Introduction of the MDM2 T309G Mutation in Primary Human Retinal Epithelial Cells Enhances Experimental Proliferative Vitreoretinopathy

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PURPOSE. The murine double minute (MDM2) is a critical negative regulator of the p53 tumor suppressor, and MDM2 SNP309G is associated with a higher risk of proliferative vitreoretinopathy (PVR); in addition, the MDM2 T309G created using clustered regularly interspaced short palindromic repeats (CRISPR)/associated endonuclease (Cas9) enhances normal rabbit vitreous-induced expression of MDM2 and survival of primary human retinal pigment epithelial (hRPE) cells in vitro. The goal of this study was to determine whether this MDM2 T309G contributes to the development of experimental PVR.

METHODS. hRPE cells expressing MDM2 T309G or T309T only were treated with vitreous from human PVR donors (HV). The expression of MDM2 and p53 in the treated cells was examined by Western blot. The in vitro vitreous-induced cellular responses, such as contraction were assessed, and PVR was induced by intravitreal injection of the hRPE cells with MDM2 T309G or T309T only into rabbit eyes.

RESULTS. Western blot analyses indicated that treatment of hRPE cells with HV led to a significant increase (1.7 ± 0.2-fold) in the expression of MDM2 and a significant decrease in p53 in the cells expressing MDM2 T309G compared with those with MDM2 T309T. In addition, HV promoted contraction of the hRPE cells expressing MDM2 T309G significantly more than those with MDM2 T309T only. Furthermore, MDM2 T309G in the hRPE cells enhanced the development of PVR in a rabbit model.

CONCLUSIONS. The MDM2 SNP309 in RPE cells enhances their potential of PVR pathogenesis. Keywords: MDM2, CRISPR/Cas9, SNP309, RPE, PVR

The major cause of failure in surgery to correct rhegmatogenous retinal detachment (RRD) is proliferative vitreoretinopathy (PVR),1–3 which is characterized with formation of fibroctic-cellular epiretinal membranes (ERMs) composed of extracellular matrix proteins and cells, including RPE cells, retinal glial cells, fibroblasts, and macrophages. Indeed, RPE and glial cells in these vitreal membranes appear to play an especially critical role in the pathogenesis of PVR.4

PVR occurs in 5% to 10% of patients who undergo the surgical repair of RRD, and accounts for approximately 75% of all primary failures after the surgery.5,5,10 Currently, repeat surgery is the only option for PVR;5, however, the visual outcome of the surgery is poor because of the retinal damage resulting from recurrent detachment and the PVR process itself.11 Adjuvant pharmacotherapies that have been evaluated thus far have neither blocked the formation of PVR nor reduced the rates of retinal redetachment secondary to PVR consistently.11 Incomplete understanding of the PVR pathogenesis is a major obstacle for developing new therapeutic options for individuals who are afflicted by this potentially blinding disease.

The oncogenic protein murine double minute 2 (MDM2), an E3 ubiquitin-protein ligase, with a human homologue called Hdm2, is an important negative regulator of the p55 tumor suppressor.12–13 We have previously shown that vitreous from experimental rabbits or PVR patients can trigger the signaling pathway of phosphoinositide 3 kinase (PI3K)/Akt, which phosphorylates MDM2. The phosphorylated MDM2 enhances p53 degradation15; in addition, blocking MDM2 binding to p53 with a small molecule nutlin-3 protects rabbits from retinal detachment in a PVR rabbit model.5

Importantly, the G allele of single nucleotide polymorphisms (SNPs) (rs2279744) in the MDM2 intron promoter locus between exons 1 and 2 attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans.16 Intriguingly, this SNP is also associated with a higher risk of
PVR for RRD patients, but whether this MDM2 G309 SNP contributes to the development of PVR is still unknown.

