Collagen Phagocytosis by Fibroblasts Is Regulated by Decorin*

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Decorin is a small, leucine-rich proteoglycan that binds to collagen and regulates fibrillogenesis. We hypothesized that decorin binding to collagen inhibits phagocytosis of collagen fibrils. To determine the effects of decorin on collagen degradation, we analyzed phagocytosis of collagen and collagen/decorin-coated fluorescent beads by Rat-2 and gingival fibroblasts. Collagen beads bound to gingival cells by α2β1 integrins. Binding and internalization of decorin/collagen-coated beads decreased dose-dependently with increasing decorin concentration (p < 0.001). Inhibition of binding was sustained over 5 h (p < 0.001) and was attributed to interactions between decorin and collagen and not to decorin-collagen receptor interactions. Both the non-glycosylated decorin core protein and the thermally denatured decorin significantly inhibited collagen bead binding (~50 and 89%, respectively; p < 0.05). Mimetic peptides corresponding to leucine-rich repeats 1–3, encompassed by a collagen-binding ~11-kDa cyanoan bromide fragment of decorin and leucine-rich repeats 4 and 5, previously shown to bind to collagen, were tested for their ability to inhibit collagen bead binding. Although the synthetic peptide 3 alone exhibited saturable binding to collagen, neither peptides 3 nor 1 and 2 markedly inhibited phagocytosis. Leucine-rich repeat 3 bound to a triple helical peptide containing the α2 integrin-binding site of collagen. When collagen beads were co-incubated with peptides 3 and 4, inhibition of collagen phagocytosis (55%) was equivalent to intact native/recombinant core protein. Thus a novel collagen binding domain in decorin acts cooperatively with leucine-rich repeat 4 to mask the α2β1 integrin-binding site on collagen, an important sequence for the phagocytosis of collagen fibrils.

The intracellular phagocytic pathway in fibroblasts contributes to the physiological remodeling of collagen by lysosomal degradation of internalized collagen fibrils (1–4), but the mechanisms that regulate this pathway in vivo are poorly characterized. Previous morphological studies have shown that collagen fibrils are “decorated” by proteoglycans (5); consequently, decorin may affect the binding step of collagen phagocytosis (6).

Decorin (DCN)1 is a matrix proteoglycan that belongs to the small leucine-rich proteoglycan family (7). The mature form of DCN (~100 kDa) consists of an ~45-kDa core protein, a single dermatan or chondroitin sulfate glycosaminoglycan chain, cysteine loops near the N and C terminus, and either two or three asparagine-bound oligosaccharides. The central part of the core protein consists of 10 leucine-rich repeat (LRR) sequences in tandem array (8). Rotary shadowing-electron microscopy and molecular modeling studies suggest that the DCN core protein is horseshoe-shaped (9, 10) and that the inner concavity accommodates and may provide a binding site for type I collagen (10). Indeed, DCN binds not only to type I but also to collagen types II, III, VI, and XIV (11–15). DCN binding to collagen molecules is thought to influence collagen fibrillogenesis and the final diameter of fibrils. The DCN core protein may mediate these interactions (16), but the impact of DCN-collagen binding interactions on collagen phagocytosis is not known.

Triple helical collagen type I possesses a specific DCN core protein-binding site at the d-band in each D-period (15, 17) and a second DCN core protein-binding site located in a narrow region ~25 nm from the central region of type I collagen in a zone that coincides with the c1 band of the collagen fibril D-period (18). However, the structural elements of DCN that mediate binding to collagen type I are not completely defined. Although analyses of the DCN core protein show that neither the N-terminal half nor the central LRR repeats can, by themselves, bind tightly to fibrillar collagen (19), more recent work indicates that LRRs 4–6 may contain putative high affinity binding site(s) for collagen type I (20–22).

Despite these reports describing putative structural determinants that determine the interactions between DCN and collagen type I, there is no consensus model for binding. Furthermore, previous studies describe interactions between DCN and collagen in the context of regulation of fibrillogenesis. Currently, there are no reports describing a role for DCN as a modulator of collagen degradation by phagocytosis. Here we examined the effect of DCN on the collagen binding and internalization steps of phagocytosis by using quantitative flow cytometry (23). Based on the data obtained with DCN digestion and DCN mimetic peptides, we have identified putative collagen-binding sites in DCN that may be involved in the regulation of collagen phagocytosis.

