Discoidin Domain Receptor 1 Controls Growth and Adhesion of Mesangial Cells

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Abstract. Various types of collagen are known as modulators of mesangial cell proliferation. Here the function of the collageen-binding tyrosine kinase receptor discoidin domain receptor 1 (DDR1) in mesangial cells is investigated. The expression of DDR1 in the mouse kidney is confirmed by Northern analysis. In primary mesangial cells isolated from wild-type and DDR1-null mice, tyrosine phosphorylation in response to collagen-stimulation, adhesion to collagen, and cellular proliferation were measured. DDR1 phosphorylation was induced after overnight incubation of cells with type I collagen. Compared with wild-type cells, the adhesion of DDR1-null cells was drastically reduced. In contrast, DDR1-knockout cells showed significantly enhanced proliferation compared with wild-type cells. Both effects were largely independent of the collagen-binding α1/β1 integrin function. This study found that the increased proliferation rate of DDR1-null cells is caused by a constitutive upregulation of p42/p44 and p38 mitogen-activated protein kinases (MAPK) activity. This is the first evidence indicating that DDR1 could be involved in the proliferative stage of renal disorders.

Alterations in the expression pattern of collagens are a hallmark of glomerulosclerosis. Renal fibrosis is characterized by the differentiation of interstitial cells into myofibroblasts and by deposition of fibrillar collagens (1–3). The signaling pathways that trigger these processes are largely unknown. In the course of disease progression, mesangial cells respond with unrestrained growth and uncoordinated extracellular matrix (ECM) remodeling. Soluble growth factors as well as components of the ECM can mediate the proliferation of mesangial cells (4). Whereas some factors, such as platelet-derived growth factor (PDGF), induce growth, others, such as transforming growth factor-β (TGF-β), are inhibitory (5). During the progression of glomerulosclerosis, mesangial cells have an increased proliferation rate and show changes in α-smooth muscle actin, osteopontin, and type I collagen expression (6). Similar changes are also seen in glomerular cells, where the accumulation of excessive extracellular matrix proteins, particularly collagen type IV, causes thickening of the basement membrane and a decreased filtration surface.

In mammalian cells, signaling pathways downstream of growth-factor receptors are regulated by at least three different groups of mitogen-activated protein kinases (MAPK): the p42/p44 (Erk), JNK (c-Jun NH2-terminal kinase), and p38 families. Whereas JNK is commonly activated by environmental stress, such as UV light, heat, strain/relaxation, or hypertonicity (7), p42/p44 respond to mitogens, such as PDGF or vascular endothelial growth factor (8). Both mitogens and stress can trigger p38. In mesangial cells, members of all three MAPK were found to be expressed (9,10).

Receptor tyrosine kinases (RTK) are specialized cell surface receptors that bind extrinsic factors and transmit signals through the plasma membrane. RTK-mediated signaling regulates cell proliferation, differentiation, transformation, senescence, and apoptosis (11). Of the 53 RTK in the human genome, two members constitute a novel subfamily called discoidin domain receptors (DDR). DDR are distinguished from other receptor tyrosine kinases by a discoidin domain in their extracellular domain, which is a homology region originally identified in the protein discoidin I from Dictyostelium discoideum (12–14). If nutrients are withdrawn from Dictyostelium, it executes a developmental program that converts solitary living amoebae into a multicellular organism (15). In the fruiting body of the slime mold, it is thought to be important for the maintenance of cell morphology and cytoskeletal organization.

A unique feature of DDR1 and DDR2 is the fact that both receptors have collagens as their cognate ligands. Whereas DDR1 activation is achieved by type I to type VI, and type VIII collagens, DDR2 is only activated by fibrillar collagens (16,17). The 160-amino-acid-long discoidin domain is followed by an approximately 200-amino-acid-long stalk region. Whereas the discoidin domain is essential for collagen binding of DDR1, both domains are necessary for receptor signaling (18). In adherent cells, maximal DDR1 phosphorylation is reached after several hours of collagen stimulation, whereas this takes place somewhat faster in suspension cultures (19). Collagen-induced shedding of the ligand-binding domain of DDR1 is found in several cell lines, including human mammary carcinoma cells (20).
The cDNA coding for human DDR1 has been cloned from several tissues or carcinoma cells (12,14). Expression of DDR1 is predominantly seen in epithelial cells, particularly from kidney, lung, gastrointestinal tract, and brain, but also in corneal and dermal fibroblasts (14,21–23). Upregulated DDR1 expression has been reported from breast, ovarian, esophagus, and brain tumors as well as injured arteries (24–29).

