The matrilin-3 VWA1 domain modulates interleukin-6 release from primary human chondrocytes

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ARTICLE INFO

Article history:
Received 29 August 2012
Accepted 6 March 2013

Keywords:
Matrilin
VWA domain
EGF domain
Coiled-coil domain
Cartilage

SUMMARY

Objective: We previously demonstrated the ability of matrilin-3 to modulate the gene expression profile of primary human chondrocytes (PHCs) toward a state favoring cartilage catabolism. The structure within matrilin-3 responsible for the induction of these catabolic genes is unknown. Here, we investigated the potential of matrilin-3 (MATN3) and truncated matrilin-3 proteins, in both monomeric and oligomeric form, to stimulate interleukin (IL)-6 release in PHCs.

Methods: We expressed full-length matrilin-3 oligomers, matrilin-3 von Willebrand factor A (VWA) domain oligomers, matrilin-3 four epidermal growth factor (EGF) domain oligomers, matrilin-3 monomers without oligomerization domains, matrilin-3 VWA domain monomers, and matrilin-3 4EGF monomers. We then incubated PHCs in the absence or presence of full-length matrilin-3 or one of the truncated matrilin-3 proteins and finally determined the release of IL-6 in cell-culture supernatants.

Results: The addition of full-length matrilin-3 oligomers, matrilin-3 VWA domain oligomers, and, less pronounced, matrilin-3 monomers without oligomerization domains, and matrilin-3 4EGF-oligomers to the cell-culture medium led to a significant induction of IL-6 in PHCs.

Discussion: Based on recombinant expression of different matrilin-3 domains in both monomeric and oligomeric form, this work demonstrated that the VWA1 domain of matrilin-3 is primarily responsible for the induction of IL-6 release and that the oligomerization of the VWA1 domain markedly promotes its activity.

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Introduction

We previously demonstrated the ability of matrilin-3 to modulate the gene expression profile of primary human chondrocytes (PHCs) toward a state favoring cartilage catabolism. Moreover, stimulation of PHCs with matrilin-3 resulted in a significant induction of the proinflammatory cytokines interleukin (IL)-6, IL-8, IL-1 and tumor-necrosis factor (TNF)α, the matrix metalloproteinase (MMP)1, 3, and 13, the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). However, the structure within matrilin-3 responsible for the induction of these catabolic genes is unknown.

The matrilins form a four-member family of modular, oligomeric extracellular matrix proteins that are most strongly expressed in cartilage, but are also present in many other forms of extracellular matrix. Matrilins show a distinct structural heterogeneity caused by alternative splicing, the formation of homo- and heterooligomers and proteolytic processing, which all affect their interactions. They are thought to serve as adaptor proteins mediating interactions between the major cartilage collagen fibrils, collagen VI microfibrils and the aggrecan gel. Except for matrilin-3, mature matrilins consist of subunits with two von Willebrand factor A (VWA)-like domains that are connected by a variable number of epidermal growth factor (EGF)-like domains, and these subunits form trimers or tetramers via a C-terminal z-helical coiled-coil domain. Matrilin-3 is the smallest family member and is mainly expressed in cartilage. Its subunits consist of a single VWA-like domain, followed by four EGF-like domains and a C-terminal coiled-coil domain. A common feature of VWA domains is their involvement in the formation of intra- or extracellular multiprotein complexes (for review see). EGF-like domains and a C-terminal coiled-coil domain. A common feature of VWA domains is their involvement in the formation of intra- or extracellular multiprotein complexes (for review see).
domains are about 40–50 amino acid residues long, and are present in many membrane-bound or secreted proteins. Both VWA and EGF domains could potentially modulate the gene expression profile of PHCs after binding to cell-surface receptors.

In the present study, we aimed at determining which domain within matrilin-3 has the ability to induce catabolic genes in PHCs. To accomplish this, we recombinantly expressed different matrilin-3 domains in both monomeric and oligomeric form (Fig. 1) and investigated their potential to stimulate IL-6 release in PHCs.

