Biomarkers, 2011; 16(5): 426–433

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.3109/1354750X.2011.584131

informa
healthcare

RESEARCH ARTICLE

Immunological detection of the type V collagen propeptide fragment, PVCP-1230, in connective tissue remodeling associated with liver fibrosis

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Abstract

Aim: Liver fibrosis involves excessive remodeling and deposition of fibrillar extracellular matrix (ECM) components, which leads to malfunction of the organ, causing significant morbidity and mortality. The aim of this study was to assess whether levels of a type V collagen fragment, the propeptide CO5-1230, indicate the amount of collagen deposited during liver fibrosis.

Methods: A specific competitive enzyme-linked immunosorbent assay (ELISA) was developed to measure CO5-1230 levels. The sequence TAALGDIMGH located at the start of the C-terminal propeptide between amino acid position 1230′ and 1239′ (CO5-1230) of the α2 chain was selected as the immunogen. Monoclonal antibodies were raised against this fragment. An assay developed using the biotin–streptavidin system was evaluated in two rat models of liver fibrosis: bile duct ligation (BDL) and carbon tetrachloride (CCl_4)-treated rats, for up to 20 weeks.

Results: The ELISA was capable of measuring CO5-1230 in serum specifically, with an intra-assay variation of 3.46% and inter-assay variation of 5.09%. Mean CO5-1230 levels were significantly elevated in CCl_4 rats compared with controls [8 weeks: 57.4 ng/mL, controls 45.5 ng/mL ($P = 0.0020$); 12 weeks: 81.3 ng/mL, controls 50.2 ng/mL ($P = 0.0020$); 16 weeks: 85.1 ng/mL, controls 51 ng/mL ($P = 0055$); 20 weeks: 92 ng/mL, controls 47.8 ng/mL ($P = 0.0033$)]. CO5-1230 levels correlated with the total amount of collagen in sections from the injured livers, quantified from Sirius red stains (Spearman, $R^2 = 0.5580$). In BDL rats, serum levels of CO5-1230 were also elevated compared with controls [2 weeks: 160.1 ng/mL, controls 78.9 ng/mL ($P = 0.0007$); 4 weeks: 111.3 ng/mL, controls 62.2 ng/mL, ($P = 0.0068$)] and showed a linear correlation to the total collagen content (Spearman, $R^2 = 0.3305$).

Conclusions: Increased serum levels of CO5-1230 were associated with the extent of collagen deposition in two different models of fibrotic processes in the liver. The data indicate that formation of type V collagen may be of value as a disease-specific diagnostic biomarker that reflects the total burden of disease. The amino acid sequence selected is located in the first 10 amino acids of the C-terminal propeptide section, which is a formation-specific region.

Keywords: Biochemical markers, type V collagen, liver fibrosis, propeptide, bile duct ligation, N-terminal, carbon tetrachloride

Introduction

The extracellular matrix (ECM) is the most prominent element of connective tissue, offering the necessary structural support for cells to adhere to and migrate across. The ECM consists of collagens, proteoglycans, and glycoproteins, all of which play important, unique, and interrelated functions in maintaining the physicochemical structure of the tissue. ECM remodeling is a normal process in which formation and degradation of healthy tissue occurs synergistically, in a balanced way. However, in disease states an imbalance of ECM elements can result in various pathologies. Excessive deposition

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(Received 02 March 2011; revised 19 April 2011; accepted 22 April 2011)

of fibrillar ECM components, especially collagens, may occur in all tissues and organs as either a disease-specific fibrogenesis or as scar formation. During the fibrosis progress, the formation of ECM molecules—particularly those comprising collagen—occurs more rapidly than their degradation. Due to the consequential architectural changes in the ECM, extensive tissue remodeling and fibrosis ultimately lead to organ failure. This is evident in end-stage chronic liver disease, where cirrhosis is associated with significant morbidity and mortality (Friedman 2003; Wynn 2008).

Fibrosis may begin in response to various acute or chronic stimuli, including infections, autoimmune reactions, toxins, radiation, and mechanical injury (Veidal et al. 2010) that damage tissue. Tissue injury is followed by acute or chronic tissue repair, which is believed to be a dynamic series of events involving complex cellular and molecular mechanisms (Schuppan et al. 2001). In the case of liver fibrosis, a series of events that involve several cell types working in synergy to ultimately cause irreversible damage of the liver (Copple et al. 2010).