Thus far, five isoforms of DDR1 (dubbed with the affixes a to e) have been cloned as a result of alternative splicing (30). Compared with the longest c-isoform, the b-isoform lacks 6 amino acids inserted in the kinase domain between exons 13 and 14 (14). The α-, δ-, and e-isoforms arise through alternative splicing in the juxtamembrane region. The deletion of exon 11 coding for 37 amino acids gives rise to DDR1a, and the deletion of exons 11 and 12 results in DDR1d. In DDR1e, the first half of exon 10 and all of exons 11 and 12 are missing (30). Deletion of DDR1 in the mouse germ line resulted in viable animals that are significantly smaller than their littermates (31). Female DDR1-null mice show defects in blastocyst implantation and mammary gland development. Furthermore, in primary vascular smooth muscle cells cultured from DDR1-null mice, decreased proliferation, collagen-attachment, and migration have been observed (32,33).

Here, we used primary mesangial cells derived from knock-out mice to test the role of DDR1 in mesangial cell adhesion and proliferation. We found that DDR1 is necessary for collagen attachment and is a negative regulator of cell growth potentially by inhibiting MAPK pathways.

Materials and Methods

Animals, Antibodies, and Reagents

The generation of DDR1–null mice has been described earlier (31). All mice used were from a mixed 129/Sv and ICR background and were free of pathogens. All animal procedures were in accordance with the Declaration of Helsinki and the Guide for the Care and Use of Laboratory Animals (NIH). [32P]-DATP and [3H]-thymidine were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). RPMI-1640 medium, glutamine, penicillin/streptomycin, and fetal calf serum were obtained from Biowhittaker Europe (Verviers, Belgium). Rat type I collagen was purchased from Collaborative Technology, Inc. (Santa Cruz, CA), antibodies to p38 and pp38 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), -smooth muscle actin from Sigma, Inc. (Munich, Germany), anti-phosphotyrosine (4G10) and anti-MAPK antibodies from Upstate Biotechnology, Inc. (Lake Placid, NY), antibodies to α-smooth muscle actin from Sigma, Inc. (Munich, Germany), anti-CD49a (α1 integrin) and anti-CD29 (β1 integrin) from BD Biosciences Pharmingen (Heidelberg, Germany), antibodies to p38 and pp42/44, and pp42/44 inhibitor (U0126) from NEB (Frankfurt am Main, Germany). SB 203580 for 60 min or with 10 μM SB 203580 for 90 min or with 10 μM U0126 for 90 min. Cells were lysed with 1% Triton X-100 (pH 7.5), 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μM aprotinin. Cellular lysates were centrifuged at 4°C and 13,000 rpm for 10 min. Lysates were adjusted to contain equal protein concentrations by using the BCA Protein assay (Pierce, Rockford, IL) and subjected to SDS-polyacrylamide gel electrophoresis or further analyzed by immunoprecipitation. For immunoprecipitation, lysates were mixed with an equal volume of HNTG (20 mM HEPES [pH 7.5], 150 mM NaCl, 0.1% Triton, 10% glycerol) and incubated with anti-DDR1 antibodies and protein A-agarose on a rotating wheel at 4°C for 4 h. For lectin-affinity purification, lysates were incubated with concanavalin A-Sepharose beads (Sigma). The sepharose complexes were washed three times with HNTG, boiled

RNA was determined by ethidiumbromide staining. RNA was transferred to Hybond-N+ membrane (Amersham Pharmacia Biotech). Samples were UV crosslinked onto the membrane and hybridized at 50°C with ULTRAhyb (Ambion, Cambridgeshire, UK) containing a 32P-labeled DDR1-specific cDNA probe (330-bp fragment of mouse 3’-untranslated cDNA). After overnight hybridization, the blot was washed twice in 2× SSC/0.1% SDS, twice in 0.1× SSC/0.1% SDS at 50°C and exposed to Hyperfilm (Amersham Pharmacia Biotech). Experiments were repeated three times.

