Degradation of decorin by matrix metalloproteinases: identification of the cleavage sites, kinetic analyses and transforming growth factor- β 1 release

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Decorin (DCN) is a ubiquitous proteoglycan comprised of a core protein attached to a single dermatan/chondroitin sulphate glycosaminoglycan chain. It may play a role in regulation of collagen fibrillogenesis and function as a reservoir of transforming growth factor β (TGF- β) in the extracellular milieu. We have examined the susceptibility of DCN to five different matrix metalloproteinases (MMPs): MMP-1 (tissue collagenase), MMP-2 (gelatinase A), MMP-3 (stromelysin 1), MMP-7 (matrilysin) and MMP-9 (gelatinase B). MMP-2 and MMP-3 digest DCN into seven major fragments in a similar pattern. The N-terminal sequence of the two fragments generated by MMP-2 and MMP-3 is Leu²¹¹-Lys-Gly-Leu-Asn, but that of the others is Asp¹-Glu-Ala-Ser-Gly. MMP-7 cleaves DCN into three major fragments

which have the N-termini Asp¹-Glu-Ala-Ser-Gly, Glu²-Ala-Ser-Gly-Ile and Leu²⁴⁴-His-Leu-Asp-Asn. Activities of MMP-1 and MMP-9 against DCN are negligible. The values of K_m for the MMPs capable of degrading DCN are very similar (10–12 μ M), but the k_{cat}/K_m value for MMP-7 (30.5 μ M⁻¹·h⁻¹) is 4.5-fold higher than those for MMP-2 and MMP-3. Incubation of a DCN-TGF- β 1 complex with MMP-2, -3 or -7 results in release of TGF- β 1 from the complex. These data indicate proteolytic degradation of DCN by MMP-2, MMP-3 and MMP-7, and suggest the possibility that, under pathophysiological conditions, the digestion by the MMPs may induce tissue reactions mediated by TGF- β 1 released from DCN in the connective tissues.

INTRODUCTION

Proteoglycans carrying one or more glycosaminoglycan (GAG) chains form a large gene family that may be classified into five groups according to the structural properties of the core protein. One of the groups is the small Leu-rich proteoglycan family comprised of decorin (DCN), biglycan, fibromodulin and lumican [1]. These are characterized by 40 kDa core proteins that contain Leu-rich repeats of approx. 12 amino acids [1]. DCN [2] is a prototype of the group and is also referred to as PG-S2 [3], PG40 [4], proteodermatan sulphate [5] and DS-PGII [6]. It contains one dermatan/chondroitin sulphate GAG chain covalently linked to Ser⁴ of the mature core protein and is considered to be a multifunctional proteoglycan [1]. The proposed functions of DCN include regulation of collagen fibrillogenesis, maintenance of tissue integrity via binding with fibronectin and thrombospondin, and a reservoir of transforming growth factor β (TGF- β) [1,7]. The latter function of DCN is achieved through its core protein sequestering the growth factor in the extracellular milieu from receptors expressed on the cell surface [8]. Thus degradation of DCN might liberate TGF- β from extracellular matrix, leading to expression of TGF- β activities *in vivo*. However, little is known about the enzymic digestion of DCN.

The matrix metalloproteinases (MMPs) are structurally and functionally related zinc endopeptidases, of which there are at least 14 members. They can be classified into five major groups: interstitial collagenases, gelatinases, stromelysins, membrane-type MMPs and others [9–11]. MMPs are key enzymes during normal physiological remodelling and pathological degradation of the extracellular matrices [9]. Although large series of studies

have shown that they degrade various extracellular macromolecules including proteoglycans [9], their ability to digest DCN has not been studied.

In the present study, we have examined susceptibility of DCN to five different MMPs: MMP-1 (tissue collagenase), MMP-2 (gelatinase A), MMP-3 (stromelysin 1), MMP-7 (matrilysin) and MMP-9 (gelatinase B). The data indicate that MMP-2, MMP-3 and MMP-7 digest DCN into fragments by cleaving within the Leu-rich region at multiple sites and that MMP-7 activity is higher than the other two. These three MMPs can also release TGF- β 1 from DCN-TGF- β 1 complexes.

