Decorin, a Novel Player in the Insulin-like Growth Factor System*

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Decorin is a multifunctional proteoglycan that is expressed by sprouting endothelial cells. Its expression supports capillary formation and cell survival. Previously, it was shown that some effects of decorin are mediated by protein kinase B and the cyclin-dependent kinase inhibitor, p21. However, the cell surface receptor responsible for these effects was unknown. We demonstrate that decorin binds to the insulin-like growth factor-I (IGF-I) receptor on endothelial cells with an affinity in the nanomolar range ($K_D = 18 \text{nM}$), which is comparable with IGF-I ($K_D = 1.2 \text{nM}$). Furthermore, decorin can bind IGF-I itself, but with a lower affinity ($K_D = 190 \text{nM}$) than classical IGF-I-binding proteins. Decorin addition causes IGF-I receptor phosphorylation and activation, which is followed by receptor down-regulation. These effects are caused by the core protein of decorin, and the binding region could be mapped to the N-terminus of the molecule. The physiological relevance of the decorin/IGF-I receptor interaction was corroborated in two animal models (e.g. inflammatory angiogenesis in the cornea and unilateral ureteral obstruction). In both models the IGF-I receptor was up-regulated in decorin-deficient mice compared with controls and the up-regulation could not compensate the decorin deficiency in the disease models. These data indicate that decorin is an important player in the IGF system and its loss cannot fully be compensated in different types of diseases.

Decorin is the most thoroughly investigated member of the still growing family of small leucine-rich proteoglycans. Core proteins of these proteoglycans are characterized by leucine-rich repeat motifs flanked by cysteine-rich clusters. In addition, they carry at least a single glycosaminoglycan chain. Recently, a crystal structure for bovine decorin has been published (1) that together with earlier x-ray scattering data (2) suggests that decorin is a dimeric protein with 12 leucine-rich repeat motifs that include the N- and C-terminal cysteine-rich regions. This numbering of leucine-rich repeats is subsequently used in this report. Each monomer adopts a curved structure, and an antiparallel dimerization occurs through the β-sheet on the concave surface of the monomer. Decorin has been shown to have a variety of different functions that can be mediated by the core protein as well as the glycosaminoglycan chain. It regulates collagen fibril formation and stabilization, and it modulates cell adhesion as well as transforming growth factor-β activity (for review see Refs. 3 and 4). More recently it was found to influence directly the behavior of several types of cells. It can interact with members of the ErbB receptor family in tumor cells where it leads to a more differentiated phenotype (5) or to apoptosis of these cells (6). Decorin expression in endothelial cells, hepatocytes, fibroblasts, or macrophages causes differentiation, but it does not lead to apoptosis. In contrast, it protects cells from programmed cell death (6–8).

Endothelial cells cultured inside a collagen lattice can form capillary-like structures. We showed that both capillary formation and protection from apoptosis were only observed in decorin-synthesizing cells. A decorin-containing matrix was not sufficient to induce these changes. Adenoviral gene transfer of decorin into endothelial cells confirmed that decorin was instrumental for tube formation and cell survival (7). The physiological and pathophysiological relevance of these observations in culture was further supported by the finding that interleukin-6 and -10, two cytokines that are released during inflammation, induce decorin synthesis in endothelial cells growing in a collagen lattice (9). These cell culture results corroborate earlier observations in vivo, where decorin was found during inflammation-induced angiogenesis, i.e. in newly formed capillaries in granulomatous tissue (7) or in neovessels in temporal arterialis, but not in capillaries of the ovary in different phases of follicle and corpus luteum formation (10).

The importance of decorin in angiogenesis could also be demonstrated in a model of inflammatory angiogenesis in the cornea, as blood vessel growth was significantly reduced in decorin knock-out mice compared with wild-type mice (11). However, decorin signaling is involved not only in angiogenesis but also in renal inflammation and fibrosis, as enhanced apoptosis and atrophy of tubular epithelial cells as well as an increased infiltration of mononuclear cells were observed in unilateral ureteral obstruction (UUO)$\dagger$ in decorin-deficient mice (12).

