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Characterization of an Anti-Decorin Monoclonal Antibody, and Its Utility[1]

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6B6 is a monoclonal antibody raised against a purified small dermatan sulfate proteoglycan from human ovarian fibroma capsule. Although it has been widely used as an anti-decorin monoclonal antibody, its epitope has not yet been characterized at the molecular level. Here, we show that 6B6 is specific to decorin. The antibody recognized human, mouse, and bovine decorin core protein, but not biglycan. Using recombinant decorin domains, we determined that the epitope lies within the region of amino acid residues 50–65, termed the cysteine cluster region. Cross-reactivity among species further narrowed it down to a primary sequence of residues 57–65. We also established the conditions for immunostaining. 6B6 stained both frozen and fixed sections. Whereas the glycosaminoglycan chain of decorin inhibited access of the antibody in immunoblotting, pretreatment of tissue sections with chondrotinase ABC did not affect the intensity of staining, suggesting that the glycosaminoglycan chain is integrated and the Cys cluster region oriented outside of the collagen fibrils in the tissue. When 6B6 was applied to enzyme-linked immunosorbent assay, a concentration as low as 0.5 μg/ml of decorin was detectable by either direct or sandwich ELISA. 6B6 is thus a sensitive and reliable antibody to study functions of decorin from various aspects.

Key words: antibody, decorin, extracellular matrix, proteoglycan.

Decorin is a leucine-rich proteoglycan (PG) with a protein core of 36 kDa and a glycosaminoglycan chain attached to the N-terminus (*1*). Decorin contributes to fibril stability of several collagen types *in vivo*, including types I, II, V, VI, and XIV, by binding through leucine-rich repeat regions (*2–6*), and influences cell adhesion by interacting with extracellular adhesive molecules such as fibronectin (*7*) and thrombospondin (*8*). In some cells, decorin activates the epidermal growth factor receptor, thereby triggering a signaling cascade that leads to phosphorylation of mitogen-activated protein kinases, induction of p21, and suppression to growth (*9*). Decorin also interacts with transforming growth factor (TGF)-β and modulates its activity. It may have a curative effect on glomerulonephritis by inhibiting TGF-β activity (*10*), or it may increase TGF-β activity on osteoblasts (*11*).

Several decorin-specific antibodies have been used for biochemical and immunohistochemical analyses, including LF series (*12*), polyclonal antibodies raised against recombinant peptides of decorin core protein, and monoclonal antibodies such as 7B1, 5D1, 3B3, 6D6, 1XA (*13, 14*), CB-1 (*15*), and DS1 (*16, 17*), some of whose detailed epitope regions have not been determined yet.

6B6 is a monoclonal antibody prepared with a purified small dermatan sulfate PG from human ovarian fibroma capsule as an antigen (*18*). Immunoblot analyses demonstrated that the antibody recognizes both intact dermatan sulfate PG and a core protein of ~40 kDa. Immunohistochemical analyses indicated that 6B6 stains various tissues such as venous wall, adventitia and atheromatous plaque of large arteries, reticular dermis, perichondral tissue and nerve sheath, suggesting that the antigen is associated with collagen fibers (*19, 20*). Immunohistochemical studies of chondroitin sulfate and dermatan sulfate proteoglycans in tumor tissues using various antibodies including 6B6 revealed that the interstitial fibrous elements within the cancer cell nests contain chondroitin 4-sulfate, whereas the surrounding connective tissue and the preexisting fibrous connective tissue involved in the tumor growth consists mostly of dermatan sulfate proteoglycans (*18, 21*). As these results suggest that 6B6 may recognize decorin core protein, 6B6 has widely been used as an anti-decorin antibody. However, it is not certain that 6B6 is specific to decorin, and its exact epitope structure has not been determined yet. To better interpret previous results using 6B6, detailed analysis of this antibody is absolutely required.

In this study, we identified the antigen for 6B6 as the decorin core protein, and further determined an epitope structure as the primary amino residues of the cysteine cluster region. Furthermore, we reexamined the conditions

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; PG, proteoglycan; PMSF, phenylmethanesulfonyl fluoride; TGF, transforming growth factor.

for immunoblotting, immunohistochemical analyses, and enzyme-linked immunosorbent assay (ELISA), and conclude that 6B6 is useful for studies on decorin functions.

