Cell Injury, Repair, Aging, and Apoptosis

Prevention of Age-Related Macular Degeneration–Like Retinopathy by Rapamycin in Rats

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Age-related macular degeneration, a neurodegenerative and vascular retinal disease, is the most common cause of blindness in the Western countries. Evidence accumulates that target of rapamycin is involved in aging and age-related diseases, including neurodegeneration. The target of rapamycin inhibitor, rapamycin, suppresses the senescent cell phenotype and extends life span in diverse species, including mice. Rapamycin decreases senescence-associated phenotypes in retinal pigment epithelial cells in culture. Herein, we investigated the effect of rapamycin on spontaneous retinopathy in senescence-accelerated OXYS rats, an animal model of age-related macular degeneration. Rats were treated with either 0.1 or 0.5 mg/kg rapamycin, which was given orally as a food mixture. In a dose-dependent manner, rapamycin decreased the incidence and severity of retinopathy. Rapamycin improved some (but not all) histological abnormalities associated with retinopathy. Thus, in retinal pigment epithelial cell layers, rapamycin decreased nuclei heterogeneity and normalized intervals between nuclei. In photoreceptor cells, associated neurons, and radial glial cells, rapamycin prevented nuclear and cellular pyknosis. More important, rapamycin prevented destruction of ganglionar neurons in the retina. Rapamycin did not exert any adverse effects on the retina in control disease-free Wistar rats. Taken together, our data suggest the therapeutic potential of rapamycin for treatment and prevention of retinopathy. *(Am J Pathol 2012, 181:472–477; http://dx.doi.org/10.1016/j.ajpath.2012.04.018)*

Age-related macular degeneration (AMD) and diabetic retinopathy are the leading causes of blindness in the Western countries. There are few treatment options.[1] Wet AMD (the more severe type of AMD) and proliferative retinopathy are associated with neovascularization (angiogenesis), in part because of secretion of vascular endothelial growth factor (VEGF) by retinal pigment epithelial (RPE) cells.[2–6] Therefore, anti-VEGF therapy has been recently approved for these conditions. In RPE cells, VEGF and other cytokines are induced by the target of rapamycin (mTOR)/hypoxia-inducible factor-1 pathway by insulin, glucose, growth factors, and inflammatory cytokines.[7–15] Rapamycin prevents mitogen-induced hypoxia-inducible factor-1 and hypoxia-inducible factor-1–dependent transcription and secretion of VEGF.[15,16] Based on these effects, rapamycin can be considered for therapy of wet AMD and proliferative retinopathy.[15,17,18] Also, there is one fundamental reason why rapamycin may be indicated for AMD: rapamycin is a potential anti-aging drug that may postpone age-related diseases.[19,20] In post-mitotic quiescent cells, the mTOR pathway drives the acquisition of senescent phenotype and rapamycin suppresses the senescent phenotype, preventing conversion of quiescence to senescence (geroconversion).[21,22] The mTOR pathway may link cellular aging to organismal aging and age-related diseases, such as AMD.[19,20] Furthermore, inhibition of mTOR slows down aging and extends life span in worms, flies, and mice.[23–28] By slowing down aging, rapamycin should delay age-related diseases, including AMD.[29] The clinical application of rapamycin for AMD treatment has been hindered by the lack of appropriate animal models for human

AMD. Recently, we have developed a rat model of AMD similar to human AMD.[30–35] Herein, we tested whether oral administration of rapamycin can delay AMD in OXYS rats. We found that rapamycin prevented development of AMD in AMD-prone rats. More important, there was no retinal adverse effect in healthy Wistar rats used as a control for potential adverse effects. Given that rapamycin is a clinically available drug, our data suggest systemic administration of rapamycin to prevent and treat AMD in humans.

