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Regulation of Intracellular Decorin via Proteasome Degradation in Rat Mesangial Cells

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ABSTRACT

Decorin (DCN) is a member of small leucine-rich proteoglycan family that neutralizes the bioactivity of transforming growth factor-beta1 (TGF-β1). It has been proven to be a promising anti-fibrotic agent to treat glomerulonephritis. But the underlining mechanism for regulating and degrading intracellular DCN is still not fully understood. In this study, we investigated the roles of ubiquitination in the regulation of cytoplasmic DCN metabolism in rat mesangial cells (MC) by immunoprecipitation and Western blot. The results showed that a proportion of cytoplasmic DCN was ubiquitinated in normal MC and was enhanced in *N*-glycosylation inhibitor (tunicamycin)-treated MC. After being treated with the proteasome inhibitor MG132, ubiquitinated DCN accumulated and displayed a prolonged half-life, accompanied by decreased TGF-β1 expression and reduced collagen IV mRNA level in MC. This study demonstrated that the stability and function of cytoplasmic DCN can be regulated by ubiquitin-proteasome system (UPS) in MC, which implies that regulating the ubiquitination and degradation of DCN might be a novel approach for modulating MC bioactivity. J. Cell. Biochem. 111: 1010–1019, 2010.

KEY WORDS: DECORIN; PROTEASOME DEGRADATION; GLYCOSYLATION; MESANGIAL CELL; TGF-B1

Decorin (DCN) is a member of the small leucine-rich proteoglycan family [Hocking et al., 1998; Iozzo, 1999]. Its core protein interacts with extracellular matrix (ECM) components, growth factors, and plasma membrane-located receptors. For instance, it is well known that DCN has the ability to form complexes with transforming growth factor-beta1 (TGF-β1) [Border et al., 1992], and plays a key role in the development of fibrosis and sclerosis of various tissues [Asakura et al., 1999; Häkkinen et al., 2000; Dudás et al., 2001; Schaefer et al., 2002]. DCN can neutralize the prosclerosic effect of TGF-β1 and acts as a natural regulator of TGF-β1 and inhibits the development of glomerulonephritis and glomerulosclerosis, in which TGF-β1 is overproduced. In rat anti-thy1.1 glomerulonephritis, administration of DCN could alleviate ECM accumulation and proteinuria [Border et al., 1992]. Up-regulation of DCN by DCN transfection in human mesangial cells (MC) and rat MC down-regulates TGF-β1 mRNA and decreases expression of collagen IV and fibronectin [Costacurta et al., 2002]. Using the HVJ-liposome method to transfer a DCN plasmid vector into rat skeletal muscle, Isaka showed the result with increased amounts of DCN protein in the skeletal muscle and in the kidney and a marked therapeutic effect of the protein on renal fibrosis induced by glomerulonephritis [Isaka et al., 1996]. In our previous study, the DCN-transfected cloned MC was transferred to rat glomeruli of the anti-thy1.1 glomerulonephritis model by a left renal artery injection. Over-expression of DCN in diseased glomeruli, resulted in remarkably reducing TGF-β1 activity, suppressing proliferation of MC and exhibiting a therapeutic effect on progression of glomerulonephritis [Wang et al., 2003, 2005]. Moreover, DCN may also interact with epidermal growth factor receptor (EGFR) and suppress the growth of tumor cells [Moscatello et al., 1998; Iozzo et al., 1999; Csordás et al., 2000; Santra et al., 2002; Hu et al., 2009]. DCN over-expression can induce cell growth inhibition and apoptosis of MC in vitro [Wu et al., 2008].

Because of these properties, it has been suggested that DCN is an important factor in mediating bioactivity of TGF-β1 in glomerulonephritis. Therefore, it is evident that the regulation of DCN

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stability and activity are of great physiological importance. The metabolism of extracellular DCN has been studied most intensively in cultured fibroblasts, in which DCN represents the major proteoglycan species and is secreted into the culture medium. Fibroblasts and other mesenchymal cells are known to efficiently internalize DCN by several receptors-mediated endocytosis such as low-density lipoprotein receptor [Truppe and Kresse, 1978; Hausser et al., 1992] or EGFR [Feugaing et al., 2007], then followed by targeting to the lysosomal degradation [Brandan et al., 2006; Feugaing et al., 2007]. However, the mechanisms for intracellular DCN degradation are still not fully understood. Since the inhibitor of proteasome MG132 could increase the level of cytoplasm DCN in ovarian tumors [Nash et al., 2002], it is supposed that the degradation of intracellular DCN may be regulated by the ubiquitin-proteasome system (UPS) in addition to lysosomal proteolysis.

It is now appreciated that UPS plays a significant role in regulating turnover and function of different proteins involved in many cellular processes. It is recognized that the level and function of proteins could be reduced or enhanced via controlling this pathway, consequently lead to a change of cellular activities [Passmore and Barford, 2004]. Therefore, the clinical importance of the UPS is rapidly expanding. For example, dysfunction of proteasome may contribute to neurological diseases, such as Alzheimer diseases and Parkinson's disease [Ardley et al., 2004; Layfield et al., 2005]. Blocking the ubiquitination of p53 by Herpesvirus-associated ubiquitin-specific protease (HAUSP), a specific deubiquitinating enzyme of p53, has effects of regression of cancer growth [Li et al., 2002].

The aim of the present study is to explore roles of ubiquitination in the stability of intracellular DCN, factors that control the process, and possible effects of the process on the function of DCN. Our study demonstrated that degradation of DCN is regulated by the UPS in MC in vitro. Inhibition of *N*-glycosylation enhances the ubiquitination and degradation of DCN, and accumulation of DCN by MG132 treatment decreased expression of TGF-β1 and reduced the mRNA level of collagen type IV.

MATERIALS AND METHODS

ANTIBODIES AND REAGENTS

Mouse anti-DCN monoclonal antibody was from R&D Systems (Minneapolis, MN). Mouse anti-ubiquitin and rabbit anti-TGF-β1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal antibody against β-actin was from Sigma–Aldrich (St. Louis, MO). Peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were from Jackson ImmunoResearch (West Grove, PA). Proteasome inhibitor MG132 (Calbiochem, San Diego, CA), *N*-glycosylation inhibitor tunicamycin (Sigma), and cycloheximide (Beyotime, Haimen, Jiangsu, China) were dissolved in sterile dimethylsulfoxide (DMSO), and lysosome inhibitors NH_4Cl (Calbiochem) were dissolved in sterile distilled water. Protein-A Sepharose CL-4B was purchased from Amersham Biosciences Limited (Piscataway, NJ). Super Signal West Pico stable peroxide solution enhanced chemiluminescence (ECL) system and BCA Protein Assay Reagent Kit were obtained from Pierce Biotechnology (Rockford, IL). Trizol reagent was purchased from Invitrogen Corporation (Carlsbad, CA). All other reagents were from Sangon Biological Engineering Technology & Service Co. Ltd. (Shanghai, China).

MESANGIAL CELL CULTURE

MC were prepared from cortex of male SD rats kidney as previously described [Zhang et al., 1995]. The cells were used between passages 6 and 15 and maintained in DMEM medium containing 10% newborn bovine serum at 37°C in a humidified atmosphere containing 5% CO_2/95% air. MC was treated with different reagents containing 2.5 μM MG132, 20 mM NH_4Cl, 5 μg/ml tunicamycin, and cycloheximide (10 μg/ml).

