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Published in:
Biochimie

DOI:
10.1016/j.biochi.2024.03.011

Publication date:
2024

Document version
Version created as part of publication process; publisher's layout; not normally made publicly available

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Citation for published version (APA):
Matrix metalloproteinase landscape in the imiquimod-induced skin inflammation mouse model

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A R T I C L E   I N F O

Article history:
Received 9 January 2024  
Received in revised form 29 February 2024  
Accepted 18 March 2024  
Available online xxx

Handling Editor: Dr B Friguet

Keywords:  
Parallel reaction monitoring  
Pre-clinical animal model  
Proteases  
Psoriasis  
Skin inflammation  
Targeted proteomics

A B S T R A C T

Inflammation and autoimmunity are known as central processes in many skin diseases, including psoriasis. It is therefore important to develop pre-clinical models that describe disease-related aspects to enable testing of pharmaceutical drug candidates and formulations. A widely accepted pre-clinical model of psoriasis is the imiquimod (IMQ)-induced skin inflammation mouse model, where topically applied IMQ provokes local skin inflammation. In this study, we investigated the abundance of a subset of matrix metalloproteinases (MMPs) in skin from mice with IMQ-induced skin inflammation and skin from naïve mice using targeted proteomics. Our findings reveal a significant increase in the abundance of MMP-2, MMP-7, MMP-8, and MMP-13 after treatment with IMQ compared to the control skin, while MMP-3, MMP-9, and MMP-10 were exclusively detected in the IMQ-treated skin. The increased abundance and broader representation of MMPs in the IMQ-treated skin provide valuable insight into the pathophysiology of skin inflammation in the IMQ model, adding to previous studies on cytokine levels using conventional immunochimical methods. Specifically, the changes in the MMP profiles observed in the IMQ-treated skin resemble the MMP patterns found in skin lesions of individuals with psoriasis. Ultimately, the differences in MMP abundance under IMQ-induced inflammation as compared to non-inflamed control skin can be exploited as a model to investigate drug efficacy or performance of drug delivery systems.

1. Introduction

Inflammatory skin diseases affect millions of people worldwide. To provide better treatments to people suffering from inflammatory skin diseases, several disease-relevant models have been suggested and evaluated. For instance, murine models mimicking human psoriasis have been developed and extensively characterized to improve translation from animal to human [1]. A highly relevant and well-established model is the imiquimod (IMQ)-induced skin inflammation mouse model. In this model, topical IMQ is applied onto mouse ear or back skin repeatedly, which induces rapid skin inflammation with a phenotype with similarities to human psoriasis characterized by skin erythema, scaling, and thickening [2]. Moreover, the specific cytokine expression pattern

https://doi.org/10.1016/j.biochi.2024.03.011
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and cellular infiltrates provoked by the topical IMQ treatment resemble aspects seen in skin from psoriatic patients [3]. Since its establishment in 2009 [2], the IMQ model has been recognized for its convenience, as well as its rapid and robust skin response. The model has mainly been utilized in fundamental research, screening of drug candidates, but more recently also for assessment of drug delivery systems (e.g., topical formulations, polymeric nanoparticles, and lipid-based delivery systems) [3–7]. So far, the model has extensively been characterized using conventional immunochromatographic methods to determine cytokine levels in IMQ-treated mouse skin [2,4,8,9], however, the MMP landscape in the IMQ mouse model has not been investigated yet.

IMQ is a nuclease analogue of the imidazquinoline family, and its primary biological effects are mediated through agonistic activity on toll-like receptor (TLR) 7 and 8 [10]. IMQ-induced skin inflammation is characterized by elevated production of cytokines and chemokines (e.g., IL-1α, IL-6, IL-17A, IL-17F, IL-22, IL-23, CCL-2, and MIP-3α), increased cellular infiltration (e.g., T-cells, macrophages, neutrophils, and dendritic cells), and a subsequent increase in epidermal thickness [3,8]. During skin inflammation, proteases play an essential role by degrading extracellular matrix (ECM) components, leading to the release and activation of chemotactic fragments (e.g., TNF-α, IL-1β, IL-6, CCL-2), which attract immune cells and exacerbate the inflammatory process [11–13]. In skin lesions from psoriatic patients, increased expression and higher concentrations of proteolytic enzymes, in particular matrix metalloproteinases (MMPs), have been reported [14]. MMPs are a family of zinc- and calcium dependent endopeptidases [15]. Under physiological conditions, the activity of MMPs in skin is tightly regulated by tissue inhibitors of metalloproteinases (TIMPs) [16]. However, investigation of inflated skin lesions from patients with psoriasis has shown a significant increase in MMP-2, MMP-3, MMP-7, MMP-8, and MMP-9 on mRNA and protein level [17–19].

