MMP Mediated Degradation of Type VI Collagen Is Highly Associated with Liver Fibrosis – Identification and Validation of a Novel Biochemical Marker Assay

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Abstract

Background and Aims: During fibrogenesis, in which excessive remodeling of the extracellular matrix occurs, both the quantity of type VI collagen and levels of matrix metalloproteinases, including MMP-2 and MMP-9, increase significantly. Proteolytic degradation of type VI collagen into small fragments, so-called neo-epitopes, may be specific biochemical marker of liver fibrosis. The aim of this study was to develop an ELISA detecting a fragment of type VI collagen generated by MMP-2 and MMP-9, and evaluate this assay in two preclinical models of liver fibrosis.

Methods: Mass spectrometric analysis of cleaved type VI collagen revealed a large number of protease-generated neo-epitopes. A fragment unique to type VI collagen generated by MMP-2 and MMP-9 was selected for ELISA development. The CO6-MMP assay was evaluated in two rat models of liver fibrosis: bile duct ligation (BDL) and carbon tetrachloride (CCl4)-treated rats.

Results: Intra- and inter-assay variation was 4.1% and 10.1% respectively. CO6-MMP levels were significantly elevated in CCl4-treated rats compared to vehicle-treated rats at weeks 12 (mean 30.9 ng/mL vs. 12.8 ng/mL, p = 0.002); week 16 (mean 34.0 ng/mL vs. 13.7 ng/mL, p = 0.0018); and week 20 (mean 35.3 ng/mL vs. 13.3 ng/mL, p = 0.0033) with a tight correlation between hepatic collagen content and serum levels of CO6-MMP (R² = 0.58, p < 0.0001) in CCl4-treated rats. In BDL rats, serum levels of CO6-MMP were significantly elevated compared to the levels in sham-operated animals both at 2 weeks (mean 29.5 ng/mL vs. 14.2 ng/mL, p = 0.0001) and 4 weeks (mean 33.0 ng/mL vs. 11.8 ng/mL, p = 0.0003).

Conclusions: This novel ELISA is the first assay enabling assessment of MMP degraded type VI collagen, allowing quantification of type VI collagen degradation, which would be relevant for different pathologies. The marker was highly associated with liver fibrosis in two liver fibrosis animal models, suggesting type VI turnover to be a central player in fibrogenesis.

Introduction

Liver fibrosis due to viral or alcohol-induced injury is one of the leading causes of death worldwide [1]. To date no curative treatment for liver fibrosis is available and patients are dependent on the success of inactivation or removal of the injurious agent or in the case of end-stage cirrhosis, on liver transplantation. Assessment of liver fibrosis is important to estimate the prognosis for the progression to liver cirrhosis and to determine surveillance strategies. At present, liver biopsy is the most commonly used method for fibrosis assessment, but it is invasive, associated with patient discomfort and, in rare cases, with serious complications [2]. In addition, the accuracy of liver biopsy is limited due to sampling error and significant intra- and inter-observer variability in histological staging [3,4]. Therefore, research has focused on the evaluation of non-invasive methods for the assessment of liver fibrosis [3].

Fibrosis may be described as extensive scar formation, observed as increased deposition and abnormal distribution of extracellular matrix (ECM) components such as collagens and proteoglycans. ECM remodeling is a key process of tissue homeostasis [6–8], and specific proteolytic activities are a prerequisite for a range of
cellular functions and interactions during the process [9]. The specific proteolytic activities are precisely coordinated under physiological situations, with a specified sequence of events resulting in controlled tissue turnover. In pathological situations, including inflammations, fibrosis and cancer, the normal damage/repair balance is displaced [10], leading to excessive remodeling. As a consequence of this tissue turnover, there is a release of several protein degradation fragments specific for the combination of the involved proteases, the affected organ and the disease. The fragmentation results in exposure of new peptide ends (so-called neo-epitopes) to which specific antibodies can turn over, there is a release of several protein degradation fragments [10], leading to excessive remodeling. As a consequence of this tissue remodeling, with a specified sequence of events resulting in controlled proteolytic activities are precisely coordinated under physiological cellular functions and interactions during the process [9].