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1 The abbreviations used are: DCN, decorin; COL, collagen; HGF, human gingival fibroblasts; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; ANOVA, analysis of variance; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PBS, phosphate-buffered saline; cpDCN, DCN core protein; MALDI, matrix-assisted laser desorption ionization.
EXPERIMENTAL PROCEDURES

Reagents—Bovine type I collagen solution (Vitrogen®) was from Co- hesion Technologies (Palo Alto, CA). Recombinant human decorin (38 kDa by SDS-PAGE; >95% purity) was from EMP Genetech (Germany). Bovine articular cartilage DCN was from Sigma (catalog number D-8428; lot number 22K4024; 300 μg/mg; >100 kDa; purified according to Ref. 24). Chondroitin sulfate, bovine serum albumin (BSA), and FITC-conjugated goat anti-mouse antibody were from Sigma. Proteinase-free chondroitinase ABC (from Proteus vulgaris, EC 4.2.2.4) and dermatan sulfate were from Seikagaku (Falmouth, MA). Mouse anti-bovine DCN monoclonal antibody (DS-1) was from Developmental Studies Hybridoma Bank (University of Iowa). FITC-streptavidin was from Cederlane (Hornby, Ontario, Canada). Antibody to β1 integrin (4B4) was from Beckman Instruments (Burlington, Ontario, Canada). Type I collagen-derived triple helical peptides from type I collagen residues α1(I)(496–507) were obtained from Gregg Fields (Florida Atlantic University, Boca Raton, FL). Ligand binding to the α2 integrin subunit has a K<sub>D</sub> = 1.1 × 10<sup>-6</sup> M. A second collagen-derived triple helical peptide from type I collagen residues α1(I)(772–786) was also obtained from Gregg Fields as a second DCN-binding region within collagen.

Cell Culture—Rat-2 cells (CRL 1674, ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum and antibiotics (0.017% penicillin G; 0.01% gentamicin sulfate). Rat-2 cells exhibit several phenotypic traits in common with gingival fibroblasts, including rapid collagen phagocytosis (3), and were used to model collagen binding and internalization processes. In some experiments, human gingival fibroblasts (HGF; passages 5–12) cultured in minimal essential medium with antibiotics and 10% (v/v) fetal bovine serum were used in bead binding experiments with cells grown to confluence. All bead incubations were conducted for 1 h at 37 °C in serum-free conditions.

Collagen and DCN Bead Coatings—Carboxylate-modified, yellow-green polystyrene beads (2 μm diameter; Molecular Probes, Eugene, OR) were coated with collagen, and fibril formation was induced as described previously (24). In some experiments, monomeric collagen was bound to beads using nonpolymerizing conditions during the bead preparations. Nonspecific binding sites were blocked with 0.1% (w/v) BSA. For preparation of DCN/collagen beads, collagen beads were prepared, but instead of resuspending the bead pellet in PBS, the pellet was resuspended in varying concentrations of DCN (0.01, 0.1, and 1 μg/ml), sonicated, incubated at 37 °C for 1 h, and rinsed with PBS at 37 °C. Bead binding to DCN was confirmed by surface staining of DCN.

Collagen Bead Binding—We optimized concentrations of DCN that affect bead binding by directly loading cells with either collagen or collagen/collagen-coated beads for 1 h at 37 °C. Bead binding to cells was quantified by flow cytometry (6, 23, 25, 26). In some experiments, two-color flow cytometry was used to analyze simultaneously the binding of collagen- and collagen/collagen-coated beads to cells. Red and blue fluorescence beads (2.0 μm; Molecular Probes; red beads, Ex<sub>max</sub> = 580 nm and Em<sub>max</sub> = 605 nm; blue beads, Ex<sub>max</sub> = 365 nm and Em<sub>max</sub> = 460 nm) were used to identify bead preparations. Nonspecific binding sites were blocked with 0.1% BSA. In some experiments, collagen surfaces were further coated with DCN (1 μg; 500 μl) for 1 h at 37 °C. Cells were plated at 1 × 10<sup>4</sup> cells/cm<sup>2</sup> in serum-free medium, allowed to attach and spread for 1 or 3 h, washed, fixed with formaldehyde, permeabilized with 0.03% Triton X, and stained with rhodamine phalloidin to visual- ize actin filaments. Cell attachment and spreading of individual cells was imaged at the substrate-cell interface by confocal microscopy.

To determine whether DCN affects cell attachment to collagen, 8-well tissue culture surfaces were prepared with DCN coating as described above. Cells (4 × 10<sup>4</sup> cells/well) were allowed to attach for 1 or 3 h in serum-free medium. At each time point, the medium was removed, and the cells were gently jet-washed in PBS to remove any loosely bound cells. Adherent cells were trypsinized, suspended in iso- tonic solution, and counted.