The current literature on liver fibrosis biomarkers contains a sizable number of experimental studies. However, utilization of biomarkers for diagnosis and monitoring of fibrosis, fibrogenesis, and fibrolysis is relatively limited (Gressner et al. 2007). Our group recently described noninvasive biomarkers for the evaluation of liver fibrosis, including a matrix metalloproteinase 9-generated type III collagen type fragment (Barascuk et al. 2010), and the N-terminal propeptide of type I collagen (PINP) (Veidal et al. 2010). Both markers emphasize the potential advantage of assessing chronic liver disease by measurements of ECM imbalance.

Fibril-forming collagens are synthesized as precursor molecules with large propeptide extensions at the N- and the C-termini of the molecule (Gelse et al. 2003). Propeptides are cleaved from procollagen by N- or C-terminal proteinases, and the mature collagen is integrated into the ECM (Garnero et al. 2003). Type V collagen constitutes about 1–5% of the total collagen in a healthy human, but it seems to rapidly proliferate during ECM remodeling due to its structural position (Ruggiero et al. 1994; Fichard et al. 1995). Even though the exact functions of type V collagen are elusive, it is known to be highly expressed during tissue development, wound repair, and fibrogenesis, and its interaction with other cells such as fibroblasts seems to also affect ECM stiffness and cell behavior during development (Gressner et al. 2007; Breuls et al. 2009). Type V collagen effects on cell behavior have been utilized in experimental models of ECM remodeling such as lung fibrosis (Braun et al. 2010). However, no markers of type V collagen formation have been developed for assessing fibrosis in both physiological and pathological conditions. The lack of a clear understanding of the collagen type V-specific roles in conjunction with its relative scarce levels relatively to collagen type I might have lead in overlooking a potentially informative for fibrosis pathology biomarker.

Bile duct ligation (BDL) in rats has been used as a model of chronic liver injury due to its similarity to hepatocyte injury, 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 (CCl_4) can induce liver cirrhosis by centrilobular parenchymal destruction (Tugues et al. 2007; Muñoz-Luque et al. 2008). Liver fibrosis induced by CCl_4 exhibits most of the features observed in human cirrhosis, including widespread hepatic fibrosis and nodule formation.

We set out with the hypothesis that a type V collagen propeptide marker may be useful for monitoring liver fibrosis due to its implication to wound repair and could potentially indicate a different disease stage and state than that detected by our previous collagen-based assays (Tsukadaa et al. 2006). The increasing need for biomarkers that could facilitate diagnosis, prognosis, and drug development ultimately presents the challenge to classify and validate biomarkers according to their different properties as described by the BIPED criteria (Karsdal et al. 2009; Veidal et al. 2010). The scope of the present work was to measure levels of a C-terminal propeptide of type V collagen, CO5-1230, using a novel enzyme-linked immunosorbent assay (ELISA), and test whether those levels correlated with type V collagen formation observed in two rat models of liver fibrosis.

Materials and methods

Reagents

All reagents used for experiments were standard high-quality chemicals from 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.

Selection of peptide for immunization

The sequence of peptide selected for the assay was chosen from propeptide fragments of type V collagen. Peptide fragments were identified using Uniprot (accession number P05997). The sequence TAALGDIMGH located at the start of the C-terminal propeptide between amino acid position 1230′ and 1239′ (CO5-1230) of the α2 chain was selected as immunogen. The first 10 amino acids of each free end of the sequences identified were regarded as a target sequence. All relevant sequences were analyzed for homology 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 (TAALGDIMGH-GGC-OVA). Immunizations were performed at 2-week intervals

until stable titer levels were obtained. At each bleeding, the serum antibody titer was measured and the mice with the highest antibody titer and best reactivity toward serum and urine were selected for fusion. The selected mice were boosted intravenously with 50 µg immunogen in 100 µL 0.9% sodium chloride solution 3 days before surgical removal of the spleen for cell fusion.

Fusion and antibody screening

The fusion procedure has been described elsewhere (Gefter et al. 1977). In brief, mouse spleen cells were fused with SP2/0 myeloma fusion partner cells. The hybridoma cells were cloned using a limiting dilution method and transferred into 96-well microtiter plates for further growth. Standard limited dilution was used to promote monoclonal growth. Supernatants were screened using an indirect ELISA, whereas the biotinylated peptide TAALGDIMGH-GGC-Biotin was used as a catcher peptide on streptavidin-coated microtiter plates.