Materials and Methods

Ethics Statement

The BDL experiments were approved by the Experimental Animal Committee of the Danish Ministry of Justice and were performed according to the European Standard for Good Clinical Practice (2008/561-1450).

The CCl4 study was approved by the Ethical Committee of Animal Experimentation of the University of Barcelona (B-NNP-233/09) and was performed according to the criteria of the Investigation and Ethics Committee of the Hospital Clinic Universitari (Barcelona, Spain).

Reagents

All reagents used for the experiments were standard high-quality chemicals from companies such as Merck (Whitehouse Station, NJ, USA) and Sigma Aldrich (St.Louis, MO, USA). The synthetic peptides used for monoclonal antibody production were purchased from the Chinese Peptide Company, Beijing, China.

In vitro cleavage

Purified type VI collagen from human placenta (cat. no. ab7538, Abcam, Cambridge, UK) was cleaved with pro-MMP-2 or pro-MMP-9 (cat. no. 444213; 444231; Calbiochem, Merck, Whitehouse Station, NJ, USA). Fifty μg MMP-2 or MMP-9 was activated with 20 μl 1 mM 4-aminophenylmercuric acetate (AMPA) in dimethyl sulfoxide and incubated at 37°C for 3 hours. Type VI collagen was delivered dissolved in 0.5 M acetic acid. To facilitate MMP cleavage, the protein was dialyzed for two days to remove the acetic acid. The liquid was filtered to remove proteins below 10 kDa (Microcon Ultracec YM-10, cat. no. 42407, Millipore, Billerica, MA, USA). Each MMP cleavage was performed separately by mixing 100 μg type VI collagen and 1 μg of either MMP-2 or MMP-9 in MMP buffer (100 mM Tris-HCl, 100 mM NaCl, 10 mM CaCl2, 2 mM Zn acetate, pH 8.0). As control, 100 μg of collagen was mixed with MMP buffer alone. The solutions were incubated for 24 hours at 37°C. The cleavage reaction was stopped using 50 μM ethylenediaminetetraacetic acid (EDTA) to a final concentration of 1 μM. Cleavage was verified by visualization using the SilverXpress Silver Staining Kit (cat. no. LC6100, Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

Pepsin cleavage was performed by mixing 100 μg type IV collagen and 1 μg of pepsin in pepsin buffer (0.2 M sodium acetate buffer, pH 4.0). The resultant enzyme/protein mixture was incubated at 37°C for 2 hours. At the designated time, 2 M Trizma Base was added to adjust pH to neutral to stop the reactions.

Peptide identification

Peptide fragments in the in vitro cleaved samples were identified using liquid chromatography (LC) coupled to electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS). LC-MS samples were ultra-filtrated to remove proteins above 10 kDa, the pH was adjusted to 2.0 using formic acid, and a 4 μl sample was analyzed by LC-MS/MS. LC was performed on a nanoACQUITY UPLC BEH C18 column (Waters, Milford, MA, USA) using a formic acid/acetoniitril gradient. MS and MS/MS were performed on a Synapt High Definition Mass Spectrometry quadrupole time of flight MS (QUAD-TOF; Waters, Milford, MA, USA), with an acquisition range of 350–1600 m/z.
in MS and 50–2000 m/z, in MS/MS. The software “ProteinLynx Global SERVER (PLGS)” (Waters, Milford, MA, USA) was used to analyze spectra and generate peak lists. To identify peptides, MS and MS/MS data was searched against a type VI collagen (FASTA) protein database using the Mascot 2.2 (Matrix Science, Boston, MA, USA) software with the ESI-QUAD-TOF settings and carbamidomethyl (C), oxidation of methionine (M), oxidation of lysine (K) and oxidation of proline (P) as variable modifications.

The six amino acids in the N- or C-terminal of the peptides identified by MS were regarded as a neo-epitope generated by the protease in question. All protease-generated sequences were analyzed for homology and distance to other cleavage sites and tested for homology using NPS@: network protein sequence analysis (Combet C, Blanchet C, Gourjon C, Deleage G. NPS@:network protein sequence analysis. Trends Biochem Sci 2000; 25: 147–50).

