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RESEARCH ARTICLE

A novel marker for assessment of liver matrix remodeling: An enzyme-linked immunosorbent assay (ELISA) detecting a MMP generated type I collagen neo-epitope (C1M)

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Abstract

A competitive enzyme-linked immunosorbent assay (ELISA) for detection of a type I collagen fragment generated by matrix metalloproteinases (MMP) -2, -9 and -13, was developed (CO1-764 or C1M). The biomarker was evaluated in two preclinical rat models of liver fibrosis: bile duct ligation (BDL) and carbon tetra chloride (CCL4)-treated rats. The assay was further evaluated in a clinical study of prostate-, lung- and breast-cancer patients stratified according to skeletal metastases. A technically robust ELISA assay specific for a MMP-2, -9 and -13 neo-epitope was produced and seen to be statistically elevated in BDL rats compared to baseline levels as well as significantly elevated in CCL4 rats stratified according to the amount of total collagen in the livers. CO1-764 levels also correlated significantly with total liver collagen and type I collagen mRNA expression in the livers. Finally, the CO1-764 marker was not correlated with skeletal involvement or number of bone metastases. This ELISA has the potential to assess the degree of liver fibrosis in a non-invasive manner.

Keywords: Biochemical markers, type I collagen, liver fibrosis, protease-cleaved neo-epitope, MMP-2, -9, -13, translational science, bile duct ligation, CCL4, rat model, breast cancer, prostate cancer, bone metastases

Introduction

Liver fibrosis is one of the leading causes of death worldwide mostly due to viral or alcohol-induced injury (Friedman, 2003). At present there is no curative treatment for liver fibrosis and patients are reliant on removal of the causative injury or liver transplantation in the case of liver cirrhosis. Thus the evaluation of liver fibrosis is imperative for diagnosis, and monitoring patients preferably as close as possible. 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 (Gressner et al., 2007). Furthermore, the accuracy of liver biopsy is imprecise due to sampling error and significant intra- and inter-observer variability in histological staging as well as is very limited in the number of times it may be performed (Maharaj et al., 1986; Bedossa et al., 2003). Consequently, much focus has been put forward for evaluation and development of non-invasive methods for the assessment of liver fibrosis such as biochemical markers (Gressner et al., 2007; Veidal et al., 2010). The most well studied extracellular matrix (ECM) biochemical marker for liver fibrosis is the propetide of type III collagen (PIIINP), which has shown good potential as a fibrogenesis marker, however still with limitation (Schuppan et al., 1986; Rosenberg et al., 2004; Seven et al., 2011). Others have shown that the 7S domain of type IV collagen (PIVNP 7S) may be used for the assessment of fibrogenesis during liver fibrosis and other ECM-related diseases, (Risteli et al., 1980; Risteli et al., 1985; Obata et al., 1989; Schuppan, 1991; Murawaki et al., 1996).

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The ECM is the most prominent component of connective tissue. It offers both the necessary structural support for cells to adhere and migrate to while its multifunctional proteins also play important signalling roles. The ECM mainly consists of collagens, proteoglycans and glycoproteins, all of which play important and unique functions in maintaining the physicochemical structure of tissue (Schuppan et al., 2001; Wakatsuki and Elson, 2003). ECM remodelling is a normal process in which formation and degradation of healthy tissue work synergistically in a balanced way. However, in disease an imbalance in the ECM remodelling processes exists, which manifests itself in various pathologies. Excessive deposition of fibrillar ECM components, especially collagens, may occur in all tissues and organs, and is a significant cause of morbidity and mortality. Extensive tissue remodelling and fibrosis ultimately lead to organ failure resulting in death. This is most evident in end-stage liver fibrosis, i.e. cirrhosis, a condition associated with high morbidity and mortality (Friedman, 2003; Wynn, 2008).

Endopeptidases such as MMPs play major roles in the degradation of extracellular macromolecules such as collagens and proteoglycans (Zhen et al., 2008), resulting in neo-epitopes. A neo-epitope is a protease-generated post-translational modification (PTM) of a protein [10, 11], and has potential as a biochemical marker of ECM degradation. Assays developed using specific monoclononal antibodies towards neo-epitopes, may aid the understanding of events leading to PTMs, and their potential role in liver fibrosis mechanisms. At present, no accurate markers of fibrosis diagnosis, staging and prognosis exist (Gressner et al., 2007). However, recently our group described a novel assay for the evaluation of liver fibrosis which involved assessment of a matrix metalloproteinase-9 (MMP-9) generated type III collagen fragment (Barascuk et al., 2010). During liver fibrosis there is an abnormal up-regulation in synthesis of collagen types I and III as well as MMPs (Friedman, 2003). Type I and III collagen levels increase by up to 8 times compared to their normal values (Gressner and Weiskirchen, 2006). With respect to excessive proteolytic activity in the fibrous tissue, the gelatinases MMP-2 and MMP-9 have been investigated and documented to be highly up-regulated (Kirimlioglu et al., 2008; Gieling et al., 2009).

Several animal models for liver fibrosis have been developed, most of them in small rodents (Weiler-Normann et al., 2007), each with individual strengths and weaknesses. These different rodent models are complementary as they represent different pathways to fibrosis, as also seen in human disease. Bile duct ligation (BDL) in rats has been used as a model of chronic liver injury due to its resemblance to hepatocyte damage, hepatic stellate cell (HSC) activation, and liver fibrosis observed in human cholestatic liver disease (Friedman, 2003; Osawa et al., 2006; Weiler-Normann et al., 2007). Exposure to the hepatotoxin carbon tetrachloride (CCL4) can induce liver cirrhosis and is used in a preclinical model of liver injury and fibrosis in rats (Tugues et al., 2007; Munoz-Luque et al., 2008). Liver fibrosis induced by carbon tetrachloride exhibits most of the features observed in human cirrhosis, including widespread hepatic fibrosis and nodule formation accompanied by other well known liver fibrosis pathological hallmarks. It is a highly reproducible and robust model (Friedman, 2003; Weiler-Normann et al., 2007) and 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.

We set out with the hypothesis that MMP-2 and -9 mediated degradation of type I collagen may be useful for monitoring liver fibrosis. The scope of the present work was to develop a novel enzyme-linked immunosorbent assay (ELISA) for the assessment of MMP-2 and -9 degradation of type I collagen. Since bone tissue mainly consists of type I collagen it was worthwhile to aim for elimination the potential background coming from bone, which may be the case if the main protease of the bone resorbing cells, osteoclasts, cleaved type I collagen inside the selected epitope as described for cathepsin K (Cat K) in Figure 1.