Cell-surface DCN—HGFs were incubated with DCN (10 μg solution) for 15 min at 4 °C, washed, incubated at 4 °C for 1, 10, and 60 min in medium, and washed, trypsinized, and neutralized with growth medium. Cells were pelleted, resuspended in normal goat and mouse sera (1:1000 each), incubated on ice for 10 min, washed, sedimented, resuspended in 1:50 solution of mouse anti-DCN antibody in PBS, and incubated on ice for 1 h. Cells were washed, counterstained with FITC- goat anti-mouse antibody, incubated on ice for 1 h, washed, sedimented, resuspended in PBS, and analyzed by flow cytometry to assess cell surface staining of DCN.

DCN Cleavage—For preparation of DCN core protein (cpDCN), DCN was digested with chondroitinase ABC (0.2 units/ml) in 0.1% Triton-HCl for 1 h at 37 °C. Digestion was confirmed by Blue-stained 10% SDS-PAGE. The effect of cpDCN on inhibition of binding was examined using collagen beads coated with the cpDCN (as described above) at equimolar concentrations as used for intact DCN. Cells were incubated with these beads for 1 h at 37 °C, and bead binding was assessed by flow cytometry.

For CNBr cleavage, DCN was dissolved in trifluoroacetic acid; CNBr was added, and the mixture was incubated for 4 h at room temperature. Distilled water was added to each tube and evaporated to dryness, and the residue was rehydrated. Control samples confirmed that the cleavage occurred as a result of CNBr digestion and not as a consequence of acid hydrolysis. Collagen beads were prepared and coated with 30 μg of the CNBr digestion mixture for 1 h at 37 °C. The beads were washed with PBS, and bound DCN fragments were eluted with SDS sample buffer containing 10% Triton X-100, reduced and alkylated prior to CNBr digestion. The gel pieces were vacuumdried prior to CNBr digestion with sequencing grade trypsin (Promega, Madison, WI). Samples were analyzed on a 10–20% Tris-Tricine gel gradient and silver-stained, and the relevant bands were analyzed by mass spectrometry.

Preparation of Gel Samples for Mass Spectrometry—In-gel trypsin digests were used to prepare eluate bands for mass spectrometry. Briefly, each silver-stained gel band was minced, destained, reduced, and alkylated prior to trypsin digestion. The gel pieces were vacuumdried prior to CNBr digestion with sequencing grade trypsin (Roche Applied Science) overnight at 37 °C. After quenching with trifluoroacetic acid, the supernatant was retained, and the gel was twice extracted with acetonitrile. The combined supernatant and extracts were vacuumdried prior to MALDI-mass spectrometry protein identification and sequencing.

Mass Spectrometry—For mass spectrometry (Mass Spectrometry Facility, Faculty of Medicine, University of Toronto) to identify putative collagen-binding sequences within the DCN core protein, saturated α-cyano-4-hydroxy-cinnamic acid in 70% acetonitrile, 0.1% trifluoroacetic acid was used as the matrix solution. Protein enzymatic digestion mixtures (1 μl) were spotted on the sample target, and saturated matrix solution (1 μl) was added. After crystal formation, the sample target was analyzed by MALDI-mass spectrometry in linear mode on an Applied Biosystems Voyager-DE STR MALDI-time of flight mass spectrometer (337 nm laser). Acceleration voltage was 20 kV; grid voltage was 94%; guide wire was 0.05%; delay time was 175 ns, and low mass gate was 800 Da. The mass spectra were externally calibrated from molecular weights of a mixture of standard peptides. Masses of fragments from experimental samples that did not appear in the mass spectrometry analysis of controls were analyzed by MALDI-mass spectrometry to provide predicted sequences that were then probability matched for DCN using standard proteom- ics databases and search engines.

Mimetic Peptides—Peptides were synthesized based on the LRR se- quences 1–5 of DCN. Control peptides contained the same amino acids in the scrambled sequence, coated with carboxylate-modified nonfluorescent beads (Centre for Peptide Synthesis Facility, University of Toronto). The sequences for the LRR 1 and scrambled peptides were LGLEKVKPDLPLPDLLDNDNKLQ and GKKVDDPLPDDLLNDNLAPL, respectively. The sequences for the LRR 2 and scrambled peptides were TEIKGGDFFNNLHLLNINF and GKEFKGNLVIDIIKNGHTD, respectively. The sequences for the LRR 3 and scrambled peptides were SKISPGAPAFPLVKLERYL- SKNQL and LKIAFLPGGFVPSRELKSYNSLQ. A portion of each of
Beads (with or without collagen coating) were incubated with decorin at the indicated concentrations in solution. Decorin bound to beads was eluted and quantified by immunoblotting and densitometry comparisons to standards.