EXPERIMENTAL PROCEDURES

Purification of Native Decorin from Fibroblast Culture Medium—Decorin produced by normal skin fibroblasts was isolated according to a previously described method with slight modification (*22*). Briefly, Tris-HCl, pH 7.4, EDTA, PMSF, and NaCl were added to the conditioned medium to make final concentrations of 20 mM, 5 mM, 1 mM, and 0.25 M, respectively. The solution was applied to a DEAE cellulose mini-column (1 ml) which had been equilibrated with Solution A (20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM PMSF) containing 0.25 M NaCl. The column was washed with 5 volumes of Solution A containing 0.40 M NaCl and eluted with Solution A containing 1 M NaCl.

Expression of Decorin Core Protein as a Fusion Protein—A partial cDNA probe of human decorin was purchased from TELIOS Pharmaceuticals. The polymerase chain reaction (PCR) with a set of primers consisting of 5′-AATACTCTAGATAAAATCATGAAGGCCACTATCATCC-3′ and 5′-TTCCTGAGCTCTTCAGCGGATTGGTG-3′ and a human fetal skeletal muscle cDNA library (Clontech) as template was used to amplify an upstream region of the decorin cDNA with an *Xba*I site, which was ligated with a cDNA clone of human decorin (*1*) using the *Sac*I site. The full-length decorin cDNA was directionally inserted into a bacterial expression vector pET15b (Novagen), which encodes six consecutive histidine residues at the N-terminus of the fusion protein and thrombin cleavage site, and transfected into bacteria, BL21 (DE3). Expression was induced by addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and after 3 h, a full-length decorin core protein with 6xHis·Tag® was harvested by boiling in a 1× SDS sample buffer for 10 min. DCN1-140 was ligated into pET15b with *Bam*HI polylinker after *Xba*I and *Pvu*II digestion. DCN18-65 was ligated into pET15b after *Ava*II digestion and blunt-end formation. DCN50-359 was prepared by digestion with *Avr*II and *Eco*RI and ligation of the fragment into the expression vector with a *Bam*HI linker after blunt-end formation. For expression of DCN141-359 and DCN65-359, various segments of decorin core protein were amplified by PCR with a set of primers consisting of 5′-CCAAGGATCCGCTGAAGGAATTGCCAGAAA-3′ and 5′-AGCCGGATCCCGAATTCGAGCACATAGAGCT-3′ or 5′-ATCTGGATCCGGTCCAGTGTTCTGATTTGG-3′ and 5′-AGCCGGATCCGAATTCGAGCACATAGAGCT-3′, followed by insertion into the expression vector (Fig. 1A). For mammalian expression of decorin, a full-length decorin cDNA with *Xho*I and *Bgl*II sites attached at 5′- and 3′-ends respectively was prepared by PCR with a set of primers 5′-ACGTTACTCGAGGATGAGGCTTCTGGGATAGGC-3′ and 5′-GCAGCCAGATCTTTACTTATAGTTTCCGAGTTGAATGG-3′. The PCR product was inserted into a mammalian expression vector, pBFX (*23*), which encodes a FLAG® residue at the N-terminus. 293 cells were stably transfected with the expression construct, and the expressed protein with a FLAG-tag was purified with an anti-FLAG M2 column (1 ml) from serum-free conditioned media as previously described (*23*).

Gel Electrophoresis, Immunoblot, and Dot Blot Analyses—Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (*24*), and transferred to a nitrocellulose membrane in 0.1 M Tris-glycine/20% methanol using a semi-dry or a wet blotter (Nihon Eido). The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS)-Tween 20, and then treated with a sheep anti-decorin polyclonal antibody (Biogenesis) or 6B6 (Seikagaku Kogyo) at a dilution of 1:2,000 or 1:1,000 in the TBS-Tween 20 at 4°C overnight. After washing five times × 5 min with the same buffer, the membrane was treated with HRP-conjugated donkey anti-sheep (×60,000, Jackson) or HRP-conjugated goat anti-mouse IgG (×3,000, Cappel), followed by washing five times × 5 min and detection with ECL Plus (Amersham) chemiluminescense. Protein expression was confirmed by immunoblot analyses using an anti-4xHis antibody (×2,000, QIAGEN) or an anti-FLAG M2 antibody (×400). For dot blot analyses, 2 or 5 μl of the sample was applied onto the membrane. After drying the sample, the membrane was treated with the same process as the immunoblot.