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated nucleases (Cas) in bacteria and archaea form their adaptive immune system, in which CRISPR RNAs (crRNAs) can guide the Cas to cleave the foreign nucleic acids. In Streptococcus pyogenes (Sp) there are two nuclease domains in the SpCas9, RuvC and HNH, each of which can cleave one strand of the double-stranded target DNA when directed by the crRNA and trans-activating crRNA (tracrRNA). Importantly, this SpCas9 can be engineered which can cleave one strand of the double-stranded target DNA motif. With this CRISPR/Cas9 technology, we have created the MDM2 T309G in the genome DNA of human primary RPE (hRPE) cells and found that the MDM2 T309G mutation enhances rabbit vitreous (RV)-induced expression of MDM2 and cell proliferation, but whether this mutation contributes to the pathogenesis of PVR is still unclear. The goal of the studies presented in this article aims to resolve this question.

**Materials and Methods**

**Major Reagents and Cell Culture**

The antibodies against p53 and MDM2 were purchased from Cell Signaling Technology (Danvers, MA, USA) and from Abgent (San Diego, CA, USA), respectively. The primary antibody against β-actin and the secondary antibodies of the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescent substrate for detection of HRP was from Thermo Scientific (Waltham, MA, USA).

The hRPE cells were purchased from Lonza (Walkersville, MD, USA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM)/nutrient mixture F-12 medium (F12) (Thermo Scientific, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The hRPE cells with MDM2 T309G or T309T only were created using a system of AAV-SpCas9 D10A and AAV-SpGuide (MDM2 or IacZ) and a homology direct recombinate DNA template with MDM2 G309 as described previously. All cells were cultured at 37°C in a humidified 5% CO2 atmosphere.

**Quantitative PCR**

The hRPE cells with MDM2 T309G were plated into six-well plates at a density of 1 x 10⁵ cells per well in DMEM/F12 supplemented with 10% FBS overnight and then serum-starved for 24 hours. Subsequently, the cells were treated with or without vitreous from patients with PVR (HV) (diluted 1:3 in DMEM/F12) for 0.5, 2, 4, and 16 hours. Total RNA was respectively isolated with a RNeasy mini kit (Qiagen, Germantown, MD, USA), and cDNA was synthesized with an iScript cDNA synthesis kit (BioRAD, Hercules, CA, USA) in a program (25°C, 5 minutes; 42°C, 30 minutes; 85°C, 5 minutes; 4°C, forever). The cDNA was subjected to quantitative PCR using a Fast Start universal SYBR green Master mix (Roche, Basel, Switzerland) in a Light Cycler 480 II machine (Roche). Primers of quantitative PCR synthesized by Integrated DNA Technology (Coralville, IA, USA) were (forward: 5′-AGAAGGACAAGAATCTGAGTG-3′, reverse: 5′-GTGCATTTCATAGTGCTA-3′) for MDM2 and (forward: 5′-CCTGGCCTGTTAGACTG-3′, reverse: 5′-AGACGGT CAGTCCCTGTCATAA-3′) for a housekeeping gene hHPRT1.

**Western Blot**

hRPE cells with MDM2 T309G or T309T cultured to 90% confluence in 24-well plates were switched to serum-free medium for 24 hours and then were treated with or without HV (diluted 1:3 in DMEM/F12) for 16 hours. After rinsing twice with ice-cold PBS, cells were lysed in 1 x sample buffer, which was diluted with excretion buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF; 1% Triton X-100, 20 μg/mL aprotinin, 2 mM Na3VO4, and 1 mM phenylmethylsulfonyl fluoride) from the 5 x protein sample buffer (25 mM EDTA, pH 7.0, 10% SDS, 500 mM dithiothreitol, 50% sucrose, 500 mM Tris-HCl, pH 6.8, and 0.5% bromophenol blue). The samples were heated at 95°C for 5 minutes and then centrifuged for 5 minutes at 13,000 g. Proteins in the samples were then separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and subjected to Western blot analysis. Signal intensity was determined by densitometry using NIH Image J (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

**Cell Proliferation Assay**

The hRPE cells with MDM2 T309G or T309T were plated into 24-well plates at a density of 3.0 x 10⁵ cells per well in DMEM/F12 supplemented with 10% FBS. After the cells had attached to the plates, the medium was replaced with DMEM/F12 supplemented with HV (1:3 dilution in DMEM/F12). Three days later, the cells were counted in a hemocytometer; at least three independent experiments were performed.