Peptide conjugation

The peptide conjugation was performed using the Malecid Activated Immunogen Conjugation Kit (Sigma-Aldrich, MO, USA). Briefly, the cysteine-containing immunogenic neo-epitope (YRGPEGPQGP-GGC, 400 μl peptide at 5 mg/ml) with one free sulfhydryl (-SH) group was mixed in conjugation buffer with the maleimide-activated ovalbumin (OVA) (180 μl OVA at 10 mg/ml) as a carrier protein with an available maleimide group that could react with sulfhydryl-containing peptides and incubated for 2 hours at room temperature. Conjugated products were cleared of EDTA and sodium azide by desalting or dialysis for two days. For the biotin-conjugated peptides, the biotin-conjugated lysine of EDTA and sodium azide by desalting or dialysis for two days.

Monoclonal antibody development

4–6 weeks-old Balb/C mice were immunized subcutaneously with about 200 μl emulsified antigen and 50 μg of the neo-epitope CO6-MMP (YRGPEGPQGP-GGC-OVA). Consecutive immunizations were performed at 2-week intervals until stable sera titer levels were reached in Freund’s incomplete adjuvant. Blood samples were collected from the 2nd Immunization. At each blood sampling, the serum titer was determined and the mouse with highest anti-serum titer was selected for fusion. After the 4th immunization, this mouse was rested for 1 month and then boosted intravenously with 200 μl peptide at 5 mg/ml) with one free sulfhydryl (-SH) group was mixed in conjugation buffer with the maleimide-activated ovalbumin (OVA) (180 μl OVA at 10 mg/ml) as a carrier protein with an available maleimide group that could react with sulfhydryl-containing peptides and incubated for 2 hours at room temperature. Conjugated products were cleared of EDTA and sodium azide by desalting or dialysis for two days. For the biotin-conjugated peptides, the biotin-conjugated lysine was added in the solid-phase peptide synthesis procedure.

Fusion and antibody screening

The fusion procedure performed as described by Gefter et al [29]. Briefly, mouse spleen cells were fused with SP2/0 myeloma fusion partner cells. The hybridoma cells were cloned using a semi-solid medium method and transferred into 96-well microtiter plates for further growth and incubated in a CO2-incubator. Standard limited dilution was used to promote monoclonal growth. Supernatants were screened using an indirect ELISA with streptavidin-coated microtiter plates and YRGPEGPQGP-K-Biotin as a capture peptide.

Characterization of clones

Native reactivity and peptide binding of the monoclonal antibodies was evaluated by displacement of native samples (human/rat/mouse serum, plasma and urine) in a preliminary ELISA using 10 ng/mL biotinylated peptide coater on a streptavidin-coated microtiter plate and the supernatant from the growing monoclonal hybridoma. Specificities of the clones to a free peptide (YRGPEGPQGP), a non-sense peptide, and an elongated peptide (“YRGPEGPQGP”) were tested. Isotyping of the monoclonal antibodies was performed using the Clonotyping System-HRP kit, cat. no. 5300-05 (Southern Biotech, Birmingham, AL, USA). The selected clones were purified using protein G columns according to manufacturer’s instructions (GE Healthcare Life Science, Little Chalfont, Buckinghamshire, UK). Selected monoclonal antibodies were labeled with horseradish peroxidase (HRP) using the Lightning link HRP labeling kit according to the instructions of the manufacturer (Innovabioscience, Babraham, Cambridge, UK).