EXPERIMENTAL

Materials

Materials were obtained as follows: Brij 35, BSA, Coomassie Brilliant Blue, 2-mercaptoethanol and Nonidet P-40 from Sigma Chemical Co. (St. Louis, MO, U.S.A.); acrylamide, Amido Black 10B, EGTA and SDS from Wako Chemicals; chondroitinase ABC from Seikagaku Kogyo; N-Glycanase from Genzyme (Cambridge, MA, U.S.A.); recombinant human TGF- β 1 from King Brewing Co.; TGF- β 1 ELISA system using neutralizing antibodies from Promega (Madison, WI, U.S.A.); PVDF membrane from Bio-Rad (Hercules, CA, U.S.A.); 4-aminophenylmercuric acetate (APMA) from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); Na¹²⁵I (596 MBq/ μ g) from Amersham International, Amersham, Bucks., U.K. ProMMP-1 was purified from CCD-41SK human skin fibroblasts [13]. ProMMP-2 and -3 were purified from the culture medium of rheumatoid synovial fibroblasts as previously described [14,15],

Abbreviations used: APMA, 4-aminophenylmercuric acetate; DCN, decorin; GAG, glycosaminoglycan; MMPs, matrix metalloproteinases; TGF- β , transforming growth factor β .

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and proMMP-7 and -9 were from CaR-1 rectal carcinoma cells and HT1080 fibrosarcoma cells respectively [16,17]. Human recombinant DCN was prepared from culture medium of Chinese hamster ovary cells as reported previously [7].

Digestion of DCN by MMPs

DCN (66 μ g) was incubated for up to 24 h at 37 °C with APMA-activated MMP-1, -2, -3, -7 or -9 [16] at an enzyme/substrate ratio of 1:50 in a final volume of 147 μ l in 50 mM Tris/HCl, pH 7.5, containing 0.15 M NaCl, 10 mM CaCl₂, 0.05 % Brij 35 and 0.02 % NaN₃ (TNC + B). After incubation, 33.5 μ l of each sample was taken at 2, 6, 12 and 24 h, and the reaction was terminated with 20 mM EDTA. GAG chains were removed by treatment with 0.1 unit of chondroitinase ABC for approx. 8 h at 23 °C followed by boiling for 5 min with SDS sample buffer containing 2-mercaptoethanol. *N*-Glycanase (0.3 unit) was added to the samples in SDS sample buffer containing 1.2 % Nonidet P-40 and then incubated for about 12 h at 23 °C to remove Asn-linked oligosaccharides. The samples were subjected to SDS/PAGE.

To determine catalytic efficiency of MMP-2, -3 and -7, DCN (6.85 μ g) was incubated for 2 h at 37 °C with the APMA-activated MMPs in a final reaction volume of 40.5 μ l in TNC + B. Six different enzyme concentrations, i.e. enzyme/substrate ratios of 1:100–1:200 for MMP-2 and MMP-3 and 1:80–1:2650 for MMP-7, were used for the study. After treatment of the samples with 20 mM EDTA, chondroitinase ABC and *N*-Glycanase, they were subjected to SDS/PAGE (10 % acrylamide) under reducing conditions. The protein bands in the gels were stained with 1 % Coomassie Brilliant Blue and densitometrically scanned using computer-assisted image analysis. The reaction velocity was quantified by measuring the disappearance of a 38 kDa band of intact DCN core protein, since a single cleavage product of DCN was not obtained. The disappearance pattern was linear with time until approx. 20–25 % of the substrate was degraded. Based on the deduced molecular masses of 70952, 52220 and 27938 Da for MMP-2, -3 and -7 respectively and 36319 Da for DCN, the absorption coefficients were calculated [4,16].

