Novel specific human and mouse stromelysin-1 (MMP-3) and stromelysin-2 (MMP-10) antibodies for biochemical and immunohistochemical analyses

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Short running title: Novel specific MMP-3 and MMP-10 antibodies

Abstract

Matrix metalloproteinases (MMP) are a family of more than 25 zinc-dependent enzymes that are centrally involved in cellular migration, tissue remodelling, cancer invasion and metastasis. Besides degrading extracellular matrix proteins, MMPs are crucial for growth factor and cytokine release and activation. At the same time, they can inactivate inflammatory mediators and enzymes themselves through protein degradation. Subclasses of MMPs include collagenases, gelatinases, stromelysins, membrane-bound MMPs and others. With regard to the stromelysin subfamily, 3 members exist, e.g. stromelysin-1 (MMP-3), stromelysin-2 (MMP-10) and stromelysin-3 (MMP-11). MMP-3 and MMP-10 share extensive similarities at the amino acid level that made it difficult to develop specific antibodies distinguishing between MMP-3 and MMP-10. Scrutinizing published data on and performing different analyses with detection of both stromelysins with commercially available or lab-made antibodies showed ambiguous results with regard to specificity of antibodies used to date. We developed new specific antibodies against the most divergent parts of the active forms of both proteins. We assessed the specificity of our novel specific anti-human and anti-mouse MMP-3 and MMP-10 antibodies in cell lysates and different human and murine skin tissues. Tests analysing specificity of the novel antibodies included Western immunoblotting, immunofluorescence and immunohistochemistry on paraffin sections. Analyses demonstrated specific detection of respective protein for human or mouse samples except for the anti-human MMP-3 antibody. The aim of this summary was to call attention the MMP research community to distinguish clearly between both enzymes. Our new specific anti-mouse MMP-3 and both MMP-10 antibodies allow us to address this detection problem and to enable comparative studies between both stromelysins with regard to their respective location and function in the tissue.

Introduction

Since the discovery of the first matrix metalloproteinase (MMP), i.e. collagenase by Gross and Lapière in 1962[1] numerous other MMPs have been described. Meanwhile, this family of zinc-dependent endopeptidases has grown up to 25 currently known extracellular members.[2] Subgroups of the MMP family constitute the collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11) and membrane-bound MMPs.

Stromelysin-1 (MMP-3) was first purified from bone tissue and called proteoglycanase in 1983.[3] Due to its stromal origin and hydrolytic activity, it was named stromelysin in 1985.[4] Okada isolated two isoforms of the enzyme and named it MMP-3 in order to distinguish it from MMP-1 and MMP-2.[5] MMP-3 is secreted as a 57 kDa proenzyme and activated by cleavage of the propeptide resulting in a 45 kDa peptide. By autolytic cleavage, active MMP-3 is further processed to a 28 kDa form with indistinguishable activity and substrate specificity.[6] Substrates for MMP-3 are many extracellular matrix proteins (e.g. collagen type II and III, laminin, proteoglycans), cell surface proteins (e.g. E-cadherin) and other MMPs (MMP-1, MMP-8 and MMP-13) that it activates by pro-peptide cleavage. Furthermore, it is a potent activator of plasminogen by binding to tPA (tissue-type plasminogen activator).[6] MMP-3 enzyme expression is induced by cytokines and tumour necrosis factor-alpha (TNF-α) in synovial fibroblasts[4] and skin.[7] During skin wound healing, keratinocytes at the wound margin express MMP-3.[8] MMP-3 knock-out mice develop normally and show no specific phenotype. Challenged by wounding, cutaneous healing is marked by reduced wound contraction albeit uncompromised epithelial migration.[9] MMP-3 overexpression in cutaneous keratinocytes facilitates wound healing by accelerated wound closure (unpublished own data). Besides skin repair, MMP-3 plays an important physiological role in various organs, e.g. mammary gland, cartilage, alveolar wound healing and some tumours. Main interest focussed on MMP-3 has been due to its contribution in pathological conditions such as osteoarthritis, synovitis, cartilage destruction and promotion of metastasis by E-cadherin cleavage.[6]

An important problem in the interpretation of immunological data for MMP-3, e.g. derived from Western blots or immunohistochemistry, has been the fact that MMP-3 is extremely similar to stromelysin 2 (MMP-10). MMP-10 is secreted as 54 kDa protein in its latent form being subsequently activated to a 44 kDa protease, just like MMP-3. It was cloned and described for the first time by rat stromelysin cDNA hybridisation based on the high similarity in their mRNA sequences.[10] A comparison between MMP-3 and MMP-10 revealed that both enzymes are extremely similar and share up to 78% identity at the amino acid level in their active forms. Differences exist with regard to pH optimum,[11] enzyme activation and expression in various cells.[8] MMP-10 shares several features with MMP-3, namely the ability to cleave many extracellular matrix proteins,[12] e.g. proteoglycans[13] and to activate collagenases.[14] Further substrates are collagen type III and IV, fibronectin and aggrecan.[15]

Whilst much research has focused on the specific biological functions of MMP-3 or MMP-10, only a few groups have addressed the problem of developing specific immunodetection based tools to clearly distinguish between both enzymes.[8, 16-18] Although some of the strategies to make specific antibodies or block residual cross reactivity seem convincing, there is little published proof for the specificity of the used antibodies for direct comparison between MMP-3 and MMP-10. Due to almost identical protein migration behaviour in SDS PAGE, Western blot detection does not provide direct evidence for identification of the protein bands. It is even more problematic, if only immunohistology is applied without further biochemical identification of the antigen. Hence, we want to alert researchers to the difficulty but necessity to clearly distinguish between MMP-3 and MMP-10, and to point out that insufficiently characterised antibodies should be dealt with great care, especially as one may compensate for the absence of the other, for example in knockout animals. To overcome specificity issues with several commercially available antibodies tested, we developed new antibodies against both enzymes in mouse and in humans and verified their specificity by direct comparison between MMP-3 and MMP-10.

