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Type VIII collagen is elevated in diseases associated with angiogenesis and vascular remodeling

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ABSTRACT

Objectives: Type VIII collagen is involved in angiogenesis and remodeling of arteries. We hypothesized that type VIII collagen was upregulated in diseases associated with vascular remodeling, e.g. pulmonary fibrosis and cancer. In this paper we present the development and validation of a competitive enzyme-linked immunosorbent assay (ELISA) targeting type VIII collagen in serum and plasma.

Design and methods: A monoclonal antibody was raised against the C-terminal of type VIII collagen (C8-C) and a competitive ELISA was developed. The assay was evaluated in relation to epitope specificity, technical performance, and in relevant disease cohorts. The developed ELISA was applied for the assessment of type VIII collagen in serum from patients diagnosed with chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF) and various cancers.

Results: The C8-C ELISA was technically stable and applicable for measurements in serum and plasma samples. Concentrations of C8-C was increased in serum from patients diagnosed with COPD ($n = 68$) ($p < 0.0001$), SCC lung- ($n = 10$) ($p < 0.0001$), breast- ($n = 13$) ($p < 0.0001$), colon- ($n = 7$) ($p = 0.002$), melanoma- ($n = 7$) ($p = 0.001$), non-small cell lung- ($n = 12$) ($p < 0.0001$), ovary- ($n = 10$) ($p = 0.0001$), pancreas- ($n = 5$) ($p = 0.017$), prostate- ($n = 14$) ($p = 0.001$) and small cell lung cancer ($n = 8$) ($p = 0.0002$) when compared to controls ($n = 43$). Concentrations of C8-C were not significantly increased in serum from patients diagnosed with IPF.

Conclusions: The C8-C assay was technically robust and specific for type VIII collagen. Concentrations of C8-C were significantly elevated in serum from patients diagnosed with COPD and within 9 different cancer types, but not IPF. Further research is required to test C8-C as an efficacy marker for angiostatic treatments.

1. Introduction

Type VIII collagen is a short chain non-fibrillar collagen that is part of the endothelium of blood vessels and is present in arterioles and venules. It is found in organs such as the heart, brain, liver, lung and muscle, as well as around chondrocytes located close to the perichondrium in the cartilage [1]. The human α1 procollagen gene is located on chromosome 3, while the human α2 procollagen gene is located on chromosome 1. Each α chain has a molecular weight of approximately 60 kDa [2]. Previously, type VIII collagen was described as a heterotrimer comprised of two α1 chains and one α2 chain, but in vitro studies have shown that homotrimers of either α1 or α2 can also be formed [3].

Type VIII collagen is synthesized by aortic and corneal endothelial cells, as well as by pulmonary artery endothelial cells and microvascular endothelial cells. Not all endothelial cells express type VIII collagen, as such the collagen can be absent from large and small vessels [4]. Human mast cells have also been shown to produce type VIII collagen under normal and pathological conditions, and it has been speculated that this contributes to angiogenesis, tissue remodeling and fibrosis [5].

Angiogenesis, tissue remodeling and fibrosis are important parts of tumor development and progression [6]. The lungs have a large surface area with an associated basement membrane and interstitial matrix, and it is well known that matrix proteins such as type I, III, IV and VI collagen and elastin are elevated in patients with a pulmonary disease [7–11]. Type VIII collagen may be related to cancer since tumor angiogenesis is found in most malignancies. It is indirectly involved in tumorigenic events such as cell proliferation and metastasis due to the dependence

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on the exchange of oxygen and nutrients with tumor waste products [12]. During angiogenesis, endothelial cells are induced to proliferate and migrate as well as to activate signaling pathways that in turn drive cell shape changes and angiogenic sprouting [13]. Furthermore, the tumor blood vessels often fail to become quiescent due to an altered tissue remodeling leading to sporadic angiogenesis and formation of leaky blood vessels. As with angiogenesis, fibrosis is a phenomenon that can be observed in many malignancies. In cancer fibrosis is also known as desmoplasia. In desmoplasia, an accumulation of perpetually activated cancer associated fibroblasts (CAFs) are observed which exhibit increased and altered expression of extracellular matrix (ECM) proteins including collagens [14]. Desmoplasia is emerging as an important and active process involved in tumor initiation and progression and may, amongst other things, promote the migration of cancer cells [15].

The aim of this work was to quantify type VIII collagen in serum samples from patients diagnosed with diseases associated with vascular remodeling and angiogenesis, such as fibrosis and cancer. In order to measure type VIII collagen, we developed and validated a competitive ELISA with a monoclonal antibody that targets the C-terminal part of the α1(VIII) chain.

2. Materials and methods

2.1. 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 and assay development were purchased from the Chinese Peptide Company, Beijing, China.