Determination of K_m and k_{cat}

K_m and V_{max} of DCN degradation by MMPs were determined using the methods described by Welgus et al. [18]. Briefly, increasing amounts of DCN (2.8, 5.5, 8.2, 11.0 and 13.7 μ g) were incubated with 21.6 ng of each MMP for 2 h at 37 °C. The reaction was performed in TNC + B in a final volume of 40.5 μ l and terminated with EDTA. The samples were subjected to SDS/PAGE under reducing conditions after deglycosylation as described above. The initial velocity of disappearance of the intact DCN band in the gels was measured densitometrically.

N-Terminal sequencing of DCN degradation products

DCN (50 μ g) was incubated with MMP-2 (1 μ g), MMP-3 (1 μ g) and MMP-7 (0.25 μ g) at 37 °C for 6 h (MMP-2 and 3) or 30 min (MMP-7) in a total reaction volume of 100 μ l in TNC + B. The samples were subjected to SDS/PAGE (15 % acrylamide) under reducing conditions after treatment with EDTA, chondroitinase ABC and *N*-Glycanase. Proteins in the gels were transferred to a PVDF membrane and located by staining with 0.1 % Amido Black 10B. The bands of interest were excised and sequenced by Edman degradation using an Applied Biosystems (Foster, CA, U.S.A.) 477A sequencer.

Release of TGF- β 1 from immobilized DCN

Wells of 96-well microtitre plates (3072 Microtest III; Falcon) were coated with 100 μ l of 10 μ g/ml DCN in 50 mM Tris/HCl, pH 7.5, containing 0.15 M NaCl, 0.05 % Brij 35 and 0.02 % NaN₃ (TN + B) at 4 °C for approx. 12 h and then incubated with 120 μ l of 1 % BSA in TN + B for 90 min at 23 °C to avoid non-specific binding. After a brief wash of the wells with binding buffer (50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 5 mM MgCl₂, 5 mM KCl, 1 mM CaCl₂ and 0.02 % NaN₃) [19], 1 ng (approx. 6.5×10^5 c.p.m.) of ¹²⁵I-labelled TGF- β 1 in the buffer was incubated in the wells for 12 h at 4 °C. Specific binding of TGF- β 1 to DCN was confirmed because ¹²⁵I-labelled BSA showed no binding in the treated microtitre plates. The wells were then rinsed with TN + B, and immobilized DCN–TGF- β 1 complex was allowed to react with APMA-activated MMP-2, -3 or -7, or buffer alone at enzyme/substrate ratios of 1:50 and 1:100 at 37 °C for 0, 2 and 6 h in 50 mM Tris/HCl, pH 7.5, containing 0.15 M NaCl, 10 mM CaCl₂ and 0.02 % NaN₃. At each time point, radioactivity in the supernatants of the wells was counted. The unreleased fractions were also counted for radioactivity after treatment of the wells with 0.3 M NaOH containing 1 % Triton X-100. The percentage of TGF- β 1 released by MMP digestion was calculated after subtraction of radioactivity in the supernatant obtained using buffer alone using the following formula: [(upper supernatant counts – counts released by buffer)/(upper supernatant and unreleased fraction counts – counts released by buffer)] $\times$ 100 (%). To examine whether TGF- β 1 is degraded by the MMP treatment, the supernatants were subjected to SDS/PAGE (15 % acrylamide) and the gels autoradiographed using a Bioimage Analyzer BAS 1000 (Fuji Photo Film Co.). Iodination of recombinant human active TGF- β 1 and subsequent removal of free ¹²⁵I were performed as described previously [15].

TGF- β 1 released from the immobilized DCN–TGF- β 1 complex was also monitored by an ELISA for TGF- β 1. For this experiment, non-iodinated TGF- β 1 was allowed to bind to the immobilized DCN, and the DCN–TGF- β 1 complex was digested with MMP-2, -3, -7 or buffer alone as described above. Concentration of released TGF- β 1 was calculated by subtracting the concentration released by incubation with buffer alone according to the manufacturer's (Promega) instructions.