PROTEIN ISOLATION AND WESTERN BLOTTING ANALYSIS

Cells were scraped off the dishes and pelleted by centrifuging at 1,100 g for 10 min. The cell pellet was lysed in cold cell lysis buffer (pH 7.4) [50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 10% glycerol, 0.2 mg/ml NaN_3, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride]. Proteins were loaded with 5× SDS loading buffer and resolved by SDS–PAGE in a discontinuous (8–12%) gel, transferred onto a PVDF membrane. The membrane was incubated in blocking solution (10% nonfat milk in 1× TBST) at 20–25°C for 1 h, then immunoblotted with DCN monoclonal antibody (1:1,000 dilution), TGF-β1 polyclonal antibody (1:500 dilution), and β-actin monoclonal antibody (1:1,000 dilution). Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL; Pierce Biotechnology). The housekeeping protein β-actin was used as a control.

IMMUNOPRECIPITATION

Cells were harvested and lysed in the TNT buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1% Triton X-100, 10 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, and 1% aprotinin). Identical amounts (1.5 mg of protein) of precleared cell lysates were immunoprecipitated with 1 μg antibodies against either DCN or ubiquitin by being incubated at 4°C for 12 h after adjusting the volumes to 0.5 ml with NET buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P (NP)-40, 1 mM ethylene diamine tetra-acetic acid, 0.25% gelatin, 0.02% sodium azide, 1 mM PMSF, and 1% aprotinin). The immune complexes were precipitated by Protein-A Sepharose CL-4B and washed three times with TNT buffer, once with NET buffer, and once with PBS. Immunoprecipitated proteins were eluted with 5× SDS loading buffer and resolved by standard SDS–PAGE. The following steps were the same as for Western blotting.

DCN siRNA TRANSFECTION

RNAi transfection was performed according to the procedure as recommended by Qiagen Corporation. In brief, shortly before transfection, 0.5×10^6 cells were seeded in 60 mm dishes with 4 ml culture medium containing serum and incubate the cells under 37°C and 5% CO_2 for the short time until transfection. Dilute 256 ng DCN siRNA in 100 μl culture medium without serum. Add 20 μl of HiperFect Transfection Reagent to the diluted siRNA and mix by vortexing. The mixtures were incubated for 5–10 min at 15–25°C. Then the complexes were added by drops onto the cells. Gently swirl

the dishes to ensure uniform distribution of the transfection complexes. Change the medium after 24 h and culture the cells for about 72 h after transfection. Before protein extraction, transfected cells were treated with MG132 (2.5 μM) for the final 20 h of incubation [Awasthi and Wagner, 2006]. The sequences of DCN-siRNA used were: sense: 5′-GGGCGGCAACCCACUGAAATT-3′, anti-sense: 5′-UUUCAGUGGGUUGCCGCCCAG-3′.

TOTAL RNA EXTRACTION AND RT-PCR

Total RNA was extracted from the lysate of MC using Trizol reagent. Reverse transcription (RT) was performed in 25 μl reaction volume containing 2 μg total RNA, 1 μl M-MLV (200 U/μl) reverse transcriptase, and 3 μl Oligo $(dT)_{18}$ (100 μg/μl) (synthesized by Sangon Biological Engineering Technology & Service Co. Ltd). The conditions for polymerase chain reaction (PCR) were 95°C for 12 min, followed by 30 cycles of 95°C for 45 s, 56°C for DCN, 57°C for Col IV, 58°C for TGF-β1 and β-actin for 30 s, and 72°C for 40 s and a final cycle of 72°C for 10 min. The PCR products were electrophoresed in a 1% agarose gel. The PCR primers for DCN were: 5′-TGGCAGTCTGGCTAATGT-3′ and 5′-ACTCACGGCAGTG-TAGGA-3′; for Col IV 5′-TCCTTGTGACCAGGCATAGT-3′ and 5′-TTGAACATCTCGCTCCTCTC-3′; for TGF-β1 5′-TGCGCCTGCAGA-GATTCAAG-3′ and 5′-AGGTAACGCCAGGAATTGTTGCTA-3′; and for β-actin: 5′-AGGATGCAGAAGGAGATTACTGC-3′, and 5′-AAAACGCAGCTCAGTAACAGTGC-3′. The sizes of the PCR products were 199, 592, 60, and 220 bp for DCN, Col IV, TGF-β1, and β-actin, respectively.

IMMUNOSTAINING OF DCN

MC were seeded onto 18 mm^2 glass coverslips in 35-mm dishes and fixed with a mixture of methanol and ethyl alcohol at a ratio of 3:1 for 15 min at 4°C. Fixed cells were then treated with very low concentration of trypsin (0.05% trypsin diluted 1,000-fold) (Sigma–Aldrich) for 15 min to increase membrane permeability. After washing thrice with PBS, DCN antibody and then fluorescence TRITC-conjugated secondary antibody were added onto the glass coverslips and observed using fluorescence microscopy.

FLOW CYTOMETRY ANALYSIS

Analysis of the cell cycle was performed by flow cytometry analysis. MC were released from culture plates by 0.05% trypsinization, and treated with citrate buffer for 30 min before being incubated with RNase for few minutes followed by propidium iodide (PI) for 15 min. PI is capable of binding and labeling DNA, which makes it possible to evaluate the stages of the cell cycle by flow cytometry [Riccardi and Nicoletti, 2006] and analyze by CellQuest software (BD Bioscience, Mississauga, Ontario, Canada) in Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Each group of MC was repeated for three times.

STATISTICAL ANALYSIS

The experiments were all repeated at least three times in independent biological replicates. Results are expressed as means ± SD. One-way analysis of variance (ANOVA) was used to assess the differences between multiple groups, and least significant difference (LSD) was used as the post-test to analyze difference between any two means. $P < 0.05$ was designated as statistically significant. The density of the bands on the Western blots was quantified by a densitometry and analyzed using Gel-Pro Analyzer from Media Cybernetics (Bethesda, MD).

RESULTS

DCN IS UBIQUITINATED IN MC

To investigate whether DCN is ubiquitinated in MC, cell lysate was immunoprecipitated with an anti-DCN antibody and blotted with the anti-ubiquitin antibody. As shown in Figure 1A (the top bands), Poly-ubiquitination of DCN appeared in smear pattern in MC.

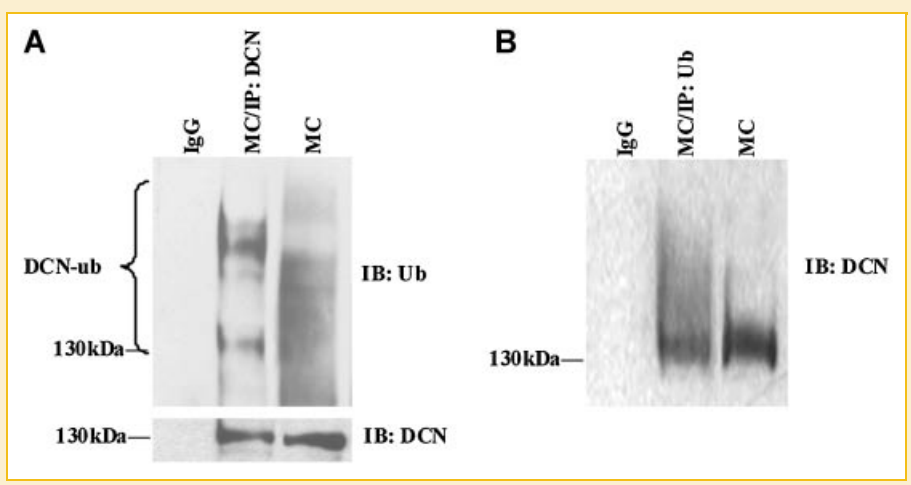

Fig. 1. Analysis of DCN ubiquitination in MC. A: Cell lysates were subjected to immunoprecipitation with anti-DCN antibody (IP: DCN) followed by immunoblotting with an anti-ubiquitin antibody (top bands). The polyubiquitination of DCN was observed with smear pattern in the MC/IP lane. The bands at the bottom were the same blot re-probed by the anti-DCN antibody to confirm the specificity of ubiquitin combining to the DCN. B: By a reciprocal approach, the proteins were immunoprecipitated with an anti-ubiquitin antibody (IP: Ub) and were immunoblotted with an anti-DCN antibody, which also showed that the DCN was co-binded with polyubiquitin. Lysate from MC (MC lane) used as a positive control and isotype-match nonspecific IgG (IgG) used as a negative control, respectively. Results are representatives of at least three replicated experiments.