How decorin affects differentiation and apoptosis is not completely understood. In tumor cells, decorin binds to the epithelial growth factor (EGF) receptor or ErbB4 and leads to activation of the mitogen-activated kinase (MAPK) pathway, Ca$^{2+}$ influx, induction of the cyclin-dependent kinase inhibitor, p21, $\dagger$

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† The abbreviations used are: UUO, unilateral ureteral obstruction; Akt, protein kinase B; DCN, decorin; EGF, epithelial growth factor; IGF, insulin-like growth factor; IGF-IR, IGF factor I receptor.
and subsequently to down-regulation of the receptor (13–15). In endothelial cells decorin affects different pathways. It enhances the phosphorylation of protein kinase B (Akt) and subsequently induces p21 by a MAPK-independent pathway. In addition to p21 the related cyclin-dependent kinase inhibitor, p27, is also induced, but by an Akt- and MAPK-independent mechanism (16).

In this study we have searched for potential cell surface receptors that could be involved in decorin signaling. Inhibitor studies indicated that the insulin-like growth factor-I receptor (IGF-IR) was a likely candidate. The IGF-IR is a ligand-activated tyrosine protein kinase highly homologous to the insulin receptor. It consists of a heterotetramer (α2β2) linked by disulfide bridges. Via its extracellular α2 units, the receptor binds IGF-I and IGF-II with high affinity. This interaction leads to autophosphorylation of the receptor and downstream proteins, including insulin receptor substrate-1. In the next step phosphoinositide 3-kinase is activated, which increases the levels of phosphoinositide-3,4,5-trisphosphate. Phosphoinositide-3,4,5-trisphosphate binds to the pleckstrin homology domains of phosphoinositide-dependent kinase I and Akt, thereby initiating the phosphorylation of Akt and several other signaling molecules such as protein kinases C and A. These signaling events finally lead to the inhibition of proapoptotic factors, the activation of survival factors, and cell differentiation or proliferation, depending on the cell type and tissue investigated (Ref. 17 and references therein). The general importance of the IGF-IR is implicated by the phenotype of IGF-IR knock-out mouse, which exhibits severe growth retardation and dies at birth (18). IGF-I is mainly produced by the liver, but expression in other types of tissue has been shown (19). The availability of IGF-I and IGF-II for receptor binding is regulated extracellularly by six high affinity-binding proteins and several low affinity-binding proteins. In addition, some of the IGF-binding proteins can exert biological functions independently of their role in the IGF system (20).

In this report we show for the first time that decorin can bind the IGF-IR and also interacts with IGF-I itself. This interaction regulates IGF signaling and may be responsible for the retardation of angiogenesis in the cornea of decorin knock-out mice and for the atrophy in later stages of tubulointerstitial fibrosis observed after UUO in these mice.

EXPERIMENTAL PROCEDURES

Materials—Antibodies used were sc-713 (Santa Cruz Biotechnologies) against the β-chain of the IGF-IR, rabbit anti-phospho-Akt (T308), rabbit anti-Akt (Cell Signaling Technologies, Beverly MA), and rat anti-CD31 (ERMP12; BMA Biomedical, Augst, Switzerland). Secondary antibodies labeled with horseradish peroxidase used were porcine anti-rabbit-IgG (DAKO, Hamburg, Germany), goat anti-rabbit-IgG (Bio-Rad), and horse anti-mouse IgG (Alexis, Grünberg, Germany). Tyrothostin AG1048 and AG1478 were obtained from Alexis. IGF-I and chemicals not specifically indicated were purchased from Sigma. Decorin A-Sepharose was added to remove non-specifically binding proteins. Then 10 μg of the anti-IGF-I-IR were added, and after 16 h the antibodies were precipitated with protein A-Sepharose. As control, a similar amount of protein A-Sepharose was added to decorin to remove non-specifically binding proteins. Then 10 μg of the anti-IGF-I-IR were added, and after 16 h the antibodies were precipitated with protein A-Sepharose. As control, a similar amount of protein A-Sepharose was incubated with the antibody but without cell lysate. After washing (3 × lysis buffer, 2 × phosphate-buffered saline), the material was divided in 7 aliquots and incubated in 250 μl of Tris-buffered saline, 0.1% bovine serum albumin, 0.1% Tween 20 for 1 h, followed by 1 ml of chemical lysis buffer (4 h, 37 °C). The bound material was released and measured by scintillation counting. Dissociation constants were determined as described above.