RESULTS

Degradation of DCN by MMPs

Purified human recombinant DCN from CHO cells showed a broad band ranging from approx. 120 to 70 kDa on SDS/PAGE with and without reduction. It migrated as a doublet of 48 and 45 kDa after treatment with chondroitinase ABC, and additional digestion with *N*-Glycanase further reduced the size to 38 kDa with a minor band at 40 kDa (Figure 1). The electrophoretic patterns are consistent with the data reported by Sawhney et al. [20]. The doublet at 48 and 45 kDa is considered to be differently glycanated forms of the DCN core protein, and the 38 kDa species is the mature core protein. The minor component at 40 kDa may correspond to the procure domain-containing core protein [20].

DCN was incubated at 37 °C with five different MMPs, MMP-1, -2, -3, -7 and -9 at an enzyme/substrate ratio of 1:50, and the degradation products were analysed after 2, 6, 12 and 24 h incubation by SDS/PAGE of the samples treated with chondroitinase ABC and *N*-Glycanase. Of the five MMPs, MMP-7 degraded DCN most efficiently (Figure 2A). MMP-2 and -3 also

Figure 1 Changes in molecular mass of DCN after digestion with chondroitinase ABC and *N*-Glycanase

Human recombinant DCN (10 μ g) was treated with or without chondroitinase ABC and/or *N*-Glycanase and subjected to SDS/PAGE (10% acrylamide) under reducing conditions. The molecular mass of native DCN (lane 1) is reduced by treatment with chondroitinase ABC (lane 2), migrating at 48 kDa (upper arrow) and 45 kDa (lower arrow). *N*-Glycanase treatment reduces the molecular mass further to 40 kDa (upper arrowhead) and 38 kDa (lower arrowhead) (lane 3). Protein standards were phosphorylase *b* (94 kDa), human transferrin (77 kDa), BSA (68 kDa), IgG heavy chain (55 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and soya-bean trypsin inhibitor (21 kDa).

digested DCN (Figure 3), but negligible digestion was observed with MMP-1 or -9 using the same conditions (Figure 2B). MMP-2 and MMP-3 yielded seven major fragments with similar sizes ranging from 33 to 13.5 kDa; the major fragments obtained with MMP-2 were 33, 28, 26, 24.5, 19, 14.5 and 13.5 kDa, and those obtained with MMP-3 were 33, 28, 26, 23.5, 19, 15 and 14 kDa (Figure 3). However, the digestion pattern with MMP-7 was different, showing three major fragments of 20, 15.5 and 9.5 kDa (Figure 3). Removal of GAG chains from DCN by chondroitinase ABC treatment before the incubation with these

Figure 2 Time course of degradation of DCN by MMP-7 and DCN digestion by MMP-1 and -9

(A) DCN (66 μ g) was incubated with MMP-7 (1.32 μ g) at 37 °C in a final volume of 135 μ l. Samples of the reaction mixture (33.5 μ l) were taken after 2 (lane 2), 6 (lane 3), 12 (lane 4) and 24 h (lane 5), and the reaction was terminated with 20 mM EDTA. The samples were incubated with chondroitinase ABC and *N*-Glycanase and then subjected to SDS/PAGE (15% acrylamide) under reducing conditions as described in the Experimental section. Lane 1 is the control sample incubated with buffer alone for 24 h. Protein standards are as in Figure 1 except for lysozyme (14 kDa). (B) DCN (15 μ g) was incubated with buffer alone (lane 1), 300 ng of MMP-1 (lane 2) or 300 ng of MMP-9 (lane 3) at 37 °C for 24 h. The samples were then subjected to SDS/PAGE as described above. Note that DCN digestion is negligible after treatment with these MMPs.

Figure 3 Digestion products of DCN by incubation with MMP-2, -3 and -7

To show the degradation fragments, native DCN (15 μ g) was incubated at 37 °C with buffer alone for 6 h (lane 1), MMP-2 (300 ng) for 6 h (lane 2), MMP-3 (300 ng) for 6 h (lane 3) or MMP-7 (300 ng) for 0.5 h (lane 4). After termination of the reaction with EDTA, the digestion products were treated with chondroitinase ABC and *N*-Glycanase and analysed by SDS/PAGE (15% acrylamide) under reducing conditions. The gels were stained with 1% Coomassie Brilliant Blue. Detectable fragments (a to q) which were subjected to N-terminal sequence analysis are indicated.

