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Murine Double Minute-2 Inhibition Ameliorates Established Crescentic Glomerulonephritis

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Rapidly progressive glomerulonephritis is characterized by glomerular necroinflammation and crescent formation. Its treatment includes unspecific and toxic agents; therefore, the identification of novel therapeutic targets is required. The E3-ubiquitin ligase murine double minute (MDM)-2 is a nonredundant element of NF-κB signaling and the negative regulator of tumor suppressor gene *TP53*-mediated cell cycle arrest and cell death. We hypothesized that the MDM2 would drive crescentic glomerulonephritis by NF-κB—dependent glomerular inflammation and by p53-dependent parietal epithelial cell hyperproliferation. Indeed, the pre-emptive MDM2 blockade by nutlin-3a ameliorated all aspects of crescentic glomerulonephritis. MDM2 inhibition had identical protective effects in *Trp53*-deficient mice, with the exception of crescent formation, which was not influenced by nutlin-3a treatment. *In vitro* experiments confirmed the contribution of MDM2 for induction of NF-κB—dependent cytokines in murine glomerular endothelial cells and for p53-dependent parietal epithelial cell proliferation. To evaluate MDM2 blockade as a potential therapeutic intervention in rapidly progressive glomerulonephritis, we treated mice with established glomerulonephritis with nutlin-3a. Delayed onset of nutlin-3a treatment was equally protective as the pre-emptive treatment in abrogating crescentic glomerulonephritis. Together, the pathogenic effects of MDM2 are twofold, that is, p53-independent NF-κB activation increasing intraglomerular inflammation and p53-dependent parietal epithelial cell hyperplasia and crescent formation. We therefore propose MDM2 blockade as a potential novel therapeutic strategy in rapidly progressive glomerulonephritis. *(Am J Pathol 2016, 186: 1442—1453; http://dx.doi.org/10.1016/j.ajpath.2016.01.017)*

Rapidly progressive glomerulonephritis (GN) is clinically defined by rapid loss of glomerular filtration rate together with proteinuria and hematuria as biomarkers of glomerular filtration barrier disruption.[1] This clinical syndrome can be a manifestation of *ANCA*-associated vasculitis, anti-glomerular basement membrane (GBM) disease, or immune complex GN,[2] which all trigger glomerular vascular necrosis by neutrophil extracellular trap formation and the local cytotoxicity of extracellular histones and complement.[3,4] Glomerular loop necrosis results in plasma leakage into Bowman space, causing hyperplasia of parietal epithelial cells (PECs), that is, glomerular crescent formation.[5] Current treatments of rapidly progressive GN include unspecific, and therefore, toxic immunosuppressant and antiproliferative agents such as steroids and cyclophosphamide. It remains an unmet medical need to identify more specific molecular targets that mediate both glomerular necroinflammation and PEC hyperproliferation in rapidly progressive GN.[6]

The E3 ubiquitin ligase murine double minute-2 (MDM2) regulates proteasomal degradation of the tumor suppressor gene and cell cycle regulator p53 but also facilitates NF-κB signaling.[7,8] MDM2 promotes the survival and proliferation of malignant cells by p53 inhibition.[9—11] Vice versa, MDM2 blockade promotes cell cycle arrest or cell death by enhanced p53 activation. To use these properties, specific small molecule MDM2 inhibitor nutlins were developed and are currently in clinical trials for cancer treatment.[11] We have

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previously reported that MDM2 drives NF-κB signaling-related sterile inflammation in acute tubular injury, whereas its proregenerative effect in the healing phase was exclusively p53 dependent.[7] In podocyte injury, MDM2 promotes glomerular inflammation and podocyte loss by overcoming cell cycle G_2/M arrest and entering mitosis (ie, mitotic catastrophe).[12] The anti-inflammatory and antiproliferative potential of MDM2 inhibition can elicit additive effects, in particular in lymphoproliferative autoimmune diseases, such as systemic lupus erythematosus and lupus nephritis.[13]

We therefore hypothesized that MDM2 would drive crescentic GN by NF-κB–dependent glomerular inflammation and by p53-dependent PEC hyperproliferation and that, vice versa, MDM2 blockade could be a potent and innovative strategy to abrogate crescentic GN.