CO6-MMP ELISA methodology

In preliminary experiments, we optimized the reagents, their concentrations and the incubation periods by performing several checkerboard analyses. The CO6-MMP ELISA was developed as follows: A 96-well streptavidin plate was coated with biotinylated synthetic peptide YRGPEGPQGP-K-Biotin dissolved in assay buffer (25 mM Tris, 1% BSA, 0.1% Tween-20, pH 7.4) and incubated 30 minutes at 20°C. Twenty μl of peptide calibrator or sample were added to appropriate wells, followed by 100 μl of conjugated monoclonal antibody and incubated 1 hour at 20°C. Finally, 100 μl tetramethylbenzidine (TMB) (Kem-En-Tec cat. no. 430OH) was added and the plate was incubated 15 minutes at 20°C in the dark. All the above incubation steps included shaking at 300 rpm. After each incubation step the plate was washed five times in washing buffer (20 mM Tris, 50 mM NaCl, pH 7.2). The TMB reaction was stopped by adding 100 μl stopping solution (1% HCL) and measured spectrophotometrically at 450 nm with 650 nm as the reference. A standard curve was performed by serial dilution of the CO6-MMP peptide and plotted using a 4-parametric mathematical fit model. Standard concentrations were 0, 0.39, 7.8, 15.6, 31.3, 62.5, 125 250 ng/mL.

Technical evaluation

From 2-fold dilutions of pooled serum and plasma samples, linearity was calculated as a percentage of recovery of the 100% sample. The lower detection limit (LDL) was calculated from 21 determinations of the lowest standard (the zero standard) and calculated as the mean ±3x standard deviation. The inter- and intra-assay variation was determined by 10 independent runs of 5 QC samples, with each run consisting of two replicates of double determinations of the samples. Finally, for each assay, a master calibrator prepared from synthetic peptides accurately quantified by amino acid analysis was used for calibration purposes.

The analyte stability was determined for six serum samples (three rat and three human) for 10 freeze and thaw cycles.

ELISA characterization

The developed CO6-MMP ELISA was evaluated using 20 μl of the cleavage–samples: type VI collagen, type VI collagen cleaved with MMP-2, type VI collagen cleaved with MMP-9 described under “In vitro cleavage”. The negative control was in vitro cleaved type VI collagen with fibroblast activation protein (FAP). Cross-reactivity was tested using intact or in vitro cleaved type I or IV collagen using 20 μl peptide solution of 1000 ng/mL for each test in the assay. Neo-epitope specificity was tested using cleaved (by either MMP-2 or MMP-9) and non-cleaved type VI collagen and by an elongated CO6-MMP amino acid sequence (GYRGPEGPQGP).

Bile duct ligation

A total of 40 female Sprague-Dawley rats aged 6 months were housed at the animal research facilities at Nordic Bioscience,
Denmark. The rats were kept in standard type III-H cages at 18–22°C with bedding and nest material (Altromin 1324; Altromin, Lage, Germany) and water ad libitum. Rats were kept under conditions of a 12-hour light: dark cycle. Experiments began after 1 week of acclimatization. Bile duct ligation (BDL) was performed in anaesthetized rats by ligation of the bile duct in two places and dissection between the ligations in an open-surgery procedure. In sham-operated rats, the abdomen was closed without BDL. BDL- or sham-operated rats were sacrificed after 2 or 4 weeks.

**CCl₄ inhalation**

The study included 52 3-months old male Wistar rats treated with CCl₄ and 28 Wistar control rats (Charles-River, Saint Aubin les Elsief, France). Complete details of the study are described elsewhere (Segovia-Silvestre T et al.). Liver damage was induced as previously described [30], and in short included administration by inhalation of CCl₄ twice weekly. Phenobarbital (0.5 g/l) was added to the drinking water. Animals were stratified into groups receiving 8, 12, 16 or 20 weeks of CCl₄ (n = 13 for CCl₄; n = 7 control for each group). Control rats received Phenobarbital only. Four animals from the CCl₄ groups died during the study. After control for each group). Control rats received Phenobarbital only. Four animals from the CCl₄ groups died during the study. After the stated weeks of CCl₄ administration the rats were weighed, anaesthetized with pentobarbital (30 mg/kg) and terminated by decapitation.

**Blood and tissue sampling**

Blood samples were taken under light CO₂/O₂ anesthesia at baseline and at termination from the retro-orbital sinus of rats which had fasted for at least 14 hours. The collected blood was left for 30 min at room temperature to clot, followed by centrifugation at 3000 g for 10 min. All clot-free liquid was transferred to new tubes and centrifuged again at 3000 g for 10 min. The serum was then transferred to clean tubes and stored at −80°C.