MMPs appeared to decrease the susceptibility of the substrate to MMP-2 and MMP-3: both MMP-2 and MMP-3 showed weak activity with the GAG-chain-free DCN core protein under the conditions in which the two MMPs readily digested the native DCN core protein (Figures 4A and 4B). However, no definite reduced degradation of GAG-free DCN was observed with MMP-7 (Figure 4C).

Cleavage sites of DCN by MMP-2, -3 and -7

N-Terminal sequence analysis of the DCN fragments generated by incubation with MMP-2, -3, or -7 was performed. As shown

Figure 4 Digestion of GAG-chain-depleted DCN with MMP-2 (A), -3 (B) and -7 (C)

A, GAG-free DCN (5 μ g) was prepared by treatment with chondroitinase ABC (0.1 unit) for 8 h at 23 °C, and then incubated with buffer alone (lane 1) or 200 ng of MMP-2 (lane 2) for 24 h at 37 °C. The digestion was compared with native DCN (5 μ g) digested with 200 ng of MMP-2 for 24 h at 37 °C (lane 3). After termination of the reaction with EDTA, all the samples were treated again with chondroitinase ABC and subjected to SDS/PAGE (10% acrylamide) under reducing conditions. (B) GAG-free DCN (lane 2) and native DCN (lane 3) were incubated with 200 ng of MMP-3 for 24 h at 37 °C, and the digestion compared. Lane 1 is GAG-free DCN incubated with buffer alone. (C) Comparison of the digestion of GAG-free DCN (lane 2) and native DCN (lane 3) with 50 ng of MMP-7 for 8 h at 37 °C. Lane 1 is GAG-free DCN incubated with buffer alone.

Table 1 N-Terminal sequence of DCN fragments generated by digestion with MMP-2, MMP-3 or MMP-7

MMP	Fragment		N-Terminal sequence
	Species	Size (kDa)	
MMP-2	a	33	X ¹ EAXGIXPEV
	b	28	D ¹ XAXXIXPEV
	c	26	D ¹ EAXGIGPEV
	d	24.5	D ¹ EAXGIGPEV
	e	19	D ¹ XAXGIGPEVP
	f	14.5	L ²¹¹ KGLNNLAKL
	g	13.5	L ²¹¹ XXLNNLAXL
MMP-3	h	33	D ¹ EAXGIGPEVPDD
	i	28	D ¹ EAXXIXPXVP
	j	26	D ¹ EAXGIXPEV
	k	23.5	D ¹ EAXGIGPEV
	l	19	D ¹ EAXGIGPEV
	m	15	D ¹ EAXGIGPEV
	n	14	L ²¹¹ KGLNNLAKL
MMP-7	o	20	E ² AXGIGPXV
	p	15.5	D ¹ EAXGIGPXV
	q	9.5	L ²⁴⁴ HLDNNKLTR

Table 2 Kinetic parameters of MMP-2, -3 and -7 on DCN

MMP	K_m (μM)	k_{cat} (h^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot h^{-1}$)
MMP-2	10	66	6.6
MMP-3	12	78	6.5
MMP-7	11	336	30.5

in Table 1, the 14.5 and 13.5 kDa species obtained by MMP-2 treatment and the 14 kDa fragment by MMP-3 had the same N-terminus of L²¹¹KGLNNL. However, the N-terminal sequence of the other fragments produced by MMP-2 and MMP-3 (33, 28, 26, 24.5 and 19 kDa species by MMP-2, and 33, 28, 26, 23.5, 19 and 15 kDa species by MMP-3) was D¹EASGIG. On the other hand, three major fragments obtained by MMP-7 treatment showed different N-termini: E²ASGIGD, D¹EASGIG and L²⁴⁴HLDNNK for the 20, 15.5 and 9.5 kDa fragments respectively.