Considering the remarkable upregulation of MMPs associated with psoriasis and skin inflammation, it is highly relevant to investigate, if the IMQ model displays similar features, potentially allowing it to be used as a simpler model to describe these conditions. We therefore sought to investigate the MMP abundance in IMQ-treated skin lesions by mass spectrometry, and developed a novel, antibody-free parallel reaction monitoring (PRM) mass spectrometric assay for MMP quantification [20,21]. We chose a subset of MMPs, selecting members from the four major groups of MMPs: stromelysins, gelatinases, collagenases, and matrilysins [18,19,22].

PRM provides a much higher sensitivity and specificity of the protein detection compared to conventional techniques such as Western blot, immunohistochemistry, and enzyme-linked immunosorbent assay (ELISA). In this study, we set out to develop and optimize a novel PRM MS assay, which was subsequently used to analyze the presence of a subset of MMPs in both IMQ-treated and non-treated skin samples. Overall, we aimed to investigate if the MMP abundance in IMQ-treated skin differs from that of control skin, thereby exploring the resemblance to inflamed skin from patients with inflammatory skin diseases such as psoriasis. Beyond evaluation of drug candidates, mapping the MMP landscape in IMQ-treated skin as compared to untreated skin revealed potential of the IMQ mouse model is envisioned to provide a useful tool for evaluating drug delivery systems responsive towards skin disease-related flare-ups of MMP activity.

2. Materials and methods

2.1. Materials

Altrimoin 1329 was purchased from Brogaarden (Lyngby, Denmark), whereas Imiquimod (IMQ) 5% cream was obtained from Meda AB Pharmaceuticals (Aldara; Solna, Sweden). Signaling lysis buffer (CST-9803S) and Halt™ phosphatase inhibitor cocktail (PIE78420) were obtained from ThermoFisher Scientific, (Rockford, USA), while protease inhibitor cocktail tablets (REF 11 873 580 001) were from Roche Diagnostics GmbH (Mannheim, Germany). Sodium Ortho Vandate (NEB-P0758L) from New England Biolabs (Ipswich, USA), and micronic tubes from Micronic (Lelystad, Netherlands).

Moreover, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS), acetonitrile, C18 Empore disks for STAGE pipette-tip columns, chloroacetamide, Dulbecco’s Phosphate Buffer Saline, guanidine hydrochloride, Lys-c, methyl, and trypsin were all obtained from Sigma Aldrich (Darmstadt, Germany). PCT micropestles and PCT microtubes were obtained from Pressure BioSciences (Boston, USA), whereas Bradford reagent (B6916) was purchased from Bio Rad (Copenhagen, Denmark).

2.2. Animals

Female BALB/cAnNCrl mice were purchased from Charles River Laboratories (Charles River, Sulzfeld, Germany) at 7 weeks old, weighing approximately 20 g upon arrival. The mice were housed in the animal facility at LEO Pharma A/S (Ballerup, Denmark) in a controlled environment (20°C–23°C, 30%–60% humidity, 12 h night/day cycle) and provided with ad libitum water and Altrimoin 1329 feed (Brogaarden; Lyngby, Denmark). The experimental procedures were ethically approved by the Danish Animal Experiments Inspectorate (Permission Number 2018-15-0201-01447) and performed in line with relevant guidelines and regulations including the EU Directive 2010/63/EU, the Danish Animal Experimentation Act LBK No 474’, the PREPARE guidelines and the 3Rs (Refinement, Reduction, Replacement) [23].

2.3. Imiquimod-induced skin inflammation mouse model

An overview of the IMQ study outline can be seen in Fig. 1. The back area of the mice was shaved 4 days prior to application of IMQ (5% IMQ, Aldara™, Meda) at 0 h and 24 h. IMQ was applied in a volume of 20 µl on a 1.5 cm × 1.5 cm shaved area on the dorsal back of the mice using a Gilson pipette. Naïve mice with clipped back area were used as controls (n = 3). All mice were terminated by intracardial heart puncture under isoflurane anesthesia, followed by cervical dislocation. An 8-mm biopsy from the IMQ-treated skin was collected for further analysis at indicated timeslots after a second application of IMQ (Table 1). The two separate IMQ studies (study I and II) were originally conducted to assess the cytokine and chemokine levels after treatment with a potential drug candidate for treatment of psoriasis. Skin samples from animals receiving no treatment (with potential drug candidate) but with IMQ-induced skin inflammation were utilized for the PRM analysis described here. Therefore, there is a variation between the studies in the group size and termination time of the mice.