Materials and Methods

Ethics Statement

All animal procedures were in compliance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research and the European Communities Council Directive 86/609/EES. All manipulations with animals were approved by Scientific Council 9 of the Institute of Cytology and Genetics Siberian Branch of the Russian Academy of Sciences, according to The Guidelines for Manipulations with Experimental Animals (the decree of the Presidium of the Russian Academy of Sciences of April 2, 1980, 12000-496).

Animals and Diet

Male senescence-accelerated OXYS ($n = 45$) and age-matched male Wistar ($n = 45$) rats (as controls) were obtained from the Breeding Experimental Animal Laboratory of the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences (Novosibirsk, Russia). At the age of 4 weeks, the pups were weaned, housed in groups of five animals per cage (57 × 36 × 20 cm), and kept under standard laboratory conditions (22°C ± 2°C, 60% relative humidity, and natural light), provided with a standard rodent feed (PK-120-1, Ltd, Laboratorsnab, Russia), and given water ad libitum. To study the influence of rapamycin supplementation on retinopathy development, 1.5-month-old OXYS and Wistar rats were randomly assigned to one of two groups ($n = 15$): control diet or control diet supplemented with rapamycin (Rapamune; Wyeth Pharmaceuticals Inc., Berkshire, UK), 0.1 or 0.5 mg/kg of body weight per day from the age of 1.5 to 3.5 months. Body weight was measured during the experiment. Rapamycin supplementation did not affect body weight in both OXYS and Wistar rats ($F_{2,89} = 0.57$, $P = 0.59$). An analysis of variance demonstrated that body weight depended on genotype ($F_{1,89} = 81.0$, $P < 0.000$) and was higher in Wistar rats than in OXYS rats (356 ± 32 g and 296 ± 27 g, respectively).

Ophthalmoscopic Examination

All animals were examined by an ophthalmologist twice, before and after rapamycin supplementation, at the ages of 1.5 and 3.5 months. All rats underwent funduscopy with a Heine BETA 200 TL Direct Ophthalmoscope (Heine, Herrsching, Germany) after dilatation with 1% tropicamide. An assessment of stages of retinopathy was performed

according to the Age-Related Eye Disease Study grade protocol.[36] A Kowa Genesis-D fundus camera (Japan) was used as a handheld digital fundus camera to take digital fundus photographs of the retina. The degree of retinopathy was estimated as follows: 0 arbitrary units (AU) corresponds to healthy retina; 1 AU, appearance of drusen and other pathological changes in the RPE and partial atrophy of the choroid capillary layer; 2 AU, exudative detachment of RPE and of retinal neuroepithelium, with further choroid capillary layer atrophy; and 3 AU, neovascularization and exudative-hemorrhagic detachment of RPE and neuroepithelium scarring. Five days after the last eye examination, the rats were sacrificed and the eyes were removed. To measure S6 ribosomal protein and phosphorylated S6, we used retinas obtained from rats treated with 0.5 mg/kg rapamycin (and control animals). The retina was separated from the other tissues, placed in microcentrifuge tubes for protein isolation, and frozen in liquid nitrogen. All specimens were stored at −70°C before the analysis. The left eyes of these animals were used for histological and morphometric studies.