Histological and Immunohistochemical Analyses—Skin tissue obtained from various patients was fixed with 10% buffered formalin for 48 h, then embedded in paraffin. Tissue sections (6 μm) were dewaxed in xylene, and rehydrated through decreasing concentrations of ethanol. To block endogenous peroxidase activity, rehydrated sections were treated with 0.3% H_2O_2 in absolute ethanol for 5 min, then processed for immunostaining with 6B6 (×500) and a Histofine Simple Stain Max PO (M) (Nichirei) according to a manufacturer's instructions. For immunostaining with polyclonal antibodies (Biogenesis and US biological) that detect the C-terminus of decorin core protein, tissue sections washed in phosphate-buffered saline (PBS) were incubated with 10% goat serum in PBS for 60 min, washed extensively with PBS, and incubated with antibodies at a dilution of 1:200 at 4°C overnight. After washing with PBS, tissue sections were treated with a horseradish peroxidase (HRP)–conjugated donkey anti-sheep IgG (Jackson) at a dilution of 1:1,000 for 60 min. For regular histology, tissue sections were stained with hematoxylin and eosin (HE).

Enzyme-Linked Immunosorbent Assay (ELISA)—ELISA plates (NUNC) were coated overnight at 4°C with bovine decorin (Sigma). The wells were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at RT, treated with 6B6 (×200) in 1% BSA in PBS for 1 h at RT. After washing with 0.05% Tween 20 in PBS (1 min × three times), the wells were treated with an HRP-conjugated anti-mouse IgG (×200, Cappel) for 1 h at RT. After washing wells, detection was performed using tetramethylbenzidine (TMB) for 1 h.

For sandwich ELISA, microtiter plates (NUNC) were coated for 1.5 h with 50 μl of 20 μg/ml 6B6 in PBS. After washing wells with PBS two times, wells were blocked with 100 μl of immunostabilizer (ABI) for 1 h. After washing with 0.05% Tween 20 in PBS, 0.05% procline, the wells were treated with 50 μl of bovine decorin diluted with 1% BSA in PBS, 0.05% procline at a concentration indicated for 1 h at RT. After washing with the same buffer two times, the wells were treated with 50 μl of 0.8 μg/ml Biotin-Ly111 (Seikagaku Kogyo) for 1 h at 37°C. After washing, 50 μl of ×1,000 HRP-streptavidin (Vector) was added, and incubation was continued for 1 h at 37°C. After washing, detection was performed using TMB.

RESULTS

As previous findings suggested that 6B6 is an antibody against decorin core protein, we first confirmed that 6B6 recognizes the core protein in immunoblots. Decorin was partially purified from the conditioned media of human primary fibroblasts. When the sample treated with chondroitinase ABC was applied to immunoblot analysis using a specific polyclonal antibody that recognizes the C-terminus of decorin core protein, two bands with apparent molecular mass of 44 and 46 kDa were observed (Fig. 1A), confirming the presence of decorin in the sample. 6B6 stained those bands, indicating that it recognizes human decorin core protein. We also tested whether 6B6 recognizes the recombinant decorin core protein expressed as fusion proteins with 6xHis and with FLAG in bacterial and mammalian expression systems, respectively. Decorin core protein expressed in the bacterial system was detected by an anti-4xHis antibody as a single homogenous product with a molecular size of 45 kDa, which was also detected by 6B6 (Fig. 2B). Expression of decorin with a FLAG-tag in the mammalian system was confirmed by immunoblotting with an anti-FLAG antibody. Both the polyclonal antibody and 6B6 recognized the protein after chondroitinase ABC treatment, but did not do so without the treatment. The antibody recognized commercially available bovine decorin core protein after treatment with chondroitinase ABC, but not without treatment. When the immunoblot analyses were performed after SDS-PAGE under reducing conditions, 6B6 stained the core protein band, suggesting that it recognizes a primary amino acid sequence of decorin core protein. Since an untreated sample gave no band in the immunoblotted membrane, we examined the possibility that the glycosaminoglycan chain interferes with the epitope-antibody interaction by dot blot analysis. 6B6 detected decorin without treatment, but the intensity of staining was diminished to approximately 30% compared with decorin with chondroitinase ABC treatment (Fig. 1B, lower panel).