**Apoptosis Assay**

The hRPE cells with MDM2 T309G or T309T were seeded into 6-cm dishes (1 x 10⁵ cells/dish) in DMEM/F12 with 10% FBS. After cell attachment, the medium was replaced with DMEM/F12 supplemented with HV (1:3 dilution in DMEM/F12). On day 3, the cells were harvested and stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) following the instructions provided with the apoptosis kit (BD Biosciences, Palo Alto, CA, USA). The cells were analyzed using fluorescence-activated cell sorting (FACS) in a Coulter Beckman XL instrument. At least three independent experiments were performed.

**Collagen Contraction Assay**

The hRPE cells with MDM2 T309G or T309T only were resuspended in 1.5 mg/mL of neutralized collagen I (NAMED, Fremont, CA, USA) (pH 7.2) on ice at a density (1 x 10⁶ cells/mL). The mixture was transferred into wells of 24-well plates that had been preincubated overnight with 5 mg/mL BSA/PBS. Ninety minutes later at 37°C, 0.5 mL DMEM/F12 or HV (1:3 dilution in DMEM/F12) was added. On day 3 after being photographed, the gel diameter was measured, and the gel area was calculated using a formula 3.14 x r², where r is the radius of the gel. At least three independent experiments were performed.

**PVR Patient Vitreous**

As previously described, human vitreous from patients with PVR (HV) (1.0 to 1.5 mL) was obtained during pars plana vitrectomy before initiating the pars plana infusion at the
Vancouver Hospital. An ethical approval was obtained before the initiation of this project from the Vancouver Hospital and University of British Columbia Clinical Research Ethics Board and from Nova Scotia Health Research Ethics Board, Dalhousie University. The University of British Columbia Clinical Research Ethics Board and the Nova Scotia Health Research Ethics Board policies comply with the Tri Council Policy and the Good Clinical Practice Guidelines, which have their origins in the ethical principles in the Declaration of Helsinki. Written informed consent was obtained from patients.

Experimental PVR in Rabbits

As described previously,26–28 PVR was induced in right eyes of Dutch Belted rabbits (6 months old, 2–3 kg, male and female) (Covance, Denver, PA, USA). Briefly, a gas vitrectomy was performed by injecting 0.1 mL perfluoropropane (C₃F₈) (Alcon, Fort Worth, TX, USA) into the vitreous cavity 4 mm posterior to the corneal limbus. One week later, all rabbits were injected with 0.1 mL of platelet-rich plasma (PRP) from rabbits and 0.1 mL DMEM/F12 containing either 3.0 × 10⁵ cells of hRPE cells with MDM2 T309G or T309T only (10 rabbits per group) under an operative microscope. The retinal status was examined with an indirect ophthalmoscope through a +30 diopter (D) fundus lens on days 1, 3, 5, 7, 14, 21, and 28 by two ophthalmologists without knowing the injected cell types. PVR was graded using a standard of the Fastenberg classification from 0 through 5.27,29 On day 28, the rabbits were killed. All surgeries adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and a protocol for the use of animals was approved by the Schepens Animal Care and Use Committee (Boston, MA, USA).

Statistics

The data were analyzed using an unpaired t-test or a Mann-Whitney test. A power (P) value less than 0.05 was considered statistically significant.