Immunoblot and Antibodies

Frozen tissues of retina were homogenized in protein lysis buffer radioimmunoprecipitation assay (50 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS; and 1 mmol/L EDTA) supplemented with protease inhibitor cocktail (P8340; Sigma-Aldrich, St. Louis, MO). After incubation for 20 minutes on ice, samples were centrifuged at 9660 × g at 4°C for 30 minutes and the supernatants were transferred to new tubes. Total proteins were measured with a Bio-Rad Bradford kit (Bio-Rad Laboratories, Hercules, CA). Samples were resolved on 12% SDS-PAGE on Tris-glycine buffer (25 mmol/L Trisbase, 190 mmol/L glycine, and 0.1% SDS) and transferred to nitrocellulose membranes. Membranes were probed with specific antibodies: S6 Ribosomal Protein and Phospho-S6 Ribosomal Protein (1:1000; Cell Signaling Technology, Danvers, MA) at 4°C, overnight, and with anti-actin (1:1000; Abcam, Cambridge, MA). Signals were scanned, and the intensity of the emission bands was measured using ImageJ version 1.41 (NIH, Bethesda, MD).
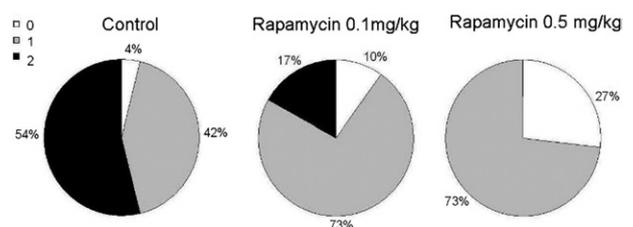

Figure 1. Stages of retinopathy in 3-month-old control and rapamycin-treated OXYS rats. Treatment (0.1 or 0.5 mg/kg per day of rapamycin) is started at 1.5 months. In each group, 30 eyes of 15 animals are examined. Data are presented as percentage of eyes with 0, 1, and 2 stages of retinopathy.
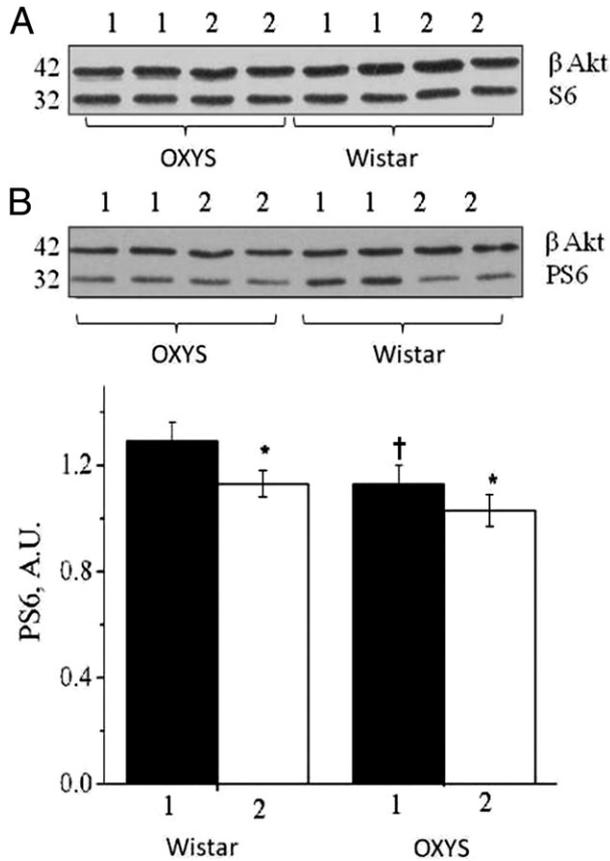
Figure 2. Effects of rapamycin (0.5 mg/kg per day) on phosphorylation status of S6 ribosomal protein in the retina OXYS versus Wistar rats. **A:** Levels of S6 by immunoblot. **B:** Levels of phosphorylated S6 (PS6) by immunoblot. **Bottom panel:** The phosphorylation status of S6 is calculated by intensity of PS6/intensity of β-actin ($n = 5$). *$P < 0.05$, a statistically significant effect of rapamycin; †$P < 0.05$ between OXYS and Wistar rats. Data are given as mean ± SEM. 1, control; 2, rapamycin.