| DCN solution | DCN bound to bare beads | DCN bound to collagen beads |
|--------------|--------------------------|-----------------------------|
| µM           | pg/bead; n = 3           | pg/bead; n = 1              |
| 0.01         | 0.003 ± 0.001            | 0.003                       |
| 0.1          | 0.006 ± 0.002            | 0.006                       |
| 1.0          | 0.025 ± 0.01             | 0.023                       |

The degree of DCN-induced inhibition of binding was sharply reduced using monomeric collagen (only 20\% inhibition at 0.1 µM DCN on collagen beads compared with 90\% inhibition with fibrillar collagen).

The \( \alpha_2 \beta_1 \) integrin is the major collagen receptor on human gingival fibroblasts and mediates collagen phagocytosis by these cells; the other \( \beta_1 \) integrins do not make a major contribution to phagocytosis of fibrillar collagen (24). We examined the impact of blocking collagen bead binding in HGF with the use of an antibody to the \( \beta_1 \) integrin (clone 4B4; 1 µg/ml) followed by incubation with collagen beads, a protocol that abrogates collagen binding to the \( \alpha_2 \beta_1 \) integrin in these cells. Collagen bead binding was reduced by >4-fold with antibody, but binding of DCN-collagen beads was not reduced any additional amount with antibody treatment (Table III).

**Internalization of Collagen**—As DCN significantly inhibited collagen binding, we assessed whether this effect would impact on the subsequent internalization step (28). Cells were post-loaded with either FITC-collagen or DCN/FITC-collagen-coated nonfluorescent latex beads for 1, 3, and 5 h. At each specified time point, 0.2\% trypan blue was added to the culture medium to quench extracellular FITC fluorescence, thereby allowing us to quantify the proportion of internalized beads for individual cells. For collagen bead con-
**FIG. 1. Effect of DCN on collagen bead binding and internalization.** A, immunostaining of DCN/collagen-coated beads to confirm DCN binding ($p < 0.01$ between control and each of the DCN concentrations; $n = 10,000$ beads per analysis). B, dose-dependent inhibition of DCN/collagen binding compared with cells incubated with collagen beads alone ($p < 0.001$; ANOVA). C, E, and G, confocal microscopy; D, F, and H, flow cytographs of Rat-2 cells loaded with collagen-coated, DCN-coated, or DCN/collagen-coated beads at a 4:1 bead:cell ratio (cells counterstained with rhodamine phalloidin). YG, yellow green. C and D demonstrate bead binding to cells (yellow particles in confocal images) and corresponding flow cytographs show four peaks representing the proportion of cells with 1–4 beads bound, respectively. E and F show cells with no bound DCN-coated beads as DCN does not bind to cells appreciably. G and H, cells loaded with DCN/collagen-coated beads show that cells have only limited bead binding. Flow cytograph demonstrates only one small peak of cells with one bound bead. I, time course of collagen and Decorin Inhibition of Phagocytosis.
Collagen beads were incubated with vehicle or the indicated decorin preparations in solution and then added to Rat-2 cells or HGF for 1 h. The % of cells with bound beads was determined by flow cytometry. The data are the % reduction of bead binding compared with vehicle-treated collagen bead controls. There were no significant differences between inhibition of collagen bead binding of Rat-2 cells and human gingival fibroblasts (p > 0.2) or between bovine articular cartilage decorin (1 μM) and human recombinant decorin (1 μM; p > 0.2).

| Cell type      | DCN preparation | % reduction of binding to collagen (n = 3) |
|----------------|-----------------|------------------------------------------|
| Rat-2 cells    | Bovine cartilage (1 μM) | 88 ± 3                                   |
| HGF            | Bovine cartilage (1 μM) | 82 ± 3                                   |
| HGF            | Human recombinant (1 μM) | 80 ± 4                                   |
| HGF            | Human recombinant (0.1 μM) | 60 ± 5                                   |

Integrin blocking of collagen bead binding

Collagen beads or DCN collagen beads were incubated with human gingival fibroblasts at 6 beads/cell for 1 h in the presence of vehicle (PBS) or with 1 μg/ml 4B4 antibody, a β1 integrin blocking antibody. The % of cells with bound beads was measured by flow cytometry.

| Treatment                        | % cells with bound beads (n = 4) |
|----------------------------------|----------------------------------|
| Collagen beads + vehicle         | 38 ± 2                           |
| Collagen beads + 4B4 antibody    | 9 ± 3                            |
| DCN-collagen beads + 4B4 antibody| 8 ± 3                            |

Collagen beads were incubated with vehicle or the indicated decorin preparations in solution and then added to Rat-2 cells or HGF for 1 h. The % of cells with bound beads was determined by flow cytometry. The data are the % reduction of bead binding compared with vehicle-treated collagen bead controls. There were no significant differences between inhibition of collagen bead binding of Rat-2 cells and human gingival fibroblasts (p > 0.2) or between bovine articular cartilage decorin (1 μM) and human recombinant decorin (1 μM; p > 0.2).