Materials and methods

Reagents

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

In vitro cleavage

Purified type I collagen from human placenta (cat. no. ab7533, Abcam, Cambridge, UK) was cleaved with pro-MMP-2,-3,-8,-9 and -13 (cat. no. 444213-5; PF063-10; 444229-5; 444231-5; 444287-5; Calbiochem, Merck, Whitehouse Station, NJ) and pro-Cat K (cat. no. 342001-10, Calbiochem, Merck, Whitehouse Station, NJ). Pro-Cat K was activated using a solution of 50 µL of 100 mM

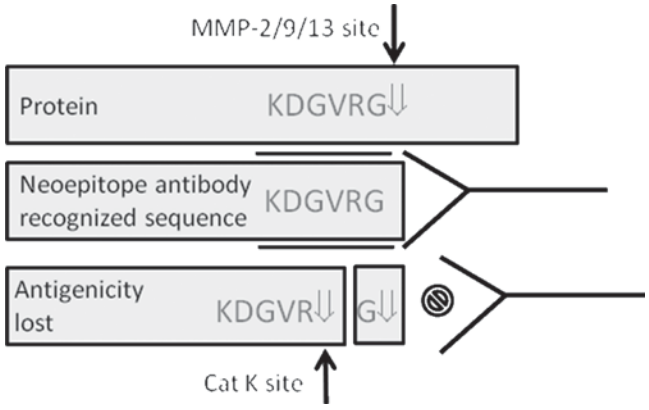

Figure 1. The principle behind the double-neo-epitope approach is that a neo-epitope generated by MMP-2 -9 or -13 is recognized by a monoclonal antibody and the antigenicity is destroyed by Cat K.

NaAcetate/10 mM DL-dithiothreitol (DTT)/5 mM ethylenediaminetetraacetic acid (EDTA) pH 3.9. 50 µL of this solution was mixed with 50 µL pro-Cat K and incubated for 40 min at room temperature. 50 µg MMP was activated with 20 µL 1 mM 4-aminophenylmercuric acetate (APMA) in dimethyl sulfoxide (DMSO) and incubated at 37°C for 2 h. To facilitate MMP and Cat K cleavage of type I collagen, 1 mg/mL type I collagen diluted in 0.5 M acetic acid was dialyzed for 2 days to remove the acetic acid. The liquid was filtered to remove proteins below 10,000 kDa (Microcon Ultracel YM-10, cat. no. 42407, Millipore, Billerica, MA). Each protease cleavage was performed separately by mixing 100 µg type I collagen and 1 µg of enzyme (MMP-2, -9, -13 or Cat K) in either MMP buffer (100 mM Tris-HCl, 100 mM NaCl, 10 mM $CaCl_2$, 2 mM Zn acetate, pH 8.0) or Cat K buffer (50 mM Na-Acetate, 20 mM L-cysteine, pH 5.5). As a control, 100 µg of type I collagen was mixed with MMP or Cat K buffer only. The type I collagen cleaved by Cat K was incubated for 24 h at 37°C. Each type I collagen aliquot cleaved by different MMPs was incubated for 3 days at 37°C. All cleavages were terminated using 1 µM cysteine protease inhibitor E64 (cat. no. 219377, Calbiochem, Merck, Whitehouse Station, NJ). Next, the pre-cleaved MMP-9, which was subsequently cleaved by Cat K: 125 µL of the pre-cleaved MMP-9 sample was mixed with 16.5 µL of activated Cat K (150 µg/mL in 100 mM Na-Acetate, 10 mM DL-dithiothreitol (DTT), 5 mM EDTA, pH 3.9) and 237 µL activation buffer (50 mM Na-Acetate, 20 mM L-cystine, pH 4) to reach a final pH of 5.5. The mixture was incubated for 72 h at 37°C. The cleavage was terminated using 1 µM cysteine protease inhibitor E64 (cat. no. 219377, Calbiochem, Merck, Whitehouse Station, NJ). Finally the cleavage was verified by visualization using the SilverXpress® Silver Staining Kit (cat. no. LC6100, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cleavages were repeated for three additional batches.

Peptide identification

Peptide fragments in the *in vitro* cleaved samples were identified using matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) and liquid chromatography coupled to electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS). MALDI-TOF samples were purified using C_{18} zip-tips (cat. no. ZTC18SO24, Millipore, Billerica, MA) according to specifications and 0.1 µg of material was eluted onto a MTP 384 ground steel target plate (Bruker-Daltonics, Bremen, Germany). MALDI tandem mass spectra were recorded on a Bruker ultraflex MALDI-TOF/TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany) in positive ion reflector mode. Mass spectra were externally calibrated in the m/z range of 800–4000 using peptides generated by tryptic digestion of bovine β-lactoglobulin. The m/z software "Flexanalysis" (Bruker-Daltonics, Bremen, Germany) was used to analyze spectra. LC-MS samples were ultra-filtrated to remove proteins above 10kDa, 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 C_{18} column (Waters, Milford, MA) using a formic acid/acetonitril gradient. MS and MS/MS were performed on a Synapt High Definition Mass Spectrometry quadruple time of flight MS (QUAD-TOF; Waters, Milford, MA), with a aquisition 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) was used to analyze spectra and generate peak lists. To identify peptides, MS and MS/MS data was searched against a type I collagen (FASTA) protein database using the Mascot 2.2 (Matrix Science, Boston, MA) software with either the MALDI-TOF/TOF or ESI-QUAD-TOF settings with carbamidomethyl (C), oxidation of methionine (M), oxidation of lysine (K) and oxidation of proline (P) as variable modifications.

Selection of peptide for immunizations

The first six amino acids of each free end of the sequences 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 then blasted for homology using the NPS@: network protein sequence analysis (Combet et al., 2000).

Immunization procedure

Six 4–6 week-old Balb/C mice were immunized subcutaneously in the abdomen with 200 µL emulsified antigen (50 µg per immunization), using Freund's incomplete adjuvant (OVA-CGG-GSPGKDGVRG). Immunizations were performed at two-week intervals until stable titer levels were obtained. At each bleeding, the serum titer was investigated and the mouse with the highest titer was selected for fusion. The selected mice were boosted intravenously with 50 µg immunogen in 100 µL 0.9% sodium chloride solution three days before isolation of the spleen for cell fusion.

Fusion and antibody screening

The fusion procedure has been described elsewhere (Gefter et al., 1977). 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. Here standard limited dilution was used to promote monoclonal growth. Supernatants were screened using an indirect ELISA, while the biotinylated peptide Biotin-K-GSPGKDGVRG was used as a catcher peptide on streptavidin-coated microtitre plates.

Characterization of clones

Native reactivity and peptide binding of the monoclonal antibodies was evaluated by displacement of human serum, plasma and urine; rat serum and urine; and mouse serum, plasma and urine, in a preliminary competitive ELISA using 10 ng/mL biotinylated peptide coater on a streptavidin coated microtitre plate

and the supernatant from the growing monoclonal hybridoma. Clones were tested against the free peptide (GSPGKDGVRG), a non-sense peptide, and an elongated peptide (GSPGKDGVRGL). Isotyping of the monoclonal antibodies was performed using the Clonotyping System-HRP kit, cat. no. 5300-05 (Southern Biotech, Birmingham, AL). Due to high homology, cross reactivity toward a collagen type V sequence (GQKGDDGVRG) and collagen type VII sequence (TPVLDGVRG) was tested using synthesized peptides. The selected clones were purified using Protein G columns according to manufacturer's instructions (GE Healthcare Life Science, Little Chalfont, Buckinghamshire, UK).