Characterization of clones

Native reactivity and peptide binding of the monoclonal antibodies in human serum and urine, and in rat serum and urine, was evaluated using a preliminary ELISA with a 10 ng/mL biotinylated peptide coater on a streptavidin-coated microtiter plate and the supernatant from the growing monoclonal hybridoma. Clone specificity was tested against a free peptide (TAALGDIMGH) and a nonsense peptide. Isotyping of the monoclonal antibodies was performed using the Clonotyping System-HRP kit, cat. 5300-05 (Southern Biotech, Birmingham, AL). The selected clones were purified using Protein G columns according to the manufacturer's instructions and dialyzed (GE Healthcare Life Science, Little Chalfont, Buckinghamshire, UK).

CO5-1230 assay protocol

The following competitive ELISA protocol was optimized for use with the CO5-1230 monoclonal antibody. The selected monoclonal antibodies were labeled with horseradish peroxidase (HRP) using the Lightning-Link HRP antibody labeling kit according to the manufacturer's instructions (Innova Bioscience, Babraham, Cambridge, UK). A 96-well streptavidin plate (Roche diagnostics, Basel, Switzerland) was coated with 2.5 ng of the biotinylated synthetic peptide, TAALGDIMGH-GGC-Biotin, dissolved in assay buffer (50 mM Tris, 1% bovine serum albumin (BSA), 0.1% Tween-20, adjusted to pH 7.4 at 20°C) and incubated for 30 min at 20°C. Twenty microliters of the peptide calibrator or sample were added to appropriate wells, followed by 100 µL of 125 ng conjugated monoclonal antibody and incubated for 1 h at 20°C. Finally, 100 µL tetramethyl benzinidine (TMB) (Kem-En-Tec cat.438OH, Taastrup, Denmark) was added, and the plate was incubated for 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 four-parametric mathematical fit model with a starting concentration of 200 ng for the standard peptide following a 2-fold dilution.

Technical evaluation

From 2-fold dilutions of pooled human serum and urine samples, linearity was calculated as a percentage of recovery of the 100% sample. The lower limit of detection (LLD) was determined from 21 zero samples (i.e. buffer) and calculated as the mean + 3× standard deviation. The inter- and intra-assay variation was determined by 10 independent runs of eight samples run in duplicate.

Rat bile duct ligation

CO5-1230 levels were measured in a BDL rat model of liver fibrosis. The Danish ethical board for animal studies approved the study (2008/561-1450). Seventeen female Sprague-Dawley rats aged 6 months were stratified into four groups. Liver fibrosis was induced in anesthetized rats by standard BDL [24], in which the bile duct was ligated in two places and dissected between the ligations in an open-surgery procedure. In sham-operated rats, the abdomen was closed without BDL surgery. BDL- or sham-operated rats were sacrificed after 2 or 4 weeks. Blood samples were taken under CO_2/O_2 anesthesia at baseline and at termination from the retro-orbital sinus of rats that had fasted for 14 h. The collected blood was left for 30 min at room temperature to clot, followed by centrifugation twice at 1500 *g* for 10 min. The serum was then transferred to clean tubes and stored at –80°C.

Rat CCl_4 liver fibrosis model

CO5-1230 levels were also measured in a CCl_4 inhalation rat model of liver fibrosis. Fifty-two male Wistar rats (Charles-River, Saint Aubin les Elseuf, France) were treated with CCl_4, whereas another 28 male Wistar rats served as controls. Induction of liver fibrosis was performed as previously described (Clarià and Jimenez 1999; Segovia-Silvestre et al. 2010). In brief, CCl_4 was administered by inhalation twice weekly and phenobarbital (0.3 g/L) was added to the drinking water. Control rats received phenobarbital only. Animals receiving CCl_4 were stratified into groups treated for 8, 12, 16, or 20 weeks of CCl_4 ($n = 13$ for each group). Seven control animals were terminated at each of the four treatment durations. This animal study was performed according to the criteria of the Investigation and Ethics Committee of the Hospital Clinic Universitari (Barcelona, Spain). Blood was collected at termination and was allowed to stand at room temperature for 20 min to clot, before centrifugation at

2500 rpm for 10 min. Samples were stored at –80°C. Liver sections 4 µm thick were stained with 0.1% Sirius red (F3B) in saturated picric acid (Sigma-Aldrich). From each animal, the amount of fibrosis expressed as a percentage of total collagen levels against the total liver area was measured by quantitative histology and the average value per animal presented (Muñoz-Luque et al. 2008). Each field was acquired at 10× magnification (E600 microscope, Nikon) and RT slider SPOT digital camera (Diagnostic instruments, Inc., Sterling Heights, MI). Results were analyzed using Bioquant life science morphometry system. Fibrosis area was evaluated by measuring collagen area divided by the net field area and then multiplied by 100. Subtraction of vascular luminal area from the total field area was also added in the calculation of the net fibrotic area. From each animal analyzed the amount of fibrosis as percentage was measured and the average value is presented. Classification of groups according to their fibrosis levels was done by determining the percentage of Sirius red positive liver area (1st Quartile: <5%, 2nd Quartile: 5–10%, 3rd Quartile: 10–15%, 4th Quartile: >15%. Exclusion criteria included animal with bacteremia and spontaneous bacterial peritonitis.