DCN in solution for 1, 10, or 60 min at 4 °C to block internalization. Cell surface binding was measured by immunostaining for DCN and quantified by flow cytometry. Cell surface DCN staining for HGFs was not significantly higher than controls (Fig. 2E; p = 0.6 by ANOVA). This effect was not because of trypsin-induced degradation of DCN during cell dissociation because trypsin did not significantly reduce immunostaining of DCN-coated beads (DCN-collagen beads without trypsin treatment (Fig. 2E; p > 0.2). Furthermore, the lack of binding of soluble DCN was not because of internalization because cells at 4 °C that were incubated with DCN, fixed, and immunostained for surface DCN showed no fluorescence above background levels. Thus, binding of DCN to human gingival fibroblasts was experimentally insignificant.

Cell Attachment and Spreading—As cell attachment and spreading are critical early steps in collagen phagocytosis (6), we asked if DCN affects these processes when cells were plated on collagen substrates. Cell attachment to DCN/collagen-coated substrates was reduced with increasing DCN concentration. Compared with cells plated on collagen, there were significant reductions in cell attachment for cells plated on DCN/collagen substrates with 0.1 and 1.0 μM DCN (~2-fold, p < 0.05; Fig. 3A). For spreading studies, cells were plated on collagen-coated surfaces and allowed to attach and spread for 1 h or 3 h in the absence of serum followed by staining with rhodamine phalloidin to visualize actin filaments (Fig. 3, B–E). Cells that attached and spread in the absence of DCN showed abundant actin filaments and exhibited spreading within 1 h. At 3 h, there was further enhancement of actin filament staining and increased cell spreading. Cells that were plated on DCN/collagen-coated substrate ([DCN] in solution,1.0 μM) were able to adhere to the substrate; however, spreading and actin filament formation were minimal.

Effect of DCN Structure on Collagen Binding—We assessed if the DCN core protein mediates DCN-induced inhibition of collagen binding. DCN core protein was produced by digestion with chondroitinase ABC, and the efficiency of this treatment was examined by SDS-PAGE. Whole, undigested DCN exhibited a molecular mass of ~100 kDa, whereas the digested sample exhibited bands at ~45 and 48 kDa, corresponding to the DCN core protein (Fig. 4A). With this digestion protocol, we assessed whether the DCN core protein could inhibit bead binding by human gingival fibroblasts. DCN core protein (1 μM) significantly inhibited collagen bead binding (~50% of controls, p < 0.01; Fig. 4B). This reduction was not because of large reductions of DCN core protein binding to the collagen beads as SDS-PAGE showed little difference in the abundance of whole DCN or DCN core protein that was eluted from the beads. Compared with DCN core protein, recombinant DCN (1 μM) inhibited collagen bead binding by ~30% more (Table II). At coating concentrations of 10 μM core protein, there was more inhibition of collagen bead binding (75 ± 7% reduction).

We also considered the importance of the glycosaminoglycan chain of DCN in the inhibition of collagen bead binding. Cells incubated with collagen beads coated with either 1 μM dermatan sulfate or 1 μM chondroitin sulfate showed no inhibition of bead binding compared with collagen bead controls (dermatan sulfate 98 ± 4% of controls, p > 0.2; chondroitin sulfate, 99 ± 3% of controls, p > 0.2). Incubation of beads with Alcian blue showed increased staining when collagen beads were coated with glycosaminoglycans, indicating that dermatan sulfate and
chondroitin sulfate could bind to the collagen beads. We next determined the importance of DCN tertiary structure in mediating inhibition of collagen binding by irreversibly denaturing DCN (heating at 70 °C for 30 min). The efficacy of the heat treatment was evaluated by circular dichroism of DCN at 25 and 70 °C. There was loss of ellipticity at 205 nm as described previously for heat-denatured DCN (see Ref. 27; results not shown). Heat-denatured DCN (0.5 μM) inhibited collagen bead binding almost as effectively as native DCN (by ∼89%; p < 0.01; Fig. 4B). Because the native structure of DCN did not appear to be required for collagen binding, we used CNBr to digest the DCN core protein into smaller peptides. Collagen beads were coated with CNBr-digested DCN (1 μM), and bead binding to cells was compared with collagen beads. The CNBr DCN fragments inhibited bead binding by 88 ± 3% (n = 4 independent samples) compared with collagen beads, indicat-

