Matrix Biology 30 (2011) 275–280

Contents lists available at ScienceDirect

Matrix Biology

journal homepage: www.elsevier.com/locate/matbio

Matrilin-4 is processed by ADAMTS-5 in late Golgi vesicles present in growth plate chondrocytes of defined differentiation state

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ARTICLE INFO

Article history:
Received 8 February 2011
Received in revised form 7 April 2011
Accepted 8 April 2011

Keywords:
Cartilage
Growth plate
ADAMTS
Proteolytic processing
Matrilin

ABSTRACT

The two aggrecanases ADAMTS-4 and ADAMTS-5 have been shown to not only play roles in the breakdown of cartilage extracellular matrix in osteoarthritis, but also mediate processing of matrilins in the secretory pathway. The matrilins are adaptor proteins with a function in connecting fibrillar and network-like components in the cartilage extracellular matrix. Cleavage resulting in processed matrilins with fewer ligand-binding subunits could make these less efficient in providing matrix cohesion. In this study, the processing and degradation of matrilin-4 during cartilage remodeling in the growth plate of the developing mouse long bones were studied in greater detail. We show that ADAMTS-5 and a matrilin-4 neoepitope, revealed upon ADAMTS cleavage, colocalize in prehypertrophic/hypertrophic chondrocytes while they are not detected in proliferating chondrocytes of the growth plate. ADAMTS-5 and the cleaved matrilin-4 are preferentially detected in vesicles derived from the Golgi apparatus. The matrilin-4 neoepitope was not observed in the growth plate of ADAMTS-5 deficient mice. We propose that in the growth plate ADAMTS-5, and not ADAMTS-4, has a physiological function in the intracellular processing of matrilins and potentially of other extracellular matrix proteins.

1. Introduction

Proteolysis mediated by members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family of metalloproteinases plays many and important roles in connective tissues. Some ADAMTS family members participate in the physiological processing of procollagens and other matrix proteins, while some have been implicated in the pathogenesis of different forms of both inherited and acquired connective tissue diseases (Apte, 2009). Studies in cartilage have mainly focussed on ADAMTS-4 and -5, which are considered to be the two major aggrecanases that process the cartilage proteoglycan aggrecan in extracellular space. Aggrecan has a modular structure and ADAMTSs cleave its core protein at distinct sites either in the interglobular domain, located between the N-terminal globular domain G1 and domain G2, or in the long region of the core protein substituted with chondroitin sulfate chains (Fosang et al., 2008; Rogerson et al., 2008). Aggrecan proteolysis is of particular relevance in osteoarthritis, where interleukin 1 signaling results in increased extracellular aggrecanase activity leading to degradation of the cartilage extracellular matrix. Studies in which the catalytic domains in ADAMTS-4 and -5 were targeted by homologous recombination in mouse showed that only diminished ADAMTS-5 activity protects the cartilage matrix in experimental models of osteoarthritis, pointing to ADAMTS-5 as the functionally most important aggrecanase in mouse (Stanton et al., 2005). However, neither ADAMTS-4 nor -5 is required for normal skeletal development or aggrecan turnover in cartilage (Majumdar et al., 2007; Rogerson et al., 2008).

The matrilins constitute a four-member family of modular, multi-subunit extracellular matrix proteins, which are expressed not only in cartilage but also in many other forms of connective tissue (Klatt et al., 2011). Matrilins interact with a variety of molecules present in the cartilage matrix, prominently aggrecan (Paulsson and Heinegård, 1979; Hauser et al., 1996), fibrillar collagens (Winterbottom et al., 1992), the fibril associated collagen IX (Budde et al., 2005), the collagen binding protein COMP (cartilage oligomeric matrix protein, thrombospondin 5) (Mann et al., 2004) and the small leucine-rich proteoglycans decorin and biglycan (Wiberg et al., 2003). It is believed that matrilins connect fibrils and molecular networks present in cartilage matrix, thereby acting as adaptor proteins. In matrilin subunits two potentially ligand

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doi:10.1016/j.matbio.2011.04.002

binding VWA (von Willebrand factor A) domains are connected by a varying number of EGF (epidermal growth factor) like domains. These are followed by a hinge region and a C-terminal α-helical coiled-coil domain which allows the oligomerization of the single subunits in a bouquet-like fashion. Only matrilin-3 lacks the second VWA domain (Klatt et al., 2011). Matrilins in tissues are often proteolytically processed and therefore carry a varying number of ligand binding subunits, a fact that will influence the avidity of the interactions. A proteolytic cleavage site, first identified in matrilin-4 (Klatt et al., 2001), is conserved in all matrilins and for matrilin-3 and -4 it was shown that this site is used by ADAMTS-4 and -5, but not by ADAMTS-1 (Hills et al., 2007; Ehlen et al., 2009). It was found that the cleavage occurs already in the secretory pathway and that it depends on the activation of ADAMTS-4 and -5 by proprotein convertases (Ehlen et al., 2009). The present study focuses on three important aspects of protein cleavage by ADAMTS-5. We show that *in vivo* pronounced matrilin processing occurs only in subpopulations of growth plate chondrocytes during endochondral ossification of mouse long bones and we define the intracellular compartment where cleavage takes place. Further, we show that matrilin-4 is specifically cleaved by ADAMTS-5 and not by ADAMTS-4 in the growth plate.