Histological Examination

For light microscopic analysis, the cornea and lens were removed, and the posterior wall of the eye was collected and immersed in 10% neutral formaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). The posterior wall of the eye was collected and fixed. Serial frontal sections (4 to 5 μm

thick) were cut, stained with H&E, and examined with a photomicroscope (Axiostar Plus, Carl Zeiss, Germany). The morphometric parameters were measured using quantitative analyses of the images performed with Axiovision software (Zeiss, Thornwood, NY). Estimation was performed by examination of the five fields of view for each retina, with magnification of 10 × 100 using a frame area of 900 μm. The specific area of choroid vessels (open, with stasis, blood cell aggregation, or thrombosis) and the specific area of RPE cells were measured, and their ratio to the total area of choroid or RPE, respectively, was calculated. The number of ganglion neurons with central and total chromatolysis and nuclear pyknosis was calculated separately. The percentage of photoreceptors with nuclear pyknosis was calculated per 1000 photoreceptors, percentage of radial glial cells, and neurons in the inner nuclear and ganglionar layer per 200 corresponding cells of the retina.

Statistical Analysis

Data were analyzed using repeated-measures analysis of variance with the statistical package Statistica 6.0 (StatSoft, Tulsa, OK). Two-way analysis of variance was used to evaluate the effects of treatment. The independent variables were genotype (Wistar or OXYS) and treatment (control or rapamycin). A Newman-Keuls post hoc test was applied to significant main effects and interactions to estimate the differences between particular sets of means. One-way analysis of variance was used for individual group comparisons. To assess the therapeutic effectiveness, we performed a dependent pairwise comparison of the eye states before and after treatment (*t*-test for dependent samples). Data are represented as means ± SEM. Results were considered statistically significant if $P < 0.05$.

Results

Rapamycin Inhibits mTOR and Clinical Retinopathy

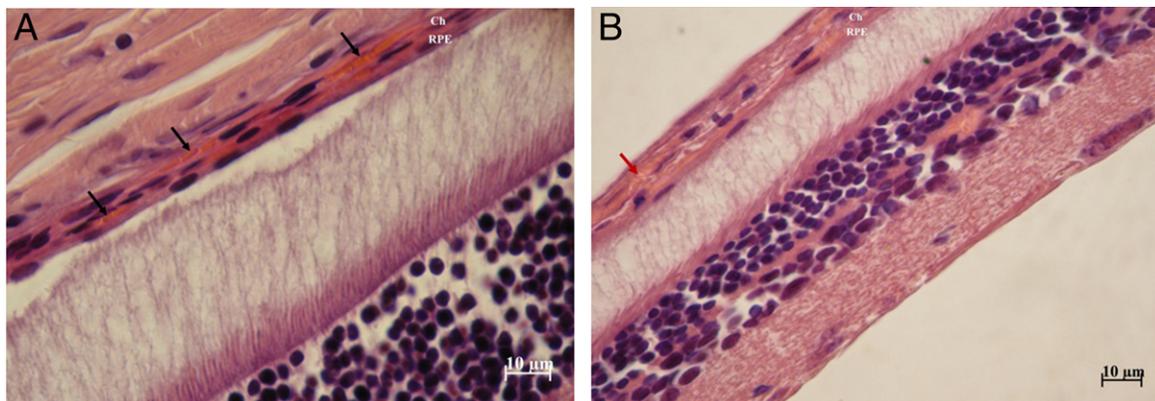There was no difference between 1.5-month-old OXYS rats assigned to experimental and control groups: 22%

Figure 3. Morphological analysis of the choroid. **A:** In Wistar rats, choroid capillaries are functionally active, with a few blood cells (**black arrows**). **B:** In OXYS rats, plethora, stasis, and sludge of the blood cells in capillaries of the choroid (**red arrow**). Staining with H&E. Ch, choroid.