Primary Mesangial Cell Culture

The isolation of mouse mesangial cells was performed as described previously (34). Glomeruli were prepared by mechanical sieving of the renal cortex isolated from age-matched male mice weighing 25 to 33 g. The kidneys were removed under anesthesia from the mice, and the cortex was dissected and minced into a paste-like consistency. The suspended homogenate was pushed successively through 180-μm and 106-μm sieves. The glomeruli were retained on the top of a 50-μm sieve and centrifuged 10 min at 60 × g. The resuspended pellet was incubated with 1 mg/ml collagenase for 30 min and then transferred onto tissue culture plates. Mesangial cells were cultured in RPMI-1640 medium supplemented with 5 mM glutamine, 15 mM N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20% fetal calf serum in an atmosphere of 5% CO2/95% air. After the cells had reached confluence, they were trypsinized and passaged. For the proliferation experiment, cells were cultured in 6-well plates for various periods of time, detached with trypsin/EDTA and counted with a Coulter Particle Counter Z1 (Coulter, Miami, CA). All cells used for experiments were from the second or third passage.

Immunocytochemistry

Mouse mesangial cells were grown on coverslips for 3 d, fixed with 3% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Cells were incubated with DDR1 or α-smooth muscle actin antibody in a 1:50 dilution. While the α-smooth muscle actin antibody was already FITC-labeled, a rabbit FITC-labeled secondary antibody from goat (Jackson Immunoresearch, West Grove, USA) was used for DDR1 immunocytochemistry. Cells were counterstained with propidium iodide and visualized using a Fluorescence DM IRB Leica confocal microscope (Leica Microsystems, Heidelberg, Germany).

Western Blot Analyses

Semi-confluent mouse mesangial cells were kept in serum-free medium and stimulated with 10 μg/ml collagen overnight. In some experiments, cells were incubated with 10 μg/ml integrin α1 or β1 blocking antibodies for 30 min, with 5 μM SB 203580 for 60 min or with 10 μM U0126 for 90 min. Cells were lysed with 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.5), 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μM aprotinin. Cellular lysates were centrifuged at 4°C and 13,000 rpm for 10 min. Lysates were adjusted to contain equal protein concentrations by using the BCA Protein assay (Pierce, Rockford, IL) and subjected to SDS-polyacrylamide gel electrophoresis or further analyzed by immunoprecipitation. For immunoprecipitation, lysates were mixed with an equal volume of HNTG (20 mM HEPES [pH 7.5], 150 mM NaCl, 0.1% Triton, 10% glycerol) and incubated with anti-DDR1 antibodies and protein A-agarose on a rotating wheel at 4°C for 4 h. For lectin-affinity purification, lysates were incubated with concanavalin A-Sepharose beads (Sigma). The sepharose complexes were washed three times with HNTG, boiled

Northern Blot Analysis

Mouse kidney tissue was lysed in 4 M guanidinium hydrochloride. Total RNA was extracted by ultracentrifugation into a layer of 5.7 M CsCl. Poly(A)+ RNA was selected by affinity chromatography (Qiagen, Germany). RNA samples were fractionated by electrophoresis using a 1.2% agarose/formaldehyde gel. The integrity of the purified
with Laemmlli-buffer, and analyzed by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) and immunoblotted with antibodies diluted 1:1000 (4G10, p42/p44, pp42/pp44), 1:500 (DDR1, pp38), or 1:250 (p38) in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 0.5% gelatin overnight. Western blots were incubated with mouse or rabbit peroxidase-coupled secondary antibodies, respectively (Bio-Rad, Munich, Germany) and developed using Enhanced Chemiluminescence (Amersham). Western blots were quantified using Quantity-One software (Bio-Rad).