2.4. Protein extraction from murine skin lysates and proteolytic digestion

Skin biopsies were snap-frozen in CK14 homogenizing tubes, and a Precellys 24 tissue homogenizer (both from Bertin Instruments, Montigny-le-Bretonneux, France) used to lyse the skin before protein extraction. The lysis buffer consisted of cell signaling lysis buffer (Cell Signaling Technology, Leksand, Netherlands) with a complete mini protease inhibitor cocktail added [Roche diagnostics, Mannheim, Germany], Halt™ phosphate inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA) and sodium
diluted with a sample volume of dH2O. The protein concentration was carefully decanted. The remaining pellet was air-dried for 30 min at room temperature before being slowly resuspended with 2 mM tris(2-carboxyethyl)phosphine (TCEP) 1:20 (v/v) and incubation on shaker at 65 °C overnight at 350 rpm. The digestion was stopped the next day by adding trifluoroacetic acid (TFA) to a final concentration of 1% (v/v).

2.5. Targeted LC-MS/MS analysis (parallel reaction monitoring)

Prior to MS analysis, samples were pre-treated using liquid-solid phase extraction (SPE) carried out on stop and go extraction (STAGE) pipette tip columns (C18 resin) following the manufacturer’s description. Targeted proteomics experiments were performed using a Q Exactive mass spectrometer (ThermoFisher Scientific, Bremen, Germany). A PepMap™ RSLC analytical column (2 µm C18 beads, Thermo Fisher ES803A) was used for separation. After sample loading, the column was flushed with solvent A (0.1% FA) with an increasing gradient of solvent B (80% ACN, 0.1% FA) with a flowrate of 250 nL/min. Solvent B went gradually from 6% to 60% over 60 min, followed by a washing step going from 60% to 95% over 3 min, and remained at 95% for 7 min to finalize the washing step. A parallel reaction monitoring (PRM) assay optimized for detection of a subset of MMPs (MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, and MMP-13) was utilized. All experiments were conducted in PRM mode for 70 min, with a MS1 resolution of 70,000, the AGC target at 3,000,000, a maximum injection time set to 20 ms, and a scan range going from m/z 375 to 1500 for a complete MS scan. MS2 scans were conducted with a resolution of 70,000, the AGC target at 3,000,000, a maximum injection time of 256 ms, and an isolation window of m/z 1.2 with a normalized collision energy of 30. The instrument was operated in profile mode. MS performance was verified for consistency by running complex cell lysate quality control standards, and chromatography was monitored to check for reproducibility.

The PRM data was analyzed using Skyline (Version 22.2.0.351, MacCoss Lab, Seattle, USA). First the filter parameters at the MS1 orbitrap was defined at a resolution of 60,000, at m/z 400, and MS2 targeted filtering at 60,000 resolution at m/z 200, before importing the targeted peptides. Endogenous peptides had fixed modifications of carboxamidomethylation of cysteines (+57.021 Da) and each precursor mass has at least 4 transitions. To determine the transition peak areas, a peak scoring model from the mProphet algorithm was trained using the second best peaks utilizing the mProphet algorithm, and their boundaries were manually adjusted when necessary [24]. The area under the curve (AUC) of independent injections were normalized based on the total ion current and plotted as log2 transformed values for all detected MMPs in the

**Table 1**

Overview of study design and time of termination.

<table>
<thead>
<tr>
<th>Description</th>
<th>Study</th>
<th>No. of animals</th>
<th>Challenge</th>
<th>Time of termination after 2nd IMQ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy skin</td>
<td>Ctrl</td>
<td>3</td>
<td>–</td>
<td>72</td>
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...
non-treated skin samples (control study) and IMQ-treated skin samples (IMQ study I and II).

2.6. Statistical analyses

Statistical analyses and graphing were performed using GraphPad Prism (8.1.1, GraphPad, Boston, USA). A student’s t-test was conducted to compare two experimental groups, while one-way ANOVA was used for comparing more than two groups. Data are presented as mean ± standard deviation (*p-value <0.05, **p-value <0.01, ***p-value <0.001 and ****p-value <0.0001) with n ≥ 3.