Material and methods

Expression and purification of recombinant matrilin-3 and recombinant matrilin-3 domains
cDNA constructs coding for murine full-length matrilin-3 and different matrilin-3 domains were generated by Reverse transcription polymerase chain reaction (RT-PCR) and cloned with 5’-terminal SpeI and 3’-terminal BamHI restriction sites using oligonucleotide primers (Supplementary Material). The amplified PCR products were inserted into a modified pCEP-Pu vector containing an N-terminal BM-40 signal peptide5 and a C-terminal One-STrEP-tag (IBA GmbH) downstream of the restriction sites. Each of the expression constructs was transfected into 293EBNA cells with Lipofectamine™ 2000 (Invitrogen), according to the manufacturer’s instructions. The cell lines were selected with puromycin (1 μg/ml) and cultured under serum-free conditions prior to harvesting, conditioned cell-culture supernatants. Cells expressing full-length matrilin-3, as well as the VWA1 domain with coiled-coil domains (A1cc) and the 4EGF domains with coiled-coil domains (4EGFcC) were cultured with 10% SeraPlus (PAN) to reduce proteolytic processing, thereby increasing the yield of intact matrilin-3 oligomers. One-STrEP-tagged matrilin-3 and matrilin-3 domains were affinity purified from conditioned supernatants using a Strep-Tactin Superflow column (IBA).

Isolation, cultures and stimulation of PHCs

Articular cartilage taken from femoral condyles and tibial plateaus of human knee joints was obtained from patients suffering from osteoarthritis at the time of total joint replacement surgery. Patient age at the time of surgery ranged from 52 to 78 years. Chondrocytes were taken from whole condyles. To isolate PHCs, cartilage samples were rinsed with Dulbecco’s Modified Eagle Medium (DMEM)/F12 medium (Gibco), minced, and digested with Pronase® (0.4%, 90 min, Calbiochem) and Collagenase-P® (0.025%, 16 h, Roche) in DMEM/F12 medium containing 5% BD-NU serum (BD Biosciences). PHCs were filtered with a 100 μm and a 40 μm filter, seeded (1 x 10^6 cells/well) in six-well multiwell plates ( Falcon) in DMEM/F12 (PAN) containing 10% BD-NU serum (BD Biosciences) and 50 μg/ml gentamicin/ml (Gibco) and then cultivated in a CO2 incubator (5% CO2, 37 °C).

Cells were incubated in the absence or presence of full-length matrilin-3 or matrilin-3 domains at different concentrations. For this purpose, matrilin-3 and matrilin-3 domains were dialyzed against culture medium. The dialysis buffer (devoid of full-length matrilin-3 and matrilin-3 domains) was used as a negative control.

The concentrations were determined by using the Bio-Rad Protein Assay (Bio-RAD). The viability of the cells was verified by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Thiazolyl Blue Tetrazolium Bromide) test (Sigma), according to the manufacturer’s instructions. IL-6 release was related to the values of the negative controls and depicted as fold induction. To further demonstrate the specificity of stimulation, prior to stimulation culture medium was depleted for matrilin-3, matrilin-3 monomer without oligomerization domains (dCT), and A1cc, respectively, by incubation with Strep-Tactin Superflow resin (IBA) for 1 h and subsequently centrifugation (at 84 g).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

Cell-culture supernatants were separated on a 4–12% Bis-Tris gel (Invitrogen) and stained by Coomassie Brilliant Blue (Merck). For immunoblotting, the proteins were transferred to a Polyvinylidene fluoride (PVDF) membrane (0.45 μm) (Invitrogen). Membranes were blocked with 5% milk powder, incubated in an appropriate dilution of an affinity-purified polyclonal rabbit anti-matrilin-3 antisera1, and finally incubated in a 1/5,000 dilution of horseradish peroxidase-conjugated polyclonal donkey anti-rabbit IgG antisera (Amersham Pharmacia Biotech). Membranes were treated with ECL Plus according to the manufacturer’s instructions (Amersham Pharmacia Biotech), and visualized on Hyperfilm (Amersham Bioscience).

Enzyme-linked immuno sorbent assay (ELISA)

Release of IL-6 was determined in cell-culture supernatants using a commercial IL-6 ELISA kit (BD Biosciences) in accordance with the manufacturer’s protocol.

Statistical analysis

The term dose dependency as used in this text refers to the magnitude of a biological effect as a function of the concentration of the inducing agent. The biological effect changes when the concentration of the inducing agent changes, both are correlated. Therefore tests of correlation can be performed to estimate dose dependency. Here, Pearson’s correlation coefficient analysis was used. To test for differences between effects, irrespective of potentially underlying effects of dose dependency. Analysis of variance between groups (ANOVA) was used. To test for normality, the Shapiro–Wilk test was used (not shown). All measurements were normally distributed, a prerequisite for applying ANOVA or Pearson’s correlation coefficient analysis. P-values less than 0.05 indicate significant results, while P-values less than 0.01 indicate
highly significant results. Abbreviations used: $P$ = $P$-value; $n$ = number of patients; $F$ = test statistic (ANOVA); and $r$ = correlation coefficient (Pearson).

