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A decorin-deficient matrix affects skin chondroitin/dermatan sulfate levels and keratinocyte function☆

Katerina Nikolovska [a,1], Jana K. Renke [a,1], Oliver Jungmann [a], Kay Grobe [a], Renato V. Iozzo [b], Alina D. Zamfir [c], Daniela G. Seidler [a,*]

[a] Insitute of Physiological Chemistry and Pathobiochemistry, Waldeyerstr. 15, University Hospital Münster, University of Münster, D-48149 Münster, Germany
[b] Department of Pathology, Anatomy and Cell Biology, and the Cancer Cell Biology and Signaling Program, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, 19107, USA
[c] Department of Chemical and Biological Sciences, "Aurel Vlaicu" University of Arad, Romania and Mass Spectrometry Laboratory, National Institute for Research and Development in Electrochemistry and Condensed Matter, Timisoara, Romania

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ABSTRACT

Decorin is a small leucine-rich proteoglycan harboring a single glycosaminoglycan chain, which, in skin, is mainly composed of dermatan sulfate (DS). Mutant mice with targeted disruption of the decorin gene ($Dcn^{-/-}$) exhibit an abnormal collagen architecture in the dermis and reduced tensile strength, collectively leading to a skin fragility phenotype. Notably, Ehlers–Danlos patients with mutations in enzymes involved in the biosynthesis of DS display a similar phenotype, and recent studies indicate that DS is involved in growth factor binding and signaling. To determine the impact of the loss of DS-decorin in the dermis, we analyzed the glycosaminoglycan content of $Dcn^{-/-}$ and wild-type mouse skin. The total amount of chondroitin/dermatan sulfate (CS/DS) was increased in the $Dcn^{-/-}$ skin, but was overall less sulfated with a significant reduction in bisulfated ΔDiS2,X (X = 4 or 6) disaccharide units, due to the reduced expression of uronyl 2-O sulfotransferase (Ust). With increasing age, sulfation declined; however, $Dcn^{-/-}$ CS/DS was constantly undersulfated *vis-à-vis* wild-type. Functionally, we found altered fibroblast growth factor (Fgf)-7 and -2 binding due to changes in the micro-heterogeneity of skin $Dcn^{-/-}$ CS/DS. To better delineate the role of decorin, we used a 3D $Dcn^{-/-}$ fibroblast cell culture model. We found that the CS/DS extracts of wild-type and $Dcn^{-/-}$ fibroblasts were similar to the skin sugars, and this correlated with the lack of uronyl 2-O sulfotransferase in the $Dcn^{-/-}$ fibroblasts. Moreover, Ffg7 binding to total CS/DS was attenuated in the $Dcn^{-/-}$ samples. Surprisingly, wild-type CS/DS significantly reduced the binding of Fgf7 to keratinocytes in a concentration dependent manner unlike the $Dcn^{-/-}$ CS/DS that only affected the binding at higher concentrations. Although binding to cell-surfaces was quite similar at higher concentrations, keratinocyte proliferation was differentially affected. Higher concentration of $Dcn^{-/-}$ CS/DS induced proliferation in contrast to wild-type CS/DS. 3D co-cultures of fibroblasts and keratinocytes showed that, unlike $Dcn^{-/-}$ CS/DS, wild-type CS/DS promoted differentiation of keratinocytes. Collectively, our results provide novel mechanistic explanations for the reported defects in wound healing in $Dcn^{-/-}$ mice and possibly Ehlers–Danlos patients. Moreover, the lack of decorin-derived DS and an altered CS/DS composition differentially influence keratinocyte behavior.

1. Introduction

Decorin belongs to the family of the small leucine-rich proteoglycans (Iozzo and Murdoch, 1996) and is covalently linked with one glycosaminoglycan chain (GAG). Depending on the tissue decorin is covalently linked with either chondroitin or dermatan sulfate (CS/DS). Decorin is a multifunctional proteoglycan involved in several biological processes, like matrix organization (Seidler and Dreier, 2008), myogenesis (Brandan and Gutierrez, 2013), inflammation and cancer (Schaefer and Iozzo, 2008, 2012). The biosynthesis of GAGs is a multi-step process that begins with the formation of a specific carbohydrate–protein linkage region and continues with the alternating addition of D-glucuronic acid (D-GlcA) and either D-N-acetylglycosamine (D-GlcNAc) or D-N-acetylgalactosamine (D-GalNAc). CS/DS, the two types of galactosaminoglycans, is distinguished by the absence or presence of L-IdoA residues, respectively. The dermatan sulfate epimerase (*Dse*) together with several sulfotransferases modifies the CS/DS chain thus introducing a micro-heterogeneity along the chain. It is known that DS and CS show

Abbreviations: SLRP, small leucine-rich proteoglycan; $Dcn^{-/-}$, decorin-null mouse; GAG, glycosaminoglycan; DS, dermatan sulfate; CS, chondroitin sulfate; *Dcn*, mouse decorin gene; Fgf, fibroblast growth factor; ECM, extracellular matrix; EDS, Ehlers–Danlos syndrome; $Dse1^{-/-}$, dermatan sulfate epimerase 1-null mice.