**Fig. 2. DCN competitive inhibition of collagen bead binding.** A–C, dual color bead, flow cytometry experiments to assess if DCN (1 μM) competitively inhibits collagen bead binding. Scatter plot and flow cytographs in which Rat-2 cells were loaded simultaneously with COL-RED and DCN/COL-BLUE beads. A, ∼5-fold difference in bead binding between COL-RED and DCN/COL-BLUE beads as shown in D. A very small percentage of cells bound both types of beads simultaneously. B and C, note the marked reduction of DCN/COL-BLUE fluorescence (FL) compared with the COL-RED fluorescence (n = 10,000 cells/analysis). D, rat-2 cells loaded simultaneously with COL-RED and COL-BLUE beads exhibited similar amounts of bead binding (p > 0.5); cells loaded simultaneously with COL-RED and DCN/COL-BLUE demonstrated a ∼5-fold discrepancy in bead binding (p < 0.005); DCN/COL-RED and DCN/COL-BLUE beads loaded simultaneously were bound in comparable amounts. E, cell surface staining of DCN. Cells were incubated with DCN (1 mM) for indicated times, immunostained for DCN, and analyzed by flow cytometry for surface binding of DCN. Controls are cells stained with secondary antibody only. Data in graphs are mean ± S.E.
ing that CNBr-derived peptide sequences within the DCN core protein can inhibit collagen binding.

CNBr digestion generated distinct DCN fragments that were resolved by silver staining of Tris-Tricine gradient gels (Fig. 5A). Based on the predicted cleavages from the primary amino acid sequence of DCN, we found a mixture of complete and incompletely digested DCN fragments ranging in size from 2.6 to 11 kDa (Fig. 5A). We determined which CNBr-digested fragments bound to collagen-coated beads by incubating CNBr-digested DCN with collagen beads. An 11-kDa fragment eluted from the collagen beads was analyzed by mass spectrometry and sequencing. The sequences from the tryptic digest that were positively matched to the bovine decorin sequences were VVQCSDLGLEK and ISPGAFAPLVK which correspond to LRR regions 1–3 in the 11-kDa CNBr-digested fragment.

Peptide Binding Experiments—To assess the collagen binding ability of the LRR 1–3 peptides, we synthesized biotinylated peptides corresponding to each LRR and detected peptide binding to collagen-coated beads by incubating CNBr-digested DCN with collagen beads. An 11-kDa fragment eluted from the collagen beads was analyzed by mass spectrometry and sequencing. The sequences from the tryptic digest that were positively matched to the bovine decorin sequences were VVQCSDLGLEK and ISPGAFAPLVK which correspond to LRR regions 1–3 in the 11-kDa CNBr-digested fragment.

Competitive Inhibition of Peptide Binding—As the LRR 3 peptide bound more abundantly to collagen than the LRR 1 and 2 peptides, it was used to assess the specificity of binding to collagen in more detailed binding and competition experiments. Dose-response experiments demonstrated large and significant increases in biotinylated LRR 3 peptide binding with increasing concentrations (1–100 μM peptide), after which a plateau was observed (Fig. 6A; difference in mean fluorescence/bead between 1 and 10 μM peptide was 8-fold; \( p < 0.005 \)). For competitive inhibition of peptide binding, beads were prepared, blocked with BSA, coated with biotinylated peptides (10 μM), and incubated with 100× excess of unlabeled peptide. The difference in mean fluorescence/bead between the positive control and competition group was 3.4-fold (\( p < 0.0005 \); Fig. 6B), indicating that the biotinylated peptide was released and that the unlabeled peptide effectively competed binding by the labeled peptide.

We measured binding of LRR 3 and scrambled sequence LRR 3 peptide to type I collagen-derived triple helical peptides from type I collagen as well as to BSA. The first triple helical peptide...
included residues α1(I)-(496–507) (high affinity ligand binding to the α2 integrin subunit; $K_D = 1.1 \times 10^{-6}$ M), and the second peptide included residues α1(I)-(772–786) which may mimic a DCN-binding site. Peptides (at 100 μM) were attached to 2 M nonfluorescent, carboxylate-modified beads by carbodiimide coupling, and elution experiments followed by amino acid analysis showed that both triple helical peptides were bound to beads. With the use of biotinylated LRR 3 and the scrambled LRR 3 peptide, followed by streptavidin-FITC staining and measurement by flow cytometry, we found nearly 3-fold higher binding of LRR 3 to the triple helical collagen peptide (residues α1(I)-(496–507) containing the binding to the α2 integrin subunit) than that exhibited by the scrambled LRR 3 sequence (Table IV).