Methods

First, commercially available MMP-3 and MMP-10 antibodies were used for Western Immunoblotting. Because of cross-reactivity of antibodies between both enzymes, novel peptides were designed as tailor-made antigens to generate novel antibodies selective for either MMP-3 or MMP-10. The "Method" section is divided into a) methods that describe the analyses with the commercially available antibodies, then b) the design to develop novel antibodies and finally, c) the methods for testing all novel antibodies.

a) Methods for analyses with commercially available antibodies

Skin tissue provenience and ethics

Patients undergoing plastic surgery for reduction plasty or wound excision donated skin after giving their written consent. The Ethics Committee of the Medical Chamber of Bremen (No. 336/12 and RA/RE-336) approved the study. The skin was kept at 4°C in 0.9% NaCl (saline) and used within 4 hours after removal.

Animals, husbandry and ethics

MMP-3 and wildtype animals

All animals were kept in in cages with a ground area of 440 cm^2 under controlled standardised conditions: temperature 21 ± 2°C, relative humidity 50 ± 5%, and artificial light (14 h light, 10h dark, light on at 06:00 a.m.). Sterilised commercial softwood granulate bedding was used (Lignocel, Altromin; Lage, Germany). Autoclaved commercial pellet diet (Altromin 1314) and autoclaved water were provided *ad libitum*.

Whenever possible, mice were kept in sibling groups. The microbiological status was examined as recommended by FELASA and absence of listed microorganisms, except *Pasteurella pneumotropica*, *Helicobacter sp*., and *Norovirus* was confirmed.

The experiments were in accordance with the German Animal Welfare Legislation and approved by the local Institutional Animal Care and Research Advisory Committee and permitted by the Lower Saxony State Office for Consumer Protection and Food Safety (reference number 42500/1H, 13A404 and 522-27-11/02-00/118).

Mice of the colony Stock *Mmp3*^{tm1Mol}, kindly provided by GA Rosenberg, Albuquerque, NM, USA were delivered on a non-standardized genetic background. After SNP marker assisted backcrossing to C57BL10.RIIIH2^{r} (N7) C57BL10.RIIIH2^{r}-*Mmp3*^{tm1Mol} /Ztm mice were maintained as congenic inbred strain. Informative SNPs were rs13459107, rs13459122, rs13459145, rs13459160, rs13475760, rs13475913, rs13476182, rs13476801, rs13476956, rs13477019, rs13477132, rs13477622, rs13477746, rs13477824, rs13477863, rs13478000, rs13478995, rs13479233, rs13479522, rs13479733, rs13479929, rs13479999, rs13480014, rs13480122, rs13480362, rs13480759, rs13480829, rs13480835, rs13481014, rs13481039, rs13481117, rs13481439, rs13481573, rs13481634, rs13481676, rs13482397, rs13482551, rs13482990, rs13483208, rs13483296, rs13483369, rs13483883, rs29359333, rs31233932, rs3687811, rs3711383, rs4165065, rs4225661, rs6371316. Genotype of the *Mmp3* locus was controlled by PCR using oligonucleotides MMP3-null neo cassette sense: 5`- CGG ATT TGC CAA GAC AGA GTG – 3` and MMP3-null neo cassette antisense: 5`- GGC TAC CCG TGA TAT TGC TGA A – 3`. Genetic background of the inbred mice was regularly controlled.[19] The strain C57BL10.RIIIH2^{r}-*Mmp3*^{tm1Mol} /Ztm is abbreviated as BL10.

MMP-3 deficient mice were crossed back to a homozygous BL10 background for 10 generations as described above. Male MMP-3 knock-out and BL10 wildtype mice were used for this experimental setting. Full-thickness, excisional wounds in MMP-3 knock-out and BL10 wildtype mice were made as described

elsewhere.[20] Mice were sacrificed by decapitation four or eight days after wounding. The skin was excised and kept in cold PBS buffer for immediate use.

Experimental model setting for MMP-10 experiments

Full-thickness excisional wounds in non-diabetic C57BL/KsOlaHsd-Db/+ mice (henceforth referred to as Db+ mice; Harlan UK Ltd) were made essentially as described previously[21] under Project Licence no. PPL80/1799. For the study reported here, two wounds were made in the dorsal surface of Db+ mice, which were killed under Schedule 1 of the 1986 Animal Scientific Procedures Act (UK) 7 days post-wounding. Non-wounded skin was excised at 2 cm distance from the wound site. Skin tissue was cut into small explants and cultured for 24 hours in DMEM, (supplemented with mM L-glutamine, 100 units/ml Penicillin/Streptomycin and 10% foetal calf serum). Monensin interferes with protein glycosylation in the Golgi apparatus and extracellular secretion.[22] To prevent MMP secretion from cells/explants, Monensin (2μM) was added to samples of MMP-10 experiments for the final 3h of culture.[23] At the end of the culture period tissue was snap frozen in liquid nitrogen.

Western Immunoblotting using commercially available antibodies

Human or mouse skin was homogenized in a modified RIPA lysis buffer (0.1M Tris/HCl, 0.15M NaCl, 1% Triton X-100, 0.1% SDS, pH 7.4) containing complete protease inhibitor cocktail without EDTA (Roche, Mannheim, Germany). Protein content was quantified using the BCA method (Pierce, Rockford, IL, USA). To analyse skin wound extracts by Western blot, 10μg of protein were separated in a 12.5% SDS-PAGE gel and transferred into a PVDF membrane. Proteins were analysed by using different commercially available MMP-3 or MMP-10 antibodies available (Suppl Table 1) and lab-made antibodies (Suppl Table 2).[24] Donkey antibodies to rabbit, goat, rat, and sheep (all from Dianova, Hamburg, Germany) were used as secondary antibodies. The reaction was developed by using ECL Prime Western Blotting Detection kit from Amersham (GE Healthcare, UK).