Catalytic efficiency and kinetic parameters

Relative catalytic efficiency of MMP-1, -2, -3, -7 and -9 for the cleavage of DCN was quantified by subtraction densitometry at several enzyme concentrations, since no single product was obtained by lowering temperature and enzyme concentrations. The number of molecules of each MMP required to produce 50 % substrate digestion was determined by linear extrapolation. MMP-7 was found to be the most efficient enzyme (0.8 pmol) with 6.85 μg of DCN, and the efficiency was about 5- and 4-fold higher than MMP-2 (4.0 pmol) and MMP-3 (3.1 pmol) respectively. DCN digestion by MMP-1 and MMP-9 was considered to be minimal since the 50 % digestion by these MMPs was at least 10-fold less than that of MMP-2.

The kinetic parameters were also determined for MMP-2, -3 and -7 (Table 2). After incubation of DCN (2.8–13.7 μg) with each MMP for 2 h at 37 °C, the samples were subjected to SDS/PAGE and the stained gels were scanned densitometrically for the disappearance of intact DCN molecule. In these experi-

Table 3 Release of TGF- β 1 from DCN–TGF- β 1 complex by MMP-2, -3 and -7

Results are means $\pm$ S.D.

MMP	Release of labelled TGF- β 1 (%)			Amount of TGF- β 1 released (pg/ml)		
	0 h	2 h	6 h	0 h	2 h	6 h
MMP-2	0 $\pm$ 0	32 $\pm$ 25	57 $\pm$ 5	0 $\pm$ 0	170 $\pm$ 46	90 $\pm$ 61
MMP-3	3 $\pm$ 4	41 $\pm$ 18	49 $\pm$ 5	0 $\pm$ 0	155 $\pm$ 28	150 $\pm$ 36
MMP-7	0 $\pm$ 0	44 $\pm$ 17	62 $\pm$ 6	0 $\pm$ 0	146 $\pm$ 80	238 $\pm$ 11

ments, degradation of DCN was kept to less than 25 % to measure initial velocity even at the lowest concentration of DCN employed, and plotted by the method of Lineweaver and Burk [21]. The K_m for each of these MMPs was approximately the same. Values for k_{cat} were within the range 66–336 mol of DCN/mol of MMP per h. k_{cat}/K_m , an index of specificity, ranged from 6.5 to 30.5 $\mu M^{-1} \cdot h^{-1}$. The value of k_{cat}/K_m for MMP-7 was approximately 4.5-fold higher than that for MMP-2 and MMP-3.

Release of TGF- β 1 from immobilized DCN–TGF- β 1 complex of MMPs

DCN is known to bind to TGF- β and to possibly act as a reservoir of active TGF- β 1 in the extracellular milieu [7]. To examine whether digestion of DCN by MMPs releases TGF- β 1 from the DCN–TGF- β 1 complex, we measured TGF- β 1 liberated from the complex immobilized on microtitre plates after incubation with MMP-2, -3 or -7 by two different assays. First, radioactivity of ¹²⁵I-labelled TGF- β 1 released into the supernatant after incubation with each MMP was counted. Since incubation of the DCN–TGF- β 1 complex with buffer alone also released some radioactivity, the values were subtracted from those obtained by incubation with MMP-2, -3 or -7. Subsequently, the percentage of ¹²⁵I-labelled TGF- β 1 released was calculated. As shown in Table 3, release was significantly greater in the samples incubated with each MMP for 2 and 6 h than in those incubated for 0 h. ¹²⁵I-labelled TGF- β 1 was detected by autoradiography after SDS/PAGE in the supernatants of the wells incubated with each MMP for 2 and 6 h, but no definite degradation of the 12.5 kDa growth factor was seen (results not shown).