**Discussion**

The intracellular phagocytic pathway in fibroblasts is an important step in the degradation of collagen under physiological conditions (1, 4). Collagen fibrils are coated by proteoglycans in vivo (5, 29), and decorin-deficient mice exhibit enhanced collagen degradation (30). Consequently, we considered that decorin may affect collagen phagocytosis. Our central finding is that DCN, at densities as low as 6 μg/mol/m² of DCN on bead surfaces, can inhibit internalization of collagen. These findings may have important implications for physiological connective tissue remodeling and wound healing because they point to an important role for decorin, and possibly other proteoglycans that bind to collagen, as critically important inhibitors of the phagocytosis step in the collagen degradation pathway. This proteoglycan-mediated inhibitory mechanism provides a novel corollary to the matrix metalloproteinase pathway of collagen degradation and its inhibition by tissue inhibitors of matrix metalloproteinases.

The biological significance of our data depends on the validity of the DCN/collagen bead model system for phagocytosis. As immunostaining demonstrated that DCN bound to collagen-coated beads, this model system mimics the in vivo situation in which proteoglycans, possibly including DCN, are thought to “decorate” collagen fibrils (5, 31). Furthermore, data obtained with the in vitro collagen phagocytosis bead model likely reflect

**Fig. 4. Effect of DCN structure on collagen bead binding.** A, 10% SDS-PAGE shows bands corresponding to native DCN at ~100 kDa (lane a is untreated mature DCN; lane b is DCN treated with the chondroitinase ABC vehicle). Lane cpDCN shows doublet core protein bands at ~45 and 48 kDa after chondroitinase ABC digestion. MWM, molecular weight markers. B, inhibition of collagen bead binding by heat-denatured DCN (hdDCN; 0.5 μM equivalent to native DCN) and DCN core protein (cpDCN; 1 μM equivalent to native DCN). Binding of heat-denatured DCN/collagen and cpDCN/collagen beads was reduced by 89 (% < 0.01) and 51% (p < 0.05), respectively (n = 10,000 cells/analysis). Data are mean % bead binding inhibition ± S.E.
authentic regulatory systems in vivo as previous studies (23, 26, 32) have demonstrated remarkable parallels between reduced collagen bead binding and drug-induced inhibition of collagen degradation in vivo. Notably, the amount of DCN on beads that was required to produce inhibition of collagen binding by cells was very low \((3 \times 10^{-20} \text{ mol of DCN bound/bead})\) and approached an equimolar ratio \((0.34)\) with the amount of collagen on beads \((8.7 \times 10^{-20} \text{ mol of collagen bound/bead})\).

**DCN and Collagen Binding**—Previous studies on DCN-collagen interactions have largely focused on modulation of collagen fibrillogenesis (9, 10, 20, 32–34). The novelty of the current report is that DCN inhibits the collagen binding step of phagocytosis as demonstrated by a consistent and marked reduction in collagen bead binding. Although previous studies (20–22) examined how DCN binding to collagen fibrils regulates fibrillogenesis, these results may also relate to the inhibitory effect of DCN on the collagen binding step of phagocytosis because DCN regulation of both fibrillogenesis and phagocytic degradation requires binding to collagen. Indeed, a three-dimensional model of decorin (10), as well as observations that several small leucine-rich proteins may lie within the concavity of the putative DCN structure (9), predicts close interactions between DCN and fibrillar collagen. Whereas these interactions may stabilize and orient fibrils during fibrillogenesis (35), in the context of collagen phagocytosis they may also inhibit the binding of collagen to integrins. The inner surface of the putative arch-shaped DCN molecule contains a series of charged residues that may facilitate adhesive interactions with triple helical type I collagen (10, 20) and possibly with collagen molecules. Notably, the bead surface in our model system contains a mixture of both fibrils and collagen molecules so there are likely heterogeneous interactions between DCN and the collagen on the bead, an issue that has become more complex with recent reports showing that DCN may exist as dimers (36).