For MMP-10 detection in Db+ mouse skin, snap frozen tissue was minced finely with scissors in the extraction buffer (10mM Tris-HCL ph7.6, 10mM NaCl, 3mM $MgCl_2$, 1% NP-40 and protease inhibitors) prior to full disruption in the TissueLyser II (Qiagen). Following protein estimation (BCA assay; Pierce) proteins (1.9μg/track) were loaded onto 10% SDS-PAGE gels and transfer to PVDF membrane and Western blotting was performed as described above. Recombinant human MMP10 (kind gift from Professor Andrew Rowan, University of Newcastle) served as a positive control.

b) Development of novel antibodies

RNA isolation and cDNA preparation

Total RNA was isolated by homogenization of the human keratinocyte cell line HaCat, according to manufacturer's protocol (peqGOLD TriFast kit, PEQLAB, Erlangen, Germany) and as described elsewhere[25] using 1 mL TriFast for every 50-100 mg of cell material. cDNA synthesis was carried out according to manufactures protocol (RevertAid™ First Strand cDNA Synthesis Kit, Thermo Scientific, Schwerte, Germany).

PCR and Production of recombinant proteins

Hu/m MMP-3 and MMP-10 were amplified and plasmids were constructed using standard recombinant cloning techniques and integrity of amplification products were verified by DNA sequencing. MMPs were amplified from cDNAs derived from primary mouse keratinocytes or from the human keratinocyte cell line HaCat using the following primers:

Nested sense mMMP-3 5´ GTGGTACAGAGCTGTGGGAAG

sense mMMP-3 5´ CGGGATCCATGAAAATGAAGGGTCTTCCG

Nested anti-sense mMMP-3 5´ CTCAGGATGCCTTCCTTG

Anti-sense mMMP-3 5´ CGGAATTCACAATTAAACCAGCTATTGCT

Nested sense huMMP-3 5´ CAACATAGAGCTAAGTAAAGCC

sense huMMP-3 5´ CGGGATCCATGAAGAGTCTTCCAATCCTACTG

Nested anti-sense huMMP-3 5´ GCCCATATTGTGCCTTCTAC

Anti-sense huMMP-3 5´ CGGAATTCACAATTAAGCCAGCTGTTACT

Nested sense mMMP-10 5´ CCAGGTGCTAGAGAAGGATCG

sense mMMP-10 5´ CGAGATCTATGGAGCCACTAGCCATCCTG

Nested anti-sense mMMP-10 5´ TACAGGGTTGTATATGTCTTG

Anti-sense mMMP-10 5´ CGGAATTCGCACAGCAGCCAGCTGTT

Nested sense huMMP-10 5´ GTAAGGGCAGTGAGAATG

sense huMMP-10 5´ CGGGATCCATGATGCATCTTGCATTCCTTGTG

Nested anti-sense huMMP-10 5´ ACACCCATATCTGTCTTCCCC

Anti-sense huMMP-10 5´ CGGAATTCGCAATGTAACCAGCTGTTACT

containing BamHI and EcoRI (m/hu MMP-3 and hu MMP-1) and BglII and EcoRI (m MMP-10) restriction sites. Amplification products were cloned in frame with the *Strep*Tag coding sequence into BamHI/EcoRI digested pcDNA3 Amp *Strep*Tag vector as described previously.[26]

Novel peptide MMP-3 and MMP-10 antibodies

Specific peptide antibodies raised against human and mouse MMP-3 and MMP-10 were purchased from Biomatik (Cambridge, Canada - SA0033 peptide polyclonal antibody package) using specific peptides for mMMP-3 (CASPDVLVVPTKSNSLEPETSP – peptide1 and CAEDFPGVDSRVDAVFEA – peptide 2), huMMP-3 (CDSPETPLVPTEPVPPEPGTPA – peptide 1 and CAEDFPGIDSKIDAVFEE – peptide 2), mMMP-10 (CSSDATVVPVLSVSPRPETPD – peptide 1 and CTDDFPGIEPQVDAVLHE – peptide 2) and huMMP-10 (CASTEEPLVPTKSVPSGSEMPA – peptide 1 and CADDFPGVEPKVDAVLQA – peptide 2) for immunization of rabbits for each peptide using the manufacturer`s service. The anti-sera were affinity

purified against the respective isoform specific peptide used for immunization and the titre was determined by ELISA by the manufacturer.

c) Analyses for comparison between commercial and designed antibodies

Cell culture and transfection

HepG2 and COS-7 were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U x mL^{-1} penicillin and 50 µg x mL^{-1} streptomycin at 37°C in a humidified atmosphere of 5% (vol/vol) CO_2. Transient transfection with plasmids encoding C-terminally Strep-tagged hu/m MMP-3 and MMP-10, respectively was carried out as described elsewhere.[25] For protein expression, HepG2 and COS-7 cells were transfected with the indicated amount of the different plasmids and were harvested after 24 h.[25]

Western blot analysis

HepG2 cells were lysed 24 h post-transfection using TNE buffer (50mM Tris, 140mM NaCl, 5mM EDTA adjusted to pH 7.5). Wound tissues were homogenized in RIPA buffer as described above [27] at pH 7.4. Homogenates were then centrifuged at 12,000g for 10 min and supernatants of the homogenized tissues collected and stored at -20°C until further analysis. Aliquots of the cell lysates or tissue extracts were loaded on 10% SDS Polyacrylamide gel and electrotransferred onto PVDF membranes. The membranes were incubated for 1 hour in a blocking buffer (20mM Tris-HCl buffered saline containing 3% nonfat milk powder and 0.1% Tween 20). Blots were incubated overnight either with our peptide specific antibodies against (human and mouse) MMP-3 and MMP-10 (Biomatik; 1:200) or commercial antibodies as follows: anti-MMP-3 antibody from Acris Antibodies GmbH (AP22793PU-N) (1:125), Assay Biotech (CO272) (1:500), MBL international (JM3523) (1:200), Novus Biologicals (NB100-91878) (1:500), Novus Biologicals (EP118Y) (1:500) (Suppl Table 1) and D248/6 sheep-anti human MMP-10 [17] (Suppl Table 2). After additional washes, the blots were incubated with the corresponding secondary antibody conjugated

with peroxidase detected by addition of chemiluminescent substrate according to the kit used (ThermoScientific ECL Western blot detection kit).