Livers were carefully dissected, weighed, fixed in 4% formaldehyde for a minimum of 24 hours, cut into appropriate slices and embedded in paraffin. Liver sections (4–5 μm) were de-paraffinised, hydrated and further stained with AEC substrate, according to the supplier’s instructions (Biogenex, Taby, Sweden). Sections were counterstained with Mayer’s haematoxylin. Digital photographs were taken using an Olympus B×60 microscope with ×40 magnification and an Olympus 5050-zoom digital camera (Olympus, Tokyo, Japan).

**Histology image analysis**

Relative fibrosis area (expressed as a percentage of total liver area) was assessed by analyzing 36 fields of Sirius Red-stained liver sections per animal. Each field was acquired at 10× magnification [E600 microscope (Nikon) and RT-Slider SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, Michigan, US)]. Results were analyzed using a computerized Bioquant Life Science morphometry system. To evaluate the relative fibrosis area, the measured collagen area was divided by the net field area and then multiplied by 100. Subtraction of vascular luminal area from the total field area yielded the final calculation of the net fibrosis area. From each animal analyzed, the amount of fibrosis as a percentage was measured and the average value presented [31].

**Immunohistochemistry**

Liver sections (4 μm) were de-paraffinised, hydrated and further peroxidase activity was blocked with the addition of 0.4% hydrogen peroxide. Sections were then incubated with a polyclonal antibody against type VI collagen (1:100; Abcam, Cambridge, UK). Sections were then rinsed and the antibody binding was depicted using the Super Sensitive Polymer-HRP IHC Detection System combined with AEC substrate, according to the supplier’s instructions (Biogenex, Taby, Sweden). Sections were counterstained with Mayer’s haematoxylin. Digital photographs were taken using an Olympus B×60 microscope with ×40 magnification and an Olympus 5050-zoom digital camera (Olympus, Tokyo, Japan).

**Results**

**In vitro cleavage and selection of peptides**

High molecular bands of type VI collagen were seen by silver staining in the in vitro control sample not exposed to proteases. These bands were abolished in the MMP-2 and MMP-9 cleaved samples, indicating that type VI collagen was degraded by these proteases (data not shown). Fragments of type VI collagen cleaved by MMP-2 or MMP-9 generated neo-epitopes five sequences were selected for immunizations since blasting showed that these sequences were unique to type VI collagen, and conserved throughout species:

1. 573’.YRGPEGPGP’584; CO6A1 generated by MMP-2 or MMP-9
2. 1164’.GIGIGNADIT’1173; CO6A3 generated by MMP-2
3. 1164’.GIGIGNADIT’1173; CO6A3 generated by MMP-2
4. 2279’.GPKGIGNRG’2288; CO6A3 generated by MMP-9
5. 2176’.LGPMPVPRG’2185; CO6A3 generated by MMP-9

The sequence 573’.YRGPEGPGP’584 (CO6-MMP) in the alpha 1 chain of type VI collagen generated by MMP-2 and MMP-9 was selected consequent to the best technical performance as the antibodies were able to distinguish between cleaved and uncleaved type VI collagen. In addition, this sequence is 100% homologous to human, rat and mouse (Figure 1).

**Clone characterization**

The antibody with the best native reactivity, affinity and stability in the assay was chosen from the antibody-producing clones generated after the fusion between spleen cells and myeloma cells. The clone selected was determined to be the IgG1 subtype.

The clone was reactive to human serum and plasma (Figure 2A), rat serum and plasma (Figure 2B) and mouse serum (Figure 2B).

**Technical evaluation**

The typical standard curve is presented in Figure 2A and 2B, showing a 4-parametric fit for the assay. The lower limit of detection (LLOD) for the assay was 0.30 ng/mL, Dilution recovery was within 100±20% (Table 1). The inter- and intra-assay variation was a mean 4.0 and 10.1% respectively (Table 2). The analyte stability was acceptable for 2–10x freeze/thaw cycles within 100+/−20% (Table 3).

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**Antibody bindingsite**

<table>
<thead>
<tr>
<th>Species</th>
<th>Mouse</th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO6A1</td>
<td>YRGPEGPGP</td>
<td>YRGPEGPGP</td>
<td>YRGPEGPGP</td>
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Figure 1. Alignment. Alignment of part of type VI collagen alpha 1 sequence for mouse, rat and human.