We next determined the epitope sequence for 6B6 using a series of truncated decorin core proteins (Fig. 2A). Their expression was first confirmed by immunoblotting with an anti-(4xHis) antibody. All the truncated decorin proteins, DCN1–140, DCN141–359, DCN18–65, DCN50–359, and DCN65–359, reacted with the antibody and showed the expected sizes (Fig. 2B, left panel). 6B6 recognized truncated core proteins of DCN1–140, DCN 18–65, and DCN

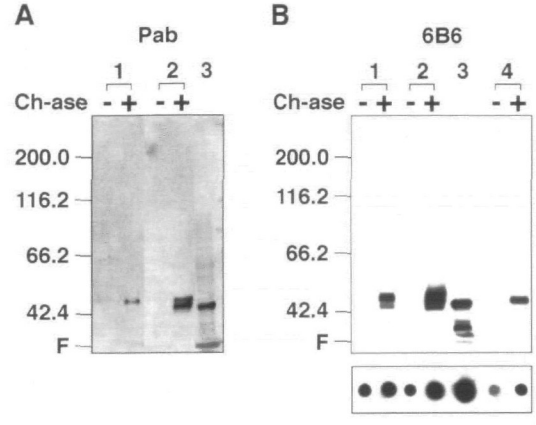
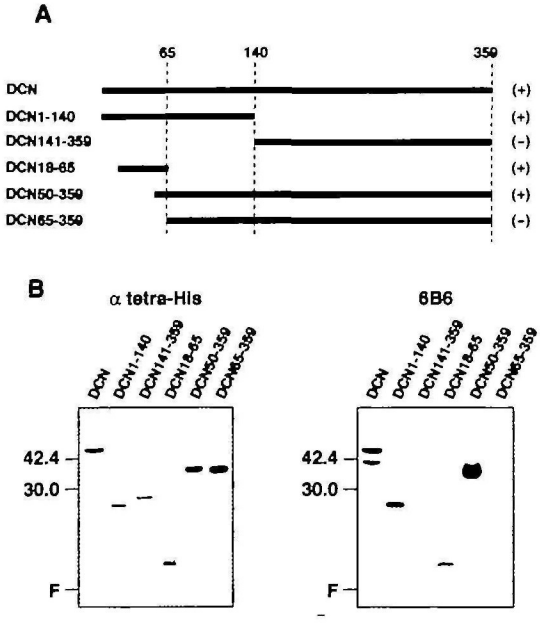


Fig. 1. **Immunoblot and dot blot analyses using an anti-decorin polyclonal antibody and 6B6.** Recombinant decorin synthesized in mammalian and bacterial expression systems, native bovine decorin, and partially purified decorin from human skin fibroblast conditioned media were applied to immunoblot analyses. Samples were pretreated with chondroitinase ABC (+) or not (–), applied to a SDS-PAGE gel under reducing conditions, and transferred to a nitrocellulose membrane. As a primary antibody, a polyclonal one that recognizes the C-terminus of decorin core protein (A) or 6B6 (B) was used at a dilution of 2,000 or 1,000. Lane 1, decorin from human skin fibroblasts conditioned medium; lane 2, recombinant human decorin; lane 3, decorin core protein expressed in a bacterial expression system; lane 4, bovine decorin purified from bovine articular cartilage (Sigma). The lower panel of (B) shows dot blot analysis using 6B6 of identical samples as indicated on the immunoblot.



C

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h DCN  50 LGPVCPFRCQCHLRVV 65
          ****************
b DCN     MGPVCPFRCQCHLRVV
          *  . **.**********
m. DCN    LISMCPYRCQCHLRVV
            .*** *.******
h. BGN    YSAMCPFGCHCHLRVV
            ...*** * *******
b BGN     YSAMCPFGCHCHLRVV
            ....***   ******
m. BGN    FSAMCPFGWHCHLRVV
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Fig. 2. **Immunoblot analyses of various recombinant decorin polypeptides.** Human full-length decorin (Decorin), and its various truncated forms (*DCN1–140*, *DCN141–359*, *DCN18–65*, DCN50–359, and DCN65–359) were expressed in a bacterial expression system. (A), Schematic diagram of human decorin expression constructs. The numbers are those of amino acid residues. Reactivity to 6B6 is indicated as (+) or (–). (B) Immunoblot analysis of the full-length decorin and its various truncated polypeptides. Bacteria [BL21 (DE3)] were transformed with expression constructs. After induction by IPTG for 3 h, bacterial lysates were collected and applied to a SDS-PAGE gel under reducing conditions, then transferred to a nitrocellulose membrane. As a primary antibody, an anti-4xHis (×2,000) (left) or 6B6 (×1,000) (right) was used. An anti-mouse IgG (×3,000) was used as the secondary antibody. Detection was performed using ECL. Note that polypeptides containing a stretch of residues 50–65 reacted with 6B6. Protein marker positions and the front line (F) are indicated. **(C)** Amino acid sequence containing the 6B6 epitope in human, bovine, and mouse.