CO1-764 assay protocol

We labeled selected monoclonal antibodies with horseradish peroxidase (HRP) using the Lightning link HRP labeling kit according to the instructions of the manufacturer (Innovabioscience, Babraham, Cambridge, UK). A 96-well streptavidin plate was coated with biotinylated synthetic peptide Biotin-K-GSPGKDGVRG dissolved in assay buffer (50 mM Tris, 1% BSA, 0.1% Tween-20, pH 7.4 adjusted at 20°C) and incubated 30 min at 20°C. 20 µL of peptide calibrator or sample were added to appropriate wells, followed by 100 µL of conjugated monoclonal antibody 4D3-HRP and incubated 1 hour at 20°C. Finally, 100 µL tetramethylbenzinidine (TMB) (Kem-En-Tec cat. no. 438OH) was added and the plate was incubated 15 min 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 of stopping solution (1% HCl) and measured at 450 nm with 650 nm as the reference. A calibration curve was plotted using a 4-parametric mathematical fit model.

Technical evaluation

From 2-fold dilutions of quality control (QC) samples consisting of serum, urine and plasma samples, linearity was calculated as a percentage of recovery of the 100% sample. The lower limit of detection (LDL) was determined from 21 zero samples (i.e. buffer) 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 replicas 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 three human serum samples and three rat serum samples for up to seven times freeze and thaw cycles and storage of human serum samples for 2–48 h at 4 and 20°C. All sample assessed in the CO1-764 assay in double determinations.

ELISA characterization

The developed CO1-764 (C1M) ELISA was evaluated using the cleaved materials described under "*In vitro* cleavage". 20 µL from a sample diluted 1:20 was used for the assay. Cross reactivity was tested using the collagen type V and VII synthetic peptides using 20 µL 1000 ng/mL peptide solution for each test in the assay.

Rat bile duct ligation liver fibrosis model

CO1-764 was assessed in a BDL rat model of liver fibrosis. The Danish animal welfare board "Dyreforsøgstilsynet" in Copenhagen, DK, had approved and given an written informed consent of the study; approval #2008/561-1450. The study has been described in detail elsewhere (Veidal et al., 2010). Briefly, 17 female Sprague–Dawley rats aged 6 months were stratified into 4 groups: BDL- or sham-operated rats sacrificed after 2 or 4 weeks. Liver fibrosis was induced in anaesthetized rats by standard BDL, in which the bile duct was ligated in two places and dissected between the two ligations prior to closing the abdomen. In sham-operated rats, the abdomen was closed without BDL surgery. Blood samples were taken under CO_2/O_2 anaesthesia at baseline and at termination from the retro-orbital sinus of rats which had fasted for at least the previous 14 h. The collected blood was left for 30 min at room temperature to clot, followed by centrifugation 2x at 1500*g* for 10 min. The serum was then transferred to clean tubes and stored at–80°C until use. The validity of the BDL model was demonstrated by (Veidal et al., 2010).

Rat CCL4 liver fibrosis model

CO1-764 levels were measured in a CCL4 inhalation rat model of liver fibrosis. Complete details of the study are described elsewhere (Segovia-Silvestre et al., 2010). The study included 52 male Wistar rats treated with CCL4 and 28 male Wistar control rats (Charles-River, Saint Aubin les Elseuf, France). Induction of liver fibrosis was performed as previously described (Clarià and Jimenez, 1999). Briefly, CCL4 was administered by inhalation twice weekly and phenobarbital (0.3 g/l) added to the drinking water. Control rats received phenobarbital only. Animals were stratified into groups receiving 8, 12, 16 or 20 weeks of CCL4 or control treatment ($n=13$ for CCL4; $n=7$ control for each group). The study was performed after written informed consent by the Investigation and Ethics Committee of the Hospital Clinic Universitari (Barcelona, Spain), approval #B-NNP-233/09. Four animals from the CCL4 groups died during the study. Blood was collected at termination and allowed to stand at room temperature for 20 min to allow clotting, before centrifugation at 2500 rpm for 10 min. Samples were stored at –80°C prior to biomarker assessment. Liver sections (4 µm thick) were stained in 0.1% Sirius red F3B (Sigma-Aldrich, St. Louis, MO) in saturated picric acid (Sigma-Aldrich). Type I collagen immunohistochemistry the liver tissue was processed following standard histological procedures, embedded in paraffin and cut into 5 µm thick sections. Sections were then melted at 60°C, de-paraffinized, hydrated and demasked 2 times by microwaving at 700 W in citrate buffer pH 6.0 for 10 min. Unspecific protein

binding was blocked with the addition of 1% BSA in TBS buffer (pH 8) for 20 min at room temperature, followed by washing and the addition of primary antibody: Rabbit polyclonal towards collagen I (cat. no. ab292; Abcam, UK) as well as a negative control (pre-immune rabbit serum). Both were diluted 1:200 and incubated with the sections for 2 hours at room temperature, followed by washing. Immuno-reactivity was detected using the Super Sensitive Polymer-HRP Detection System (Biogenex, Norway). A Biotin-Free Detection System aminoethylcarbazole (AEC) as chromogen. The nucleus was counterstained with Mayers acidic hematoxylin. Pictures were acquired with a digital camera on a microscope using a 20x magnification.

Fibrosis quantification in CCL4 livers

Liver sections (4 μm) were stained in 0.1% Sirius red F3B (Sigma-Aldrich, St. Louis, MO) in saturated picric acid (Sigma-Aldrich). 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 10X magnification [E600 microscope (Nikon) and RT-Slider SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI)]. 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 percentage was measured and the average value presented (Munoz-Luque et al., 2008).

Type I collagen gene expression in the BDL and CCL4 livers

Type I collagen transcript (Col1a1) levels in liver tissue were determined by quantitative real-time polymerase chain reaction (qPCR). Total RNA was extracted from frozen liver samples using Absolutely RNA Miniprep kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions and its quality assessed with RNA Nano chips using a 2100 Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA). Immediately after RNA isolation, complementary DNA (cDNA) was synthesized with Transcriptor First Strand cDNA Synthesis kit (Roche, Basel, Switzerland) using 1 μg of RNA as template. The gene of the purine recycling enzyme hypoxanthine phosphoribosyltransferase 1 (Hprt1) was used as a housekeeping gene for qPCR analysis in liver samples. PCR primers for Col1a1 and Hprt1 cDNA amplification were designed using NCBI Reference sequence NM_053304.1, and GenBank X62085.1, respectively, as templates. The primers sequences used were: Col1a1-Reverse: 5′-TCCggTgTgACTCgTgCAgC-3′; Hprt1-Forward: 5′-gTTggATACAggCCAgACTTTgT-3′ and Hprt1-Reverse: 5′-CCACAggACTAgAACgTCTgC-3′. Real time fluorescence data were collected in the Mx3000P (Stratagene) qPCR system. Messenger RNA values for each gene were extrapolated from their respective standard curves plotting qPCR crossing points vs. level of dilution.