Tissue handling

After the rats were put down, their livers were carefully dissected, weighed, fixed in 4% formaldehyde for a minimum of 24 h, cut into appropriate slices and embedded in paraffin. Sections 5 µm thick were cut, mounted on glass slides, and stained with Sirius red and α-SMA. The liver sections were evaluated histologically by assessment of the architecture, presence of inflammation, proliferation of bile ducts, and fibrosis.

Statistical analysis

Comparison of serum CO5-1230 levels was performed using the unpaired *t*-test with Welch correction. Correlations were measured 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

Clone characterization

The clone selected for ELISA development was determined to be the IgG1 subtype. The native reaction of this clone was high against human and rat serum but low against human and rat urine (Figure 1).

Technical evaluation

The typical standard curve is presented in Figure 1, showing a four-parametric fit for the assay. The LLD for the assay was 2.162 ng/mL. Dilution recovery was within 100 ± 15% (Table 1). The inter- and intra-assay variation was below 8% (Table 2).

CCl_4 study

The effect of CCl_4 treatment was evaluated by Sirius red staining and α-SMA immunostaining for all time points (Figure 2). Lung slices were also evaluated by Sirius red staining, showing no collagen increase in any treatment time point. Mean CO5-1230 levels at termination time

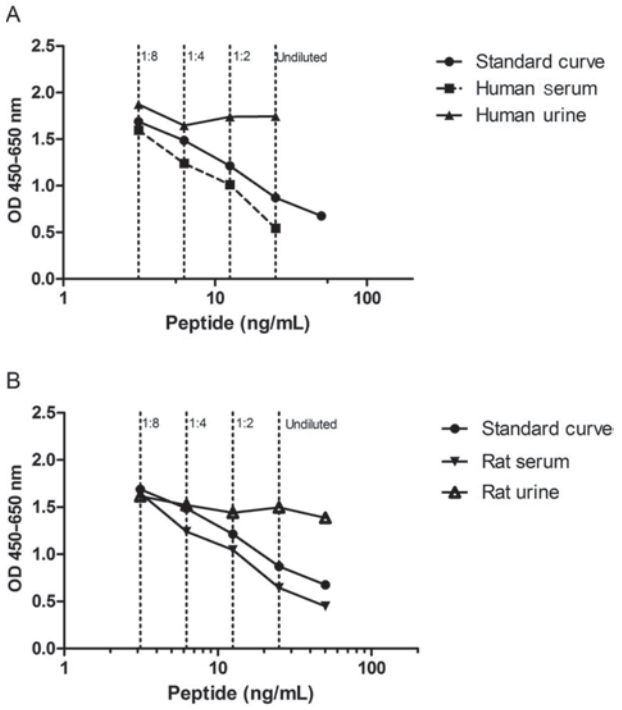

Figure 1. Representative standard curves and native reactivity against human (A) and rat (B) serum and urine. Native material was run undiluted and diluted 1:2, 1:4, or 1:8 as indicated. The signal was assessed as the optical density at 450 nm, subtracting the background at 650 nm, as a function of peptide concentration. LDL was calculated and found to be 2.162. Point zero represents increased signal inhibition and therefore elevated levels of the epitope in biological fluid.

Table 1. Percentage of the dilution recovery of the CO5-1230 assay.

PVCP-1230 ng/mL	HS1 60.51	HS2 89.8	HS3 79.7	HS4 91.7	HS5 108.17	RS1 53.1	RS2 1.57
Undiluted	100%	100%	100%	100%	100%	100%	100%
Dilution 1:2	122	107	115	110	106	111	114
Dilution 1:4	99	103	102	97	98	102	104
Dilution 1:8	89	55	77	81	87	91	90
Mean	**103**	**88**	**98**	**96**	**97**	**101**	**102**

HS = human serum; RS = rat serum.
Measurements refer to duplicates. Undiluted sample represents 100% recovery, from which recovery is calculated for the remaining dilution steps from 1:2 up to 1:8.