**Cells Growth Assay**

Mouse mesangial cells were seeded at the density of 2 × 10⁴ cells per well in 12-well plates using RPMI medium supplemented with 10% fetal calf serum. After cells were adherent, they were labeled with [³H]-thymidine (5 Ci/mmol) and cultured for various periods of time. Some samples were pre-incubated with 10 µg/ml α, or β, integrin blocking antibodies for 30 min. Nonadherent cells were removed by three washes with phosphate-buffered saline (PBS). After incubating with 3.75 mM p-nitrophenol-N-acetyl-β-D-glucosaminide in 0.25% Triton X-100 for 4 h, the assay was developed by adding 50 mM glycine and 5 mM EDTA (pH 10.4). The absorbency at 405 nm was measured. Assays were performed in triplicate.

**Hexosaminidase Assay**

The cell adhesion assay was performed as described earlier (35,36). An equal number of mouse mesangial cells were plated on collagen-coated dishes or tissue culture plastic for various periods of time. Some samples were pre-incubated with 10 µg/ml α, or β, integrin blocking antibodies for 30 min. Nonadherent cells were removed by three washes with phosphate-buffered saline (PBS). After incubating with 3.75 mM p-nitrophenol-N-acetyl-β-D-glucosaminide in 0.25% Triton X-100 for 4 h, the assay was developed by adding 50 mM glycine and 5 mM EDTA (pH 10.4). The absorbency at 405 nm was measured. Assays were performed in triplicate.

**Results**

**Expression and Collagen-Induced Activation of DDR1 in Mesangial Cells**

To determine the expression level of DDR1 in the kidney, we isolated total RNA from mouse renal cortex. After purification of poly(A)⁺ RNA, we subjected 10 µg of total and 2 µg of poly(A)⁺ RNA to Northern blot analyses (Figure 1). Using a cDNA probe derived from the mouse DDR1 cDNA, a single 4.2-kb transcript was detected in both RNA preparations. Compared with total RNA, the amount of DDR1 message was significantly enriched in poly(A)⁺ RNA.

Because many cell/matrix contacts in the kidney are found in the mesangium, we purified primary mesangial cells from the mouse. Glomeruli were isolated from the kidney cortex by sequential size fractionation, dispersed by collagenase treatment, and seeded on tissue culture plastic. Cells were kept in selection medium for up to 2 wk until they reached confluence (Figure 2A). To verify that a homogeneous culture was obtained, we stained for smooth muscle actin, a marker protein characteristic for mesangial cells. Analysis by confocal microscopy showed that all cells stained positive for α-smooth muscle actin (Figure 2B). Next, we wanted to verify that DDR1 is expressed and signaling-competent in primary mesangial cells. Confluent cells were stimulated with 10 µg/ml type I collagen overnight and lysed. To enrich for membrane proteins, we subjected the cell lysate to concanavalin A lectin chromatography. The lectin-bound fraction was analyzed by Western blot using phosphotyrosine antibody. An intense signal with a molecular weight of 125 kD was observed, which corresponds to the size of DDR1 (Figure 2C). Because other proteins with similar molecular weight might be tyrosine phosphorylated concomitantly with DDR1, the blot was reprobed with an antibody specific to DDR1 (Figure 2D). Indeed, the phosphorylated 125-kD protein was identical with DDR1. To further define the expression of DDR1 in mesangial cells, we immunostained cells with the DDR1-specific antibody. The expression was localized to the plasma membrane in a granular pattern (Figure 3A). To confirm the expression pattern, we also stained mesangial cells derived from DDR1-null animals. These cells showed no detectable staining of the plasma membrane but a relatively weak peri-nuclear staining, which we assume to be nonspecific cross-reactivity of the polyclonal anti-DDR1 serum (Figure 3B).