Cancer study design

CO1-764 levels were assessed in urine samples collected in a cross-sectional study of patients with prostate, lung or breast cancer, with or without bone metastases (BM). The study design has been published previously (Leeming et al., 2006). Briefly, 90 breast-cancer patients (45 +BM) and 45 -BM), 30 lung cancer patients (16 +BM and 14–BM) and 42 prostate cancer patients (25 +BM and 17 -BM) were referred to the Cancer Institute Hospital, Tokyo, Japan, between October 2002 and April 2004. All patients underwent bone scans using a radionuclide (Technetium-99m), as well as computer tomography (CT) and/or magnetic resonance imaging (MRI) to verify and quantify the presence of BM. The number of BM was recorded and the skeletal load was graded, as previously proposed by Soloway et al. (Soloway et al., 1988). All participants signed a written informed consent form and the study was performed in accordance with the Helsinki Declaration II and Standards of Good Clinical Practice. The Ethics Committee of Cancer Institute Hospital, (Ariake 3-10-6, Koto-ku) Tokyo, Japan approved the study protocol.

Statistical analyses

In the rat models the comparison of serum CO1-764 in the BDL/sham and CCL4/control rats at each time point was performed using the unpaired t-test with Welch correction. For the cancer study the comparison of CO1-764 levels for each Soloway score against the level in patients without metastasis was performed by analysis of variance (ANOVA) using the General Linear Models Procedure (GLM). Dunnett's adjustment of the level of significance was employed to correct for multiple comparisons. Correlations were performed using the Spearman correlation. Differences were considered statistically significant if $p < 0.05$. GRAPH PAD PRISM 5 (Graph Pad Software, La Jolla, CA) was used for calculations.

Results

In vitro cleavage and selection of peptides

Between 30 and 51 fragments of type I collagen cleaved by MMP-2, -9, -13 or Cat K, were identified with a statistically significant Mascot score ($p < 0.05$). For type I collagen cleaved by MMP-3 and -8 the number of fragments identified with a statistically significant score was 5–7 (data not shown). All protease-generated neo-epitopes were tested for homology. The sequence 755′GSPGKDGVRG⇓′764 (CO1-764) in the alpha 1 chain of type I collagen generated by MMP-2, -9, -13 (⇓) was identified by LC-MS and selected for immunizations since it is unique to type I collagen and it is further cleaved by Cat K (#) on the second position from the C-terminus of the peptide (GSPGKDGVR#G⇓). This sequence is 100% homologous to human, rat, mouse and bovine type I collagen.

Clone characterization

The clone selected for ELISA development was determined to be a IgG2b subtype. The native reactivity of this clone was high against both human serum and urine; rat serum and urine; and mouse serum (Figure 2). The signal was almost completely inhibited in all undiluted urine materials, and in human serum and plasma. For rodent serum and plasma the inhibition of the signal was around 70%.

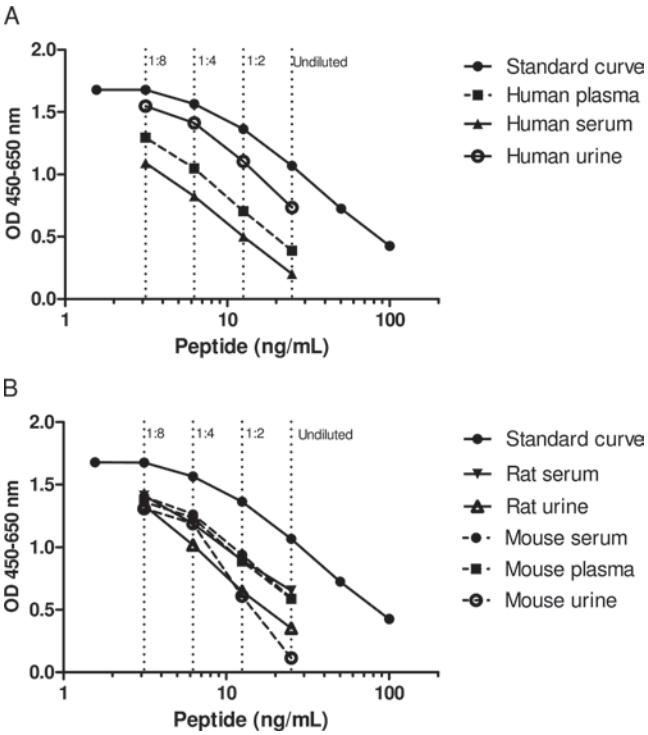

Figure 2. ELISA run showing typical standard curves and native reactivity against A) Human serum, plasma and urine, B) Rodent: Rat serum and urine; mouse serum, plasma and urine. Native material was run undiluted, 1:2, 1:4, 1:8 as indicated (---). The signal is seen as the optical density at 450 nm, subtracting the background at 650 nm, as a function of peptide concentration.

Technical evaluation

The typical standard curve is presented in Figure 2, showing a 4-parametric fit for the assay. The lower limit of detection (LDL) for the assay was 0.83 ng/mL. Dilution recovery was within 100 ± 15% except for mouse urine (mean 118%) and mouse plasma (mean 125%) (Table 1). The inter- and intra-assay variation was around or below 10% for assessments in both assays (Table 2). Analyte stability in human serum samples were acceptable; percent recovery was between 100 ± 20% for samples values after storage 2–48 h at 4- and 20°C (Table 3A). Finally, the analyte stability was acceptable for 2–7x freeze/thaw cycles: all were within 100 ± 20% in human- and rat serum (Table 3B).

ELISA characterization

From the ELISA characterization it was observed that MMP-2, -9 and -13 were able to generate the CO1-764 fragment (Figure 3A). In contrast, MMP-3, -8 and Cat K were not able to generate this fragment. The fragment was not found in intact type I collagen. These findings were consistent in all three repeated cleaved batches. The test of whether Cat K destroys the CO1-764 fragment showed that a MMP-9 cleaved sample lost approximately 50% of its reactivity following subsequent cleavage by Cat K (Figure 3B). Finally, no cross reactivity was seen to the collagen type V, VII and elongated synthetic peptide sequences that have high homology with the immunization sequence of type I collagen (Figure 3C).

Evaluations performed in the BDL study

Levels of CO1-764 did not statistically change from baseline in either the sham or BDL rats at the 2 week termination

Table 1. Percentage dilution recovery for the CO1-764 assay.

CO1-764ng/mL	HS59.0	HU23.8	HP39.7	RS60.5	RU71.1	MS48.1	MU53.0	MP45.1
Undiluted	100%	100%	100%	100%		100%	100%	100%
Dilution 1:2	100	98	100	99	100%	111	110	116
Dilution 1:4	97	90	118	94	91	122	124	131
Dilution 1:8	79	65	118	101	90	101	120	127
Mean	**92**	**85**	**115**	**98**	**91**	**112**	**118**	**125**

HS = human serum; HU = human urine; HP = human plasma; RS = rat serum; RU = rat urine; MS = mouse serum; MU = mouse urine; MP = mouse plasma.