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* Corresponding author. Tel.: +49 251 8355586; fax: +49 251 8355596.
E-mail address: dgseidle@uni-muenster.de (D.G. Seidler).

[1] These authors contributed equally.

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different biological functions (Sugahara et al., 2003) which is primarily due to a greater conformational flexibility of L-IdoA-containing GAGs (Inoue et al., 1990; Mulloy and Forster, 2000) and more frequently 2-O-sulfated than D-GlcA as the hexuronic acid moiety (Kobayashi et al., 1999). There are several DS proteoglycans and the most prominent in skin is decorin (Iozzo, 1997). Decorin contains about 60% L-IdoA in the dermis (Malmström et al., 2012) in contrast to the lack of L-IdoA in bone (Cheng et al., 1994). The lack of decorin in mice ($Dcn^{-/-}$) results in abnormal collagen fibrillogenesis and skin fragility (Danielson et al., 1997). Interestingly, tendons of $Dcn^{-/-}$ mice were less affected by aging compared to wild-type tendon (Dunkmann et al., 2013). The $Dcn^{-/-}$ mice however show a delayed epidermal wound closure and a significantly delayed dermal wound healing (Järveläinen et al., 2006). The loss of decorin and the impact on fibroblasts are well studied: $Dcn^{-/-}$ fibroblasts proliferate and adhere better compared to wild-type and express more β1 integrin (Ferdous et al., 2010). Addition of decorin proteoglycan but not decorin protein core can rescue the phenotype of fibrillar collagens (Seidler et al., 2005; Rühland et al., 2007) and the adhesive phenotype by reducing the amount of β1 integrin and stabilizing the vimentin intermediate filament system during matrix synthesis (Jungmann et al., 2012). These results indicate that the DS chain of decorin could also play a major role during regeneration and wound healing. This concept is further supported by studies utilizing $Dse1^{-/-}$ mice, which also exhibit heterogeneous collagen fibrils and skin fragility presumably as a consequence of reduced amount of L-IdoA in CS/DS of decorin and the disrupted L-IdoA clusters in the dermis (Maccarana et al., 2009). Consistent with these findings, reduced cell surface L-IdoA content of aortic smooth muscle cells leads to delayed wound healing and inability of directional migration (Bartolini et al., 2013), underlying the importance of DS in mediating proliferation, migration and adhesion (Malmström et al., 2012).

There are several mutations described in enzymes involved in the post-translational modifications of proteoglycans (Seidler, 2012). For example: mutation in the gene *B4GALT7*, an enzyme involved in the synthesis of the linkage region of GAGs leads to an Ehlers–Danlos syndrome with skin fragility and wound healing deficiency (Kresse et al., 1987; Faiyaz-Ul-Haque et al., 2004). Notably, the fibroblasts from the affected patients display defective biosynthesis of decorin characterized by release of decorin partially lacking a GAG chain (Kresse et al., 1987; Seidler et al., 2006) and reduced amount of L-IdoA in the partially processed GAG (Seidler et al., 2006). Mutations in *CHST14*, the gene that encodes the dermatan-4-O sulfotransferase, leads to an Ehlers–Danlos syndrome categorized either as EDS Kosho-type or kyphoscoliosis-type with a delayed wound healing (Miyake et al., 2010; Shimizu et al., 2011). The loss of DS in the dermis leads to abnormal fibrillar collagen due to the defects in post-translational modifications of decorin (Miyake et al., 2010; Shimizu et al., 2011).

Growth factors require GAGs for their function (Sarrazin et al., 2011) and DS can affect interferon-γ (Fernandez-Botran et al., 1999; Bocian et al., 2013) and fibroblast growth factors (FGFs) (Trowbridge and Gallo, 2002; Taylor et al., 2005). In a contact allergy model, loss of decorin leads to a reduced edema formation due to a disturbed cytokine and chemokine expression profile (Seidler et al., 2011). Wound fluid contains GAGs, and the DS derived from wound fluid promotes FGF2 signaling (Penc et al., 1998). Indeed, oligosaccharides derived from skin fibroblast DS decorin can bind FGF2 (Zamfir et al., 2003). FGF7-induced proliferation is DS dependent (Trowbridge and Gallo, 2002). However, the specificity of DS interaction with growth factor is still debated. Recently, it has been shown that FGFs display a clear preference for distinct structural features of heparin (Xu et al., 2012). Therefore, the delayed wound healing of $Dcn^{-/-}$ mice could partially occur due to the lack of DS in skin resulting in an altered Fgf7 function on keratinocytes.