Animal Experiments—All animal work was done in accordance with the German Animal Protection Law. Decorin-deficient mice were generated as described previously (23). Angiogenesis assays in mice were performed by chemical cauterization of the cornea as described (11). The eyes were removed, and fixed in 4% phosphate-buffered paraformaldehyde for paraffin embedding. Unilateral urethral obstructions were done as in previous experiments (12). For the quantification of the IGF-IR, whole kidneys were homogenized in 1 ml of extraction buffer (final volume), and equal volumes were applied for SDS-PAGE. The protein content/albumin of ligated kidneys was ~50 μg, and the protein content/albumin of unligated kidneys was ~80 μg/albumin. Equal volumes were applied because the kidneys change their weight during the procedure and equal volumes reflect more accurately the situation in the organ. Western blots were carried out as described elsewhere (12) with the exception that the blots were incubated with the antibody sc-713 (1:5000) against IGF-IR as primary antibody overnight at 4 °C. Immunohistochemistry was performed as described elsewhere (11, 12).

RESULTS

Previous experiments have shown that decorin can induce tube formation in endothelial cells and inhibit apoptosis. The phosphorylation of Akt was one step in the pathway(s) (16), but the cell surface receptor for decorin in endothelial cells was unknown. To analyze how decorin activates Akt, different re-
Decorin Affects IGF-I Signaling

Fig. 1. Effects of decorin on the IGF-IR in endothelial cells. A, endothelial cells (EA.hy 926) were grown on fibrillar collagen in the presence of 0.5% heat-inactivated fetal calf serum for 48 h. After preincubation with the indicated inhibitors for 1 h, decorin (80 μg/ml) was added for 10 min. Cells were harvested, and equal amounts of protein were separated by SDS-PAGE. Phosphorylation of Akt at Thr-308 was determined by Western blotting. The fragment DCN-Q153 is sufficient to induce Akt phosphorylation. B, EA.hy 926 cells were plated as in Fig. 1A. Wild type decorin (40 μg/ml, 1 μM) or DCN-Q153 (25 μg/ml, 2 μM) were added for 10 min. Akt phosphorylation was determined as in Fig. 1A. The mutant DCN-E180K induces p21 synthesis and translocation to the nucleus as efficiently as wild type decorin.

To investigate whether decorin can down-regulate the IGF-IR, we used a replication-deficient adenovirus containing the human decorin cDNA to induce decorin expression. Previous studies had shown that decorin-mediated effects were observed 2 days after infection and subsequent culture of endothelial cells in a collagen lattice (16). Therefore, endothelial cells treated with a decorin-containing adenovirus or control virus were preincubated at 4 °C with decorin or IGF-I and then the alternative ligand was added for 10 min at 37 °C. Neither of these experiments (n = 2) led to a significant decrease or increase in IGF-IR or Akt phosphorylation compared with the addition of a single ligand at optimal concentration (result not shown).

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demonstrate that decorin is involved in signal transduction via the IGF system in endothelial cells.

IGF-I led to a dose-dependent induction of p21 mRNA in EA.hy 926 cells after 16 h with maximal expression levels (5-fold) at a concentration of 100 ng/ml (result not shown). Therefore, 100 ng/ml IGF-I were used in the following experiments. Immunoprecipitations of the IGF-IR from endothelial cells in a collagen lattice. The IGF-IR was expressed in cells treated with the alternative ligand was added for 10 min at 37 °C. Neither of these experiments (n = 2) led to a significant decrease or increase in IGF-IR or Akt phosphorylation compared with the addition of a single ligand at optimal concentration (result not shown).

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To characterize which part of the decorin core protein is important for signaling, Akt phosphorylation and/or p21 induction were analyzed after decorin mutants were applied to endothelial cells. For these experiments the following mutants were used: DCN-Q153, a mutant that contains the N terminus and the first 5 leucine-rich repeats of decorin, and DCN-E180K, a mutant with a point mutation reducing its affinity to collagen type I (21). These two mutants were chosen because previous studies (15) had shown that the N-terminal part of decorin is not involved in binding the EGF receptor but leucine-rich repeat 7, which contains the point mutation (DCN-E180K). Our investigation demonstrated that DCN-Q153 alone was still able to induce Akt phosphorylation (Fig. 2A) and p21 expression (result not shown), albeit to a lesser extent than wild type decorin. DCN-E180K induced p21 as strongly as wild type decorin, and it also triggered its translocation to the nucleus (Fig. 2B). These results imply that a different part of the decorin core protein interacts with the IGF-IR than with the EGF receptor.