Table 2. Inter- and intra-assay variation for the CO1-764 assays using human serum and urine quality control samples. The variation was calculated as the mean variation between 10 individual determinations of each sample.

CO1-764 Sample	Amount (ng/mL)	Inter-assay variability %	Intra-assay variability %
HS1	19.7	11.1	5.3
HS2	22.2	11.8	7.5
HS3	22.9	11.4	6.7
HS4	24.4	10.1	6.8
HS5	28.4	10.3	5.5
HS6	29.2	9.7	6.2
HS7	38.0	8.7	6.8
HS8	54.9	6.8	8.4
Mean		**10.1**	**6.7**

point. However, at the 4 week termination point CO1-764 levels were significantly elevated in BDL rats compared with baseline levels (+75% increased, $p<0.05$) and compared with sham termination levels (+97% increased, $p<0.01$) (Figure 4A). From the qPCR data it was observed that BDL provoked a massive 100-fold increase of type I collagen gene expression in the liver (Figure 4B). However CO1-764 serum levels were not correlated to increases in Col1a1 expression in BDL rats (Figure 4C).

Evaluations performed in the CCL4 study

In the CCL4 rat model it was seen that the levels of CO1-764 were statistically elevated at week 12, 16 and 20 in CCL4 treated rats compared to controls (week 12: +32% increase, $p=0.008$; week 16: +50% increased, $p=0.0008$, week 20: +58% increased, $p=0.02$) (Figure 5A). When CO1-764 was stratified into quartiles by the total amount of collagen in the liver by histology (Sirius Red) (Figure 5B), it was observed that the marker was elevated in the lowest quartile of total collagen (quartile 1), as well as in quartiles 3 and 4 (Q1: +13% increased, $p>0.05$; Q3: +32% increased, $p<0.05$; Q4: +72% increased, $p<0.0001$) compared to all control animals. The elevation of CO1-764 in quartile 2 was borderline significant ($p=0.056$). The correlation between CO1-764 and total collagen was highly significant in CCL4 treated rats ($p<0.001$, $r=0.63$), however this was not seen in control rats ($r=-0.22$). CO1-764 and type I collagen formation seen as alpha 1 chain mRNA (Figure 5E) were also highly correlated ($p<0.0001$, $r=0.53$). Histology sections of the CCL4 livers stained by Sirius red (Figure 6A–H) and type I collagen (Figure 6I–P) clearly showed that type I collagen was only present in small amounts in livers of healthy control rats (0.5% Sirius red staining). Staining for type I collagen increased with increasing severity (4, 8 and 20% Sirius red). Furthermore, staining indicated that type I collagen was mainly found in the fibrotic bands generated during fibrosis and around the portal veins in which total collagen staining also were found.

Table 3. Analyte stability. A) Analyte stability in three serum samples stored at 4- or 20°C for 2–48 h. B) Analyte stability in three human- or rat serum samples in seven freeze/thaw cycles. All data are shown as mean percent recovery compared to time 0 or 1 freeze/thaw cycle.

A		
Analyte stability human serum	Percent recovery compared to time 0	
Test duration	4°C	20°C
2 h	116	98
4 h	104	96
24 h	97	96
48 h	110	102
B		
Analyte stability	Percent recovery compared to	1 x freeze/thaw
Freeze/thaw cycles	Human serum	Rat serum
2x	109	102
3x	102	95
4x	98	90
5x	100	97
6x	93	95
7x	110	97

Evaluations in the cancer study for bone metastases

Demographics of 161 cancer patients stratified according to cancer type and the presence or absence of BM have previously been reported (Leeming et al., 2006). There were no statistically significant differences in age and body mass index (BMI) between patients with or without BM. Figure 6A shows the mean values of CO1-764 in patients stratified according to cancer type and presence or absence of BM. There were no statistically significant

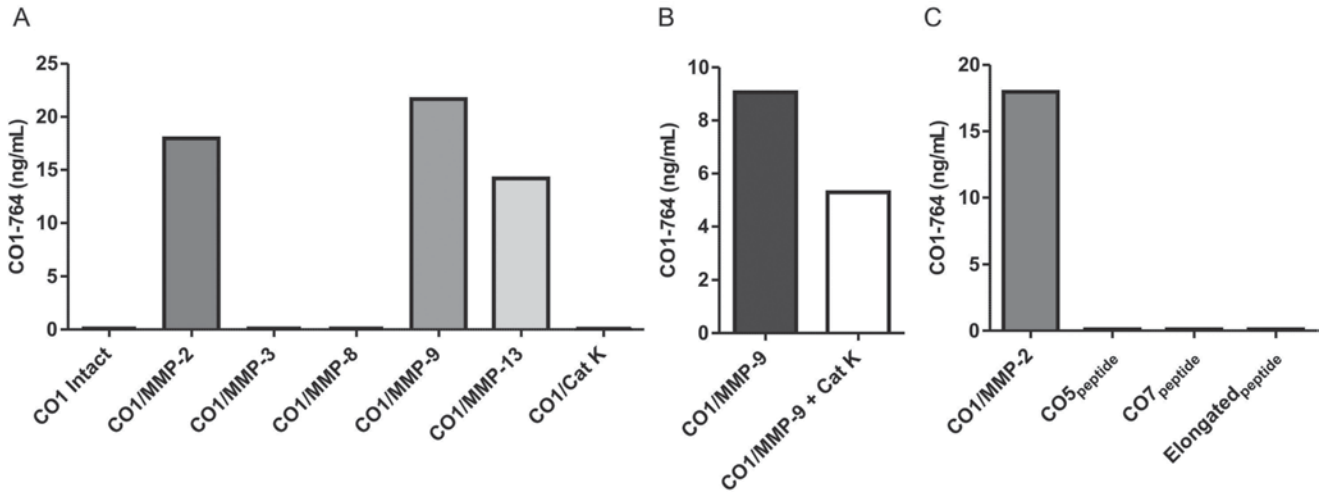

Figure 3. Characterization of the CO1-764 assay with regards to reactivity against A) intact type I collagen (CO1 intact), type I collagen cleaved by MMP-2 (CO1/MMP-2), type I collagen cleaved by MMP-3 (CO1/MMP-3), type I collagen cleaved by MMP-8 (CO1/MMP-8), type I collagen cleaved by MMP-9 (CO1/MMP-9), type I collagen cleaved by MMP-13 (CO1/MMP-13), type I collagen cleaved by Cat K (CO1/Cat K); B) type I collagen cleaved by MMP-9, type I collagen cleaved first by MMP-9 and then Cat K (CO1/MMP-9 + Cat K); C) type I collagen cleaved by MMP-2, elongated peptide and synthetic peptides corresponding to potential collagen type V ($CO5_{peptide}$) and collagen type VII ($CO7_{peptide}$) cross reactivity.