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ELISA characterization

To characterize the analytes detected in the assay, different collagens were cleaved with different proteases. Both MMP-2 and MMP-9 were able to generate the CO6-MMP fragment from

Table 1. Percentage dilution recovery for the CO6-MMP assay.

<table>
<thead>
<tr>
<th>CO6-MMP Sample</th>
<th>Amount (ng/mL)</th>
<th>Intra-assay variability</th>
<th>Inter-assay variability</th>
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</thead>
<tbody>
<tr>
<td>HS1</td>
<td>7.6</td>
<td>4.5</td>
<td>10.1</td>
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<td>Mean</td>
<td><strong>13.6</strong></td>
<td><strong>4.1</strong></td>
<td><strong>10.1</strong></td>
</tr>
</tbody>
</table>

The variation was calculated as the mean variation between 10 individual determinations of each sample.

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Figure 2. Assay characteristics. (A+B) ELISA run showing typical standard curves and native reactivity against (A) Human serum, plasma, (B) Rodent: Rat serum and plasma; mouse serum, plasma and urine. Native material was run undiluted, 1:2, 1:4, and 1:8 as indicated (—). The signal is seen as the optical density at 450 nm, subtracting the background at 650 nm, as a function of peptide concentration; (C+D) Characterization of the CO6-MMP assay with regards to reactivity against (C) intact type VI collagen (CO6 intact), type VI collagen cleaved by MMP-2 (CO6/MMP-2), type VI collagen cleaved by MMP-9 (CO6/MMP-9), type VI collagen cleaved by fibroblast activation protein (FAP) (CO6/FAP), elongated peptide with extension of one amino acid at the neo-epitope site; (D) Intact type I collagen (CO1), type I collagen cleaved by MMP-2 (CO1/MMP-2), type I collagen cleaved by MMP-9 (CO1/MMP-9), intact type IV collagen (CO4), type IV collagen cleaved by MMP-2 (CO4/MMP-2), type IV collagen cleaved by MMP-9 (CO4/MMP-9), type IV collagen cleaved by pepsin (CO4/pepsin).

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Table 2. Inter- and intra-assay variation for the CO6-MMP assays using human serum quality control samples.
collagen type VI (Figure 2C). In contrast, the fragment was not found in either intact or FAP-cleaved type VI collagen. Finally, no cross-reactivity was seen between CO6-MMP and intact or cleaved type I or IV collagens, which have high homology with the immunization sequence of type VI collagen (Figure 2D). No reactivity was seen against the elongated synthetic peptide, as expected proving neo-epitope reactivity (Figure 2C).

Evaluations performed in the BDL study

During the 4 weeks, 3 of 40 rats, 2 BDL and 1 Sham, were put down due to excessive weight loss.

Levels of CO6-MMP were significantly elevated in BDL rats compared to sham levels at both the two-(mean BDL 29.5 ng/mL, mean sham: 14.2 ng/mL, p = 0.0001) and four week (mean BDL: 33.0 ng/mL, mean sham: 11.8 ng/mL, p = 0.0003) termination point (Figure 3).

By immunohistochemistry, collagen type VI deposition was found exclusively in the venous wall of healthy rats (Figure 4B). In contrast, in BDL rats in which marked ductal proliferation was seen around the portal tract with the formation of multiple neo-bile ducts, more extensive type VI collagen was found (Figure 4B).