Table 1.   Effects of Rapamycin on the Morphometric Parameters of the Retina of OXYS Rats

Parameters	Wistar rats	OXYS rats	OXYS rats given rapamycin
Open choroid vessels	18.53 ± 1.26	11.47 ± 2.56*	11.24 ± 2.05*
Specific area of choroid vessels with stasis and thrombosis in the section	4.20 ± 0.53	18.58 ± 2.81*	15.22 ± 1.34*
Specific area of the intraretinal vessels	0.850 ± 0.118	0.687 ± 0.084	0.840 ± 0.094
Average area of RPE cells in the section	5.29 ± 0.043	2.29 ± 0.165*	2.47 ± 0.250*
Photoreceptors with nuclear pyknosis	0.391 ± 0.048	0.887 ± 0.160*	0.200 ± 0.058*†
Associative neurons with nuclear pyknosis	0.429 ± 0.052	0.637 ± 0.063*	0.414 ± 0.077†
Radial glial cells with nuclear pyknosis	1.33 ± 0.19	4.53 ± 1.06*	1.31 ± 0.25†
Ganglion neurons with central chromatolysis	7.14 ± 0.90	6.29 ± 0.89	3.43 ± 0.81*†
Ganglion neurons with total chromatolysis	2.17 ± 0.46	3.24 ± 0.85*	2.86 ± 0.51
Ganglion neurons with pyknosis	2.10 ± 0.38	6.43 ± 0.87*	1.14 ± 0.37*†

Data are given as mean ± SEM. Rapamycin was given at 0.5 mg/kg per day from 1.5 to 3.5 months.
*$P < 0.05$ compared to control Wistar rats.
†$P < 0.05$ compared to OXYS rats.

and 20% of the rats had signs of stage 1 retinopathy, respectively. By the age of 3.5 months, 42% and 54% of control OXYS rats developed stages 1 and 2 retinopathy, respectively (Figure 1). Diagnosis was based on ophthalmoscopic examination, followed by digital fundus photographs of the retina (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). In a dose-dependent manner, rapamycin decreased the incidence and severity of retinopathy (Figure 1). Thus, only 17% of the rats treated with 0.1 mg/kg rapamycin developed stage 2 retinopathy and 10% of the rats remained disease free. At a higher rapamycin dose, the preventive effect was even more prominent. None of the rats treated with 0.5 mg/kg rapamycin developed stage 2 retinopathy, and 27% of the rats were disease free. In Wistar rats that do not naturally develop retinopathy, repeated inspections did not reveal pathological alterations in the retina of rapamycin-treated rats. Next, we investigated phosphorylation of S6, a target of the mTOR pathway and a marker of mTOR activity. There was no significant difference between levels of S6 in the retina of OXYS and Wistar rats; S6 levels were not affected by the treatment (Figure 2). More important, rapamycin decreased phosphorylation of S6 in OXYS and Wistar rats ($P > 0.031$ and $P > 0.007$, respectively), indicating a decrease in mTOR activity caused by rapamycin (Figure 2).

Rapamycin Prevents Neurodegeneration as Assessed by Histological Examination

We next compared the histological features of the retina in OXYS and Wistar rats. In OXYS rats, there were prominent abnormalities of the choroidal vasculature, RPE cells, photoreceptors, associated and ganglion neurons, and radial glial cells. Unlike the choroid of Wistar rats (Figure 3A), the choroid of OXYS rats was characterized by incident aggregation of blood cells, stasis, and thrombosis of small vessels (Figure 3B). Consequently, the specific area of vessels with signs of partial occlusion of retinal vessels was significantly greater in OXYS rats compared with Wistar controls. Treatment with rapamycin did not prevent vessel abnormalities in OXYS rats (Table 1).

In normal Wistar rats, RPE cells possessed a prismatic shape with oval nuclei (Figure 4A). The cellular monolayer was dense with normal contacts, suggesting a functional blood-retinal barrier. In OXYS rats, RPE cells were flat, with a variable size and shape of their nuclei. We have detected vacuolization of the cytoplasm: nuclear pyknosis. The distance between cells was increased, suggesting increased permeability of the blood-retinal barrier (Figure 4B). In rapamycin-treated OXYS rats, RPE cells retained a uniform shape and close contacts, consistent with the functional barrier, although nuclei remained flat (Figure 4C). In OXYS rats, there was nuclear