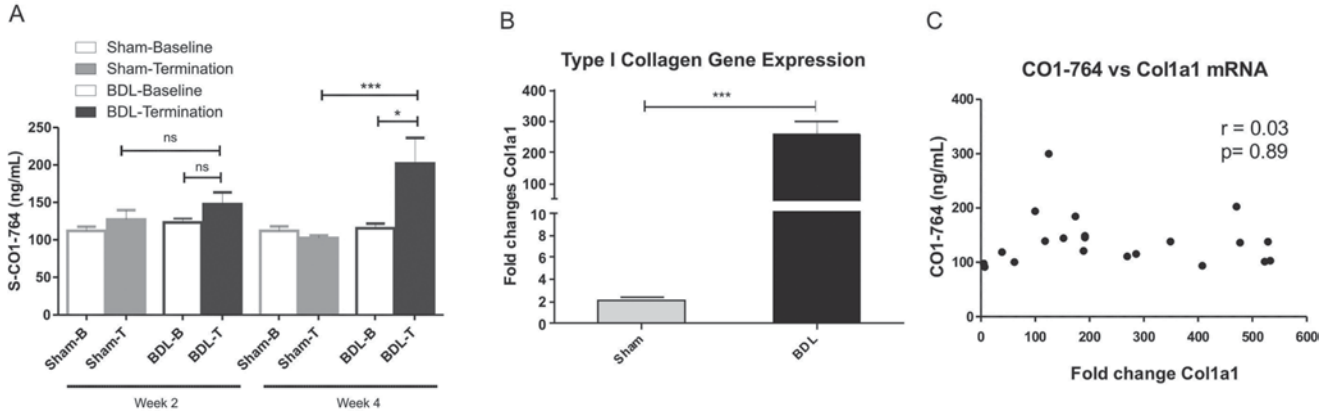

Figure 4. BDL rat liver fibrosis model: A) Serum CO1-764 was assessed in sham operated rats at baseline (sham-B) and termination (sham-T) as well as in BDL operated rats at baseline (BDL-B) and termination (BDL-T). Termination time points were 2 and 4 weeks after surgery; B) Comparison between Col1a1 expression levels (relative to housekeeping gene Hprt1) in BDL operated rats; C) Scatter diagram of serum CO1-764 and Col1a1 mRNA data pairs from treated animals. Asterisks indicate statistical significance as indicated by bars. (**$p < 0.05$; ***$p < 0.001$, ns = non-significant difference). Results shown are mean ± standard error of the mean (SEM).

differences in CO1-764 levels between patients without BM and patients with BM in any of the three cancer types.

Data from all cancer patients were pooled for subsequent analyses using the Soloway scoring system for BM, as previously reported. There was no relationship between advancing extent of the metastatic involvement of the skeletal system and CO1-764 levels (Figure 6B)

Discussion

This is, to our knowledge, the first study to present the development of an assay detecting a fragment of type I collagen generated by MMP-2, -9 and -13, which subsequently was destroyed by Cat K. The cleavage by Cat K in the epitope indicates that this collagen fragments does not derive from bone. Our main findings were: 1) The monoclonal antibody selected for assay development was highly specific towards the CO1-764 fragment and had a good native reactivity towards native materials; 2) A technically robust assay was developed with acceptable inter-, and intra-assay variation, dilution recovery, analyte stability and a low limit of detection; 3) CO1-764 levels were significantly elevated in the BDL rat compared to baseline and in CCL4 treated rats compared to controls; 4) CO1-764 levels were not related to skeletal involvement in patients with prostate, lung or breast cancer.

The CO1-764 marker

Characterization of the selected monoclonal antibody revealed strong reactivity towards human, mouse and rat body fluids as well as the CO1-764 peptide indicating that the antibody recognizes this amino acid sequence for type I collagen in native samples. Characterizations using the final ELISA format showed that the recognized peptide fragment indeed was generated by MMP-2, -9, and -13 but not MMP-3, -8 or Cat K. Furthermore, it was seen that the antibody was specific against the neo-epitope since no response was detected towards intact type I collagen or the elongated peptide. Even though the immunization sequence had a high homology toward a human collagen type V and type VII sequence, we did not detect any cross reactivity to these sequences, further verifying the specificity toward the CO1-764 neo-epitope.

Finally, we investigated the generation of the fragment by MMP-9 in intact collagen type I and subsequently tested if the antigen was cleaved by Cat K after the MMP-9 treatment. Cat K was able to destroy the antigen in our *in vitro* study, which again was in complete alignment with our MS data. The only 50% reduction in signal by Cat K may be attributed to technical issues since we were not able to switch to a Cat K buffer for the Cat K cleavage. Technical evaluations of the competitive ELISA revealed that the assay was technically stable with a good dilution recovery, and inter- and intra variation. Additionally it was seen that the analyte was stable in human- and rat serum.

Another MMP generated type I collagen marker destroyed by Cat K has been evaluated for ECM-related disease and liver fibrosis; the marker of C-telopeptide of type I collagen (ICTP) (Elomaa et al., 1992; Moller et al., 1999; Ylisirnio et al., 2001; Garnero et al., 2003). This marker has been found to be elevated in patient with cirrhosis (Moller et al., 1999), however this marker was developed using polyclonal antibodies and thus is not neo-epitope specific. Furthermore, it is generated by multiple MMPs and the specificity is not well established (Risteli et al., 1993; Garnero et al., 2003).

CO1-764 for the evaluation of liver fibrosis in the BDL study

In the BDL model of liver fibrosis, serum CO1-764 was elevated 4 weeks after BDL surgery compared to baseline and sham levels. These data are in agreement with the literature stating that especially type I collagen and III are highly elevated (Gressner et al., 2007; Veidal et al., 2010) and that MMP levels become elevated and unbalanced during fibrosis (Friedman, 2003). Additionally, our data