To test for a functional role of DDR1 in mesangial cells, we stimulated cells that were wild-type, heterozygous, or null for DDR1 with type I collagen. DDR1 was immunopurified from cell lysates and analyzed in a Western blot using a phosphotyrosine antibody. Wild-type cells stimulated with collagen showed a single band representing activated DDR1 at 125 kD, which was absent in unstimulated cells (Figure 3C). A signal with lower intensity was detected in stimulated heterozygous cells, but it was completely absent in DDR1-null cells. We concluded that the polyclonal antibody used, although having
some cross-reactivity when used for immunostaining, was specific in a Western blot analysis.

**Adhesion and Proliferation of DDR1-Null Cells**

Next, we wanted to explore if wild-type and DDR1-null mesangial cells show any differential properties during *in vitro* culture. Therefore, an equal number of both cell types were plated on plastic dishes for 2 to 60 min, and the number of adherent cells was measured by hexosaminidase assay. Starting at 10 min after plating, DDR1-null cells showed significantly reduced adhesion compared with wild-type cells (Figure 4A). This difference was even more pronounced when plating cells on type I collagen-coated dishes. Wild-type cells reached maximal adhesion 30 min after plating, when only half the amount of DDR1-null cells attached to collagen (Figure 4B). Incubating the cells with antibodies that either block α1 or β1 integrin had no effect on their adhesion to plastic (Figure 4C). However, the adhesion to collagen was slightly attenuated by anti-β1 integrin antibodies 30 min after plating of cells (Figure 4D). These results demonstrate that DDR1 plays a major role in mesangial cell adhesion and α1/β1 integrin only has a minor contribution to this effect.

We further wanted to determine whether DDR1 has an influence on cell proliferation. We plated an equal number of cells and measured their proliferation rate by flow cytometry for 2.5 d. We observed a much faster growth rate for DDR1-null cells compared with wild-type cells throughout the experiment, resulting in a 2.2 times higher number of DDR1-null cells after 2.5 d (Figure 5A). As an alternative method to measure proliferation, we plated an equal number of wild-type and mutant cells and recorded the [H3]-thymidine incorporation for up to 48 h. Again, we found a significantly higher proliferation rate for DDR1-null cells in comparison to wild-type cells (Figure 5B).

**Role of MAPK Downstream of DDR1**

To test whether the enhanced proliferation of DDR1-null cells was mediated by serine/threonine kinase activity commonly downstream of growth factor receptors, we tested the activation of the MAPK kinases, p42/p44, p38, and JNK, using...
phospho-specific antibodies. Cells from DDR1-null and control cells were stimulated with collagen overnight and also treated with the MAPK inhibitor U0126 (10 μM) for 90 min. Equal amounts of cellular lysates were analyzed by Western blotting using pp42/pp44 antibodies (Figure 6A). The blot was stripped and reprobed with an antibody against total p42/p44 (Figure 6B). The ratio between phosphorylated and total p42/p44 was calculated (Figure 6C). Stimulation of wild-type cells with collagen resulted in a 1.7-fold increase of p42/p44 activity. Surprisingly, compared with the wild-type, DDR1-null cells had a 2.5-fold higher pp42/pp44 level at the resting state. Upon collagen stimulation, DDR1-null cells showed a slight decrease in p42/p44 activity. The basal and collagen-induced MAPK phosphorylation was completely blocked by the U0126 inhibitor. Next, we tested the role of integrin in p42/p44 MAPK activation by measuring collagen-induced phosphorylation in the presence of α₁ or β₁ integrin blocking antibodies. Blocking integrins had no effect on the constitutive higher level of p42/p44 phosphorylation in DDR1-null cells (Figure 7). However, in contrast to cells with functional α₁/β₁ integrin, collagen stimulation of wild-type cells in the presence of integrin-blocking antibodies resulted in a slight decrease in p42/p44 phosphorylation, whereas DDR1-null cells responded with slightly elevated p42/p44 phosphorylation (Figure 7C).