Figure 4.   The retinal pigment epithelium layer. **A:** In Wistar rats, RPE cells are prismatic, with rounded nuclei (**black arrows**). **B:** In OXYS rats, RPE cells are of different size, flattened, and separated from each other (**white arrows**). **C:** Treatment with rapamycin several times improves the size and shape of RPE cells in OXYS rats (**red arrows**). Staining with H&E. RPE, retinal pigment epithelium.

pyknosis (clumped chromatin) in photoreceptors (see Supplemental Figure S2 at *http://ajp.amjpathol.org*), whereas treatment with rapamycin decreased several photoreceptors with pyknotic nuclei fourfold (Table 1). In OXYS rats, there were hyperchromatic pyknomorphic neurons, edemic neurons in association with radial glia, that were hyperchromic and pyknomorphic. Rapamycin prevented the pathological characteristics in associated neurons and radial glial cells of the inner nuclear layer (Table 1). In the ganglionar layer, in both control and rapamycin-treated OXYS rats, some neurons were pyknomorphic and chromolytic. In OXYS compared with Wistar rats, there was 49% more neurons with total chromolysis and three times more with pyknosis, indicating a decline of reserve capacity of neurons and a sign of hypoxia. Treatment with rapamycin prevented destruction of ganglion neurons in the retina of OXYS rats (Table 1). Intraretinal vessels were pathologically enlarged in both control and rapamycin-treated OXYS rats without significant difference, despite treatment with rapamycin (Table 1). Thus, rapamycin prevented significant abnormalities in RPE cells and the retinal barrier and neurodegeneration in photoreceptors and associated and ganglion neurons but did not significantly improve circulation in blood vessels.

Discussion

Herein, we demonstrated that rapamycin prevented AMD-like retinopathy in OXYS rats. More important, rapamycin suppressed disease progression, even though it was started after some rats had already developed stage 1 retinopathy. Interestingly, rapamycin did not decrease VEGF levels; instead, it slightly increased it in the retina of OXYS rats (see Supplemental Table S1 at *http://ajp. amjpathol.org*). Recently, we reported accelerated reduction of VEGF gene expression in the retina of OXYS rats compared with control (Wistar) rats during retinopathy manifestation,[32] because of early alterations in RPE cells and choroid vessels in OXYS rats. We supposed that such changes are prerequisite for the development of retinopathy, and its normalization is necessary for the prevention of disease. VEGF has protected photoreceptors and neuronal cells.[37–39] In agreement with our result, rapamycin inhibited retinal and choroidal neovascularization in mice without a reduction in VEGF.[40]

Taken together with other preclinical data, our work suggests rapamycin for the treatment and prevention of AMD and retinopathy. In fact, preliminary data suggest that rapamycin given systemically can alter the clinical course of the wet form of AMD.[17] Rapamycin is a clinically approved drug that has been used for a decade in renal transplant patients. Even at high doses, long-term administration of rapamycin for many years, in combination with immunosuppressants, had moderate adverse effects. Short-term treatment with rapamycin had no adverse effects in healthy volunteers[41] and prevented nutrient-induced insulin resistance.[42] Rapamycin has been used in pregnant women (followed by a normal birth)[43] and children.[44] Despite common misconception, rapamycin (in appropriate doses and schedules) improves

the immunity in mice,[45] including old mice.[46] Furthermore, some adverse effects of long-term doses in humans are actually beneficial effects, including starvation-like changes in lipid metabolism.[47] In humans, rapamycin prevented cancer and treated some pre-existing tumors.[48–51] In animal models, rapamycin prevented most age-related diseases, including atherosclerosis, neurodegeneration, and cancer.[19,20,26–29,52–56] Rapamycin is even indicated for treatment of chronic viral infections, such as HIV.[57] Although rapamycin can be administered for these indications, it may simultaneously prevent AMD in the same patient.

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