50–359 as well as the full-length core protein, but did not recognize DCN 141–659 and DCN 65–359 (Fig. 2B, right panel). These results indicate that the epitope is located within a stretch of residues 50–65, corresponding to the N-terminal Cys cluster region. Although biglycan contains a Cys cluster region with a high degree of amino acid sequence identity, 6B6 did not recognize biglycan with or without chondroitinase ABC treatment (data not shown).

As the amino acid sequence of residues 50–65 is highly conserved among species, including human, bovine and mouse (Fig. 2C), we tested whether 6B6 could recognize mouse decorin by immunoblot analyses. Curiously, 6B6 recognized mouse decorin core protein partially purified from conditioned media of mouse fibroblasts (data not shown).

We then performed immunohistochemical studies using skin tissue sections to evaluate the usefulness of 6B6. In normal skin, 6B6 strongly stained the entire dermis, but the epidermis, the dermal region facing the basement membrane, and appendages were not stained (Fig. 3, A and F). This staining pattern is consistent with the decorin distribution reported previously (*12, 25–27*). 6B6 stained dermis of not only fixed sections but also frozen sections (data not shown). In addition, 6B6 strongly immunostained mouse dermal tissue sections, supporting the immunoblot data that 6B6 recognizes mouse decorin (Fig. 3E). We also examined immunostaining patterns of tissue sections of diseased skin, in which the distribution of decorin may be altered. Keloid is characterized by dense collagen bundles in a nodule containing dense, broad collagen bundles located in the middle to deep dermis. 6B6 stained irregular broad bundles strongly, and adjacent collagen bundles weakly (Fig. 3, B and G). Solar elastosis is hyperplasia of the elastic tissue in sun-exposed skin, characterized by amorphous basophilic granular material separated from the epidermis by a narrow band of normal collagen in the upper dermis. 6B6 stained collagen bundles surrounding the amorphous region, but not central amorphous material (Fig. 3C). Pretreatment of tissue sections with trypsin, acetic acid, hyaluronidase, or chondroitinase ABC was found

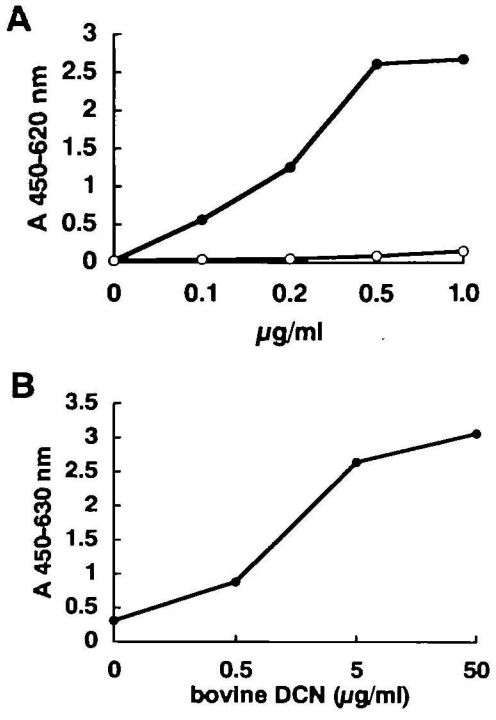

Fig. 4. **Enzyme-linked immunosorbent assay (ELISA).** Microtiter plates were coated with the indicated concentrations of bovine decorin or biglycan, and the specific 6B6 interaction was measured by using ELISA (A). Sandwich ELISA using 6B6 and Ly111. Decorin at the indicated concentrations was added to 6B6-coated plates, and captured decorin was bound with biotinylated Ly111 followed by detection with HRP-conjugated streptavidin, as described in the experimental procedures (B).