Reprobing the same membrane with anti-DCN antibody confirmed the specificity of ubiquitin combining to the DCN (Fig. 1A, the bottom band). By a reciprocal approach, DCN was detected with anti-DCN antibody after anti-ubiquitin immunoprecipitation (Fig. 1B). The result indicated that a proportion of DCN is ubiquitinated in MC.

INHIBITION OF THE PROTEASOME INCREASES DCN EXPRESSION AND ITS HALF-LIFE

To clarify whether the ubiquitinated DCN is degraded by the proteasome, MC were treated with the proteasome inhibitor MG132 (2.5 μM) for 10 and 20 h. Compared to normal MC, the DCN protein level significantly increased (Fig. 2C), and its ubiquitinated form also accumulated in MC (Fig. 2A). Meanwhile, when the cells were treated with the lysosome inhibitor NH_4Cl (20 mM) for 20 h, both the content of DCN and its ubiquitinated form in MC were not changed (Fig. 2B,D). The results demonstrated that the ubiquitinated DCN is targeted by proteasome for degradation in MC.

Moreover, the stabilities of DCN protein in the control MC and the MG132-treated MC were assessed by time-course Western blot as previously described by Rizzi [Rizzi et al., 2009; Minegishi et al., 2005]. The cells were treated with 10 μg/ml of cycloheximide, a protein synthesis inhibitor, with or without 2.5 μM MG132. Cell lysates were prepared after 0, 6, 12, 24, and 48 h. The result showed that the half-life of DCN was about 12 h with DCN 50% degradation in single cycloheximide-treated MC (Fig. 3A,C) and was prolonged with MG132 treatment (Fig. 3B,C). Meanwhile, by immunofluorescence microscopy, we demonstrated that the fluorescence staining of cytoplasmic DCN in MC was weakened by cycloheximide treatment, while the staining was intensified by both cycloheximide and MG132 treatment (Fig. 3D). The data indicated that the half-life of cytoplasm DCN is dramatically increased by blocking the proteasome.

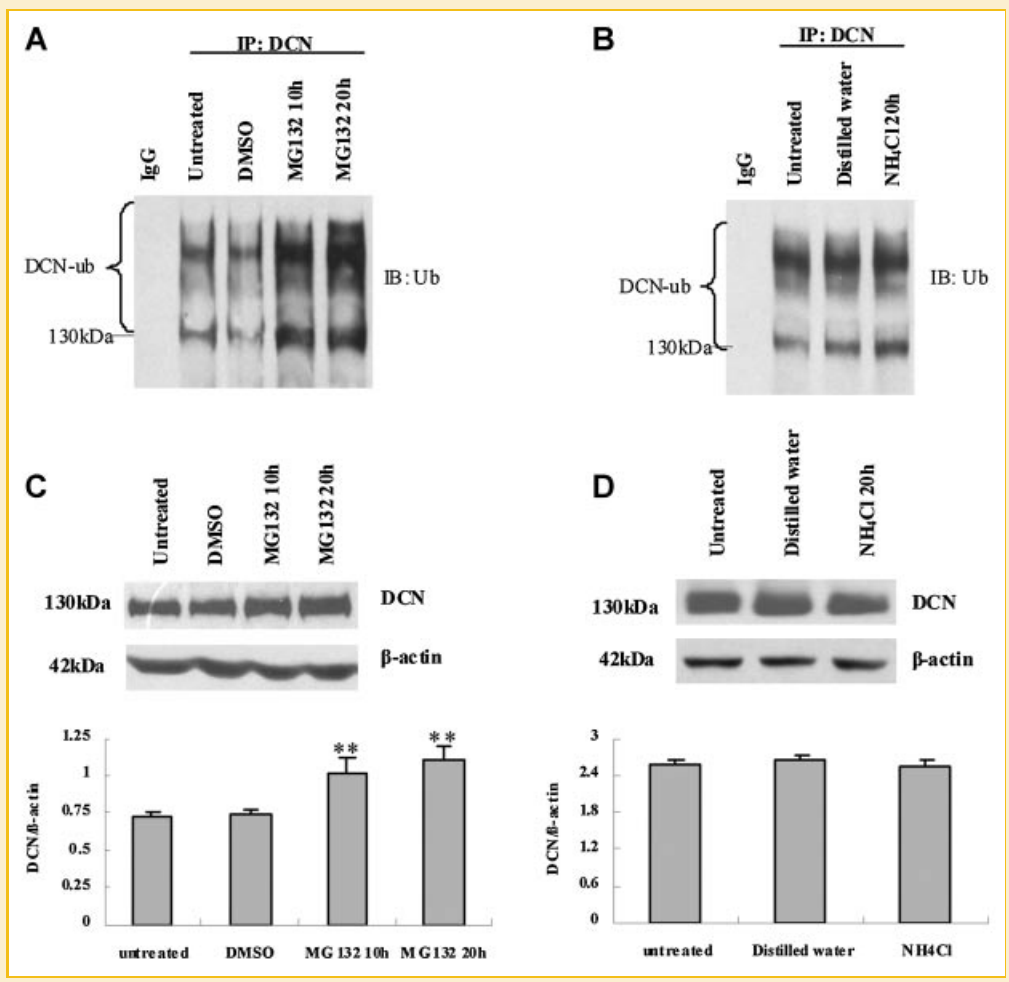

Fig. 2. Effects of the inhibitors of proteasome and lysosome on degradation of ubiquitinated DCN. MC were treated with either the proteasome inhibitor MG132 (2.5 μM) for 10 or 20 h (A,C), or the lysosome inhibitor NH_4Cl (20 mM) for 20 h (B,D). A,B: Cell lysates were immunoprecipitated with anti-DCN antibody, followed by immunoblotting with an anti-ubiquitin antibody. The ubiquitinated DCN was accumulated in MG132-treated MC, no changes in NH_4Cl-treated MC. C,D: Total protein of DCN was examined by Western blot. The densitometric analysis shows that the protein level of DCN increased significantly in MC treated with MG132 for 10 and 20 h compared with the untreated cells ($^{**}P < 0.01$), but there was no change of DCN level in NH_4Cl treating MC. The IgG used as a negative control for IP. MC with DMSO treatment (DMSO) or sterile distilled water treatment (distilled water) was used as negative controls. β-Actin was used as an internal control. Results are representative of at least three replicated experiments.