Immunofluorescence

For the immunofluorescence analysis of C-terminally Strep-tagged human and mouse MMP-3 and MMP-10 in transfected cells, COS-7 cells (3×10^4) were plated on coverslips in a 24 well plate and were grown overnight to reach 80% confluence. Fixation and permeabilisation of cells were performed as described previously.[25] COS-7 cells were immunostained using the same antibodies already used for Western blot analysis. After washing twice with PBS/2% BSA, cells were treated for 90 min in PBS/2% BSA at room temperature in the dark with donkey anti-rabbit antibody Cy3 or donkey anti-mouse antibody Cy5 conjugate (Dianova, Hamburg, Germany). Cells were washed twice with PBS, embedded in Fluoro-Gel II (*EMS*, Hatfield, USA) and images were captured with an Apotome fluorescence imager (ApoTome, Axio Imager Z1, Zeiss, Jena, Germany). Commercial and lab-made antibodies were used as stated in Suppl Tables 1 and 2. All antibodies were used at a concentration of 1:200. After washing again with PBS containing 2% BSA, the cells were incubated with the corresponding secondary antibody conjugated with Cy3 or Cy5 fluorophore.

Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde for 12 hours, dehydrated and embedded in paraffin. Four µm sections were stained using antibodies listed in Suppl Table 1. Immunostaining was performed by the avidin-biotin-peroxidase complex technique (DC137C100 Kit; DCS Innovative Diagnostic Systems, Hamburg, Germany). Diaminobenzidine was used as a chromogenic substrate and hemalaun was used as the counterstain, which were described in detail.[28] Imaging was performed with the Nikon Eclipse Ti microscope and digitized pictures analysed using Nis-Elements BR 3.2™ software (Nikon). Immunohistochemical stainings with peptide antibodies were performed on tissues from at least three

different donors, e.g. wildtype animals (n=6), MMP-3 knock-out animals (n=4), human burned skin and human normal skin (each n=3).

Sandwich Immunoassay for MMP detection

For quantitative MMP-3 and MMP-10 analysis, two different sandwich immunoassays were purchased from Meso Scale Discovery®, Gaithersburg, MD, and tested for MMP-3 and MMP-10 cross-reactivity. The human MMP-2-Plex ultra-sensitive kit detecting human MMP-2 and MMP-10 (#KI15033C) and the human MMP-3-Plex ultra-sensitive kit detecting human MMP-1, MMP-3 and MMP-9 (#KI15034C) were used according to manufacturer's instructions. Standard series of each kit were added to the plates and detection recorded.

Results

The need for novel MMP-3 and MMP-10 antibodies resulted from the disappointing attempt to demonstrate lack of MMP-3 protein in MMP-3 knock-out mice by Western blot analysis. Using different commercial antibodies, we still detected distinct bands with the same electrophoretic running behaviour in wildtype and in MMP-3 knock-out animals despite of negative results with DNA and RNA analysis (data not shown). We reasoned that the most likely explanation for this phenomenon was antibody cross-reactivity due to the extreme similarity between MMP-3 and MMP-10 proteins[6].

Analyses with commercially available and lab-made antibodies

To confirm our suspicion of unwanted cross-reactivity of anti-MMP-3 antibodies with MMP-10, we controlled the specificity of different commercial antibodies against recombinant MMP-3 and MMP-10 derived from human or mouse origin by Western blot analysis (Fig. 1A). To ease comparative analyses,

we ectopically overexpressed C-terminally Strep-tagged human and mouse MMP-3 or MMP-10 in HepG2 cells and loaded aliquots of the cell lysates for SDS-PAGE and subsequent Western-blot analysis (Fig. 1B). Interestingly, none of the commercial anti-MMP-3 antibodies that we used was able to clearly distinguish between MMP-3 and MMP-10 (Fig. 1).

To verify specific binding of commercial antibodies in tissue sections, immunohistochemistry on murine skin from wildtype (BL10) or MMP-3 knock-out mice using commercial antibodies JM3523 and MAB 548 was performed (Suppl Table 1, Fig. 2). Positive staining for MMP-3 was seen with MAB 548 in both MMP-3 wildtype and MMP-3 deficient mouse skin (Fig. 2C,D) and unspecific staining in both groups with JM3523 (Fig. 2A+B). Next, immunofluorescence staining was performed on COS-7 cells overexpressing human or mouse MMP-3 or MMP-10 with commercial antibodies M3B (Novus Biologicals, #NB110 – 57221) and M3C (Abcam, #EP1186Y) (Fig. 3). Good detection of human and mouse MMP-3 was noted with both, M3B and M3C (Fig. 3A,B). However, these antibodies showed cross-reactivity with human MMP-10 (Fig. 3C), but not with mouse MMP-10 (Fig. 3D).