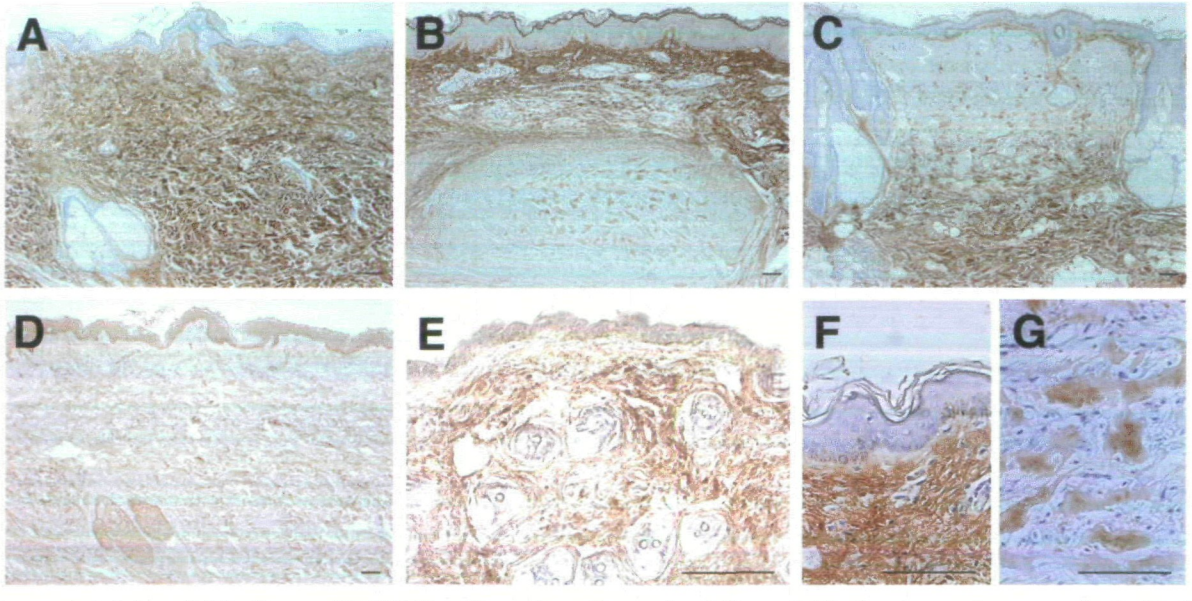

Fig. 3. **Immunostaining of skin tissue with 6B6.** Paraffin sections of normal and diseased skin tissues were immunostained with 6B6. Normal skin (A) with higher magnification (F), keloid (B) with higher magnification (G), solar elastosis (C), mouse skin (E). Normal skin immunostained with an anti-decorin polyclonal antibody (D).

not to affect the intensity of immunostaining or alter staining patterns (data not shown).

Next, we tested the ability of native decorin to interact with 6B6 in ELISA assay. Microtiter plates were coated with bovine decorin or biglycan, and the specific 6B6 interaction was measured. Binding was proportional to the amount of bovine decorin coated, with the lowest concentration of 0.1 μg/ml. In contrast, 6B6 did not recognize biglycan (Fig. 4A). To examine whether 6B6 recognizes decorin in solution, we attempted to establish sandwich ELISA, using 6B6 and Ly111, which recognizes chondroitin-4-sulfate (*28*). Microtiter plates were first coated with 6B6. After blocking, solutions containing different concentrations of decorin were added, and biotinylated Ly111 was added to detect decorin sandwiched by both 6B6 and Ly111. The degree of binding was proportional to the amount of added bovine decorin. These results of ELISA indicate that 6B6 recognizes decorin in both solid-phase and solution.

DISCUSSION

In this report, we present the evidence that the monoclonal antibody 6B6 is specific to decorin core protein. Analyses using recombinant bacterial polypeptides revealed that the epitope resides within a stretch of amino acid residues 50–65, termed the N-terminal cysteine cluster region of decorin. Immunoblot analysis demonstrated that the antibody recognizes the primary structure of human, mouse, and bovine decorin core protein. Enzyme-linked im-munosorbent assay (ELISA) revealed that the antibody recognizes decorin both in solid-phase and in solution. We also re-examined the conditions 6B6 for immunostaining of normal and diseased skin tissues with 6B6.