Results

Cloning, expression and purification of full-length matrilin-3 and truncated matrilin-3 proteins

In our previous work, we demonstrated the ability of matrilin-3 to modulate the gene expression profile of PHCs. To investigate which domain of matrilin-3 is able to stimulate gene induction, we recombinantly expressed and purified full-length matrilin-3 tetramers (flc), the single A1 domain (A1), four EGF domains (4EGF), and matrilin-3 without the C-terminal oligomerization domain (dCT) (Fig. 1). Furthermore, we expressed a matrilin-3 VWA domain oligomer (A1cc), as well as an oligomer containing only the four EGF domains (4EGFcc), by use of the C-terminal coiled-coil oligomerization domain of matrilin-3 (Fig. 1). The identity and the purity of all proteins were verified by SDS–PAGE followed by immunoblot and Coomassie staining (Fig. 2), respectively.

Matrilin-3 contains 28 cysteine residues, 26 of which form intrachain disulfide bonds. Two form disulfide bonds connecting the N- and C-terminus of the VWA domain and 24 are involved in the characteristic disulfide bond pattern of the four EGF domains. Two closely spaced cysteine residues present at the N-terminus of all matrilin-coiled-coil domains form a ring of interchain disulfide bonds that stabilizes the coiled-coil domain. This was revealed by Nuclear magnetic resonance (NMR) analysis of the structure of the matrilin-1 coiled-coil domain6. In the present work, the flc as well as the A1cc and 4EGFcc domains formed higher oligomers, which released monomers after reduction, and showed the expected migration pattern on SDS–PAGE (Fig. 2). The 4EGF protein gave a band corresponding to 40–50 kDa, which indicates dimer formation. We therefore verified the correct folding of the 4EGF protein by circular dichroism spectroscopy (data not shown).

Different ranges of yield of recombinant protein were obtained for the different protein preparations: flc 30–340 mg/ml; dCT 427–1904 mg/ml; A1 – 295–469 mg/ml; 4E – 162–467 mg/ml; A1cc – 135–287 mg/ml; 4Ecc – 428–159 mg/ml. Protein preparations were adjusted for molar concentration differences by dilution and subsequent dialysis against serum-free cell-culture medium. Resulting equimolar concentrations were double-checked to spot dialysis-associated protein loss.

Dose-dependent induction of IL-6 release primarily by full-length matrilin-3 and the oligomeric A1cc domain

To investigate which matrilin-3 domain(s) is/are responsible for induction of IL-6, we incubated PHCs with full-length matrilin-3 and the different truncated matrilin-3 proteins in various equimolar concentrations (Fig. 3) calculated on the basis of the molecular weights given in Fig. 1. Incubation with full-length matrilin-3 yielded...
a significant dose-dependent release of IL-6 ($P = 0.020, F = 4.135, n = 6$) — dose dependency: $P = 0.001, r = 0.615, n = 6$). Incubation with the monomeric dCT domain, consisting of the VWA and the four EGF domains, also led to a highly significant dose-dependent release of IL-6 ($P = 6.1 \times 10^{-7}, F = 15.234, n = 12$) — dose dependency: $P = 1.0 \times 10^{-6}, r = 0.713, n = 12$), but compared to full-length matrilin-3, the induction by dCT was less pronounced. Incubation of PHCs with the monomeric A1 domain ($P = 0.582, F = 0.668, n = 6$) — dose dependency: $P = 0.259, r = -0.240, n = 6$) and monomeric 4EGF ($P = 0.959, F = 0.100, n = 6$) — dose dependency: $P = 0.911, r = 0.024, n = 6$) domain did not result in IL-6 release, whereas the oligomeric A1cc domain caused a strong and highly significant release of IL-6 ($P = 8.8 \times 10^{-4}, F = 10.665, n = 7$) — dose dependency: $P = 2.6 \times 10^{-4}, r = 0.717, n = 7$). The oligomeric 4EGFcc domain only gave a very weak, yet highly significant release of IL-6 ($P = 6.5 \times 10^{-6}, F = 29.349, n = 6$) — dose dependency: $P = 1.8 \times 10^{-6}, r = 0.876, n = 6$) (Fig. 3).