In this study, we investigated the impact of the loss of DS of decorin in skin on growth factor binding. We found that the CS/DS chains of decorin null skin were uniformly undersulfated and this affected the binding and potentially, the biological activity of various Fgfs. Interestingly, only Fgf2 and Fgf7 binding to CS/DS was affected by the reduced amount of sulfation. Using a co-culture system of keratinocytes grown on the top of fibroblasts embedded in their own 3D matrix, we discovered that the absence of decorin caused a delay in differentiation. Our findings offer novel mechanistic explanations for the established defects in wound healing in $Dcn^{-/-}$ mice and possibly Ehlers–Danlos patients.

2. Results

2.1. $Dcn^{-/-}$ dermal CS/DS was undersulfated due to the reduced amount of ΔDi2,XS (X = 4 or 6) and ΔDi2S

Dorsal skin from male wild-type and $Dcn^{-/-}$ mice at postnatal age of 30, 45 and 75 (pools of 3–5 mice) was extracted and analyzed for total and highly-sulfated CS/DS content. $Dcn^{-/-}$ P30 mice showed significantly more uronic acid compared to the respective wild-type mice (data not shown). The values are referring to the wet weight of the skin. Analysis of highly-sulfated CS/DS obtained with further fractionation by HPLC-DEAE (Zamfir et al., 2009) showed significantly higher amount of uronic acid in P30 $Dcn^{-/-}$ mice highly-sulfated CS/DS compared to wild-type mice (Fig. 1A; n = 3; $^{**}P < 0.01$), and a concurrent significant reduction in the total sulfate content (Fig. 1B; n = 3; $^{**}P < 0.01$). With aging, both genotypes showed similar amounts of uronic acid (Fig. 1A); however, the $Dcn^{-/-}$ mice displayed reduced sulfation (Fig. 1B). The content of CS/DS GAGs in wild-type is similar to the amount described for rat skin (Jung et al., 1997) and the amount of highly-sulfated CS/DS for the decorin extraction in porcine skin (Zhao et al., 2013).

Disaccharide analysis of highly-sulfated CS/DS revealed that wild-type P30 CS/DS contained 6 differentially-sulfated disaccharides in contrast to $Dcn^{-/-}$ GAGs which contained only 4 differentially-sulfated disaccharides. In both genotypes, ΔDi4S and ΔDi6S were present to a similar extent at P30. The minor modification of ΔDi2S was detectable by FACE analysis in only one out of three CS/DS extractions in $Dcn^{-/-}$ skins. For wild-type CS/DS always contained detectable amounts of 2-O sulfated disaccharides. Our method could not distinguish between the disulfated ΔDiS2,4 and ΔDiS2,6 species, therefore we used Di2,XS (X = 4 or 6). In line with ΔDiS2, ΔDiS2,X (X = 4 or 6) was also not detectable in $Dcn^{-/-}$ CS/DS in contrast to wild-type CS/DS (Fig. 1C, D). The presence of ΔDiS2,4 was confirmed by MS/MS fragmentation of wild-type chondroitin ACI lyase digested CS/DS disaccharides (Supplementary Fig. 1). To confirm the reduction in 2-O-sulfation, we analyzed the expression of the respective enzyme in dermal extracts and fibroblasts: uronyl 2-O sulfotransferase (Ust) (Fig. 1E, F). In vivo the Ust expression was reduced in $Dcn^{-/-}$ skin extracts (Fig. 1E, upper panel). Lower panel shows the total protein stain of the extracts. Furthermore, immune blots revealed similar Ust protein expression in primary mouse wild-type and 3T3 fibroblasts; however, $Dcn^{-/-}$ fibroblasts were negative for Ust (Fig. 1F; n = 3). Therefore, the loss of decorin in the murine dermis leads to changes in uronic acid and sulfate content of highly-sulfated CS/DS GAGs.

Next, we determined the DS content of highly-sulfated CS/DS samples following chondroitin ACI lyase digestion and separation on a Superdex column (Zamfir et al., 2003). We found no difference in the amount of disaccharides derived from wild-type or $Dcn^{-/-}$ CS/DS (11–15%). However, P30 $Dcn^{-/-}$ samples contained about twice the proportion of tetrasaccharides as wild-type contained (65% vs 35% Table 1) and about thirteen fold lower hexasaccharides (~3% vs 40%, Table 1 (analysis of 6 pooled skin extracts)). The lack of DS from decorin not only displays differences in composition of the disaccharide structures but also affects oligosaccharide structures. Binding of growth factors to CS/DS also required a certain size of L-IdoA-blocks (Malmström et al., 2012). These results suggest that $Dcn^{-/-}$ CS/DS displays changes in

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