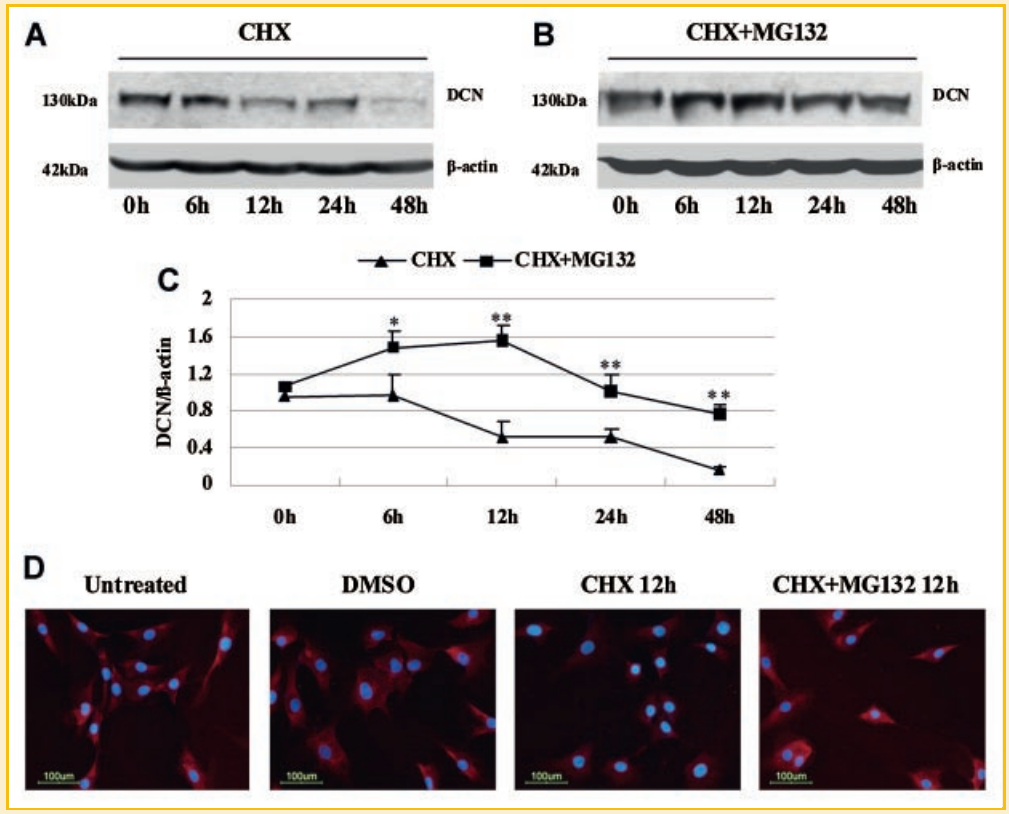

Fig. 3. Effects of proteasome inhibitor on DCN half-life. A: MC were treated with cycloheximide (CHX, 10 μg/ml), and (B) CHX (10 μg/ml) with MG132 (2.5 μM) simultaneously for 0, 6, 12, 24, and 48 h. Total protein of DCN was examined along the time course by Western blot. β-Actin was used as a loading control. The half-life of DCN was about 12 h in single CHX-treated MC and was prolonged with MG132 treatment. C: Quantitative graph obtained by densitometric scanning of DCN protein bands of three replicated experiments. Differences among CHX groups and CHX with MG132 groups were highly significant at different times points ($^{*}P < 0.05$, $^{**}P < 0.01$). D: Fluorescence immunostaining of cytoplasm DCN was examined by anti-DCN antibody in untreated MC, DMSO-treated MC, CHX-treated 12 h MC and CHX with MG132-treated 12 h MC. Blue Hoechst 33258 staining as the nuclear counterstain. Results are representative of at least three replicated experiments.

INHIBITION OF GLYCOSYLATION INCREASES DCN UBIQUITINATION AND DEGRADATION

Since *N*-linked oligosaccharide regulates DCN stability [Seo et al., 2005], we investigated whether ubiquitination of DCN is influenced by its glycosylation state. After being treated with tunicamycin, an *N*-glycosylation inhibitor, DCN ubiquitination increased in immunoprecipitation, while the DCN protein is decreased in MC on Western blot (Fig. 4A,B). Compared with normal MC, the protein level of DCN significantly decreased in tunicamycin treatment MC. In addition, the DCN half-life in MC treated with tunicamycin was measured using the same protocol of time-course Western blot. The half-life of DCN in cells with cycloheximide and tunicamycin treatment was about 6 h, which was shorter compared to 12 h in cycloheximide-treated cells (Fig. 5A–C). Moreover, when we treated MC with tunicamycin and MG132 at the same time for 20 h, DCN protein, which was decreased after exposure to tunicamycin, reincreased in treated cells (Fig. 4C). These data provide a clear link between glycosylation, ubiquitination, and degradation of DCN.

EFFECT OF UBIQUITINATION ON DCN FUNCTION

Our previous study showed that the up-regulation of DCN expression by DCN transfection decreased the mRNA level of collagen type IV and protein level of TGF-β1 in MC [Wang et al., 2003]. Here we continued to study whether the function of DCN is influenced by the regulation of its ubiquitination. With increasing protein level of DCN by being exposed to MG132, mRNA level of collagen type IV was decreased in MC while DCN mRNA expression did not change (Fig. 6A), then the mRNA level of collagen type IV was reincreased when the cells exposed to DCN siRNA following MG132 treatment (Fig. 6B). It implied that the proteasome blockade by MG132 treatment could only increase the protein level of DCN without influencing its transcription, subsequently leading to decreased collagen type IV mRNA expression. In addition, down-regulation of TGF-β1 protein expression was also observed when cells was exposed to MG132, while the mRNA level of TGF-β1 did not change (Fig. 7A), and it increased again when we treated MC with DCN siRNA following MG132 treatment (Fig. 7B). It indicated that the accumulation of DCN though proteasome blockade could, at least partially, influence the protein level of TGF-β1 and mRNA level of collagen type IV. Furthermore, Flow cytometry analysis showed that the MC treated with MG132 were arrested in G_0/G_1 stage, which concurred with our previous data that DCN over-expression could cause suppression of MC proliferation [Wu et al., 2008] (Table I).