The limited yield of primary cells, as well as the use of different concentrations and controls, made it impractical to carry out parallel investigations of PHCs from the same donor comprising the whole protein and concentration scale. Therefore, as an alternative, we compared the magnitude of the IL-6 gene induction by investigating the effect of matrilin-3 and matrilin-3 domains in parallel with PHCs from eight different donors at one defined concentration (Fig. 4). Incubation of PHCs with the A1cc domain gave the strongest effect on IL-6 release, followed by full-length matrilin-3. In comparison, the dCT and the 4EGFcc domains led to a moderate IL-6 release, while the other matrilin-3 proteins had minimal effects on IL-6 release.

**Discussion**

Our results show that the VWA1 domain of matrilin-3 is responsible for release of IL-6 and, furthermore, that it must be present in an oligomeric form to strongly stimulate IL-6 release. To this day, a receptor for matrilin-3 VWA1 domain-mediated release of IL-6 has not been identified. Earlier studies have shown that integrins weakly interact with matrilins, but the binding does not lead to formation of focal contacts and reorganization of the actin cytoskeleton. In addition, cell-surface proteoglycans have been implicated in the weak promotion of cell attachment via matrilins. Integrin activity and the cell adhesion-signaling phenotype can be regulated by clustering of the integrin receptors as well as by co-receptor interactions with cell-surface proteoglycans. Considering the lack of signal transduction by the monomeric VWA1 domain, it is possible that the oligomeric structure of matrilin-3 provides clustering of integrins and/or cell-surface proteoglycans or the activation of a still unknown receptor due to the high avidity provided by the multivalency of the matrilin oligomers.

The matrilin-3 dCT protein consists of a VWA1 and 4EGF domain. In contrast to the isolated VWA1 and 4EGF domains, incubation of PHCs with the dCT domain led to a moderate release of IL-6. However, it is uncertain why the monomeric VWA1 domain did not cause IL-6 release, while the monomeric dCT domain did. It is possible that the EGF domains binds to an as yet unidentified receptor and thus acts as a co-receptor ligand. This hypothesis is supported by the recent finding that matrilin-3 can bind to the α5β1 integrin, leading to cell adhesion and Protein kinase B (AKT) activation in an EGF1-dependent manner. So far, it has been assumed that the VWA domains are the principal interaction domains of matrilins and that the EGF domains serve as spacers. This
is based on the extensive alternative splicing of the EGF domain and that splice variants lacking EGF domains are found in zebrafish. In the light of our new results as well as the \( \gamma \delta \beta 1 \) integrin addressed above we put forward the hypothesis that both the VWA1 and the EGF domains are functional in modulating the gene expression profile of PHCs and thus cartilage homeostasis. Further studies will be aimed at showing which genes are activated by the VWA1 and the 4EGF domain or if they act in concert on the same genes.

The PHCs used in our study originate from osteoarthritic human knee joints and represent a mixture of chondrocytes from Osteoarthritis (OA)-affected and unaffected regions. The two kinds of chondrocytes probably differ in their response to matrilin-3 stimulation, a difference that was not analyzed in our experiments and the implications of which have been discussed elsewhere.

Over the past few years, it has become obvious that the Extra-cellular matrix (ECM) is more than just a rigid network for cell attachment. Many studies show that matrix proteins can influence gene expression and regulate cartilage homeostasis in addition to their function as structural components and adapters, thus playing an important role for cartilage maintenance in health and cartilage degradation in disease. As a result of its modular composition, matrilin-3 is an important tissue-specific regulator of cartilage homeostasis. This impact on cartilage homeostasis is regulated at the level of gene expression, by alternative splicing, oligomerization, matrix integration, and proteolytic processing.

**Authors contribution**

A. R. Klatt, B. Paul-Klausch and G. Klinger designed the experiments. B. Paul-Klausch, G. Klinger, G. Kuehn, U. Hillebrandt and B. Kobbe performed the experiments. J. H. Renno performed the statistical analysis. A. R. Klatt, M. Paulsson, R. Wagener, W. Johannis and J. H. Renno wrote the manuscript.

**Conflict of interest statement**

The authors declare no conflict of interest.

**Funding sources**

This work has been funded by Deutsche Forschungsgemeinschaft (WA 1338/2-6) in support of R. Wagener and M. Paulsson.

**Acknowledgment**

We thank Ann-Kathrin Becker for performing CD spectroscopy.

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**Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2013.03.005.

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