RESULTS

HV-Induced Expression of MDM2 in the hRPE Cells

MDM2 T309G is associated with a higher risk of PVR1 and the CRISPR/Cas9-created MDM2 T309G in hRPE cells enhanced RV-induced expression of MDM2 and a reduction in p53.21 Thus, we investigated whether this mutation in hRPE cells rendered the cells responsive to HV from patients with PVR. hRPE cells containing MDM2 T309G or T309T were treated with HV for 16 hours, and the cell lysates were subjected to Western blot analyses. As shown in Figure 1, HV treatment led to a 1.760.2-fold increase in the protein expression and a 1.730.15-fold increase in the mRNA of MDM2 in the hRPE cells with MDM2 T309G, compared with the cells with MDM2 T309T only. In addition, HV treatment suppressed expression of p53 by 1.75-fold more in the hRPE cells with MDM2 T309T than in the cells with MDM2 T309T only.
**MDM2 T309G Enhanced HV-Induced Cell Proliferation and Survival Against Apoptosis**

We speculated that an increase in MDM2 and decrease in p53 might enhance cell proliferation and survival. Thus, we treated the hRPE cells expressing MDM2 T309G or T309T only with HV and assessed their proliferation and survival. As shown in Figure 2A, whereas HV-induced proliferation of hRPE cells with MDM2 T309T only, the proliferative response was greater in hRPE cells with MDM2 T309G than those with MDM2 T309T. Moreover, the two types of hRPE cells were starved for serum for 3 days to induce apoptosis, and the cells were treated with or without HV, and the apoptosis was analyzed using a FITC-conjugated annexin V-based apoptosis kit by FACS. The results (Fig. 2B) showed that HV promoted survival of hRPE cells with MDM2 T309G better than those with MDM2 T309T only.

**MDM2 T309G Promoted HV-Induced Contraction of hRPE Cells and Enhanced Experimental PVR**

One of the major characteristics of PVR is the formation of epiretinal membranes, which can contract and cause the retinal detachment. Thus, we assessed the possible contribution of MDM2 T309G to the development of PVR. In a contraction assay, cells were mixed with collagen to mimic the conditions of an epiretinal membrane. As shown in Figure 3, hRPE cells expressing MDM2 T309G contracted more in response to HV than cells with MDM2 T309T only, indicating that MDM2 T309G in the hRPE cells might contribute to the pathogenesis of PVR.

Thus, we investigated whether the MDM2 T309G polymorphism influenced the pathogenesis of PVR. hRPE cells expressing MDM2 T309T only or the cells with MDM2 T309G were injected intravitreally into rabbit eyes. On the seventh day after cell injection, the cells with MDM2 T309G had begun to form fibrotic membranes, whereas all those with MDM2 T309T remained as cell aggregates in the vitreous (Fig. 4). Observation on weeks 2, 3, and 4 indicated significant differences in the development of PVR in a rabbit model between the two groups of hRPE cells with MDM2 T309T only and those with MDM2 T309G based on the standard of the Fastenberg classification from 0 through 5, as shown in Figure 4A. On week 4, for instance, the retina detached in 3 of 10 rabbits that were intravitreally injected with the MDM2 T309G hRPE cells, but none of the 10 rabbits containing MDM2 T309T only developed retinal detachment. Histologic examination indicated that there were more cells migrating into the retinas in the vitreous injected with hRPE cells expressing MDM2 T309G than those expressing MDM2 T309T (Fig. 4B). Taken together, these experiments demonstrated that MDM2 T309G polymorphism in the hRPE cells contributes to the pathogenesis of experimental PVR.
DISCUSSION

MDM2 T309G, which was generated in the hRPE cells using the CRISPR/Cas9, led to enhancement of HV-stimulated expression of MDM2 and cell proliferation, survival, and contraction in response to HV, as well as development of PVR in a rabbit model. Although the cells used in the in vivo PVR model were a mixed population of MDM2 genotypes (20% T309T, 70% T309G, and 10% G309G), they demonstrated more significant membrane formation than those expressing only MDM2 T309T.