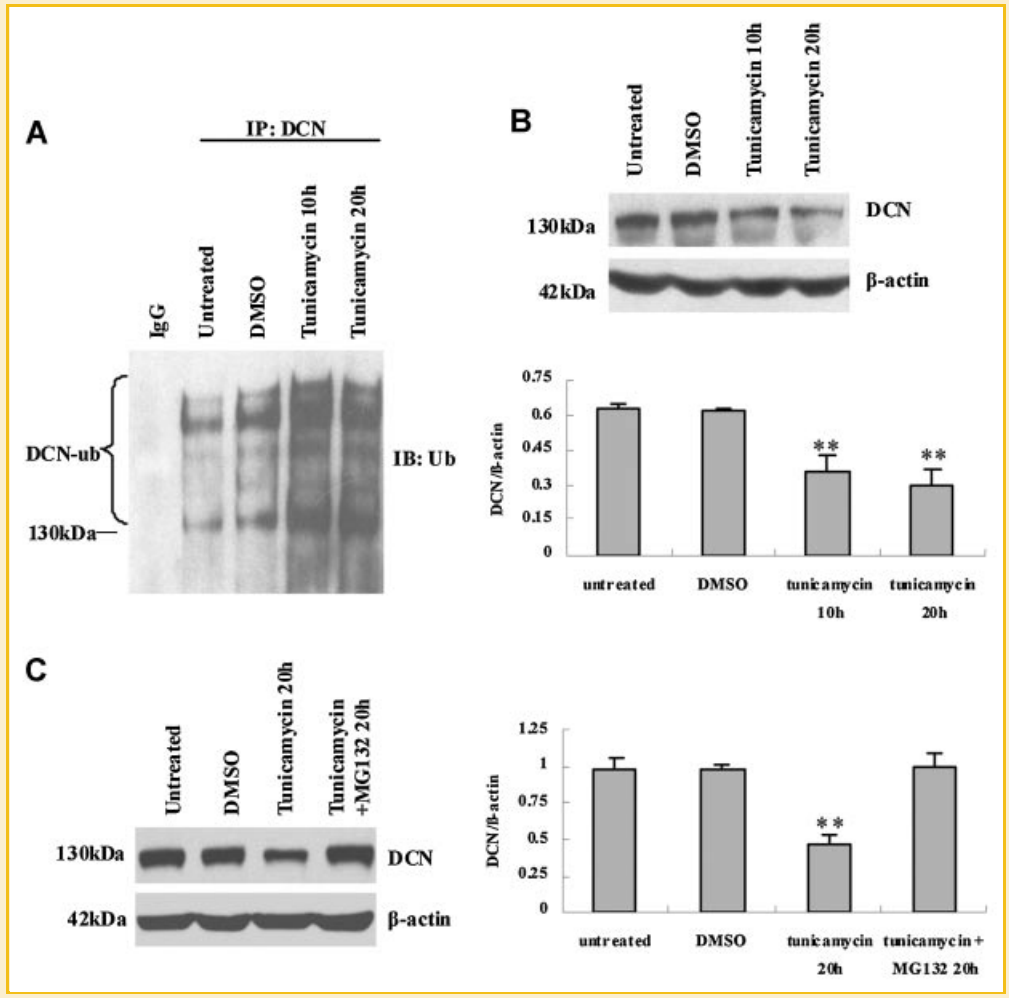

Fig. 4. Effect of tunicamycin on ubiquitination of DCN. MC was treated with tunicamycin (5 μg/ml) for 10 or 20 h. A: Equal amounts of cell lysates were immunoprecipitated with anti-DCN antibody, followed by immunoblotting with an anti-ubiquitin antibody. The result shows that the ubiquitinated DCN is increased in tunicamycin-treated MC. The IgG used as a negative control for IP. DMSO MC was used as a negative control. B: Total protein of DCN was examined by Western blot, and the protein level of DCN is decreased in tunicamycin-treated MC. C: MC were treated with tunicamycin along with or without MG132 for 20 h. Protein level of DCN was assayed by Western blot. Differences between tunicamycin treating 20 h MC and untreated MC were highly significant ($^{**}P < 0.01$). β-Actin was used as a internal control. Results are representative of at least three replicated experiments.

DISCUSSION

In the current study, we investigated the roles of ubiquitination with regard to stability and function of DCN. DCN is an important proteoglycan that neutralizes the bioactivity of TGF-β1 and is considered as a promising therapeutic agent to various mesangial proliferative glomerulonephritis [Yamaquchi et al., 1990; Border and Nobel, 1997; Costacurta et al., 2002]. We confirmed that a proportion of intracellular DCN can be degraded and removed via UPS under normal condition in MC. The DCN degradation can be inhibited by the proteasome inhibitor MG132, potentially enhancing the function of the protein in MC. The inhibition of DCN glycosylation by tunicamycin enhances the ubiquitination and degradation of DCN. These results indicate that modification of DCN via ubiquitination can affect the stability and activity of this proteoglycan, suggesting the potential for enhancing the anti-TGF-β1 effect of DCN by modulating the UPS.

Protein ubiquitination and subsequently proteasomal degradation may contribute to the degradation of abnormal, denatured, and misfolded proteins and normal turnover of many intracellular proteins [Ciechanover et al., 2000; Jiang and Beaudet, 2004]. Ubiquitination is involved in the regulation of various cellular processes including cell cycle, differentiation and development, DNA repair, immune and inflammatory response, and oncogenesis [Peters et al., 1998; Liu et al., 2007]. To date, many proteins that are known to be subjected to ubiquitin conjugation are cytoplasmic, nuclear, or membrane proteins. Our results showed that cytoplasmic DCN in MC may also be conjugated to ubiquitin and degraded by proteasome. Treated with the proteasome inhibitor MG132, the ubiquitinated form of DCN increased and half-life of DCN was prolonged in MC. Our findings are consistent with the observations reported by Nash that ovarian cancer cells treated with MG132 showed strong perinuclear immunohistochemical staining of DCN and increased DCN-specific bands in Western blot

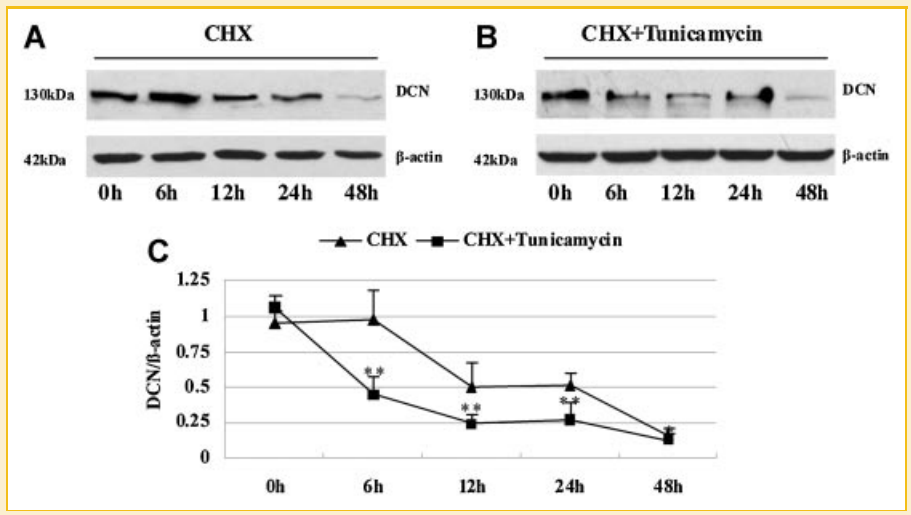

Fig. 5. Effects of the inhibitor of *N*-glycosylation on half-life of DCN. A: MC were treated with CHX (10 μg/ml), and (B) CHX (10 μg/ml) with tunicamycin (5 μg/ml) simultaneously for 0, 6, 12, 24, and 48 h. Total protein of DCN was examined with time course by Western blot. The half-life of DCN in cells with CHX and tunicamycin treatment was about 6 h, which was significantly shorter than the cells treated with CHX for 12 h ($^{**}P < 0.01$). β-Actin was used as a loading control. C: Quantitative graph obtained by densitometric scanning of DCN protein bands of three replicated experiments.

analysis [Nash et al., 2002]. Moreover, the level of DCN did not change following the treatment with lysosome inhibitor NH_4Cl in MC. The data suggest that the metabolic pathway of intracellular DCN is different from that of extracellular secreted DCN, since it is reported that the extracellular DCN is internalized by endocytosis and degraded by lysosomes in fibroblast [Brandan et al., 2006; Feugaing et al., 2007].