Evaluations performed in the CCL4 study

In the CCL4 rat model, levels of CO6-MMP were significantly elevated at all time points compared to baseline levels, except at week 8 (week 12: mean CCl4 30.9 ng/mL, mean control 12.8 ng/mL, p = 0.0015; week 16: mean CCl4 34.0 ng/mL, mean control 13.7 ng/mL, p = 0.0018; week 20: mean CCl4 33.3 ng/mL, mean control 13.3 ng/mL, p = 0.0033) (Figure 5A). When CCl4-treated rats were classified by the total amount of collagen in the liver evaluated by histology (Sirius Red) (Figure 5B), it clearly seen that the marker was elevated in the lowest quartile of total collagen (quartile 1), as well as in quartiles 2–4 compared to control animals (mean Q1: 18.1 ng/mL, p > 0.05; mean Q2: 22.0 ng/mL, p < 0.05; mean Q3: 36.7 ng/mL, p < 0.0001; mean Q4: 46.1 ng/mL, p < 0.0018; mean across all controls 13.4 ng/mL). The correlation between CO6-MMP and total collagen was highly significant in CCl4-treated rats (p < 0.0001, R² = 0.58) (Figure 5C), however this was not seen in control rats (p = ns, R² = 0.04) (Figure 5D).

By immunohistochemistry, collagen type VI deposition was found exclusively in the venous wall of control rats (Figure 4D). In contrast, in CCl4-treated rats type VI collagen was located along the fibrotic bands (Figure 4D).

Discussion

This is, to our knowledge, the first study to present the development of an assay specific for a fragment of type VI collagen generated by MMPs. Our main findings were: 1) A technically robust assay was developed with monoclonal antibodies highly specific for the CO6-MMP fragment. The assay had acceptable inter-, and intra-assay variation, dilution recovery and a low limit of detection; 2) CO6-MMP levels were assessed in two different animal models of liver fibrosis: CCl4 and BDL. In both models we found significant increased levels in liver fibrotic rats compared to controls. In addition CO6-MMP levels were significantly correlated to collagen deposition in the CCl4 model of liver fibrosis.

ECM remodeling is an essential part of tissue homeostasis. Extensive ECM remodeling is associated with a range of pathologies [7,8,20,26,32,33] in which fibrosis is of particular relevance. Biochemical markers consisting of protein fragments from pathologic tissue remodeling may be useful for diagnostic and prognostic purposes [34]. Such an approach focusing on neo-epitopes have primarily been useful for the arthritis and bone field [35], and for liver fibrosis in monitoring degradation of collagen type III [36,37].

The gelatinases MMP-2 and MMP-9 have been investigated and documented to be highly regulated in fibrous tissue [12,13]. Cleavage of type VI collagen with these two proteases generated several fragments. Among these CO6-MMP was chosen due to it being unique and conserved among species. Several assays already exist for measuring total type VI collagen in different pathologies, using polyclonal antibodies (antibodies-online GmbH) or monoclonal antibodies (Uscn Life Science). Circulating levels of total type VI collagen patients with alcoholic liver disease have been investigated by Stickel et al [24], who found a significant elevation in alcoholic patients compared to controls. In addition they concluded that total collagen type VI already was elevated in early fibrotic states and therefore seem to be an important indicator of
early fibrotic transformation [24]. These data are consistent with our data indicating CO6-MMP is an early marker for fibrosis and they are in alignment with the fact that collagen type VI may be a good candidate biomarker. The CO6-MMP assay provides additional information on protease mediated tissue destruction in face of the total collagen markers. Such a careful deconstruction of the information entailed in the precise analyte may prove important to understand the processes that are leading to increased tissue formation as well as tissue degradation, and therefore eventually resolution of diseases. This may be assisted by the measurement of protein degradation and protein formation, in face of a crude measure of total protein [11,36,38]. The competitive ELISA was technically stable with a acceptable dilution recovery, as well as inter- and intra-variation for all matrices tested. The highest sensitivity for the assay was observed in the range of 4–125 ng/mL. Characterization of the selected monoclonal antibody revealed strong reactivity towards human, mouse and rat serum as well as the CO6-MMP peptide, strongly suggesting that the antibody recognizes this amino acid sequence for type VI collagen in native samples in complicated matrices. Characterizations using the final ELISA format showed that the recognized peptide fragment was generated by MMP-2 and -9. Furthermore, it was seen that the antibody was specific against the neo-epitope generated in type VI collagen, as no response was detected when type I or type IV collagen was cleaved by MMP-2 and -9. In addition the antibody did not recognise the elongated peptide, indicating neo-epitope specificity. The analyte stability was very good for both human- and rat serum CO6-MMP all recoveries within 100+/- 20%.