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The binding properties of decorin to IGF-IR and IGF-I were investigated by using iodinated ligands. The purity of decorin for all experiments was checked by SDS-PAGE and silver staining (Fig. 3A). The binding properties of the IGF-IR from endothelial cells to decorin were not determined by direct binding to cells, because it is not known yet whether the IGF-IR is the only decorin binding receptor on the endothelial cell surface. Therefore, the receptor was immunoprecipitated with antibody against its intracellular domain (β-subunit). The same antibody was also used in the cell culture studies (Fig. 1, B and C), which confirm its specificity. 125I-IGF-I and 125I-decorin were applied as soluble ligands. Ligands bound to the receptor were quantified by scintillation counting and correlating the amount of 125I[iodine] to the protein concentration of IGF-I or decorin. K_D values of 1.2 × 10^{-9} M and K_D of 18 × 10^{-9} M were calculated for IGF-I and decorin, respectively. These determinations indicated that the affinity of decorin for the IGF-IR is only about 10-fold less than that of IGF-I (Fig. 3, B and C). In a further experiment, 125I-IGF-I was bound to the receptor and displaced by unlabeled IGF-I or decorin. Using IGF-I as competitor, an IC_{50} of 3 × 10^{-7} M was determined. The decorin proteoglycan and the decorin core protein without glycosaminoglycan chain (result not shown) gave the same IC_{50} of 5 × 10^{-7} M (n = 4), whereas the N-terminal fragment DCN-Q153 inhibited with an IC_{50} of 20 × 10^{-7} M (Fig. 3D). These data show that decorin and IGF-I can compete for the IGFR-I, and they corroborate the cell culture results that the N-terminal part of decorin is sufficient to bind and activate the IGFR-I.

To investigate whether decorin can also interact with IGF-I, decorin core protein and proteoglycan were separated by SDS-PAGE, transferred to nitrocellulose, and 125I-IGF-I was used as probe. Both bound 125I-IGF-I (Fig. 4, insert). The core protein showed increased binding compared with the proteoglycan, indicating that the glycosaminoglycan chain is not responsible for the interaction but may even inhibit binding of decorin to IGF-I. To investigate whether 125I-IGF-I also binds to decorin in solution, a Sepharose CL-4B gel filtration column was prepared and equilibrated with buffer containing different concentrations of decorin. When 125I-IGF-I was applied, it eluted in the absence of decorin in the V_{t} of the column. In the presence of decorin a dose-dependent formation of high molecular mass complexes was observed that eluted in the included volume of the column (Fig. 4). Solid phase binding assays of IGF-I with 125I-decorin gave a dissociation constant of about 190 × 10^{-9} M, which shows a 10-fold lower affinity of decorin to IGF-I itself compared with its affinity to the IGFR-I (K_D of 18 × 10^{-9}). In comparison to IGF-binding proteins this affinity is about 1000-fold lower, indicating that decorin is more likely to compete with IGF for binding to the IGFR-I than for the interaction with binding proteins that have K_D values in the range of 10^{-10} M (20).

In previous experiments (11) we could show that after chemical cauterization angiogenesis is reduced in the corneal dome of the decorin-deficient mouse compared with control mice (Fig. 5, A and B). Sections from these mice showed that after injury the invading cells including the endothelial cells contain more IGFR-I in the decorin-deficient animals compared with the wild type mice (Fig. 5, C and D). Similar observations were made 48, 72, and 96 h after injury (results not shown). In addition, an increase in expression of IGFR-I in the epithelial cell layer of the cornea was observed in the decorin-deficient animals. This was found in both the injured (Fig. 5, C and D) and non-injured epithelium (result not shown). Thus, there is a significant overexpression of IGFR-I in decorin-deficient mice.