The UPS is known to be related to many protein modifications including phosphorylation, hydroxylation, glycosylation, deacetylation, aminoacylation, and oxidation. DCN is also a glycoprotein

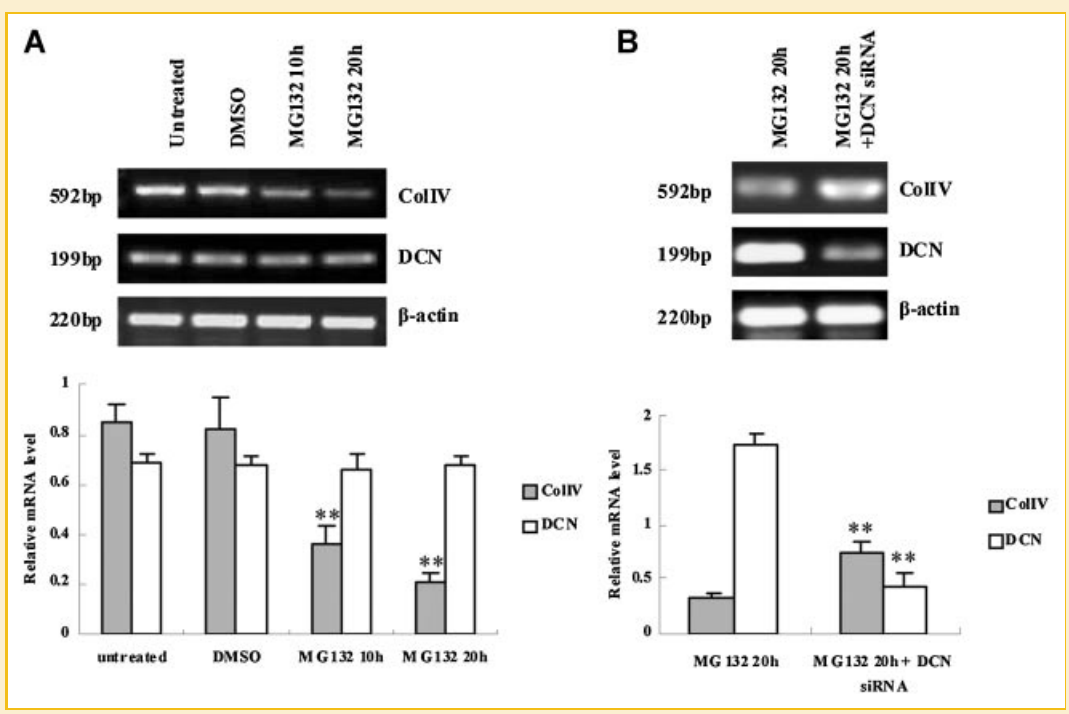

Fig. 6. Effects of MG132 on mRNA expression of Col IV and DCN in MC. MC were treated with MG132 (2.5 μM) for 10 or 20 h (A) or DCN siRNA following MG132 20 h treatment (B). MC with DMSO treatment (DMSO) was used as a negative control. mRNA level of Col IV and DCN was examined by RT-PCR. The result showed that mRNA level of Col IV in MG132-treated MC was significantly lower than untreated MC while the DCN mRNA had no changes, and it was reincreased in DCN siRNA following MG132-treated MC ($^{**}P < 0.01$). β-Actin was used as an internal control. Results are representative of at least three replicated experiments.

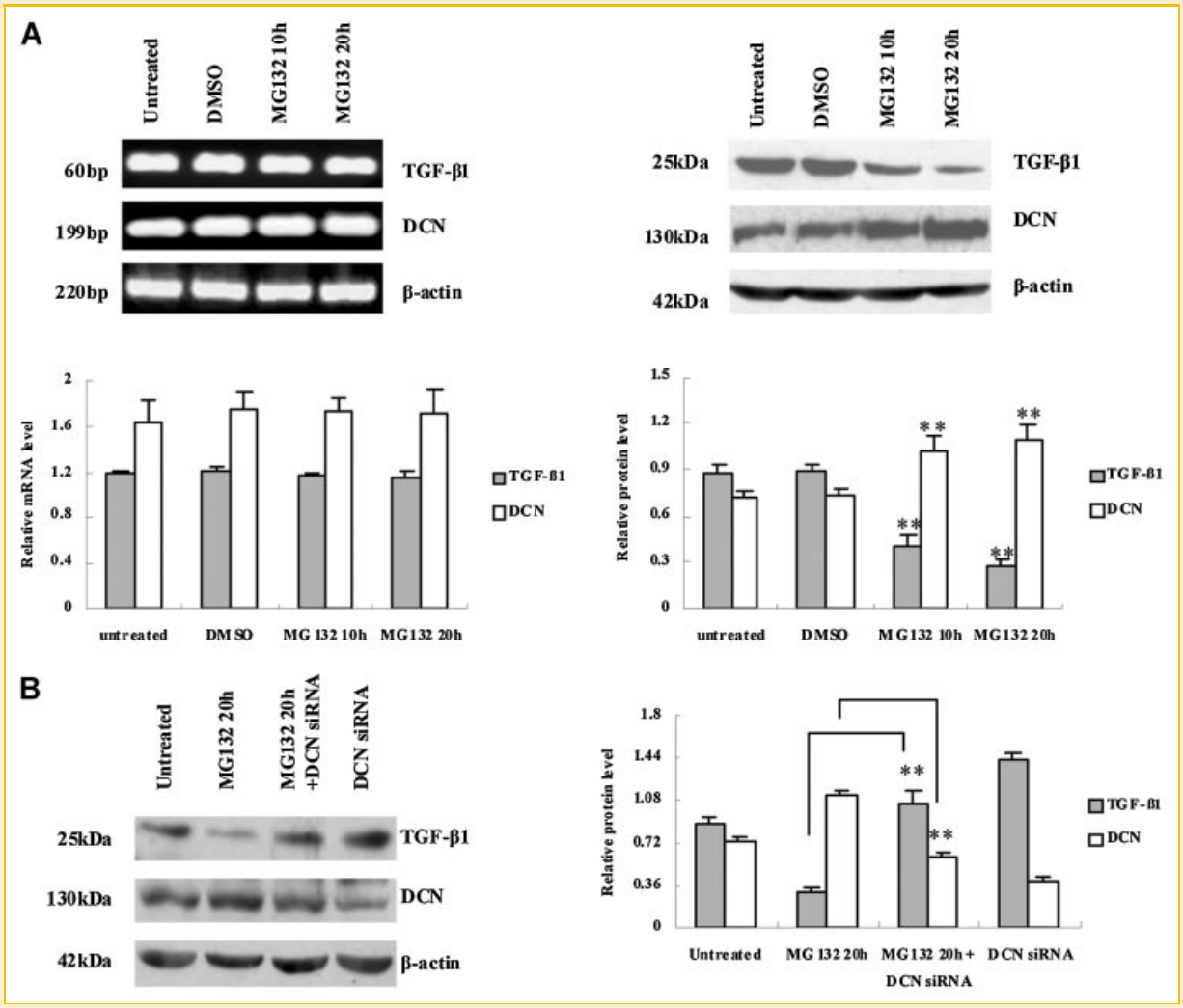

Fig. 7. Effects of MG132 on TGF-β1 expression in MC. MC were treated with MG132 (2.5 μM) for 10 or 20 h (A) or DCN siRNA following MG132 20 h treatment (B). MC with DMSO treatment was used as a negative control. mRNA level and protein level of TGF-β1 and DCN were examined by RT-PCR and Western blot, respectively. The protein expression of TGF-β1 was significantly lower with increasing DCN protein level in MG132-treated MC than that in untreated MC, and it was reincreased in DCN siRNA following MG132-treated MC ($^{**}P < 0.01$). β-Actin was used as an internal control. Results are representative of at least three replicated experiments.