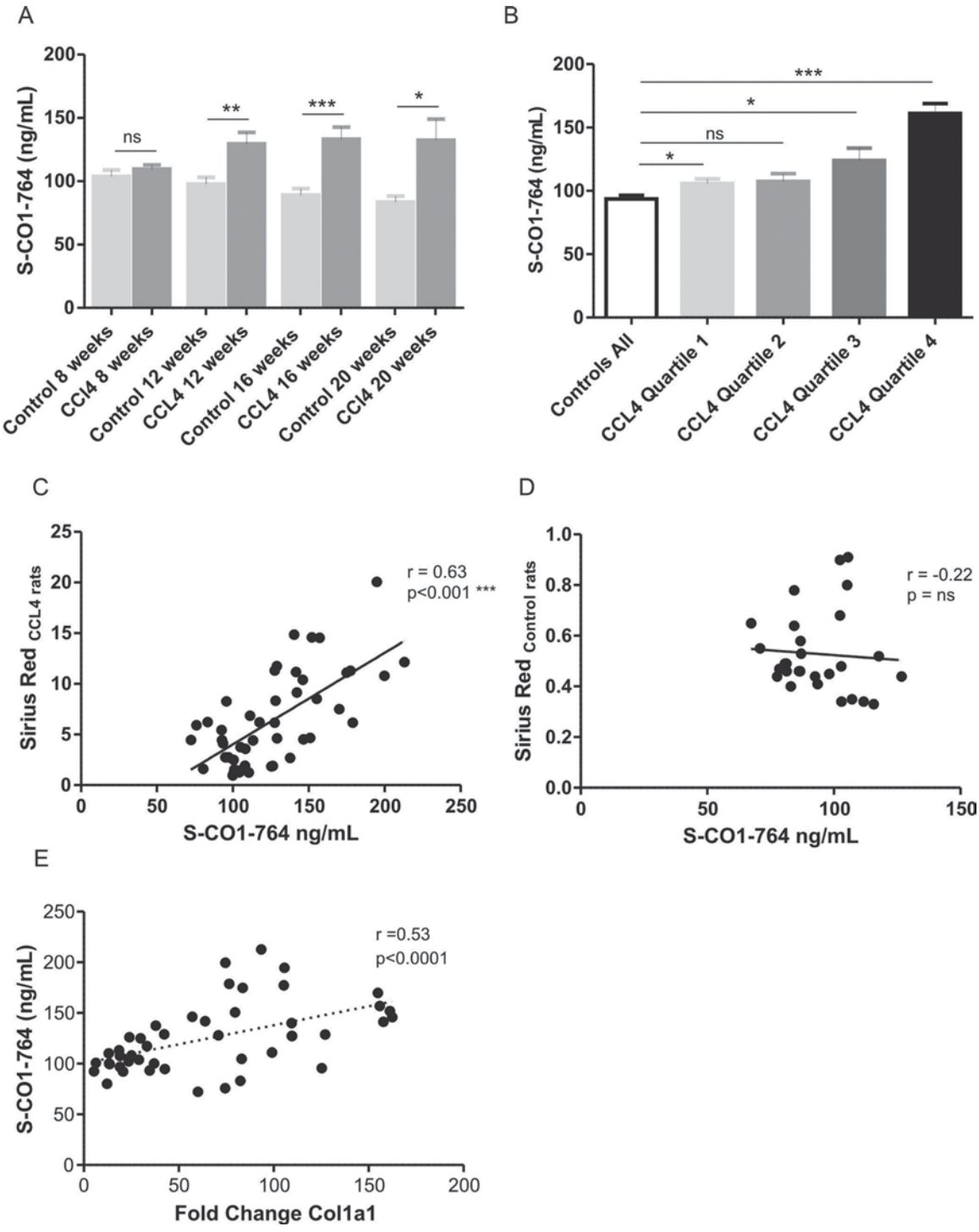

Figure 5. CCL4 rat liver fibrosis model: A) Serum CO1-764 was assessed in control rats at termination (controls) as well as in CCL4 treated rats at termination (CCL4) at week 8, 12, 16, 20. Results shown are mean ± standard error of the mean (SEM); B) Serum CO1-764 level in all control rats and CCL4 rat stratified in quartiles according to total collagen in the liver; C) Correlations between CO1-764 and Sirius red in C) CCL4 rats and in D) control rats; E) Scatter diagram of serum CO1-764 and Col1a1 mRNA data pairs from treated animals. Asterisks indicate statistical significance as indicated by bars. (**$p < 0.05$; ***$p < 0.001$, ns = non-significant difference).

are in agreement with previous findings showing that a MMP-9 generated fragment of type III collagen (CO3-610) is elevated in this BDL rat model, signifying that the BDL livers are changed towards a more collagen-related profile. Remarkably, we could not observe a correlation between the CO1-764 marker and Col1a1 mRNA in the livers. Since type I collagen is considered an index of fibrogenesis activation, these results suggest that CO1-764, contrary to other degradation fragments of liver collagens such as CO3-610 (Veidal et al., 2010), is less dependent of fibrogenesis, and potentially reflects tissue destruction caused by pathological events other than fibrogenesis. Nonetheless the CO3-610 fragment was statistically elevated at week 2 after BDL, demonstrating

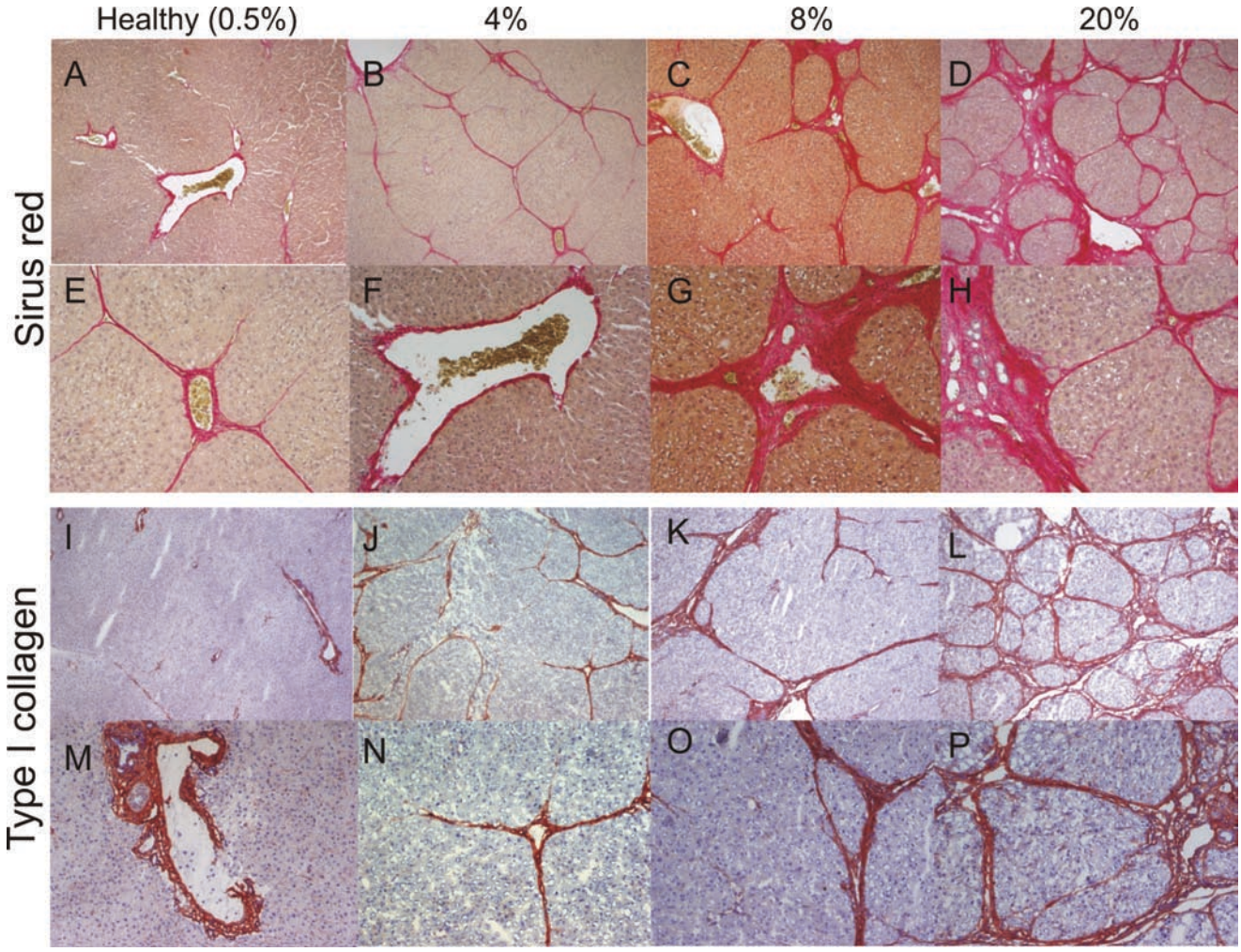

Figure 6. Histological stainings of CCL4 treated rat livers by A-H) Sirius red for total collagen and I-P) for type I collagen using a commercial polyclonal antibody. Groups were stratified according to the percent Sirius red staining: 0.5, 4, 8 and 20%.