Materials and Methods

Animals and Experimental Design

C57BL6/N mice were procured from Charles River Laboratories (Sulzfeld, Germany), and *p53*-deficient C57BL6/N mice were obtained from Taconic (Ry, Denmark). Mice were housed in groups of five in standard housing condition with a 12-hour light/dark cycle. Cages, nest lets, food, and water were sterilized by autoclaving before use, and mice were allowed unlimited access to food and water. Six- to 8-week-old mice received an intravenous injection of 75 μL of anti-GBM serum (PTX-001 sheep anti-rat GBM serum; Probetex, Inc., San Antonio, TX). Mice received intraperitoneal injections with 20 mg/kg of the MDM2 inhibitor nutlin-3a (Enzo Life Sciences, Lörrach, Germany) in 50% dimethyl sulfoxide (vehicle) or vehicle only, every alternate day. On day 7, mice were euthanized by cervical dislocation, and kidney tissues were harvested. Blood and urine were collected before sacrifice to evaluate the functional markers of the kidney damage. A part of the kidneys was stored at −80°C for protein isolation and later in RNA solution at −20°C for RNA isolation. A part of the kidney was also fixed in formalin to be embedded in paraffin for histologic analysis. The G*Power software version 3 (Heinrich Heine University, Dusseldorf, Germany) was used to calculate sample size for the experiments performed. An effect size of 0.9, α error of 0.05, and power of 0.8 was considered for these calculations. Mice were assigned different groups with the use of stratified randomization method.[14] Humane end points, as in the ethical approval for the study, were monitored throughout the study. All animal studies were conducted according to the European equivalent of the NIH's *Guide for the Care and Use of Laboratory Animals* and were approved by the local governmental authorities.[15] Urine albumin concentrations (Bethyl Laboratories, Montgomery, TX), urine and plasma creatinine concentrations and blood urea nitrogen (BUN) (DiaSys GmBH, Holzheim, Germany) concentrations were determined with commercially available kits according to the manufacturer's protocol.

Renal Structure, Immunohistochemistry, and Confocal Microscopy

Kidney tissues were fixed in 4% neutral-buffered formalin, dehydrated in graded alcohols, and embedded in paraffin. For routine histologic and morphometric analyses 4-μm sections were stained with periodic acid-Schiff reagent. For immunohistochemistry sections were deparaffinized, rehydrated, transferred into citrate buffer, and either autoclaved or microwave-treated for antigen retrieval and processed as described.[7] The following primary antibodies were used: anti-mouse Ly6G, anti-mouse F4/80 (both Serotec, Oxford, UK), anti-mouse mac-2 (Cedarlane Laboratories, Toronto, ON, Canada), pig anti-mouse nephrin (dilution 1:100; Acris Antibodies, Herford, Germany), rabbit anti-mouse WT1 (dilution 1:25; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-mouse MDM2, anti-mouse fibrinogen (dilution 1:100; Abcam, Cambridge, UK), rat anti-mouse CD31 (dilution 1:100; Dianova, Hamburg, Germany), and rabbit anti-mouse claudin-1 (dilution 1:100; Bioworld Technologies, St. Louis Park, MN). Immunofluorescence staining was evaluated with LSM 510 confocal microscope and LSM software version 4.2 (Carl Zeiss, Jena, Germany). Nuclei were counterstained with To-pro-3 (Life Technologies, Carlsbad, CA). The degree of glomerulosclerosis was assessed by observing 50 randomly selected glomeruli from each mouse kidney from periodic acid-Schiff–stained kidney sections under a light microscope (Leitz DMR; Leica Microsystems, Bensheim, Germany). These glomeruli were then further classified as having no lesions, segmental lesions (<50 of glomerulus), or global lesions (>50% of glomerulus). Cellular crescents were assessed separately, when more than a single layer of PECs was present around the inner circumference of Bowman capsule. Immunostaining positivity was quantified with Photoshop software version CS6 (Adobe Systems, San Jose, CA) and expressed as percentage of total area.

RNA Preparation and Real-Time Quantitative PCR

Total RNA was isolated from kidneys with the use of Ambion RNA extraction kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After quantification, RNA quality was assessed with agarose gels before reverse transcription with Superscript II (Invitrogen). Real-time RT-PCR was performed with SYBRGreen PCR master mix and was analyzed with a Light Cycler 480 (Roche, Mannheim, Germany). All gene expression values were normalized with 18s RNA as a house keeping gene. All primers used for amplification were from Metabion (Martinsried, Germany). Primer sequences used are listed in Table 1.

Protein Isolation and Western Blot Analysis

We extracted protein from kidney tissues with the use of RIPA buffer (Sigma-Aldrich, Hamburg, Germany) that contained protease inhibitors (Roche) and processed it for Western blot