composed of a protein core, a single GAG side chain and up to three *N*-linked oligosaccharides [Sawhney et al., 1991]. The *N*-linked oligosaccharide moieties of these proteins play a crucial role for protein folding and secretion [Helenius, 1994]. It is known that a DCN core protein mutant devoid of *N*-linked oligosaccharide attachment sites is not be secreted by CHO cells [Seo et al., 2005], and such mutant glycoproteins with improper folding are either retained in the endoplasmic reticulum lumen until they become properly folded or retro-translocated into the cytosol and degraded by the 26S proteasome [Ellgaard et al., 1999; Römisch, 2005; Moremen and Molinari, 2006]. In this respect, the mechanism of ER-associated degradation through UPS remains to be elucidated. Recently, there are accumulating studies that focus on proteasome degradation of glycoproteins [Meusser et al., 2005]. For example, during ER stress caused by tunicamycin, the unglycosylated and two immaturely glycosylated calcium-sensing receptors (CaR) are sensitive to ubiquitination and degradation in HEK293 cells [Huang et al., 2006]. ER stress was associated with aggregation and ubiquitination of alpha-actinin-4 K256E in glomerular epithelial cell (GEC), which was involved in the GEC injury in disease of focal segmental glomerulosclerosis [Cybulsky et al., 2009]. The inhibition

TABLE I. Flow Cytometry Analysis of the Effects of MG132 on MC Cell Growth

	Untreated	DMSO	MG132 (10 h)	MG132 (20 h)
G_0/G_1 (%)	60.13 ± 4.71	59.96 ± 3.73	80.11 ± 0.52**	82.19 ± 1.24**
G_2/M (%)	12.01 ± 2.14	12.69 ± 1.87	10.34 ± 1.13	10.64 ± 1.57
S (%)	22.45 ± 3.39	21.63 ± 4.06	8.56 ± 1.51**	7.08 ± 1.33**

MC was treated with MG132 (2.5 μM) for 10 or 20 h. MC with DMSO treatment was used as a negative control. Cells were then released from culture plates by trypsinization, and treated with citrate buffer. The cell growth assay of MC by flow cytometry was done and were expressed as mean ± SEM. $^{**}P < 0.01$, compared to untreated MC. Results are representative of at least three replicated experiments.

of *N*-glycosylation by tunicamycin for the MDR1 product P-glycoprotein can increase its degradation via ubiquitination and decrease drug resistance in cancer cells [Zhang et al., 2004]. In this work, we also showed that inhibition of *N*-glycosylation of DCN by tunicamycin increased its ubiquitinated forms and decreased its protein level. These results may shed some lights on the mechanism of glycoprotein degradation under ER stress.

The regulation of the stability of proteins by ubiquitination may lead to important functional consequences. So far, increasing evidence indicates that protein ubiquitination not only promotes the degradation of specific proteins in most of cellular processes, but also serves as a novel target for modulating the function of important proteins in some physiological and pathological states. It is recognized that identification of specific regulators to enhance or weaken the ubiquitination process for special protein may be an important therapeutic strategy for treating some diseases. For example, bortezomib (Velcade), the first therapeutic proteasome inhibitor for treating multiple myeloma and mantle cell lymphoma [Voorhees et al., 2003; Adams and Kauffman, 2004], can affect both growth and apoptotic signaling pathways by inhibiting proteasome function in tumor cells. It can inhibit the bioactivity of NF-κB via blocking the ubiquitination of IKKB [Chauhan et al., 2008]. Another example, HAUSP has been identified as a specific deubiquitinating enzyme of p53, it strongly blocks the ubiquitination of p53 and enhances the function of p53 in vitro and in vivo [Li et al., 2002]. Therefore, this might be a novel approach to regulate the bioactivity of certain target protein through the UPS in certain disease conditions.

In this study, we performed preliminary work to confirm the UPS regulation of intracellular DCN metabolism. We also found that MG132 treatment, leading to accumulation of DCN, was associated with decreased TGF-β1 protein expression and Col IV mRNA expression in MC, which was confirmed by DCN siRNA treatment with reverse effects. It is consistent with the result of our previous work that the over-expression of DCN with gene transfection could cause decreased protein levels of TGF-β1 and mRNA levels of Col IV in MC [Wang et al., 2003]. It revealed that blockade of proteasome degradation of DCN by MG132 could down-regulate of ECM synthesis in MC. However, the mechanism of the intracellular DCN modulation is still not fully elucidated. We speculate that when DCN accumulates in MC after MG132 treatment, it may enhance the effects of DCN via increased secretion, and it may also play critical role to modulate biological activity of the host cell itself, such as over-expression of DCN may cause apoptosis and growth inhibition of MC [Wu et al., 2008]. The present results demonstrate the possibility of enhancing the bioactivity of DCN via UPS. However, because the proteasome inhibitor MG132 is not a special regulator for DCN, now we are currently in the process to further explore some specific inhibitors for DCN degradation through UPS to bring on the prospective therapeutic effects in renal diseases.

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REFERENCES

Adams J, Kauffman M. 2004. Development of the proteasome inhibitor Velcade (Bortezomib). Cancer Invest 22:304–311.

Ardley HC, Scott GB, Rose SA, Tan NG, Robinson PA. 2004. UCH-L1 aggresome formation in response to proteasome impairment indicates a role in inclusion formation in Parkinson's disease. J Neurochem 90:379–391.

Asakura S, Kato H, Fujino S, Konishi T, Tezuka N, Mori A. 1999. Role of transforming growth factor-β1 and decorin in development of central fibrosis in pulmonary adenocarcinoma. Hum Pathol 30:195–198.

Awasthi N, Wagner BJ. 2006. Suppression of human lens epithelial cell proliferation by proteasome inhibition, a potential defense against posterior capsular opacification. Invest Ophthalmol Vis Sci 47:4482–4489.

Border WA, Nobel NA. 1997. TGF-beta in kidney fibrosis: A target for gene therapy. Kidney Int 51:1388–1396.

Border WA, Noble NA, Yamamoto T, Harper JR, Yamaguchi Y, Pierschbacher MD, Ruoslahti E. 1992. Natural inhibitor of transforming growth factor-beta protects against scarring in experimental kidney disease. Nature 360:361–364.

Brandan E, Retamal C, Cabello-Verrugio C, Marzolo MP. 2006. The low density lipoprotein receptor-related protein functions as an endocytic receptor for decorin. J Biol Chem 281:31562–31571.

Chauhan D, Bianchi G, Anderson KC. 2008. Targeting the UPS as therapy in multiple myeloma. BMC Biochem 9:1–8.

Ciechanover A, Orian A, Schwartz AL. 2000. Ubiquitin-mediated proteolysis: Biological regulation via destruction. Bioessays 22:442–451.

Costacurta A, Priante G, D'Angelo A, Chieco-Bianchi L, Cantaro S. 2002. Decorin transfection in human mesangial cells downregulates genes playing a role in the progression of fibrosis. J Clin Lab Anal 16:178–186.

Csordás G, Santra M, Reed CC, Eichstetter I, Mcquillan DJ, Gross D, Nugent MA, Hajnoczky G, Iozzo RV. 2000. Sustained down-regulation of the epidermal growth factor receptor by decorin. A mechanism for controlling tumor growth *in vivo*. J Biol Chem 275:32879–32887.

Cybulsky AV, Takano T, Papillon J, Bijian K, Guillemette J, Kennedy CR. 2009. Glomerular epithelial cell injury associated with mutant alpha-actinin-4. Am J Physiol Renal Physiol 297:F987–F995.

Dudás J, Kovalszky I, Gallai M, Nagy JO, Schaff Z, Knittel T, Mehde M, Neubauer K, Szalay F, Ramadori G. 2001. Expression of decorin, transforming growth factor-beta1, tissue inhibitor metalloproteinase 1 and 2, and type IV collagenases in chronic hepatitis. Am J Clin Pathol 115:725–735.