In the next step, we used lab-made antibodies for MMP-3 (L50/6) or MMP-10 (D248/6 or IW13, Suppl Table 2) for Western blot analysis (Fig. 4). As previously reported for human MMP-10,[24] the sheep anti-mouse MMP-3 (L50/6)[24] showed weak cross-reactivity with mouse MMP-10 (Fig. 4A) and the sheep anti-human MMP-10 antibody D248/6 [17] detected additional bands as expected (Fig. 4B). Hence, the need for specific anti-mouse and anti-human MMP-3 antibodies that could clearly differentiate between both enzymes was obvious. The problem of cross-reactivity was hitherto only addressed by the research group around Gillian Murphy and co-workers.[17, 23, 29, 30] Aside from the D248/6 sheep anti-human MMP-10 antibody, another sheep antibody IW13 was generated and reported to be highly specific for MMP-10.[31] Detection of murine MMP-10 with the anti-human MMP-10 antibody IW13 is shown with Western blot in

murine skin extracts (Fig. 4C). Additionally, IW13 detects MMP-10, but not MMP-3 in Western blot analysis (personal communication Professor Gillian Murphy, data not shown).

Antibody development

As stated above, screening commercially available antibodies against MMP-3 revealed inherent difficulties with regard to specificity (Fig. 1). Furthermore, cross-reactivity of these MMP-3 antibodies was insufficiently stated in most product sheets. It is still problematic to verify the antibody's specificity due to the high protein sequence similarity between the active forms of MMP-3 and MMP-10 (Tab. 3) and because of scarce published data showing direct comparison between both enzymes in the same study. Therefore, our first attempt was to identify peptide regions with the highest degree of dissimilarities (Fig. 5). Then, we used those regions for generating specific antibodies that are able to reliably distinguish between MMP-3 and MMP-10 with the goal to make species-specific (human or mouse specific) antibodies as well.

Analyses and tests with our new antibodies against MMP-3 and MMP-10

A series of new peptide specific affinity-purified antibodies (Biomatik, Canada) was raised against selected peptides of human and mouse MMP-3 or MMP-10 as described under Methods. With these antibodies the following enzyme and species specificities were observed by Western blot analysis of cell lysates (Tab. 4, Fig. 6). Anti-mouse MMP-3 peptide 1 showed high species and enzyme specificity (Fig. 6A) whereas peptide 2 was unspecific. Anti-human MMP-3 peptide 1 detected the respective protein with species specificity, however, with strong cross-reactivity with a peptide migrating at around 40 kDa (Fig. 6B). Attempts to identify this 40 KDa protein by immunoprecipitation and subsequent MALDI analysis were not successful (data not shown). The antibody raised against peptide 2 of human MMP-3 gave no specific signal using Western blot analysis (Fig. 6B). Anti-mouse MMP-10 peptide 1 and peptide 2 were highly specific for mouse MMP-10 (Fig. 6C). Anti-human MMP-10 peptide 1 and peptide 2 were specific

for the protein but peptide 2 also recognised murine MMP-10 (Fig. 6D). As a consequence, peptide 1 antibodies of all enzymes were used for further analysis.

Next, the new peptide-derived anti-MMP-3 and anti-MMP-10 antibodies were tested by immunofluorescence on COS-7 cells ectopically expressing strep-tagged human or mouse MMP-3 or MMP-10, respectively (Fig. 7). The binding of antibodies against mouse MMP-3, mouse MMP-10 and human MMP-10 was selective for the cells expressing the respective antigens. The antibody raised against human MMP-3 bound to cells expressing any of the four proteins (Fig. 7). In parallel, stainings with an anti-strep antibody were performed to confirm protein expression. Images of anti-strep staining are shown in Suppl. Fig. 1.

Immunohistochemistry (IHC) with peptide antibodies was performed on paraffin sections of human burn wounds and normal skin tissue (Fig. 8) and of murine excisional wounds (Fig. 9) in MMP-3 knock-out mice or wildtypes. As shown for fluorescence microscopy on MMP overexpressing cells, the anti-mouse MMP-3 antibody was also specific in tissue IHC (Fig 8A, 9A), no staining was observed in human tissue (Fig 8A). The anti-mouse MMP-3 antibody bound specifically to mouse MMP-3 (Fig. 9A) with strong staining of epidermal cells. MMP-3 specificity was further confirmed by immunohistochemistry in MMP-3 knock-out mouse skin tissue, which was not stained (Fig. 9A). With regard to anti-mouse MMP-10 (Fig 8B, 9B), mild cross-reactivity was found only in human burned but not in normal skin tissue (Fig. 8B). Strong immunopositivity was found in murine skin tissues with prominent nuclear staining for MMP-10 in both MMP-3 wild-type and MMP-3 knock-out murine skin cells (Fig. 9B). There was strong detection of MMP-3 by the anti-human MMP-3 antibody in burned skin and weak staining in the basal layer of epidermis and in the dermis of normal human skin (Fig. 8C). Distinct cross-reactivity was found with the human anti-MMP-3 antibody in murine tissue where it stained the whole epidermis in tissues of both murine strains (Fig. 9C) in contrast to the specific anti-mouse MMP-3 antibody that showed

immunopositivity only in the upper epidermal layers of MMP-3 wildtype animals (Fig. 9A). The antigen recognition was unspecific because the epidermal layer of MMP-3 knock-out tissue was stained as well by the human anti-MMP-3 antibody. The human anti-MMP-10 antibody was specific for human tissue (Fig. 8D) with only mild background staining in mouse tissue (Fig. 9D).

Sandwich Immunoassay

Despite of the fact that the manufacturer stated cross-reactivity of MMP-3 and MMP-10 using the MSD® multi-spot assay system, only a high concentration of 25 ng/mL MMP-10 was detected as 10^3-fold lower MMP-3 concentration when added to the kit that detects human MMP-1, MMP-3 and MMP-9 (arrow, Fig. 10C). No cross-reaction was detected when MMP-3 up to a concentration of 25 ng/mL was added to the kit that detects human MMP-2 and MMP-10 (Fig. 10E). Hence, this sandwich immunoassay seems to be reliable for differentiating between human MMP-3 and MMP-10 at concentrations in the range of 10 pg/mL to 10 ng/mL.