2. Results

Indirect immunofluorescence microscopy with antibodies specific for ADAMTS-5 was used on sections of tibial or femoral growth plate from mice of different postnatal stages to visualize the expression pattern of the metalloproteinase. These results were compared with the growth plate localization of a matrilin-4 neoepitope that is generated by the cleavage of matrilin-4 by ADAMTSs. Owing to two reasons, we used matrilin-4 as a prototype matrilin to determine in which chondrocyte cell populations matrilin processing by ADAMTSs occurs. First, matrilin-4 is expressed in all cartilaginous regions of the maturing mouse joint, including proliferating and hypertrophic cells of the growth plate (Klatt et al., 2002). Second, a neoepitope antibody has been generated that specifically detects the N-terminus of the C-terminal fragment of processed matrilin-4 (Ehlen et al., 2009). The application of this antibody on tissue sections allows determination of when and where matrilin processing takes place.

In newborn (P0) mice, the staining for ADAMTS-5 coincided with that for the matrilin-4 neoepitope and was seen mainly within prehypertrophic and hypertrophic chondrocytes (Fig. 1). The zone of proliferating chondrocytes was largely devoid of staining for both antigens, despite that matrilin-4 is synthesized also by the chondrocytes in this region (Klatt et al., 2002). At postnatal day 14, both ADAMTS-5 and the matrilin-4 neoepitope were detected in the upper hypertrophic zone of the growth plate (Fig. 1). At postnatal day 28, the prehypertrophic zone of the growth plate was stained for both antigens, together with scattered cells surrounding the secondary center of ossification (Fig. 1). The proliferating chondrocytes and the terminally differentiated hypertrophic chondrocytes at the cartilage-bone junction were again negative for both. In contrast to in nasal septum (Ehlen et al., 2009), almost no extracellular signal was detected in the growth plate for either the matrilin-4 neoepitope or ADAMTS-5 even after extensive enzymatic unmasking (Supplementary Fig. 1).

To determine the role of ADAMTS-5 in the processing of matrilin-4 in the growth plate and to confirm the specificity of the ADAMTS-5 antibody, immunostaining on sections of ADAMTS-5 deficient mice was performed. In such animals, no staining for ADAMTS-5 seen in the growth plate, demonstrating the specificity of the antibody. Interestingly, the C-terminal fragment of matrilin-4 could also not be detected in the growth plate of ADAMTS-5 deficient mice, indicating an essential role of ADAMTS-5 in the processing of matrilin-4 in the growth plate (Fig. 1).

Our immunofluorescence analysis reveals that ADAMTS-5 and the matrilin-4 neoepitope are localized mainly intracellularly. Therefore, we next studied the subcellular distribution of these antigens in primary mouse hind limb chondrocytes grown in monolayer (Fig. 2). These conditions allow better visualization of the intracellular distribution of the proteins. For both ADAMTS-5 and the matrilin-4 neoepitope labeled patches that had the appearance of vesicles were seen in the cells and almost no extracellular staining was detected. As

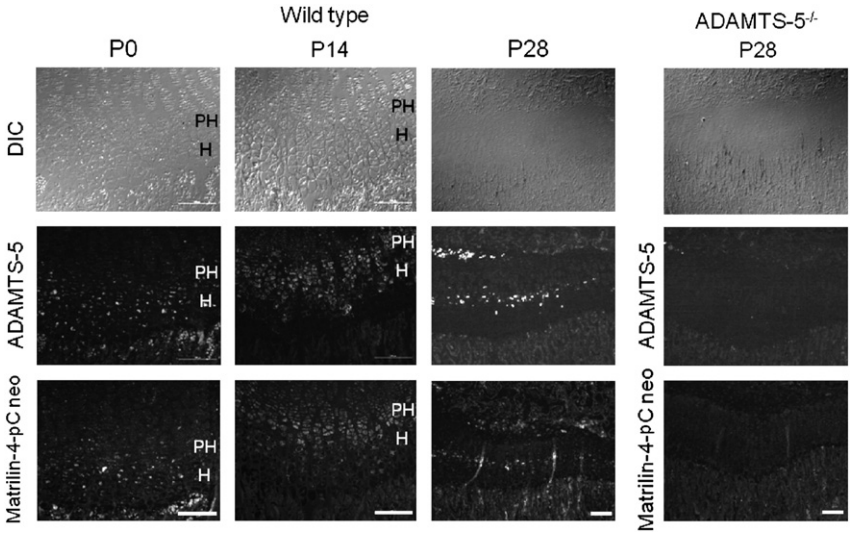

Fig. 1. Immunofluorescence staining of the proximal tibial epiphysis of newborn mice for ADAMTS-5 and the ADAMTS cleaved C-terminal fragment of matrilin-4. Differential interference contrast (DIC) images of the growth plate (upper row). Immunofluorescence staining for ADAMTS-5: In the growth plate the proliferating chondrocytes are negative for ADAMTS-5, whereas the early and late but not the terminally differentiated hypertrophic chondrocytes show an intracellular staining (middle row) at all investigated stages. For the detection of the C-terminal fragment of matrilin-4 obtained after ADAMTS cleavage, the matrilin-4-pC neoepitope antibody was used (lower row), showing an intracellular localization of the fragment in the same regions of the growth plate that are positive for ADAMTS-5. Extracellular signals for ADAMTS-5 and the matrilin-4 neoepitope were not detected. Immunofluorescence staining of the growth plate of ADAMTS-5 deficient mice shows the lack of both ADAMTS-5 and the C-terminal fragment of matrilin-4. The immunofluorescence stainings were performed on serial sections at each stage, while the DIC images depict one of these. At P0 and P14, the prehypertrophic (PH) and the hypertrophic (H) zones are indicated. The scale bar represents 100 μm.