Ellgaard L, Molinari M, Helenius A. 1999. Setting the standards: Quality control in the secretory pathway. Science 286:1882–1888.

Feugaing DD, Tammi R, Echtermeyer FG, Stenmark H, Kresse H, Smollich M, Schönherr E, Kiesel L, Götte M. 2007. Endocytosis of the dermatan sulfate proteoglycan decorin utilizes multiple pathways and is modulated by epidermal growth factor receptor signaling. Biochimie 89:637–657.

Häkkinen L, Strassburger S, Kähäri VM, Scott PG, Eichstetter I, Lozzo RV, Larjava H. 2000. A role for decorin in the structural organization of periodontal ligament. Lab Invest 80:1869–1880.

Hausser H, Ober B, Quentin-Hoffmann E, Schmidt B, Kresse H. 1992. Endocytosis of different members of the small chondroitin/dermatan sulfate proteoglycan family. J Biol Chem 267:11559–11564.

Helenius A. 1994. How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum. Mol Biol Cell 5:253–265.

Hocking AM, Shinomura T, McQuillan DJ. 1998. Leucine-rich repeat glycoproteins of the extracellular matrix. Matrix Biol 17:1–19.

Hu Y, Sun H, Owens RT, Wu J, Chen YQ, Berquin IM, Perry D, O'Flaherty JT, Edwards IJ. 2009. Decorin suppresses prostate tumor growth through inhibition of epidermal growth factor and androgen receptor pathways. Neoplasia 11:1042–1053.

Huang Y, Niwa J, Sobue G, Breitwieser G. 2006. Calcium-sensing receptor ubiquitination and degradation mediated by the E3 ubiquitin ligase dorfin. J Biol Chem 281:11610–11617.

Iozzo RV. 1999. The biology of the small leucine-rich proteoglycans. Functional network of interactive proteins. J Biol Chem 274:18843–18846.

Iozzo RV, Moscatello DK, McQuillan DJ, Eichstetter I. 1999. Decorin is a biological ligand for the epidermal growth factor receptor. J Biol Chem 274:4489–4492.

Isaka Y, Brees DK, Ikegaya K, Kaneda Y, Imai E, Noble NA, Border WA. 1996. Gene therapy by skeletal muscle expression of decorin prevents fibrotic disease in rat kidney. Nat Med 2:418–423.

Jiang YH, Beaudet AL. 2004. Human disorders of ubiquitination and proteasomal degradation. Curr Opin Pediatr 16:419–426.

Layfield R, Lowe J, Bedford L. 2005. The ubiquitin-proteasome system and neurodegenerative disorders. Essays Biochem 41:157–171.

Li M, Chen D, Shiloh A, Luo J, Nikolaev AY, Qin J, Gu W. 2002. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. Nature 416:648–653.

Liu CH, Goldberg AL, Qiu XB. 2007. New insights into the role of the ubiquitin-proteasome pathway in the regulation of apoptosis. Chang Gung Med J 30:469–479.

Meusser B, Hirsch C, Jarosch E, Sommer T. 2005. ERAD: The long road to destruction. Nat Cell Biol 7:766–772.

Minegishi N, Suzuki N, Kawatani Y, Shimizu R, Yamamoto M. 2005. Rapid turnover of GATA-2 via ubiquitin-proteasome protein degradation pathway. Genes Cells 10:693–704.

Moremen KW, Molinari M. 2006. N-linked glycan recognition and processing: The molecular basis of endoplasmic reticulum quality control. Curr Opin Struct Biol 16:592–599.

Moscatello DK, Santra M, Mann DM, Mcquillan DJ, Wong AJ, Iozzo RV. 1998. Decorin suppresses tumor cell growth by activating the epidermal growth factor receptor. J Clin Invest 101:406–412.

Nash MA, Deavers MT, Freedman RS. 2002. The expression of decorin in human ovarian tumors. Clin Cancer Res 8:1754–1760.

Passmore LA, Barford D. 2004. Getting into position: The catalytic mechanisms of protein ubiquitylation. Biochem J 379:513–525.

Peters JM, Harris JR, Finley D, editors. 1998. Ubiquitin and the biology of the cell. New York: Plenum.

Riccardi C, Nicoletti I. 2006. Analysis of apoptosis by propidium iodide staining and flow cytometry. Nat Protoc 1:1458–1461.

Rizzi F, Caccamo AE, Belloni L, Bettuzzi S. 2009. Clusterin is a short half-life, poly-ubiquitinated protein, which controls the fate of prostate cancer cells. J Cell Physiol 219:314–323.

Römisch K. 2005. Endoplasmic reticulum-associated degradation. Annu Rev Cell Dev Biol 21:435–456.

Santra M, Reed CC, Iozzo RV. 2002. Decorin binds to a narrow region of the epidermal growth factor (EGF) receptor, partially overlapping but distinct from the EGF-binding epitope. J Biol Chem 277:35671–35681.

Sawhney RS, Hering TM, Sandell LJ. 1991. Biosynthesis of small proteoglycan II (decorin) by chondrocytes and evidence for a procore protein. J Biol Chem 266:9231–9240.

Schaefer L, Macakova K, Raslik I, Micegova M, Gröne HJ, Schönherr E, Robenek H, Echtermeyer FG, Grässel S, Bruckner P, Schaefer RM, Iozzo RV, Kresse H. 2002. Absence of decorin adversely influences tubulointerstitial fibrosis of the obstructed kidney by enhanced apoptosis and increased inflammatory reaction. Am J Pathol 160:1181–1191.

Seo NS, Hocking AM, Höök M, McQuillan DJ. 2005. Decorin core protein secretion is regulated by N-linked oligosaccharide and glycosaminoglycan additions. J Biol Chem 280:42774–42784.

Truppe W, Kresse H. 1978. Uptake of proteoglycans and sulfated glycosaminoglycans by cultured skin fibroblasts. Eur J Biochem 85:351–356.

Voorhees PM, Dees EC, O'Neil B, Orlowski RZ. 2003. The proteasome as a target for cancer therapy. Clin Cancer Res 9:6316–6325.

Wang H, Zhang Z, Liu X, Chen G, Chen Q, Guo M. 2003. Influence on the growth and changes of several phenotypes of cultured rat MsC transfected by decorin gene. Fudan Univ J Med Sci 30:414–417. (in chinese).

Wang H, Chen L, Zhang Z, Jiang F, Guo M. 2005. Ex vivo transfer of the decorin gene into rat glomerulus via a mesangial cell vector suppressed extracellular matrix accumulation in experimental glomerulonephritis. Exp Mol Pathol 78:17–24.

Wu H, Wang S, Xue A, Liu Y, Liu Y, Wang H, Chen Q, Guo M, Zhang Z. 2008. Overexpression of decorin induces apoptosis and cell growth arrest in cultured rat mesangial cells *in vitro*. Nephrology 13:607–615.

Yamaquchi Y, Mann DM, Ruoslahti E. 1990. Negative regulation of transforming growth factor-beta by the proteoglycan decorin. Nature 346:281–284.

Zhang M, Guo M, Chen Q, Jin H. 1995. The culture of rat glomerular mesangial cells. J Shanghai Med Univ 22:207–209. (in chinese).

Zhang Z, Wu JY, Hait WN, Yang JM. 2004. Regulation of the stability of P-glycoprotein by ubiquitination. Mol Pharmacol 66:395–403.