2.2. Monoclonal antibody production

4–6 weeks old Balb/C mice were immunized subcutaneously with 50 μg C8-C (KLH-CGG-SFSGYLLYPM) peptide and 200 μl emulsified antigen mixed with Freund's adjuvant (incomplete). The mice were immunized with 2-week intervals until the sera titers were stable. Selection for fusion was based on the serum titers and prior to fusion the selected mouse rested for 4 weeks. 3 days prior to isolation of the spleen the mouse was given 50 μg immunogen in 100 μl 0.9% NaCl solution intravenously as a booster. Hybridoma cells were produced by fusing spleen cells with SP2/0 myeloma cells as previously described [16], in order to culture the cells by the semi-solid medium method. The hybridoma clones were then cultured for further growth in 96-well microtiter plates. Standard limited dilution was used in order to secure monoclonal growth. The supernatants were screened for reactivity with Biotin-CGG-SFSGYLLYPM which was used as a screening peptide, the free peptide SFSGYLLYPM served as calibrator and native material was used to test for specificity of the hybridoma clones.

2.3. Characterization of the clones

The monoclonal antibodies were tested for native reactivity and peptide affinity by displacement of human, mouse and rat serum, as well as with plasma and urine samples. The supernatants from the growing monoclonal hybridoma cells were tested in a preliminary indirect ELISA on streptavidin-coated microtiter plates, which were coated with 10 ng/ml biotinylated peptide (Biotin-CGG-SFSGYLLYPM). Each clone was tested for specificity towards the peptide (SFSGYLLYPM) and the elongated peptide (SFSGYLLYPMA). Clonotyping System-HRP kit, cat. no. 5300–05 (Southern Biotech, Birmingham, AL, USA) was used to isotype the monoclonal antibodies.

Selected clones were purified using protein G columns according to manufacturer's protocol (GE Healthcare Life Science, Little Chalfont, Buckinghamshire, UK). Labeling of the antibody with horseradish peroxidase (HRP) was performed using the Lightning link HRP labeling kit (Innovabioscience, Babraham, Cambridge, UK).

2.4. C8-C ELISA procedure

The finalized C8-C competitive ELISA protocol was:

1. Coat a 96-well streptavidin-coated microtiter plate (cat. no. 11,940,279, Roche Diagnostics, Mannheim, Germany) with 100 μl biotinylated peptide (Biotin-KKKFSGYLLYPM) dissolved in assay buffer (50 mM Tris-BTB, pH 7.4) for 30 min at 20 °C.
2. Wash 5 times in washing buffer (20 mM Tris, 50 mM NaCl, pH 7.2).
3. Add 20 μl standard peptide, controls or samples in duplicate to appropriate wells, then add 100 μl HRP-labeled antibody diluted in assay buffer and incubate overnight (20 h) at 4 °C.
4. Wash 5 times in washing buffer.
5. Add 100 μl tetramethylbenzinidine (TMB) (cat. no. 4380H, Kem-En-Tec, Taastrup, Denmark) and incubate for 15 min at 20 °C.
6. Finally add 100 μl stopping solution (1% H_2SO_4) in order to stop the TMB reaction.
7. Read the absorbance at 450 nm with 650 nm as reference.

The incubation steps take place in the dark on a shaker (300 rpm). The absorbance was read on an ELISA microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). The standard curve was made by a 2-fold dilution of the standard peptide (SFSGYLLYPM).

2.5. Technical evaluation of the C8-C assay

Linearity was determined by calculating recovery percentages in 2-fold dilutions of serum and plasma samples from human, rat and mouse. 21 blank samples (i.e. buffer) was used to determine the lower limit of detection (LLOD), and calculated as mean + 3x standard deviation (SD). 10 independent runs of 7 quality control (QC) samples, consisting of double determinations of the samples in duplicates, was used to determine the intra- and inter-assay variation of the C8-C assay. Mean coefficient of variance (CV %) within plates was used to calculate the intra-assay variation, whilst the mean CV % between the individual runs was used to calculate the inter-assay variation. Accuracy of the assay was determined via the use of 2 healthy human serum samples (diluted 1:2) which were spiked with the standard peptide or human serum, and was calculated as the percentage recovery of the theoretical amount of serum. Interference was ascertained by spiking lipemia (intralipid), human antibodies against mouse antigens by human anti-mouse antibody (HAMA), biotin and hemoglobin in a 2-fold dilution to a serum sample. Concentrations of lipemia, biotin and hemoglobin started at 0.56 mmol/l, 160 μg/l and 8.1 g/l, respectively. A human serum sample with high concentrations of HAMA was spiked in 2-fold dilution to a normal human serum sample. The normal serum samples served as reference in order to calculate the percentage recovery. 3 rat and 3 human serum samples were subjected to 4 freeze (−20 °C) and thaw cycles, this was carried out to determine the stability of the analyte, with the first cycle serving as reference for calculation of the percentage recovery. Acceptable range for linearity, analyte stability and inference was ±20%.

2.6. Patient serum samples

Approval was obtained from an appropriate Institutional Review Board/Independent Ethical Committee and after informed consent serum was sampled from patients and age-matched with healthy controls with no chronic or symptomatic diseases. In accordance with Danish law, it is not necessary to get ethical approval when measuring biochemical markers in previously collected samples.

The concentrations of C8-C were measured in serum samples from patients diagnosed with COPD (n = 13), IPF (n = 10) and squamous cell-carcinoma lung cancer (n = 10) obtained from Proteogenex