We subsequently monitored the activation state of p38. To enhance the response of p38 activation upon collagen stimulation, cells were treated with 100 ng/ml anisomycin for 15 min before lysis. As for p42/p44, stimulation of wild-type cells resulted in a significant (30%) increase of p38 activity (Figure 8). More importantly, basal p38 phosphorylation was increased in DDR1-null cell compared with controls. Stimulation of DDR1-null cells with collagen had no apparent effect on p38 activity, and incubation with the p38 inhibitor SB203580 resulted in complete absence of p38 phosphorylation. We concluded that both, p42/p44 and p38 activity, but not JNK are enhanced in DDR1-null cells (data not shown).
To correlate DDR1-regulated MAPK activation with the differential cell growth observed earlier, we performed the [H3]-thymidine proliferation assay in the presence of the p42/p44 inhibitor U0126 or p38 inhibitor SB203580. Although proliferation of both DDR1-null and wild-type cells was greatly diminished by the p42/p44 inhibitor, the p38 inhibitor had no effect on the previously observed discrepancy between the growth rates of DDR1-null versus wild-type cells (Figure 9).

Discussion

A diligent balance of ECM degradation and neo-synthesis is necessary to maintain the integrity of the renal basement membrane. Here we identified the receptor tyrosine kinase DDR1 as potential key element regulating mesangial cell function. First, we showed that DDR1 was highly expressed in primary mouse mesangial cells and localized to the plasma membrane. Second, stimulation of primary mesangial cells with type I collagen resulted in sustained activation of the DDR1 tyrosine kinase function. Third, we gave experimental proof that DDR1 is not only a receptor activated by collagen but also acts as an adhesion receptor, allowing mesangial cells to rapidly attach to a collagen-coated surface. Fourthly, we identified DDR1 as a negative regulator of mesangial cell proliferation. We could attribute this inhibition to a DDR1-mediated block of p38 and p42/p44 MAPK phosphorylation.

However, our results indicate that the mechanisms of DDR1 action are rather complex. We found that DDR1 is regulating MAPK signaling on two counteracting levels. Whereas the unstimulated receptor suppresses MAPK activation, activated DDR1 induces MAPK phosphorylation. The first level of action is a growth inhibitory stimulus, which is missing in the absence of DDR1. Potentially, unstimulated DDR1 interacts with molecules that are essential downstream parts of certain other growth factor-induced signaling pathways and sequesters them away from the mitogenic signaling pathways. In DDR1-
null cells, the block of p38 and p42/p44 MAPK activation observed in wild-type cells is alleviated, thereby resulting in a constitutive 2.5-fold upregulation of p42/p44 phosphorylation and a 30% increase of p38 activity (Figures 6 and 8). We did not observe any p38 phosphorylation in mesangial cells in the absence of the agonist anisomycin; therefore, proliferation seems to be mainly driven by the p42/44 pathway and to a lesser extent by p38. On the second level of action, we observed upon stimulation with collagen a mild but statistically significant induction of both p42/p44 and p38 MAPK in wild-type mesangial cells, which was absent in DDR1-null cells. Furthermore, primary mouse mesangial cells seem to express several-fold higher amounts of p42/p44 than p38 MAPK.

A number of previous studies using mesangial cells showed a suppression of mitogenic response and a block of proliferation upon collagen stimulation (6,37,38). The \( \beta_1 \) subfamily of integrins as alternative family of collagen-receptors was suggested to mediate these anti-mitotic effects (4). Although the involvement of DDR1 had not been analyzed in these studies, the present results give rather compelling evidence that DDR1-mediated growth suppression is involved in these processes. However, the role of kinases downstream of \( \beta_1 \) integrins, particularly focal adhesion kinase (FAK) or protein kinase C (PKC) that are potentially able to crosstalk with DDR1 signaling pathways, has not yet been addressed in these studies (39). We presently investigate the role of DDR1 in crosstalking to other signaling pathways. Currently, we also lack any experimental data about the role of DDR1 in other renal cells that potentially interact with collagen, such as tubular cells or podocytes (40).