It is well-appreciated that the BDL and the CCl4 models describe two different fibrotic processes in which increased ECM remodelling and excessive collagen deposition are key characteristics. The CO6-MMP was significantly related to liver fibrosis in CCL4 treated rats treated for 12–20 weeks. Furthermore, when rats were classified into quartiles according to the extent of fibrosis defined as the amount of collagen in the liver, we observed that the marker was elevated in all quartiles. The marker also correlated highly significantly to total collagen in the livers of CCL4-treated rats; however this was not the case in control rats, strongly indicating liver specific pathological relevance of the neo-epitope. In the BDL model of liver fibrosis, serum CO6-MMP was elevated 2 and 4 weeks after BDL surgery compared to baseline and sham levels. These data are in agreement with the previous studies highlighting that type VI is generated during fibrogenesis by the activated hepatic stellate cells in the liver [2,37] and that MMP levels become elevated and unbalanced during fibrosis [1]. These data suggest that liver fibrosis is a high turnover disease, not may nor exclusively be described as an accumulation disease with increased collagen formation. Additionally, our data are in alignment investigating the collagen turnover profile in fibrotic.

**Figure 4. Type VI collagen in the liver.** (A) Sirius Red photomicrographs showing the hepatic structure in rats 4 weeks after a sham operation (1), 2 weeks after BDL (2) and 4 weeks after BDL (3) (B) Immunohistochemical analysis of type VI collagen. Type VI collagen is localized around fibrotic structures. (C) Sirius Red photomicrographs showing the hepatic structure in rats 20 weeks after vehicle treatment (7), 8 weeks of CCl4 treatment (8), 12 weeks of CCl4 treatment (9), 16 weeks of CCl4 treatment (10) and 20 weeks of CCl4 treatment (11). (D) Immunohistochemical analysis of type VI collagen. Type VI collagen is localized around the fibrotic bands. Original magnification x40.

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rats, which demonstrated that a MMP-9 generated fragment of collagen type III (CO3-610) was elevated in the BDL rat model [37]. These combined data, past and present, indicate that liver fibroses may be a high collagen turnover disease with both increased collagen formation [23,38] and collagen degradation. This further emphasis the need of measuring the individual processes, enabling discovery of pathways leading to a resolution of the disease – by lowering tissue formation and increasing tissue degradation transiently. Such an investigation may not be obtained by exclusively measuring the total protein levels.

The systemic level of a biochemical marker is the result of the activity level and number/area of affected tissues. As such, levels of the CO6-MMP analyte are a systemic measurement of several local events. Type VI collagen has been identified within most tissues in different quantities [16]. In addition chronic liver diseases are associated with co-morbidities like osteoporosis, protein and calorie malnutrition [39], which may cause increased CO6-MMP levels originating from secondary tissues. The increased CO6-MMP levels in the presented two animal models may primarily derive from fibrosis in the liver but may be confounded by effects from other tissues e.g. bone loss or muscular dystrophy, albeit bone only contain minute quantities of type IV collagen [39]. The exact contribution to the systemic pool of CO6-MMP from different tissues, healthy and disease affected remains to be more carefully investigated.

This study carries some limitations. One major limitation of this study is that it is carried out in homogeneous, inbred laboratory rats with a synchronous induction of liver disease, which bear little resemblance to the highly complicated presentation of clinical description of liver fibrosis. Further investigations in clinical settings are needed to provide more information on the usefulness of CO6-MMP.

In conclusion, we have developed an assay using a specific monoclonal antibody for the detection of CO6-MMP, a collagen type VI fragment generated by MMP-2 and -9. It was demonstrated that this marker was elevated in two pre-clinical models of fibrosis, the BDL and the CCl4 rat model, indicating that there is a high potential for the use of neo-epitope biomarkers in ECM-related diseases.

**Author Contributions**

Conceived and designed the experiments: SSV MAK DJL. Performed the experiments: SSV EV AN QHTN YL. Analyzed the data: SSV MAK EV AN BY DJL. Contributed reagents/materials/analysis tools: MRL PH QZ BV. Wrote the paper: SSV MAK BV DJL.
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