6B6 was raised against small a dermatan sulfate proteoglycan partially purified from human ovarian fibroma capsule by DEAE-Sepharose chromatography and gel filtration (*18*). We have focused the epitope on the primary amino acid sequence of residues 50–65 of decorin, a member of class I SLRPs (*29*). Cross-reactivity among human, mouse, and bovine indicates that the epitope sequence could be narrowed down to the stretch of amino acid residues 57–65 (Fig. 2C). Although biglycan contains a similar sequence in the Cys cluster region, 6B6 failed to recognize biglycan, suggesting that the sequence, RCQ, is essential for the recognition. In earlier studies, series of anti-decorin antibodies with various recognition sites have been produced. Five monoclonal antibodies, 7B1, 5D1, 3B3, 6D6, and 1XA, have been reported whose epitope regions were mapped to different primary sequences of decorin by the reactivities with cyanogens bromide fragments of the core protein and synthetic peptides (*14*). Fisher *et al.* produced a series of polyclonal antibodies, the LF series, using recombinant decorin protein and specific oligopeptides (*12*). Because of difficulty of synthesizing Cys-containing peptides, they chose peptide sequences without Cys, and added Cys to the carboxy-terminus of the sequence. 6B6 is valuable because no other anti-decorin antibody recognizes the Cys cluster region.

Recent studies have revealed that both decorin and biglycan bind Zn^{2+} and that the N-terminal Cys cluster has the ability to bind two Zn^{2+} ions per core protein, although the spacing of the Cys residues is different from that of previously reported Zn^{2+}-binding motifs (*30*). Although the biological role of their Zn^{2+} binding ability remains unclear, their secondary structure may be changed by Zn^{2+} binding. Decorin and biglycan in tissues could serve as Zn^{2+} storage pools, from which the metal ions could be released to proteins that require Zn^{2+} for their stability and functions. 6B6 may serve as a functional antibody that may interrupt Zn^{2+}-binding.

We have demonstrated that 6B6 is useful for immunostaining. Among anti-decorin antibodies, 6D6 and LF-30 have been used for immunostaining, their epitope regions lying within residues 171–329, and 5–17 of the human decorin, respectively. Our observation that the mass containing amorphous material in solar elastosis was not stained with 6B6 is consistent with the previous data using LF-30 (*12*). Immunohistochemical studies of keloids using a rabbit anti-decorin anti-sera (*31*) or LF-30 (*27*) have shown that decorin expression in dermis remained unaltered. In hypertrophic scar, immunohistochemistry using 6D6 has shown little staining for decorin in early-stage samples, much stronger staining in mid-stage, and staining similar to normal skin in the late stage (*25*). Our finding that 6B6 weakly stained central dense and surrounding bundles of one-year-old keloid nodules is inconsistent with immunohistochemical patterns of mid- and late-stages of hypertrophic scar by 6D6 and those by rabbit anti-sera. These differences of staining patterns may partly be due to the different epitopes of the antibodies. It is intriguing that the intensity of immunostaining with 6B6 was unaltered by any treatment so far as examined, whereas chondroitinase ABC-treatment significantly enhanced the signal on dot blot and immunoblot analyses. Previous immunohistochemical studies have demonstrated that pretreatment does not affect the intensity of staining in normal tissues (*19*). These results suggest that the glycosaminoglycan chain of decorin may be integrated and the Cys cluster region oriented outside of the fibrils. Immunostaining with other monoclonal antibodies such as 6D6 and LF-30 may allow a comparative analysis to reflect differences in *in vivo* integration of decorin in the extracellular matrix.

As decorin is involved in inflammation and fibrosis such as repair of postoperative injury and glomerulosclerosis, regulating TGF-β activity in diseased states, it may be a good marker to monitor these diseases. Indeed, wound fluid after mastectomy has been shown to contain higher levels of dermatan sulfate proteoglycans (*32*). Quantitation of decorin in urine and plasma has demonstrated that the concentration of plasma decorin is less than 1 ng/ml in the normal state and approximately 3.2 ng/ml in advanced diabetic nephropathy. It is noteworthy that the output of urinary TGF-β1-decorin complex was approximately 24 ng/24 h in normal subjects, and 160 ng/24 h in diabetic nephropathy, respectively (*33*). As these data were obtained by partial purification of dermatan sulfate proteoglycan with DEAE-gel and semi-quantitative immunoblot analysis, decorin core protein without dermatan sulfate and partially digested core protein fragments were lost. Although the sensitivity of the ELISA presented here is much lower than the decorin concentrations in serum, urine, and exudates, the fact that the antibody recognizes decorin core protein in both solid-phase and solution suggests that 6B6 may be useful for direct quantitation without pretreatment or purification.

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