Literature overview for the use and verification of anti MMP-3 and MMP-10 antibodies

We felt that it is important to demonstrate the chronology in the use and development of antibodies raised against MMP-3 and MMP-10 to get an overview concerning the difficulties in the interpretation of results of antibodies against MMP-3 and MMP-10 (Suppl Table 5). Clearly, a small number of groups working in this field made the effort to develop specific antibodies.[24, 29, 31, 32] Unfortunately, a direct comparison of these antibodies with respect to their reactivity with MMP-3 and MMP-10 was not provided in any of these studies. Of note, MMP-10 was first discovered in 1988 and cloned by Muller et al.[10] In the following years, lab-made antibodies were then described.[8, 31, 33] At the time when Allan *et al* published their work,[29] stromelysin-2 protein was not yet available and recombinant or purified proteins from different species were not available for the analysis of inter-species cross-reactivity. Later, Hembry *et al* noticed a weak

cross-reactivity of the MMP-3 antibody with MMP-10.[31] Interestingly, while they detected many positively stained cells with the MMP-3 antibody, no cells were positive with the MMP-10 antibody[31] supporting the notion that this anti-MMP-10 antibody did not react with MMP-3. In the study of Dew *et al* [24] species and enzyme cross-reactivity of the anti-mouse MMP-3 antibody was described for human stromelysin-1 MMP-3) and stromelysin-2 (MMP-10).[24] Bord *et al*[16] used the sheep anti-human MMP-3[29] and sheep anti-MMP-10[31] antibodies from the Murphy lab for immunostaining. The authors stated that the minimal cross-reactivity of the MMP-3 antibody[29] was blocked with the MMP-10 peptide used for the immunization without, but no direct comparison was shown.[16] In support of their statement, Bord *et al* observed that different cells or regions were immunostained with either the MMP-3 or MMP-10 antibodies.[16] Lees *et al*[32] made rabbit anti-MMP-3 antibodies but checked cross-reactivity solely against MMP-1, MMP-2 and MMP-9, but not for MMP-10.

Until the early 2000s, antibodies against MMP-3 and MMP-10 were provided by the original research groups and used by several other laboratories (Suppl Table 2). We found the first reports describing the use of commercial antibodies around the year 2004.[34] However, studies directly comparing the specificities of antibodies against MMP-3 and MMP-10 are still extremely scarce (Suppl Table 5).

Discussion

MMP-3 and MMP-10 share a high degree of similarity at the amino acid level and have a similar migration behaviour in SDS PAGE after activation.[6, 15] Because of the fact that both proteins also have overlapping biological functions,[11] it is important to detect their expression with reliable tools that can clearly differentiate between both enzymes. Data postulating a distinguishable cell and/or time dependent expression of MMP-3 and MMP-10 are mainly based on commercial antibodies, which at least should be re-evaluated and verified using both proteins for comparison. A few groups addressed this problem of

potential cross-reactivity similar to our current study.[17, 31] Of note, the group around Gillian Murphy[24, 29] did intensive efforts in MMP-3 and MMP-10 research and developed peptide-derived antibodies that were selected from the peptide region itself and that were designed to yield specific MMP-3 or MMP-10 antibodies. These antibodies have also been used by other groups.[16, 17, 24] Unfortunately, in only one of the screened publications that used antibodies against MMP-3 or MMP-10 in Western blot analysis, immunofluorescence or immunohistochemistry, the protein specificity was clearly verified by direct comparison of the two proteins.[17]

Only a few of the available commercial antibodies have actually been used in published work. For some of these antibodies the manufacturer state cross-reactivity, but most of them are promoted as being specific (Suppl Table 5). We are very well aware of the fact that it is impossible to test each and every available product on the market, and that the commercial antibodies we used in our study only represent a small fraction of all available products. Nevertheless, we demonstrate clearly that these antibodies were not able to discriminate clearly between MMP-3 and MMP-10 in Western blot or immunohistochemistry (see Figs. 1-3). Strikingly, the manufacturer declared specificity for all commercial antibodies used in our study in their data sheets or cross-reactivity against MMP-10 was not mentioned at all. Because the fixation method can have an influence on epitope recognition and activity of respective antibodies, different techniques for tissue fixation may cause a problem in immunohistochemistry. In our experiment, we fixed samples in 4% PFA throughout the experiment for identical conditions for respective analytical method.

With regard to an analytical method that was able to differentiate between MMP-3 and MMP-10 we found only one exception from the previous observation. Recently, MSD® introduced ultrasensitive sandwich immunoassays for detection of multiple MMPs. [35] Despite of the manufacturer's declaration of cross-

reactivity for both enzymes, MMP-10 was only detected by the MMP-1/-3/-9 kit at concentrations above 10 ng/mL with no detection of MMP-3 by the MMP-2/-10 kit.