3. Results

The measured abundances of MMPs in control skin samples (naïve mice) and in IMQ-treated skin samples (IMQ study I and II) can be seen in Fig. 2. MMP-2, MMP-7, MMP-8, and MMP-13 were detected in control skin samples (Fig. 2A), with MMP-7 showing the highest abundance. In IMQ-treated skin samples, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, and MMP-13 were detected throughout the study duration of 72 h (Fig. 2B–C). Notably, MMP-2, MMP-7, MMP-8, and MMP-13 were detected with significantly higher abundances in IMQ-treated skin compared to control skin, while MMP-3, MMP-9, and MMP-10 were only detected in the skin after treatment with IMQ. At all time points, MMP-7 was found to

![Graph showing MMP levels](image-url)
be the most abundant MMP in the IMQ-treated skin, whereas MMP-10 and MMP-3 showed the lowest abundances. Between IMQ study I and IMQ study II, there was a significant difference in the abundance of all MMPs at 48 h/53 h, for MMP-2 at 72 h, for MMP-9 at 26 h, and for MMP-3 and MMP-10 at all time points (Supplementary Table 1).

Differences in MMP abundances between control skin samples (naïve mice) and IMQ-treated skin samples (IMQ study I) were identified for MMP-2, for MMP-7, for MMP-8, and for MMP-13 (Fig. 3). Across all four MMP subtypes, a higher abundance can be found at all timepoints for the IMQ-treated skin samples compared to the control skin samples. For MMP-2, MMP-8, and MMP-13, there were no significant change in MMP abundances across the samples from 23 h to 72 h (Fig. 3A–C). In contrast, for MMP-7 there was a significant difference in abundance across the skin samples from the IMQ-treated mice (Fig. 3B). Furthermore, MMP-3, MMP-9, and MMP-10 were not detected in skin samples from naïve mice but were present in IMQ-treated skin samples at all time points (Fig. 3E–G). MMP-3 and MMP-10 showed no change in abundance across all time points, whereas the abundance of MMP-9 was found to increase until 48 h, followed by a decrease in abundance at 72 h.

4. Discussion

As summarized above, a significant difference was found in the MMP landscape between IMQ-treated and control skin (Fig. 2). Not only did we see a significant increase in abundance but also an increase in types of MMPs present due to IMQ-induced skin inflammation. Notably, MMPs across several functional classes, including MMP-2, MMP-7, MMP-8, and MMP-13 were present in control skin, which underlines their importance in physiological processes such as maintaining the turnover of extracellular matrix (ECM) (Fig. 2A) [25]. IMQ is a potent immune activator, the action of which is mediated through interaction with TLR 7 and 8, triggering generation of pro-inflammatory cytokines and chemokines. Thus, after application of IMQ to the skin, immune cells start to migrate to the provoked site, augmenting the inflammatory response further [26]. A crucial role of the inflammatory immune cell response is to regulate the ECM degradation and remodelling, which is exerted by a release of enzymes, cytokines, and growth factors [27]. MMPs are released by immune cells, stromal cells, and epidermal cells to aid in trafficking of immune cells, generate bioactive peptides, and release ECM-anchored growth factors [28]. These inflammatory events provoked by IMQ are likely the reason for the increase in abundances of MMP-2, MMP-7, MMP-8, and MMP-13, and the additional presence of MMP-3, MMP-9, and MMP-10 in IMQ-treated skin investigated here.

The increased and broader MMP abundance in the IMQ-induced skin inflammation mouse model exhibits similarities to MMP patterns detected in inflamed skin lesions from patients with psoriasis. In fact, the upregulation of MMPs has been shown to play a key role in the pathogenesis of psoriasis [22], which is why recent research has focused on the development of MMP inhibitors to treat psoriasis [29]. The hyperproliferation of dermal cells (e.g., keratinocytes, melanocytes, and fibroblasts) in psoriasis lesions, for example, is partly caused by MMP-2, leading to both an increased epidermal thickness and secretion of further MMPs [22,30]. In addition to MMP-2, MMP-9 has been observed at elevated levels during skin inflammation in psoriasis [31–33]. Both MMPs alter the basement membrane at the epidermal and dermal interface [31,32], which correlates well with the ~20% increased abundance of MMP-2 (Fig. 3A) and the appearance of MMP-9 (Fig. 2) upon IMQ-induced skin inflammation. Beyond cleavage of collagens, it has been found that MMP-9 is important in mediating cross-talk between the neutrophils and the endothelial cells in psoriasis, enhancing inflammation [34]. Infiltrating neutrophils in psoriasis lesions, in turn, express several inflammatory mediators including not only MMP-9, but also MMP-8, which mostly cleaves collagens [35]. IMQ-treated skin displays a ~17% higher abundance of MMP-8 compared to control skin, reflecting a relevant skin inflammation-like MMP-8 profile.