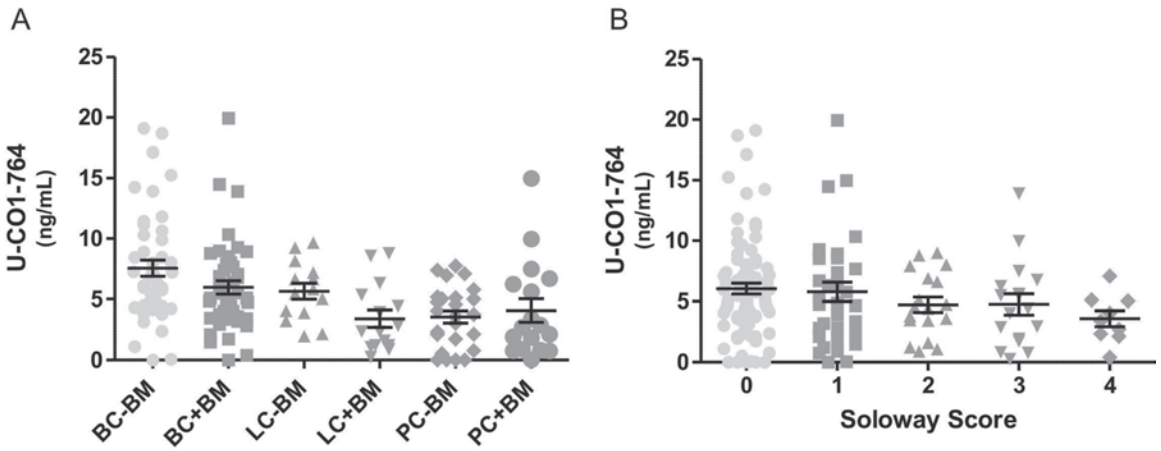

Figure 7. CO1-764 levels in 161 breast-, lung- and prostate cancer patients stratified according to A) the type of cancer and +/- BM; B) the extent of metastatic bone disease described by the Soloway score 0 (-BM), and 1–4 (+BM). Results shown are mean ± standard error of the mean (SEM). Data indicated that there was no significant difference in level in +BM patients compared to -BM, ns = non significant elevation in +BM patients as compared with–BM.

that type III collagen and MMP-9 may be elevated when fibrosis is initiated (Barascuk et al., 2010). The differences observed for the two neo-epitope assays CO3-610 and CO1-764 suggest that the two markers are not indicating the same pathologies. The data presented here indicate that the serum CO1-764 marker is a true degradation marker and not a formation marker since there is a time displace prior to its release from the liver. Furthermore, it

is known that the type I collagen formation marker PINP is elevated at the week 2 time point in this study (Veidal et al., 2010), indicating that the CO1-674 marker does not correlate to type I collagen formation.

CO1-764 for the evaluation of liver fibrosis in the CCL4 study

The CO1-764 was clearly related to liver fibrosis in CCL4 treated rats stratified by time. When rats were stratified according to the amount of total collagen in the liver, we observed that the marker was elevated in the lowest quartile of collagen. The marker also correlated very well to total collagen and type I collagen alpha 1 chain mRNA expression in the livers of CCL4 rats. However, there are limitations within this correlation since the levels of transcription and levels of protein expression are not directly comparable. Nevertheless, we sought to investigate whether there was a correlation between the transcription levels of type I collagen and the amount of CO1-764 since type I collagen is known to be highly elevated in fibrotic liver. Collectively, these data indicate that the CO1-764 marker reflects type I collagen degradation and not formation since the neo-epitope is not elevated at the early treatment time point; however becomes significant elevated when the presence of total collagen is observed by Sirius red. No correlation was observed between total collagen in the liver of control rats and CO1-764, further supporting its ability to describe type I collagen degradation in the fibrotic liver. Type I collagen stainings show that the type I collagen formation is found in the fibrotic bands and may be the source of this fragment during MMP degradation of this tissue.

CO1-764 for the evaluation of bone metastases

Bone is the most common site of tumor metastasis in breast and prostate cancer patients (Coleman, 2006). As in fibrosis, two of the key players impacting greatly on ECM remodeling in cancer proliferation are MMPs and the tissue inhibitors of metalloproteinases (TIMPs) (Sternlicht et al., 1999; Sternlicht and Werb, 2001; Wiseman et al., 2003). MMP-2, -3, -9 and -13 are known to be elevated in the microenvironment of BM (Duffy, 1996; Freije et al., 1994; Martin and Matrisian, 2007).

There were no statistical differences in urinary CO1-764 levels between patients with and without BM in any of the three cancer types, clearly indicating no relationship between this marker and BM. A similar picture was seen for CO1-764 in the same patients stratified according to the degree of skeletal involvement, using the Soloway score. We have previously shown that bone related marker such as the Cat K generated ALPHA C-telopeptide of type I collagen (CTX-I) and ICTP are highly related to number of bone metastases (Leeming et al., 2006). It is well known that the vicious cycle leads to a breakdown of type I collagen by osteoclasts (Mundy, 2000) by the production of Cat K and may explain why this marker is not related to BM. The marker was assessed in urine which may have an impact on the result since the fragment assessed may be different than that of serum.

In conclusion, we have developed an assay using a specific monoclonal antibody for the detection of the CO1-764 fragment in human, rat and mouse fluids, a type I collagen fragment derived from MMP-2, -9 and -13 activity and subsequently destroyed by Cat K *in vitro*. We demonstrated that CO1-764 was elevated in liver fibrosis but not in patients with prostate-, lung- and breast cancer with skeletal metastases, and appears not to be derived from bone breakdown. These data furthermore indicate that there is a high potential for the use of neo-epitope biomarkers for many ECM-related diseases since this is well known from the arthritis and bone field (Schaller et al., 2005), and has now been proven for liver fibrosis in the present paper and by Barascuk et al. (Barascuk et al., 2010). More studies are needed to further evaluate the potential of these markers in liver fibrosis.

Limitations

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 that 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 CO1-764.

Declaration of interest

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