Cross-reactivity between MMP-3 and MMP-10 of commercial antibodies was the initiator to address the specificity of the currently available products in more detail. Therefore, we developed our own peptide-derived antibodies and selected the most diverging peptide sequences for targeted detection. Western blot analysis, immunofluorescence of cell lysates or immunohistochemistry of skin tissue sections showed how difficult it is to obtain enzyme and species specific antibodies – especially if different analytical methods or complex tissues are used. Out of eight selected antibodies, one anti-mouse antibody showed high enzyme and species specificity, the anti-human MMP-3 antibody cross-reacted with an unknown band at 40 kDa in Western blot analysis. When used in cell immunofluorescence or immunohistochemistry of paraffin embedded tissue sections, the specificity of our peptide based antibodies got even more distinct, with both species and enzyme specificity of the anti-mouse MMP-3 and both MMP-10 antibodies but cross-reactivity of the anti-human MMP-3 antibody in murine skin. One anti-mouse and one anti-human MMP-10 antibody were specific with regard to the enzyme or species. This observation corresponds well with observations made by Murphy's group with the MMP-10 antibody.[31, 36] Especially the IW13 anti-human MMP-10 is well-known for binding specifically and exclusively to human MMP-10[31, 36] whereas some cross-reactivity with MMP-3 was found with the anti-human MMP-10 D248/6 (Prof. Gillian Murphy, personal communication; Fig. 4). Murphy's lab and associated groups, Dew[24] and Bord[16], commented on weak cross-reactivity with MMP-10 of the anti-mouse MMP-3 antibody L50/6 made by the Murphy lab.[29] Clearly, it is more difficult to manufacture specific MMP-3 antibodies without interaction with the MMP-10 protein than vice versa.

Western blot analysis yielded several bands for MMP-10 that did not correspond to the published molecular weight for active forms of MMP-10 at 24 kDa,[37] 44 kDa[15] or 47 kDa.[37] The lower band of MMP-

10 at about 37 kDa could have resulted from amino-terminal truncation of the activated or full-length latent form of MMP-10 by auto-proteolytic processing or by other proteases as described previously,[33] since EDTA was not added to our extraction buffer. Nakamura *et al* found caseinolytic activity of H_2NPhHgAc-activated MMP-10 at 40 kDa under non-reducing conditions.[37] Similar intermediate forms were reported from MMP-3 after autolytic[38] or APMA activation.[39] MMP doublets (> 50 kDa) seen for both enzymes have been ascribed to glycosylation.[33, 40] Anomalous migration behaviour of MMP-3 bands in non-reduced samples was noticed in comparison to samples run under reducing conditions.[4]

In summary, MMPs are important enzymes with multiple functions in physiological and pathological processes. The detection of specific members of the MMP family can be challenging due to similar molecular weights and electrophoretic behaviour, substrate specificity, due to intracellular re-uptake or fast extracellular degradation. The unanimous consent of research groups within the MMP field is the need for a specific determination and detection of all proteinases of this group in general, and with regard to stromelysins, in particular. Careful attention should be paid in all investigations to check for protein specific binding. By using specific antibodies and by scrutinizing carefully for cross-reactivity – within the same experimental setting – we should gain more insight in their specific functions and distributions and, increase thereby the quality of our scientific results.

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Figure legends

Fig.1 Western blot for MMP-3 of homogenized wound tissues from MMP-3 wildtype (WT) and knock-out (KO) mice (A) or overexpressed recombinant proteins (B) using different commercially available MMP-3 antibodies. A. Species specificity of the anti-MMP-3 antibody M3B but cross-reactivity in MMP-3 knock-out mouse tissue with M3D and Acris antibodies. Cross-reactivity with MMP-10 was also found in all samples analysed with MAB548 and JM3523. MW molecular weight marker, WT wild-type, KO MMP-3 knock-out mouse tissue, ns normal skin, wo wound, rmMMP-3 recombinant mouse MMP-3, rhMMP-10 recombinant human MMP-10.

B. Western immunoblotting of cell lysates from human or mouse MMP-3 or MMP-10 overexpressing HepG2 cells using commercial (B) or lab-made (C) antibodies against MMP-3. Note unspecific binding of all commercially available anti-MMP-3 antibodies to any of the overexpressed mouse or human MMP-3 or MMP-10 proteins. C. Species specificity was found with the lab-made anti-mouse MMP-3 antibody L50/6 with mild cross-reactivity against mouse MMP-10. Molecular weight markers are indicated on the left side of each blot. On top of blots respective overexpressed enzyme, e.g. mouse for mMMP-3 or mMMP-10 or human for huMMP-3 or huMMP-10.

Fig. 2. Immunohistochemistry of murine excisional skin wounds from wildtype (A,C) or MMP-3 knock-out mice (B,D) using commercial antibodies JM3523 (A,B) and MAB 548 (C,D; see also Suppl Table 1). Note unspecific staining in tissue sections of both animal strains with JM3523 and positive but enzyme unspecific staining with the anti-MMP-3 antibody MAB 548 in both MMP-3 wildtype and MMP-3 deficient mouse skin wounds. Alexa green MMP-3, red DAPI. Magnification 20x, scale bar 100µm (in D for all samples.

Fig. 3. Immunofluorescence staining of COS-7 cells overexpressing human or mouse MMP-3 or MMP-10 with commercial antibodies M3B (Novus Biologicals, #NB110 – 57221) and M3C (Abcam, #EP1186Y). Note immunopositivity with M3B and M3C for human (A) and mouse (B) MMP-3 and cross-reactivity with human MMP-10 (C) but only weak with mouse MMP-10 (D). Magnification 40x, scale bar 50µm (for all samples).

Fig. 4. Western blot using lab-made antibodies against MMP-3 and MMP-10, (A) sheep anti-mouse MMP-3 antibody (L50/6), (B) sheep anti-human MMP-10 D248/6 [17] or (C) sheep anti-human MMP-10 IW13 [31]. A,B. Tissue homogenates of wounds from MMP-3 wildtype and knock-out mice. rmMMP-3 (A) or MMP-10 standard from cell lysate (c-ly; B) to the right. A. Arrow points to weak binding of MMP-3 antibody to activated form of MMP-10 in MMP-3 knock-out mouse tissue. B. Arrows depict position of active (44 kDa) and latent (54 kDa) forms of mouse tissue MMP-10 differing from position of recombinant mouse MMP-10 from cell lysates. C. Recombinant human MMP-10 (2.5ng; left track) or tissue extract of mouse skin distant to a wound (right) were analysed with the IW13 anti-human MMP-10 antibody. Molecular weights in kDa are indicated on the left side of each blot.