Moreover, MMP-7 has been suggested to be a predictor for acute inflammation in psoriasis due to its notable increase in expression as well as positive correlation with IL-6 and psoriasis severity [19]. The upregulation of MMP-7 may trigger several of pro-inflammatory events due to cleavage of ECM substrates, exacerbating inflammatory signals, while also activating pro-MMP-9, pro-MMP-2, and TNF-α [36]. Interestingly, it was found in the current study that MMP-7 displayed an increased abundance of approximately 40% from control skin to IMQ-treated skin (Fig. 3B). In addition, MMP-3 has been found in serum from psoriasis patients with positive correlation to proinflammatory cytokines such as IL-17A, IL-12p70, and TNF-α [33]. MMP-3 is known to process other pro-MMPs, which leads to proteolytic activity and onset of a cascade of degradation processes, which might explain why we see MMP-3 only in the IMQ-treated skin. Moreover, elevated levels of MMP-13 have been found in psoriatic skin lesions [37]. Prior, MMP-13 expression has been investigated in IMQ, providing additional evidence of a higher concentration of MMP-13 was found in the IMQ-treated skin compared to the skin from naïve mice, which correlates well with our findings showing an ~22% increase in abundance of MMP-13 [37].

Overall, the increase in MMP abundance in the IMQ model is a highly relevant characteristic for the investigation of protease-responsive drug delivery systems, such as MMP-responsive hydrogels. Recently, we reported on an MMP-responsive hydrogel, releasing tofacitinib citrate in response to MMP-9 in a flare-up mimicking manner [38,39]. We plan to proceed testing of the hydrogel in vivo, for which the IMQ model is a highly relevant model. Using the IMQ model in combination with targeted proteomics, a high-throughput and sensitive readout of MMP abundance in skin can be obtained. From a broader perspective, extending the usability of the IMQ model may lead to a deeper understanding of the underlying mechanistic factors causing inflamed skin diseases, such as psoriasis. In addition, it may enable improved screening of drug candidates, not only from histopathological and immunological perspectives, but also with respect to the influence of drug candidates on specific protease subsets.

5. Conclusion

Targeted proteomics, such as PRM, offers a highly sensitive and specific analysis of complex tissue samples compared to conventional techniques, enabling the measurement of MMPs in skin samples. Here, PRM was used to investigate the abundances of a subset of MMPs in the IMQ-induced skin inflammation mouse model. We found that the MMP landscape in IMQ-treated skin compared to healthy skin differs in the abundance and presence of a number of key MMPs. The higher abundance, especially for MMP-7, and broader representation of MMPs in the IMQ-treated skin shows similarities to the MMP landscape seen in inflamed skin from patients with psoriasis. Also demonstrating the relevance of the IMQ-treated skin as a psoriasis model, MMP-3, MMP-9, and MMP-10 were only detected in the IMQ-treated skin. Taken together, this study highlights the dynamic changes in the MMP landscape after topical challenge with IMQ, providing additional insight into the intricate inflammatory events in the skin. Notably, the upregulation of MMPs during skin inflammation resembles the MMP patterns found in skin lesions from patients with psoriasis. Collectively, the alteration in the MMP landscape in the IMQ model
Fig. 3. Comparison of MMP abundance in control skin and IMQ-treated skin (IMQ study I). MMP abundance in control skin is presented in a green box and MMP abundance in IMQ-treated skin at different time points are presented in a grey box for (A) MMP-2, (B) MMP-7, (C) MMP-8, (D) MMP-13, (E) MMP-3, (F) MMP-9, and (G) MMP-10.
makes it a promising research tool to assess the efficacy not only of drugs, but also of protease-responsive drug delivery systems.

**Funding**

The work reported in this paper was funded by LEO Pharma A/S (2750 Ballerup, Denmark), as well as the LEO Foundation (Grant number LF15007: AH, M.M.). U.a.d.K. acknowledges support by a Novo Nordisk Foundation Young Investigator Award (NNF16OC0020670) and funding from the LEO Foundation (LF-OC-19-000033 and LF-OC-23-001220).

**Author contributions**


**Data availability**

All mass spectrometry-based proteomics data discussed in this study have been deposited at PanoramaWeb (https://panorama.web.org/Matrix_metalloproteinase_landscape.url) (e-mail: panorama+reviewer215@proteinms.net; password: VjnmMhvPb) with the dataset identifier PXD046049 (https://protemecentral.proteomexchange.org/cgi/GetDataset?ID=PXD046049).

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Acknowledgements**

The authors thank the research department, In Vivo Biology & Biomarkers, at LEO Pharma A/S for providing the skin samples. Moreover, the authors acknowledge Viktorija Kelandiastasi and Daniel Lopez Marcelino for preparing the samples and performing the PRM analysis during their master's project at the Section for Protein Biomarkers.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2024.03.011.

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