points were statistically elevated ($P = 0.0033$) in CCl_4 rats (72.44 ng/mL) compared with controls (42.35 ng/mL). Mean CO5-1230 levels were also statistically higher in the CCl_4-treated rats than in controls, at each termination time point: at 8 weeks: 57.4 ng/mL, controls 45.5 ng/mL ($P = 0.0020$); 12 weeks: 81.3 ng/mL, controls 50.2 ng/mL ($P = 0.0020$); 16 weeks: 85.1 ng/mL, controls 51 ng/mL ($P = 0055$); 20 weeks: 92 ng/mL, controls 47.8 ng/mL ($P = 0.0033$). A statistically significant difference ($P = 0.0001$) was also found between CCl_4-treated rats (27.13 ng/mL) versus control animals (14.15 ng/mL), whereas CO5-1230 levels were found to be significantly correlated with the total collagen increase in sections from the injured livers, as quantified from Sirius red stains ($R^2 = 0.5580$) (Figure 3).

BDL-induced fibrosis

The effect of BDL operation was evaluated by Sirius red staining and α-SMA immunostaining for all time points (Figure 4). Lung slices were also evaluated by Sirius red staining, showing no collagen increase in any treatment time point. The validity of the BDL model was demonstrated by gross examination and microscopy (Muñoz-Luque et al. 2008). At the time of sacrifice, livers of BDL animals were enlarged compared with the normal gross morphology in control animals, mean liver weights were significantly increased in the BDL rats, and semiquantitative scoring of liver sections showed significantly more structural changes at Weeks 2 and 4 in BDL animals compared with controls. Histological examination of BDL livers revealed progressive ductular proliferation from the periportal areas toward the centrilobular regions (verified by immunohistochemical staining for keratin 7). Collagen was deposited around these ductular structures as a supporting stroma. Livers of the sham-operated animals were microscopically normal without sign of fibrosis. No other signs of cholestasis were observed, including intracellular cholestasis, bile plugs, bile infarction, or hepatocytic rosette formation in either groups. Immunohistochemical staining verified at Week 4 the marked ductal proliferation around the portal tract with the formation of multiple neo-bile ducts and deposition of extensive type I collagen. In the sham-operated rats, collagen type I deposition was found exclusively in the venous wall.

Total mean serum levels of CO5-1230 were significantly elevated in the BDL rats ($P < 0.001$) (124.7 ng/mL) compared with controls (62.2 ng/mL). At the 2-week termination point, serum levels of CO5-1230 in the BDL rats compared with control animals were 160.1 ng/mL versus 78.9 ng/mL ($P = 0.0007$), and at 4 weeks, 111.3 ng/mL versus 62.2 ng/mL ($P = 0.0068$), respectively. The marker was found to be correlated to total collagen content $R^2 = 0.3305$ (Figure 5).

Table 2. Inter- and intra-assay variation for the CO5-1230 assays using human serum as quality control samples.

PVCP-1230 Sample	Amount (ng/mL)	Intra-assay variability (%)	Inter-assay variability (%)
HS1	63	4.45	5.3
HS2	76	3.97	7.5
HS3	116	3.0	6.7
HS4	133	4.88	6.8
HS5	95	2.96	4.87
HS6	75	2.09	3.70
HS7	55	2.75	3.64
HS8	75	3.59	2.28
Mean		**3.46**	**5.09**

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

Discussion

This is, to our knowledge, the first study to present the development of an assay detecting a C-terminal propeptide fragment of type V collagen. The neo-epitope CO5-1230 assay had a good native reactivity in both human serum and rat serum and was technically robust

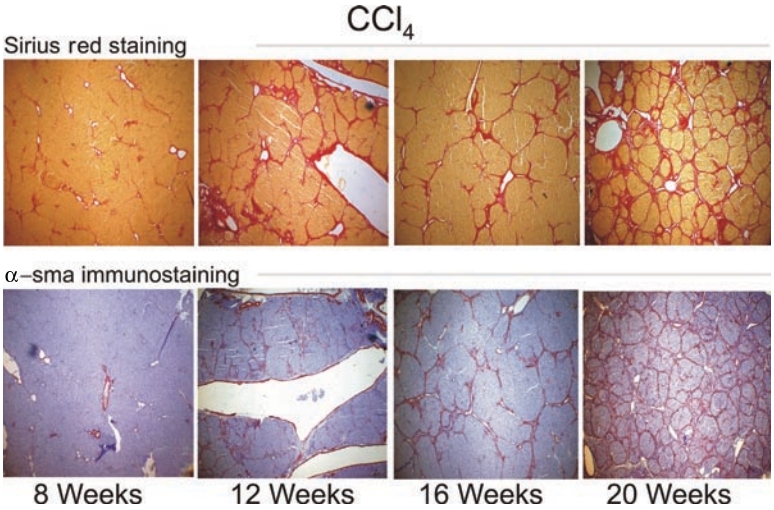

Figure 2. Representative Sirius red staining and α-SMA immunostaining for all CCl_4-treated animals.