Using a different injury model in the same type of mice, based on UUO, we found an enhanced rate of apoptosis of tubular epithelial cells in ligated kidneys from Dcn−/− compared with wild type animals up to day 14, with a maximum at day 7 (12). Immunohistochemical analysis using an antibody against the
IGF-IR in these kidneys at day 7 showed an increase of staining in the tubuloepithelium of obstructed kidneys from both $Dcn^{-/-}$ and wild type mice (Fig. 5, G and H) compared with the respective non-ligated kidneys (Fig. 5, E and F). However, ligated kidneys from $Dcn^{-/-}$ mice displayed a much stronger increase in IGF-IR (Fig. 5G) compared with ligated kidneys from wild type animals (Fig. 5H). There were no genotype-specific differences in the location and intensity of the IGF-IR staining between non-ligated kidneys (Fig. 5, E and F).

Analysis of IGF-IR expression by Western blot analysis at different time points confirmed the immunohistological findings, showing that IGF-IR is induced in the affected kidney from wild type and $Dcn^{-/-}$ mice (Fig. 6A) 7 days after UUO. The maximal difference in IGF-IR expression between obstructed kidneys from decorin-deficient compared with wild type mice was 2.1-fold $(n = 3, p < 0.00014)$ at day 7 (Fig. 6B). At day 14 the difference had declined to 1.8-fold $(n = 3, p < 0.018)$ (Fig. 6C). The expression of IGF-IR in the contralateral unligated kidney was only marginally affected with a 1.3-fold increment $(n = 3, p < 0.064)$. These data indicate that in the kidney IGF-IR is induced by UUO and that significant overexpression occurs in decorin-deficient mice. Both of these models show that IGF-IR is up-regulated, presumably as a compensatory mechanism for the decorin deficiency, and suggest that in vivo decorin and the IGF system work together during signaling in endothelial and as well as epithelial cells.

**DISCUSSION**

Our results show for the first time that decorin is involved in IGF-I signaling. It binds to the IGF-IR and activates its tyrosine kinase activity in endothelial cells. Previous results demonstrated that decorin inhibits apoptosis in endothelial cells (7). Other researchers have shown the same for IGF-I (24). In our experiments both decorin and IGF-I led to a phosphorylation of the IGF-IR after 4 min, an early stimulation of Akt phosphorylation after 10 min, and an induction of p21 after 20 h. On first view, concentrations of 100 ng/ml IGF-I compared with 80 μg/ml decorin added to the cell cultures may look very different, but on a molar basis the concentration for IGF-I (13 nM) is even higher than the concentration for decorin (2 nM). In addition, one has to consider that the endothelial cells are grown on a collagen matrix, which binds major amounts of decorin. We also found that the medium as well as the cell layer/collagen matrix contained a whole variety of IGF-I-binding proteins (data not shown). Furthermore, we could show in two separate types of experiments that at optimal concentration decorin and IGF-I do not interfere with each other’s downstream signaling. From these data we conclude that decorin stimulates the IGF-IR and does not inhibit signaling as has been shown for its interaction with receptors of the ErbB family (5, 15).

Furthermore, our binding studies show only a 10-fold lower affinity of decorin to the IGF-IR than IGF-I. This affinity was calculated with the assumption that decorin interacts as a monomer. If a dimeric form of decorin (1) binds to the receptor the dissociation constants will be even closer. Our assays using immunoprecipitated native IGF-IR from the same endothelial cells show a lower affinity ($K_D = 1.2 \times 10^{-5}$ M) for binding of IGF-I to its receptor than the values determined for IGF-I and recombinant soluble IGF-IR previously published (25, 26). These differences may be due to the different methodologies and the presence of the intracellular β-subunit that is used for...
decorin to protect tubular epithelial cells from apoptosis. Not much is known about the expression of IGF-IR protein in the UUO. In terms of IGF-IR mRNA, expression was unchanged up to 7 days of UUO in neonatal rat (33) and markedly down-regulated in hydropnephrotic kidneys with fibrotic progression in humans (34).

In conclusion, our findings indicate that decorin can bind both IGF-I and its receptor and that this interaction leads to Akt phosphorylation and p21 induction in endothelial cells. Further studies, however, are needed to clarify whether the interaction of decorin with IGF-I and its receptor is important for other types of diseases in which the IGF system is involved.

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