In the second experiment, the concentrations of released TGF- β 1 were assayed by an ELISA that detects the functionally active domain of the growth factor. Since some TGF- β 1 was released from the DCN–TGF- β 1 complex after incubation with buffer alone, TGF- β 1 release was calculated after subtraction of the values obtained by incubation with buffer alone from those with each MMP. Although negligible amounts of TGF- β 1 were detected in the samples incubated with each MMP for 0 h, 90–238 pg/ml TGF- β 1 was released from the DCN–TGF- β 1 complex after 2 and 6 h incubation. The level in the samples incubated with MMP-7 for 6 h tended to be higher than those in other samples (Table 3).

DISCUSSION

The present study is the first to demonstrate the digestion of DCN by MMPs. Of the five MMPs examined, DCN was degraded by MMP-2, -3 and -7. No digestion was detected with

Figure 5 Cleavage sites of DCN by MMP-2, -3 and -7

Schematic illustration of DCN showing the Leu-rich region between disulphide loops at the N- and C-termini, and a single GAG chain. Arrows indicate the identified cleavage sites of DCN by MMP-2, MMP-3 and MMP-7. Underlined amino acids designate Leu in the Leu-rich region.

MMP-1 or -9 under the conditions in the present study. Two pathways of DCN degradation can be considered. Previous studies [22,23] have demonstrated intracellular digestion of DCN after endocytosis mediated by receptors expressed on human skin fibroblasts. Extracellular degradation was also reported by Nakano and Scott [24], who described the susceptibility of DCN to metalloproteinases derived from gingival fibroblasts. Although they did not identify the enzymes, it can be speculated from their cellular origin and molecular mass that they correspond to MMP-2 and MMP-3. The proteolytic effect of MMP-7 on DCN is different from that of MMP-2 and MMP-3 in that MMP-7 digests the substrate about 5-fold more efficiently than the other two. Since the K_m values for DCN digestion by MMP-2, -3 and -7 are almost identical (10–12 μ M), the highest specificity constant of MMP-7 for DCN can be attributed to its catalytic activity. MMP-7 and MMP-3 were originally thought to be similar enzymes because of their specificity for various extracellular macromolecules [9]. However, recent studies on the substrates of MMP-7 have demonstrated that it has a higher specificity constant for many extracellular matrix components, such as proteoglycans, tenascin, vitronectin, entactin and cartilage link protein, and generates dissimilar degradation products [9,25–28]. The digestion of DCN is similar since MMP-7 degrades it preferentially into different fragments from those obtained with MMP-3. Aggrecan core protein is known to be susceptible to many MMPs including MMP-1, -2, -3, -7, -8 (neutrophil collagenase) and -9 [9]. Of these MMPs MMP-7 is reported to have the highest activity against cartilage proteoglycans incorporated into polyacrylamide beads [16,29]. These data suggest that MMP-7 may play a key role in the turnover and pathological degradation of proteoglycans.

It is of interest that removal of the GAG chains from DCN core protein reduces the susceptibility to MMP-2 and -3, although such an effect was not observed with MMP-7. The mechanism of this phenomenon is not clear. However, it might be possible that a conformational change of the DCN core protein caused by deglycosylation alters the accessibility of MMP-2 and MMP-3 to the DCN core protein through the interaction with their C-terminal domains. Non-glycanated forms of DCN are known to be produced in the intervertebral disc and articular cartilage, and the proportion increases with aging [30,31]. This may lead to the slower turnover of DCN by the MMPs in elderly people.

DCN core protein is unique in containing Leu-rich repeats in the central sequence spanning the disulphide looped regions at

