of probe 2, detection of active MMP-12 in BALf samples would have failed. It should also be noted that, by contrast to other reports dealing with the detection of MMPs, our approach involved no prefractionation or sample concentration (44,45). These prior steps may lead to a loss of low-abundance active forms of MMP. After the elimination of cell content by centrifugation, probe 2 was simply added to the remaining BALf after sample dilution by a factor of two. In a mouse model of lung inflammation, Cobos-Correa et al. detected, with a membrane-bound FRET probe, the active form of MMP-12 mostly on the surface of macrophages rather than in BALf (46). Here, with probe 2, active forms of MMP-12 were detected only in BALf. We cannot rule out the possibility that the active form of MMP-12 was lost during the preparation of membranes from macrophages in our assays.

Our analysis of protein adducts resulting from covalent modification by probe 2 was based on the radioimaging of proteins separated by gel electrophoresis. This approach is useful for determining the current MW of these forms, and this information could be of considerable value for MMPs, which, with the exception of MMP-7 and MMP-26, are multidomain proteins. As the hemopexin domain of MMPs has been implicated in either substrate selectivity or sensitivity to TIMP inhibition, information about the actual MW of MMPs in physiopathological contexts is potentially of great value. We show here that the active form of MMP-12 detected in BALf by probe 2 corresponds to the catalytic domain of MMP-12, consistent with the hypothesis that, after activation, MMP-12 sheds its hemopexin domain (39,40).

Having shown that Probe 2 effectively labels the catalytic domain of mMMP-12, the replacement of the radioactive tag by a clickable biotin for the affinity capture of MMP covalent adducts and the identification of MMPs by LC-MS/MS methods (47) can now be envisaged. Indeed, without the use of a specific inhibitor, it is not possible to identify the proteins modified by probe 2 formally in competition assays. We were thus unable, in the present study, to identify the protein labelled at 80 kDa by probe 2. For unclear reasons, partial blockade of probe 2 labelling by RXP470.1 was observed for this band at 80kDa, but with no dose-dependent inhibition by RXP470.1. This protein has proteolytic activity, as demonstrated by its capture by α-macroglobulin (Fig. 4B). We tried to use a potent broad-spectrum inhibitor of MMP.

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to outcompete with probe 2, but no decrease in the radioactive signal was observed. A similar lack of effect of this inhibitor was observed for the 25kDa band. This finding strongly suggests that, unlike to RXP470.1, this broad-spectrum inhibitor is not “bioavailable” under these experimental conditions. Thus, another application of probe 2 would be to determine the bioavailability of MMP inhibitors in complex media. It would also be desirable to limit the crosslinking of serum albumin by probe 2, a difficult task given the extremely high concentration of this protein in most biological samples. As serum albumin labelling was also observed with probe 1, probe 2 was designed by replacing the tryptophan in the P2’ position of the probe 1 with a tyrosine, as such a modification was previously reported to have a major impact on the binding of phosphinic peptide inhibitors to serum albumin (48). This modification did considerably reduce the binding of probe 2 to albumin, resulting in much lower levels of bindings than for probe 1, but residual binding is still observed. However, based on the similar relative radioactive signal intensities of the bands at 75 kDa and 25 kDa, we can conclude that well below 1% of the serum albumin present is actually labelled by probe 2.

Even in its current state, probe 2 should be a good tool for detecting active form of MMP-12 in different physiopathological contexts. In high-risk populations, a single-nucleotide polymorphism in the MMP-12 promoter, resulting in overexpression of the MMP-12 gene, has been linked to a loss of lung function in COPD patients (49). Attempts have also been made to detect active forms of MMP-12 in human sputum from normal, asthmatic and COPD donors. MMP-12 detection was based on FRET activity, with a specific antibody used to capture MMP-12, but conflicting results were obtained (13,14). In patients with COPD, a concentration of active MMP-12 in sputum of about 1.3 ng/mL has been reported (14), thus according to these data probe 2 should be able to detect the presence of active forms of MMP-12 using in larger volumes of such samples.