Our findings in DDR1-null cells are in agreement with previous results obtained from the analysis of DDR1 knockout mice. We found that ductal epithelial cells in the DDR1-null mammary gland from adult mice have a fourfold increase in proliferation rate compared with control animals (31). In the knockout mammary gland, enhanced proliferation and matrix deposition resulted in aberrant differentiation during pregnancy and a failure to produce milk after birth. Although DDR1 suppresses proliferation in mesangial and ductal mammary gland cells, opposing effects were found in mouse vascular smooth muscle cells (VSMC). Primary DDR1-null VSMC grew significantly more slowly than control cells (32). We reason that additional cell-type–specific elements or factors of the cellular environment are involved in the response of DDR1, either enhancing or suppressing proliferation. Interestingly, blocking of \( \alpha_1 \) or \( \beta_1 \) integrin resulted in the reversed DDR1 action: collagen stimulation suppressed the activation of p42/p44 in wild-type cells but enhanced it in knockout cells. It appears therefore that DDR1 regulates MAPK activity twofold: (1) the unstimulated receptor constitutively downregulates

**Figure 6.** DDR1 suppresses p42/p44 mitogen-activated protein kinases (MAPK) activation. DDR1-null or wild-type mesangial were stimulated with type I collagen overnight in the absence or presence of the MAPK inhibitor U0126. Equal amounts of total cell lysates were analyzed by Western blotting with an antibody against phosphorylated p42/p44 (A) or against total p42/p44 (B). Results were quantified using densitometry scanning and expressed as relative ratio between phosphorylated and total p42/p44 (C).

**Figure 7.** DDR1-induced suppression of p42/p44 MAPK activation is independent of \( \alpha_1 \) or \( \beta_1 \) integrin. DDR1-null or wild-type mesangial cells were stimulated with type I collagen overnight in the presence of blocking antibodies against \( \alpha_1 \) or \( \beta_1 \) integrin. Equal amounts of total cell lysates were analyzed by Western blotting with an antibody against phosphorylated p42/p44 (A) or against total p42/p44 (B). Results were quantified using densitometry scanning and expressed as relative ratio between phosphorylated and total p42/p44 (C).
MAPK activity and therefore cellular proliferation; and (2) stimulated DDR1 increases MAPK phosphorylation if functional α1/β1 integrins are present. It is tempting to speculate that there is a common set of signaling proteins, which simultaneously act as downstream targets of DDR1 as well as integrins and which are able to integrate the counteracting signals received from both groups of collagen-receptors.

What is the in vivo role of DDR1 in normal mesangial cells? Intact cells show a very limited rate of proliferation; we therefore hypothesize that DDR1 counteracts the growth-promoting activity of factors such as PDGF. This growth-dampening function of DDR1 could potentially be mediated by the unligated receptor, because normal mesangial cells produce very little type I collagen. Upon renal injury and induction of glomerulonephritis, DDR1 is exposed to increasing amounts of collagen, resulting in signaling-competent receptors. Activation of DDR1 is leading to increased p42/p44 and p38 MAPK phosphorylation and ultimately to cell cycle progression. Furthermore, DDR1 might directly or indirectly regulate collagen expression and matrix metalloproteinase activity. In VSMC derived from DDR1 knockout mice, we found reduced levels of active MMP2 and MMP9, the primary proteases in the basement membrane (32).

Taken together, we propose in this study that DDR1 is a key regulator of mesangial cell proliferation and adhesion. Although collagen-stimulation of DDR1 triggers MAPK activation, lack of DDR1 in knockout cells constitutively upregulates MAPK activity. Cellular events further downstream of DDR1 will be explored in future work.

Figure 8. DDR1 suppresses p38 MAPK signaling. DDR1-null or wild-type mesangial cells were stimulated with type I collagen overnight in the absence or presence of the p38 MAPK inhibitor SB203580. Equal amounts of total cell lysates were analyzed by Western blotting with an antibody against phosphorylated p38 (A) or against total p38 (B). Results were quantified using densitometry scanning and expressed as relative ratio between phosphorylated and total p38 (C).

Figure 9. Enhanced proliferation of DDR1-null mesangial cells is triggered by p42/p44 but not p38 MAPK. Proliferation of wild-type and DDR1-null cells was measured in the presence of the MAPK inhibitors U0126 or SB203580 over a period of 42 h using [H³] thymidine incorporation.

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