the N- and C-termini [4]. Our analysis of the N-terminal sequences of the DCN fragments revealed that DCN is cleaved in the Leu-rich region, i.e. at the S²¹⁰–L²¹¹ bond by MMP-2 and -3 and at the E²⁴³–L²⁴⁴ bond by MMP-7 (Figure 5). In addition to these two cleavage sites, it seems very likely that the MMPs cleave the Leu-rich repeats at multiple sites, since most of the degradation fragments, even those of very similar low molecular mass, produced by the effect of the MMPs had the N-terminal sequence D'EASGIG, i.e. the same as the intact molecule. This may be ascribed to the fact that MMPs prefer to clip bonds of aliphatic and hydrophobic residues of substrates, especially the X-Leu bond [9]. The L-X-X-L-X-L-X-X-N-X-L/I sequence found in the DCN Leu-rich repeats has been considered to be involved in DCN interaction with other proteins from an X-ray crystallographic study on the porcine ribonuclease inhibitor, which also contains the L-X-X-L-X-L-X-X-N-X-L/I repeats [32]. Recent studies by Schönherr et al. [33] indicated that there are at least two separate binding sites for the interaction of DCN with type-I collagen. Svensson et al. [34] have shown that the collagen-binding site is located in the sequence Gly¹⁶⁸ to Gly²¹³, but requires the correct surroundings for maximal binding strength to type-I collagen. On the other hand, an N-terminal peptide consisting of 17 amino acid residues including the GAG chain is shown not to be required for binding to collagen matrix [35]. These data suggest that the Leu-rich repeats play a critical role in the interaction of DCN with collagens. Thus the disruption of the Leu-rich region by MMP-2, -3 and -7 may result in the functional failure of DCN.

One of the important characteristics of DCN is the binding of active TGF- β . TGF- β 1, - β 2 and - β 3 are reported to bind to the DCN core protein with similar efficiency [8], permitting DCN to function as a reservoir of these TGF- β s in the extracellular milieu [1,7]. Takeichi et al. [36] have shown that the DCN core protein contains at least two separate TGF- β 1-binding sites with high and low affinities, which are considered to be located in the Leu-rich region [8]. Thus our data showing multiple cleavages in the Leu-rich repeats by MMP-2, -3 and -7 suggest the possibility that TGF- β may be released from the DCN–TGF- β complex after digestion with the MMPs. Supporting our expectation, the data obtained using two different assay systems showed that MMP-2, -3 and -7 liberate TGF- β 1 from the DCN–TGF- β 1 complex, and MMP-7 is the most effective. The activity of TGF- β 1 complexed with DCN is a controversial issue. Although DCN was originally reported to neutralize TGF- β 1 activity by complexing it [7], recent studies using solution-phase experiments showed no such effect of DCN on TGF- β 1 activity in osteoblastic [36] and osteosarcoma [37] cells. Assays to test TGF- β 1 activity were not performed in the present study. However, no proteolytic degradation of TGF- β 1 released from the complex was observed by SDS/PAGE. In addition, the ELISA system which recognizes the TGF- β 1 functional domain detected the release of the growth factor. Therefore it may be reasonable to speculate that the released growth factor is functionally active.

DCN is a ubiquitous extracellular matrix component of various tissues such as blood vessels, dermis, tendon, ligament, sclera and articular cartilage [38]. In human articular cartilage, it is localized within an interterritorial matrix in the superficial layer [39], and several fragments of DCN core protein have been extracted from rheumatoid and osteoarthritic cartilage tissues [40]. Since MMP-2 and MMP-3 are overproduced in the rheumatoid and osteoarthritic synovia and cartilages [9], it seems likely that these MMPs may participate in the degradation of DCN in the cartilage. In fact, the molecular mass of some of the fragments observed in the rheumatoid cartilage extracts is similar to that of the digestion fragments obtained by MMP-2 and

MMP-3 [40]. Increased levels of DCN are noted in chronic glomerulonephritis [41], where TGF- β is considered to play a pivotal role in the pathogenesis [42]. The previous experimental studies have demonstrated that cultured mesangial cells secrete MMP-2, MMP-3 and MMP-7, and the production of the latter two is down-regulated by TGF- β 1 [43]. It is also known that DCN production by mesangial cells is increased after stimulation with TGF- β 1 [41], and that intravenous injection of anti-TGF- β 1 antibody or recombinant DCN reduces the accumulation of extracellular matrix proteins in the nephritic glomeruli [42,44]. These data suggest a possible cascade in which MMPs induced in response to inflammatory factors degrade DCN and release TGF- β 1, which causes overproduction of the extracellular matrix macromolecules including DCN, leading to chronic proliferative glomerulonephritis. This possibility, however, remains to be elucidated by further studies.

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