After the disappointment of clinical trials with early broad-spectrum synthetic inhibitors of MMPs, the field is now resurging with a new focus on the development of selective inhibitors fully discriminating between different members of the MMP family, with several therapeutic applications in perspective (11,50,51). Thus, efficient MMP ABPs are clearly required to support further MMP drug development and for evaluations of the specificity of these selective inhibitors in animal models. Beyond MMP-12, Probe 2 has also been shown to crosslink in vitro other MMPs (28), and should therefore be useful for the detection of MMPs in various biological systems.
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Footnotes

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FIGURE LEGENDS

Figure 1. Covalent labelling of mMMP-12 catalytic domain with probe 2.
Radioimaging of polyvinylidenefluoride (PVDF) membranes after the transfer of proteins from 15% acrylamide SDS-PAGE gels. A) mMMP-12 (250 nM) or hMMP-12 (250 nM) was incubated with probe 2 (1 mM) for 45 min in 50 mM Tris-HCl, 10 mM CaCl₂, 0.01% Brij-35, pH 6.8 before photoirradiation. 100 fmol were loaded per samples. B) Photo-irradiation experiments with probe 2 (1 μM) of mMMP-12 (250 nM) in the absence (left lane) or in the presence (right lane) of RXP470.1 (10 μM, 45 min preincubation before the addition of probe 2). 100 fmol were loaded on the gel. C) Sensitivity of mMMP-12 detection by probe 2 labelling. Photoirradiation experiments were performed with probe 2 (100 nM) incubated with 10, 5, 2 and 1 femtomoles of mMMP-12 (1 nM, 0.5 nM, 0.2 nM and 0.1 nM respectively) in 50 mM Tris-HCl, 10 mM CaCl₂, 0.01% Brij-35, pH 6.8.

Figure 2. Protein labeling of BALf from PBS (control) or nanoparticle exposed mice with probe 2.
Radioimaging of PVDF membrane after the transfer of proteins from 12% acrylamide SDS-PAGE gels. Covalent labelling with probe 2 (100 nM) of A) BALf from mice exposed to PBS (BALf control) (1.4 μg of protein loaded) B) BALf from nanoparticle-exposed mice (BALf treated) (3.8 μg of protein loaded) C) buffered mouse serum albumin (3 μg of protein loaded). D) Radioactivity values per microgramme of proteins (mean ± SEM, n=6) in BALf from control and treated mice, for the 25 kDa protein and for the 80 kDa protein. E) casein zymography of BALf from nanoparticle-exposed mice (10.7 μg of protein loaded).

Figure 3. Competition assays with RXP470.1, a selective MMP-12 inhibitor.
Radioactivity values per microgramme of proteins (mean ± SEM) as a function of RXP470.1 concentration (final concentration 10 nM, 100 nM, 1 μM, 10 μM), for labelling with probe 2 (100 nM) of A) recombinant mMMP-12 added to BALf from control mice (n=6); B) the 25 kDa protein from wild-type (WT) nanoparticle-exposed mice (n=6); C) the 25kDa protein from MMP-12-/- knockout nanoparticle-exposed mice (n=4). Upper panels in B) and C): PVDF membranes from WT nanoparticle-exposed mice (2.4 μg of protein loaded) or MMP-12-/- KO nanoparticle-exposed mice (1.9 μg of protein loaded).

Figure 4. Labelling of α-macroglobulin-MMP-12 complexes with probe 2.
Radioimaging of PVDF membranes after protein transfer from 15% acrylamide gels. Covalent labelling with probe 2 (100 nM) of A) mMMP-12 (10 nM, 40 fmol loaded) in the absence (left lane) or presence (right lane) of α-macroglobulin (760 nM, prior incubation for 1 h). B) BALf from nanoparticle-exposed mice in the absence (left lane) or in the presence (right lane) of α-macroglobulin (760 nM with prior incubation for 1 h).
FIGURE 1

A) hMMP-12  mMMP-12
MW (kDa)

Crosslinking yield (%) 11  23

B) mMMP-12  +  +
RXP470.1  -  +
MW (kDa)

C) mMMP-12 (fmol) 10  5  2  1
MW (kDa)
FIGURE 3

A) IC$_{50}$ = 22 nM  

B) IC$_{50}$ = 57 nM  

C)
FIGURE 4

A) mMMP-12
α-macroglobulin + +
- +

MW (kDa)
250
150
100
75
50
37
25
20

B) α-macroglobulin - +

MW (kDa)
250
150
100
75
50
37
25
20
**Scheme 1**: chemical structures of probe 1 and probe 2 bearing respectively the photoactivatable azido and the trifluorodiazirine groups in the P₁' position.

![Chemical structure of probe 1](image)

MW (not activated) : 776.28 Da

![Chemical structure of probe 2](image)

MW (not activated) : 987.39 Da
Detection of endogenous matrix metalloprotease-12 active form with a novel broad-spectrum photoaffinity probe
Catherine Nury, Sarah Bregant, Bertrand Czarby, Fannely Berthon, Evelyne Cassar-Lajeunesse and Vincent Dive

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