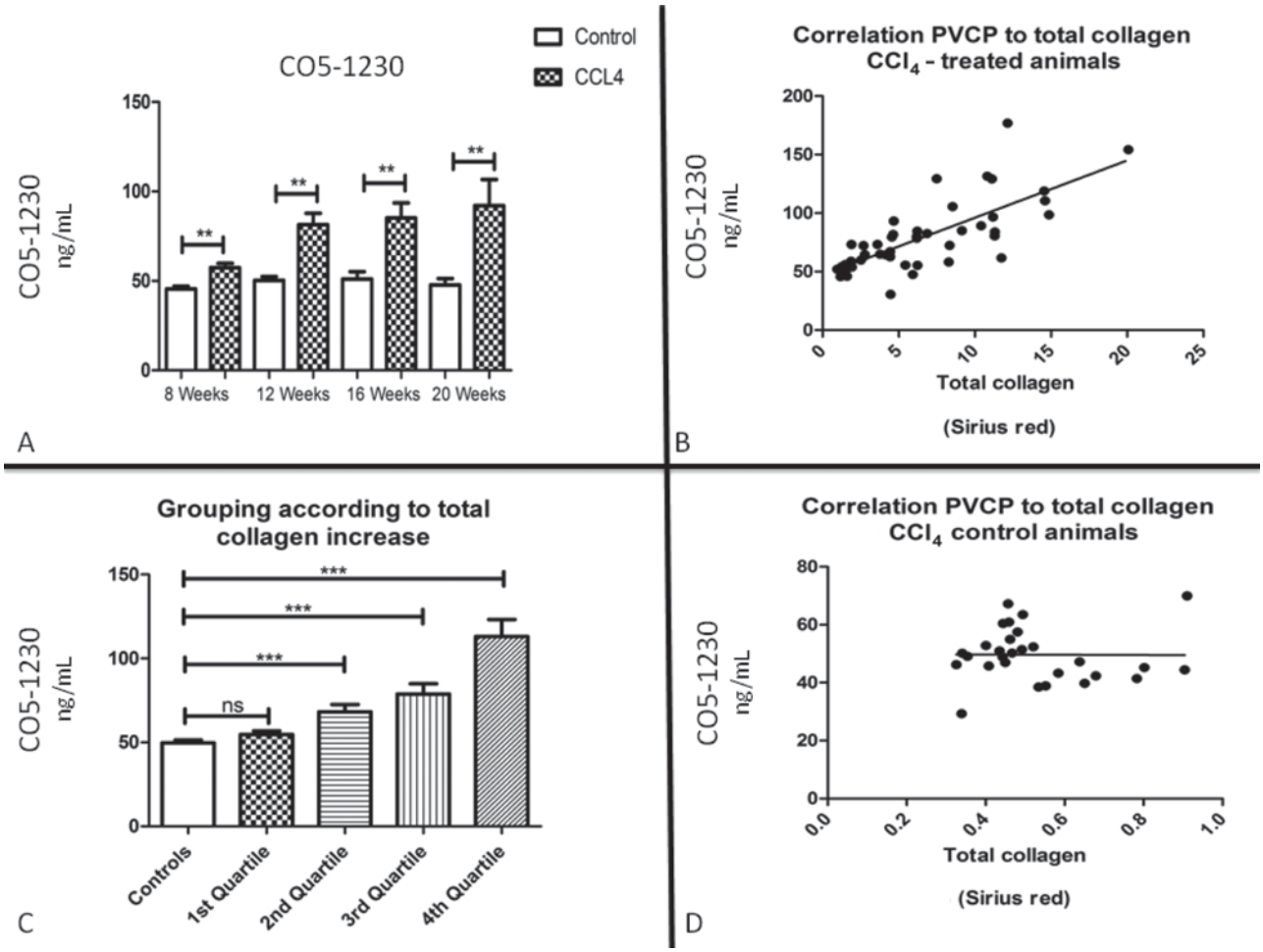

Figure 3. Mean CO5-1230 (PVCP) values revealed a progressive increase in CCl_4-treated rats for all time points. 8 weeks control: 45.5 ng/mL, CCl_4-treated: 57.4 ng/mL ($P = 0.0020$), 12 weeks control: 50.2 ng/mL, CCl_4: 81.3 ng/mL ($P = 0.0020$), 16 weeks control: 51 ng/mL, CCl_4: 85.1 ng/mL ($P = 0.0055$), 20 weeks control: 47.8 ng/mL, CCl_4: 92 ng/mL ($P = 0.0033$) (A). Correlation between PVCP and total collagen levels in CCl_4 animals, $r = 0.7470$, $R^2 = 0.5580$ (B). Stratification of mean PVCP levels according to collagen increase levels, controls: 48.6 ng/mL, first quartile: 66.7 ng/mL ($P < 0.0001$), second quartile: 71.12 ng/mL ($P < 0.0001$), third quartile: 90 ng/mL ($P < 0.0001$), fourth quartile: 135.8 