We considered that decorin may bind to cells independent of collagen, and subsequently inhibit collagen binding as a result of DCN receptor-mediated processes. The observation that very low numbers of HGF or Rat-2 cells bound beads coated with DCN alone was consistent with the finding that surface staining of HGF incubated with soluble DCN was negligible. Thus under our experimental conditions, any observed binding of DCN to cells was likely nonspecific and was of very low affinity. The apparent absence of DCN receptors in HGF is in contrast to other reports demonstrating receptor-mediated endocytosis of DCN by human skin fibroblasts (8) and of tumor cells (37, 38). In contrast, using gingival fibroblasts, DCN receptors are evidently not expressed or they are not functional.

We considered that DCN regulates collagen phagocytosis by masking the ligand-binding sites on collagen required for cell attachment and ultimately internalization. As we found that inhibition of monomeric collagen binding to cells by DCN was minimal, DCN interactions with collagen that impact on phagocytosis evidently require fibrillar collagen. Furthermore, as the \(\alpha2\beta1\) integrin is required for collagen phagocytosis by human gingival fibroblasts (6, 23, 24, 26), a likely site for DCN-induced masking of fibrillar collagen binding is the \(\alpha2\beta1\) integrin-binding site within collagen (39). Accordingly, we used DCN peptides (see below) that bind optimally to collagen, and we examined their interaction with triple helical collagen peptides mimicking the \(\alpha2\beta1\) integrin-binding site of collagen (40) and a second DCN-binding sequence within collagen (41, 42). Based on binding of the LRR 3 DCN peptide (but not the scrambled sequence peptide) to both of these triple helical collagen peptides, short collagen-binding sequences within DCN may mask the \(\alpha2\beta1\) integrin-binding site on collagen, which is required for the phagocytosis of collagen fibrils. There appear to be multiple binding sites within collagen for DCN (42), which include sequences modeled by the collagen peptide...
Whereas the LRR 3 DCN peptide apparently bound to this sequence, it is not known to mediate cell attachment via integrins and consequently may not be important in regulating collagen phagocytosis.

Importance of DCN Structure on Inhibition—In agreement with previous studies, we found that the DCN core protein is critical for binding to collagen fibrils (43, 44). Although dermatan sulfate and chondroitin sulfate bound to collagen beads, they did not, by themselves, influence collagen bead binding. Heat disruption of the tertiary structure of DCN did not substantially affect binding to collagen, indicating that binding domains within the DCN core protein (10), which are not reliant on the native structure of DCN, may be responsible for inhibition of collagen binding.

Several studies have attempted to define collagen binding domains in DCN (20–22), including putative regions in the LRRs 4–6. Currently, there are no definitive reports of binding inhibition by short peptides. Although recombinant truncated forms of DCN core protein expressed in bacteria have been used to demonstrate two putative sites that bind fibrillar collagen (20), solubilization and folding have complicated the interpretation of these studies. Accordingly, we synthesized
small peptides that correspond to sequences within LRRs 1–5. A 9-residue peptide based on the LRR 5 containing glutamate 180 weakly inhibited collagen bead binding by cells, whereas a combination of peptides (LRR 3 and LRR 4) was strongly inhibitory, suggesting that cooperativity between LRRs is required for collagen binding.

The inhibition of collagen binding and subsequent internalization indicates that DCN may block collagen phagocytosis in vivo. Thus DCN may be an important determinant of connective tissue homeostasis because DCN coatings on collagen fibrils may prevent, for example, inappropriate collagen degradation in early stages of wound healing. In addition to its collagen binding properties, DCN can bind and neutralize significant amounts of transforming growth factor-β, a potent, pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may serve a dual role by first neutralizing the effects of transform-pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may be an important determinant of connective tissue homeostasis because DCN can bind and neutralize significant amounts of transforming growth factor-β, a potent, pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may serve a dual role by first neutralizing the effects of transform-pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may serve a dual role by first neutralizing the effects of transform-pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may serve a dual role by first neutralizing the effects of transform-pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may serve a dual role by first neutralizing the effects of transform-pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may serve a dual role by first neutralizing the effects of transform-pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may serve a dual role by first neutralizing the effects of transform-pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may serve a dual role by first neutralizing the effects of transform-pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may serve a dual role by first neutralizing the effects of transform-pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may serve a dual role by first neutralizing the effects of transform-pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may serve a dual role by first neutralizing the effects of transform-pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may serve a dual role by first neutralizing the effects of transform-pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may serve a dual role by first neutralizing the effects of transform-pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may serve a dual role by first neutralizing the effects of transform-pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may serve a dual role by first neutralizing the effects of transform-pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may serve a dual role by first neutralizing the effects of transform-pro-fibrotic cytokine (45, 46). Thus in wound healing, DCN may serve a dual role by first neutralizing the effects of transform-pro-fibrotic cytokine (45, 46).