We chose rabbits for the experimental PVR because of the ease of working in these animals’ eyes. The smaller size of the rabbit lens compared with the eyeball permits manipulations to be performed within the eye, without causing any damage to the lens/retina. Rabbits lack a distinct macula; however, the medullary wing detachment simulates a retinal detachment in humans and shows PVR-like features. Retinas from rabbits are only partially vascularized, in contrast to those in humans, which are completely vascularized. However, the use of other common laboratory animals such as rats and mice, which have higher retinal vascularization, can be difficult to work with due to the difficulties in isolating and purifying the small volume of the vitreous. Indeed, the mouse lens occupies nearly 75% of the eye. It is also important to note that the cell injection into rabbit eyes to induce experimental PVR does not involve any break in the retina. Indeed, RRD models leading to the

FIGURE 4. The hRPE cells with MDM2 T309G had more potential to induce PVR in a rabbit model. (A) PVR was induced in the right eyes of Dutch Belted rabbits (10 rabbits per group) as previously described. Briefly, 1 week after gas vitrectomy, rabbits were intravitreally injected with 0.1 ml PRP and hRPE cells (3 × 10⁵) with MDM2 T309 only (T/T) or MDM2 T309 plus G309 (T/G) in 0.1 ml DMEM/F12. The rabbits were examined with an indirect ophthalmoscope and the PVR status for each rabbit was plotted on days 1, 3, 5, 7, 14, 21, and 28. The data were subjected to Mann-Whitney analysis. A P value less than 0.05 is considered a significant difference between the two groups (T/T and T/G). (B) The representative eyeballs from rabbits with PVR stages 0 and 1 (hRPE T309T), and 2 and 3 (hRPE T309G) were fixed, sectioned and stained with hematoxylin-eosin. In the eyes with PVR stages 2 and 3 injected with hRPE expressing MDM2 T309G, there were some cells (arrowheads) attaching to the retina.
development of PVR\textsuperscript{31} may be considered in future studies in an effort to more closely resemble the clinical condition.

Vitreous can induce the transcription factor specific protein (Sp)\textsuperscript{132,33} to bind the intron promoter of \textit{MDM2} in the hRPE cells with \textit{MDM2} T309G and thus increase its expression,\textsuperscript{41} resulting in a reduction in p53, and enhanced cell proliferation, viability, and contraction, leading to an increased potential for PVR. These findings might be relevant to other eye diseases, such as proliferative diabetic retinopathy (PDR), in which there are similar fibrotic-cellular membranes. Thus, it will be intriguing to determine whether this \textit{MDM2} T309G polymorphism also contributes to the development of PDR.

Both normal RV\textsuperscript{21} and HV (Fig. 1) induce expression of \textit{MDM2} as well as proliferation and survival of hRPE cells. Vitreous contains numerous growth factors and cytokines, including epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), transforming growth factor (TGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), connective tissue growth factor and interleukins (IL)-6 and -8, which are implicated in PVR.\textsuperscript{34} Some growth factors have been reported to be increased during the development of PVR, such as platelet-derived growth factor (PDGF)\textsuperscript{35} and TGF-\beta\textsuperscript{36} and these vitreal growth factors and cytokines may be essential drivers of the PVR pathogenesis.\textsuperscript{44} Moreover, the exposure of cells, such as RPE cells, to vitreous containing rich growth factors and cytokines may stimulate them to secrete growth factors and/or cytokines, increasing the local concentrations of these agents.\textsuperscript{34} A cocktail of agents neutralizing EGF, TGF-\beta, IL-8, TGF-\beta, HGF, IGF-1, and PDGFs blocks experimental PVR induced by fibroblasts,\textsuperscript{36} but whether the same approach would prevent PVR induced by RPE cells that harbor the \textit{MDM2} 309SNP is unknown.

Adeno-associated viruses (AAVs) are small viruses that are not at present known to cause any disease, and thus their derived vectors have great potential for human gene therapy.\textsuperscript{37,38} The eye is an especially ideal target organ for gene therapy, as it is easily accessible and immune privileged.\textsuperscript{39} In this study, we showed that the AAV-CRISPR/Cas9-created \textit{MDM2} T309G contributed to the development of experimental PVR in a rabbit model, further demonstrating that this CRISPR/Cas9 system is a novel powerful tool for the targeted introduction of mutations into eukaryotic genomes for the generation of reproducible disease models.\textsuperscript{40,41}

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