Fig. 5. Alignment of MMP-3 and MMP-10 from human (huMMP-3 and huMMP-10) and mouse (muMMP-3 and muMMP-10). The amino acid sequences translated from the respective coding sequences were aligned using Geneious software (version 5.5) with default settings. Black frames indicate the areas with highest sequence diversity, which were selected for the generation of peptide antibodies prepared as described under methods.

Fig. 6. Western immunoblotting of cell lysates from human or mouse MMP-3 or MMP-10 overexpressing COS-7 cells using the novel peptide antibodies against MMP-3 or MMP-10. A. Specific binding of anti-

mouse MMP-3 **peptide 1** to recombinant mouse MMP-3. Note weak band at 105 kDa. Only weak binding of anti-mouse MMP-3 **peptide 2** to mouse MMP-3 and cross-reactivity with recombinant human MMP-3 and rh- and rm-MMP-10. B. Anti-human MMP-3 **peptide 1** antibody bound specifically to rhMMP-3 but reacted also with a peptide yielding a band at 40 kDa. Unspecific binding of anti-human MMP-3 **peptide 2** antibody. C. Specific binding of anti-mouse MMP-10 **peptide 1** to rmMMP-10 albeit with very weak inter-species cross-reactivity with rhMMP-10. D. High inter-species and enzyme specificity for anti-mouse MMP-10 **peptide 2**. Anti-human MMP-10 antibodies **peptide 1** was specific to enzyme and species whereas anti-human MMP-10 **peptide 2** cross-reacted with mouse MMP-10. Molecular weights are indicated on the left side of each blot. mMMP-3 or MMP-10 recombinant mouse MMP, huMMP-3 or MMP-10 human MMP.

Fig. 7. Immunohistochemistry after incubation of novel peptide antibodies with strep-tagged murine or human MMP-3 or MMP-10 from respective enzyme overexpressing COS-7 cells. mMMP-3 mouse MMP-3, huMMP-3 human MMP-3, mMMP-10 mouse MMP-10, huMMP-10 human MMP-10, anti huMMP-3 peptide antibody, anti mMMP-3 peptide antibody, anti mMMP-10 peptide antibody, anti huMMP-10 peptide antibody; analysis of antibodies with cy2 (yellow) and nuclei with DAPI (blue) filter; magnification 40x.

Fig. 8. Immunohistochemistry after incubation of novel peptide antibodies with human skin tissue. MMP-3 and MMP-10 detection in skin after burn injury (upper row) and in non-injured normal skin (split-thickness skin graft, middle row 10x, lower row 40x). Anti-mouse MMP-3 (A) and MMP-10 (B) and anti-human MMP-3 (C) and MMP-10 (D) antibodies. E. negative control staining with secondary antibody only. Antigen recognition and background staining depended on type of tissue, e.g. wounded or normal tissue. High species specificity with no binding of anti-mouse MMP-3 (A) and weak background staining of anti-mouse MMP-10 (B) in human skin tissues. Of note, anti-mouse MMP-10 showed more staining in burned

wounds compared to normal skin. C. Distinct detection of MMP-3 by anti-human MMP-3 antibody in burned skin and weak staining in the basal layer of normal epidermis and dermis. D. Strong immunopositivity for human MMP-10 in upper layers of epidermis and dermal inflammatory cells in burned tissue (arrow upper row) or inside a vessel of the reticular dermis (arrows middle and lower row) with the anti-human MMP-10 antibody. burn, human 2nd degree burned skin wound (without epidermis), normal, normal human skin derived from split-thickness skin graft (0.2mm thickness) from the same donor. Scale bar upper and middle row 100µm (magnification 10x), scale bar lower row 50µm (40x), scale bars valid for all panels in the corresponding row.

Fig. 9. Immunohistochemistry after incubation of novel peptide antibodies with wounded murine skin tissue of MMP-3 wildtype or MMP-3 knock-out mice. Anti-mouse MMP-3 (A) and MMP-10 (B) and anti-human MMP-3 (C) and MMP-10 (D) antibodies. E. negative control staining with secondary antibody only. A. Specific staining for the anti-mouse MMP-3 antibody was observed with predominant antigen recognition the upper epidermal layers. Of note, nuclear staining of dermal inflammatory cells (arrow A). No immunopositivity with weak background staining was found in MMP-3 knock-out mouse tissue. B. Anti-mouse MMP-10 antibody showed prominent staining to the epidermis with strong nuclear staining of both epidermal and dermal cells of wildtype and MMP-3 knock-out skin tissue (arrows). C. Unspecific staining with cross-reactivity was detected with the anti-human MMP-3 antibody with prominent staining of the epidermis in both mouse strains. D. Weak epidermal background staining was noticed with the anti-human MMP-10 antibody in murine tissue. E. Negative control with tissue samples stained with the secondary antibody only. Upper row magnification of 20x, scale bar 100µm (in E for all samples of the upper row), lower row 40x, scale bar 50µm (in E for all samples in the lower row).

Fig. 10. Sandwich immunoassay results for the detection of MMP-1, MMP-2, MMP-3, MMP-9 and MMP-10 assessed by the human MMP-3-Plex ultra-sensitive kit detecting human MMP-1 (A), MMP-3 (C) and

MMP-9 (D) and the human MMP-2-Plex ultra-sensitive kit detecting human MMP-2 (B) and MMP-10 (E). Note the respective kit detects human MMPs from the pg/mL to the ng/mL range. Cross-reactivity was exclusively found for MMP-10 above concentrations of 10 ng/mL, which was detected as 10^3-fold lower MMP-3 concentration (arrow, E).