ng/mL ($P < 0.0001$) (C). Correlation between PVCP and total collagen levels in control animals, $r = 0.1058$, $R^2 = 0.011$ (D).

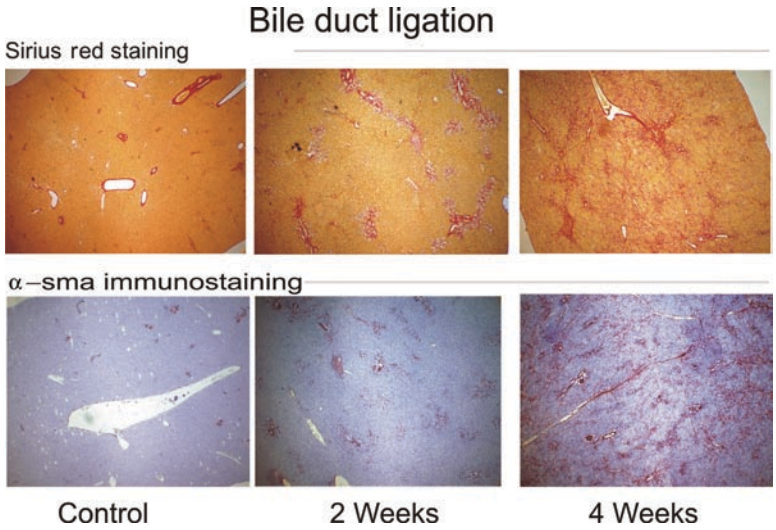

Figure 4. Representative Sirius red staining and α-SMA immunostaining for bile duct and sham-operated animals.

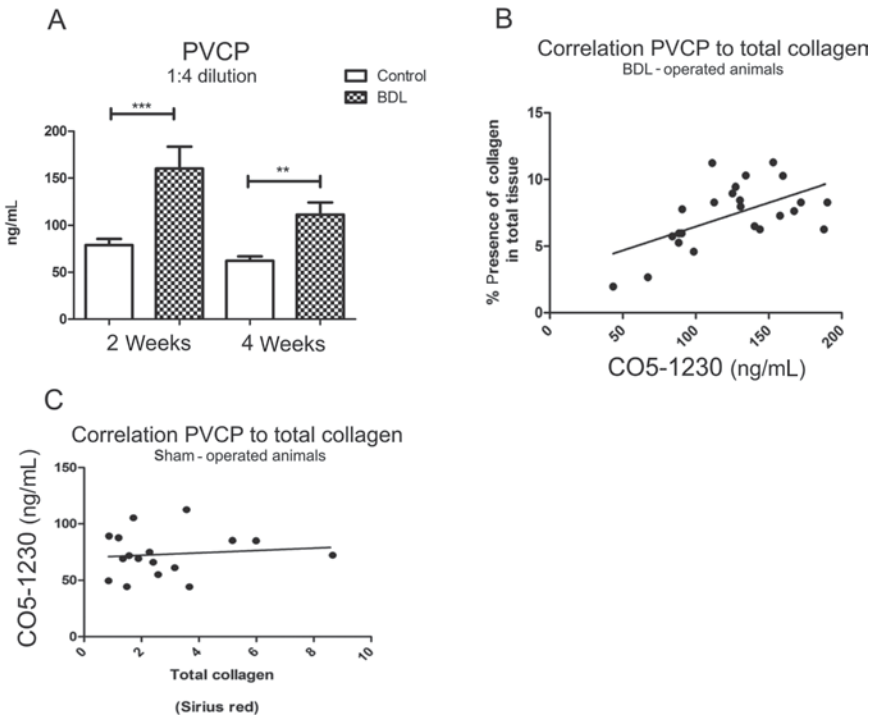

Figure 5. Mean CO5-1230 (PVCP) values revealed a progressive increase in BDL-operated rats for all time points. 2 weeks control: 78.9 ng/mL, BDL: 160.1 ng/mL (P = 0.0007). 4 weeks control: 62.2 ng/mL, BDL: 111.3 ng/mL (P = 0.0068) (A). Correlation between PVCP and total collagen levels for BDL animals $r = 0.5749$, $R^2 = 0.3305$ (B). Correlation between PVCP and total collagen levels for control animals $r = 0.1128$, $R^2 = 0.012$ (C).

with acceptable inter- and intra-assay variation, dilution recovery, and LLD. The high reactivity of the antibody in human and rat serum versus the low reactivity against urine matrice signifies that the selected fragment is found predominantly in circulation rather than metabolized and excreted with urine. Studies on these two *in vivo* fibrosis models demonstrated that CO5-1230 measurements were correlated with the total amount of collagen increase in the liver tissue. The selected amino acid sequence is located in the first 10 amino acids of the C-terminal propeptide thus providing an exact determination of the location of our measurement, as a collagen formation-specific region.

Biochemical markers consisting of protein fragments from ECM degradation may be informative of disease pathology and progression, which in turn may be useful for diagnostic and prognostic purposes. These markers could potentially detect changes resulting from intervention strategies and serve as surrogate markers of drug efficacy (Muñoz-Luque et al. 2008). Large databases containing all known protein sequences, domains, and size, in conjunction with Software query tools, enable proteins to be compared to determine the uniqueness of their amino acid sequences and important information for the understanding of protein function (Bagger et al. 2005; Muñoz-Luque et al. 2008). Collectively, these databases provide key links for biomarker identification and allow an appropriate target to be selected for a monoclonal antibody and for assay development, as was done in the present study.

Characterization of the selected monoclonal antibody revealed strong reactivity toward serum and urine in humans and rats as well as with the custom-made CO5-1230 (PVCP) peptide, which indicates that the antibody recognizes this amino acid sequence for type V collagen in native samples. These native samples could be diluted to at least 1:4 and reactivity still observed.

Even though there is not yet a study to describe the exact differences between the two rat models of liver fibrosis used in this experiment, it is acknowledged that both the BDL and the CCl_4 animal models describe two diverse fibrotic processes in which increased ECM remodeling and excessive collagen deposition are key characteristics of disease progression. In the BDL model, serum PVCP was elevated from as early as 2 weeks after BDL surgery. This indicates that the selected fragment assessed in this assay is highly up-regulated during disease-related ECM remodeling. Similar observations were seen in CCl_4-induced fibrosis, where the CO5-1230 levels could be measured from Week 8 and onward, further demonstrating type V collagen up-regulation during disease progression. Our findings indicate that these two different fibrotic processes seem to have a common up-regulating factor of type V collagen formation. A non-statistically significant difference of the marker levels in between BDL and CCl_4 control/sham groups was also observed, which we attribute to the fact that the animals in the CCl_4 study were 3 months old when treatment was initiated whereas BDL animals were 6 months old when operated. Furthermore, this can be attributed to assay

variability and to the fact that the two studies were carried out in two different sites, Copenhagen and Barcelona, by two research groups.

In conclusion, we have developed an assay using a specific monoclonal antibody for the detection of the unique sequence CO5-1230, a type V collagen fragment located at the C-terminal propeptide. The data emphasize that there is a promising potential for the use of collagen-based biomarkers for ECM-related diseases, which has been suggested also by studies on arthritis and bone and our recent research on fibrotic diseases (Bagger et al. 2005). The present study supports our line of investigations showing that collagen-derived neoepitopes can be of potential value as diagnostic and potentially prognostic markers in monitoring liver fibrosis. However, further validation in a reversible model of liver fibrosis is necessary to better characterize whether the described marker is a prognostic marker informative of early or late ECM remodeling is necessary as well its measurement in well-characterized clinical cohorts is needed.

Acknowledgements

We acknowledge the funding from the Danish Ministry of Science, Technology and Innovation and the Den Danske Forskningsfond, Centre for Clinical and Basic Research. We would also like to thank Professor Wladimiro Jimenez laboratory, Barcelona, Spain for providing the CCl_4 study material.

Declaration of interest

Efstathios Vassiliadis, Sanne S. Veidal, Dorthe V. Larsen, Xiaoliang Chen, Qinlong Zheng, Morten A. Karsdal, and Diana J. Leeming are full-time employees of Nordic Bioscience.

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