TAK1 Inhibition Accelerates Cellular Senescence of Retinal Pigment Epithelial Cells

Zeev Dvashi, Yaron Green, and Ayala Pollack

Kaplan Medical Center, affiliated with Hadassah-Hebrew University of Jerusalem, Rehovot, Israel

Correspondence: Ayala Pollack, Department of Ophthalmology, Kaplan Medical Center, 76100 Rehovot, Israel; ayala_p@clalit.org.il.

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PURPOSE. Oxidative stress and cellular senescence are known to contribute to the development of AMD; however, the mechanism is not fully understood. This study investigated the role of TGF-β-activated kinase 1 (TAK1) in the senescence of RPE cells as a model for the development of dry AMD.

METHODS. Cultured human RPE cells were treated with the TAK1 inhibitor 5Z,7-oxozeaenol for 1 hour, and then treated with 200 μM hydrogen peroxide for 1 hour. Human RPE cells that were not pretreated with TAK1 inhibitor for 1 hour served as controls. Senescence-associated β-galactosidase (SA-β-gal) activity was detected by histochemistry, and p53 expression by immunoblotting. Cell-cycle and apoptosis rate in RPE cells were determined by flow cytometry.

RESULTS. The TAK1 expression in human RPE cells was high and was altered on oxidative stress. Transforming growth factor-β-activated kinase 1 inhibition led to reduction in cell proliferation, cell-cycle arrest at G0/G1, and increased SA-β-gal expression, all known to be features of cell senescence. Exposure of cells to oxidative stress combined with inhibition of TAK1 activity decreased the expression of apoptotic proteins, such as p53, and promoted cellular senescence. Aberrant TAK1 activity in RPE cells triggered their secretion of factors that induced hypertrophy and fibrotic changes in neighboring cells.

CONCLUSIONS. The in vitro evidence indicated a role for TAK1 in the onset of senescence in RPE cells. The data shown hereby demonstrated that TAK1 activity is essential for maintaining normal function of RPE cells. Elucidation of its role in mechanisms underlying RPE cellular senescence induction may potentiate development of powerful tools for halting the development of dry AMD.

Keywords: senescence, oxidative stress, TAK1, AMD

Age-related macular degeneration (AMD) is still the leading cause of blindness in the Western world. It is widely accepted that RPE cells are important players in the pathologies in both "dry" and "wet" forms of the disease. The RPE layer is composed of nonrenewable multifunctional cells that normally form a quiescent monolayer between the photoreceptors and the vascular choroid, and is essential for the retinoid cycle, nutritional support of photoreceptors, and proper functioning of the outer blood-retina barrier. At an early stage of dry AMD, the RPE cells accumulate lipofuscin and participate in the formation of extracellular drusen deposits in the macular area. The increase in drusen enhances the risk of progression to the wet form of AMD. Geographic atrophy, or advanced dry AMD, is characterized by degeneration and loss of RPE cells and their associated photoreceptors. In contrast, advanced wet AMD is associated with activation of RPE cells, expression of inflammatory factors and angiogenic growth factors, and growth of new vessels from the choroid through the Bruch’s membrane to a site adjacent to the RPE layer. Thus, in both the early and late stages of AMD, the pathological changes target the RPE cells.

According to a current hypothesis on early AMD development, which focuses on oxidative stress affecting the RPE cells, it was found that RPE cells are subjected to high levels of oxidative stress from several sources, including visible light and contact with a high-oxygen environment. In addition, accumulation of lipofuscin, a product of lysosomal degradation, increases in aging of RPE cells and can contribute to oxidative damage. Whereas most studies of oxidative stress and AMD have focused on elucidating mechanisms of oxidative stress–inducing apoptosis, widespread death of RPE cells is not usually seen in early AMD. Retinal pigment epithelium cell death, termed geographic atrophy, occurs in only 10% to 15% of AMD patients and only in the late stages of the disease. Moreover, RPE cells can tolerate oxidative stress without initiation of cell death. These cells exhibit a distinct set of physiological responses to high levels of oxidative stress, including actin rearrangement.

At the same time, prolonged oxidative injury can affect the integrity and function of tight junctions between RPE cells, potentially leading to disruption of the blood-retinal barrier. It is therefore possible that the response of RPE cells to long periods of oxidative stress is associated with the pathophysiology of early AMD.

A major effect of oxidative stress is the induction of cellular senescence, the irreversible growth arrest of cells. Premature senescence has been implicated as a potentially important pathophysiological mediator of RPE cell atrophy in geographic atrophy. Markers of senescence, such as telomere shortening and altered gene expression, have been identified in RPE cells exposed to the advanced glycation end products and in the Bruch’s membrane in AMD. Recent in vitro studies in the human RPE cell line showed that exposure to oxidants yields four well-known senescence markers: hypertrophy,
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Materials and Methods

Materials

The human RPE cell line (ARPE-19) was maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 Nutrient Mixture (DMEM/F12) containing 2 mM glutamine, 30 µg/mL penicillin, 50 µg/mL streptomycin (Biological Industries Ltd., Beit Ha-Emek, Israel), and 10% fetal bovine serum (FBS; Beit Ha-Emek) and passaged and subcultured every 3 to 4 days. Between passages 10 and 20, the cells were seeded in 100-mm dishes and grown in DMEM/F12 medium. Treatments were applied to the cells in 60-mm or 96-well plates (Corning, Amsterdam, The Netherlands), and usually initiated 24 hours after cell plating. Before their exposure to oxidative stress, cells were treated for 1 hour with TAK1 inhibitor (5Z-7-oxozeaenol dissolved in dimethyl sulfoxide [DMSO]) according to the manufacturer's recommendation (Merck, Whitehouse Station, NJ, USA), diluted in human RPE medium with 10% FBS to a final concentration of 1 µM, or treated with DMSO only (control). The cells were then exposed to oxidative stress by treatment for 1 hour with hydrogen peroxide (200 µM, diluted in ARPE-19 medium with 10% FBS; Sigma, Rehovot, Israel). The cells were allowed to recover for the indicated times in stressor-free human RPE medium with 10% FBS.

XTT Assay

Cells (1 × 10^4 cells/well) were seeded in 96-well cell culture clusters coated with collagen. The next day, the DMEM/F12 medium was replaced with fresh medium with either TAK1 inhibitor or DMSO. The cells were then assayed for viability according to manufacturer's directions (Cell Proliferation Kit; Biological Industries Ltd.). Absorbance of the samples against a background control was performed with a spectrophotometer (ELISA reader; RayTo, Shenzhen, China) at a wavelength of 450 nm. All experiments were performed at least in triplicate.

Senescence-Associated β-Galactosidase Staining

Activity of SA-β-gal was detected as previously described, with slight modifications. Retinal pigment epithelium cells (1 × 10^5 cells) were plated on 60-mm plates. The cells were washed once with PBS (pH 7.2), fixed with 0.5% glutaraldehyde (diluted in PBS pH 7.2), and washed in PBS supplemented with 1 mM MgCl2. The cells were then stained by incubation with X-gal solution (1 mg/mL X-gal, 0.12 mM K4FeCN6, 0.12 mM K3FeCN6, Inalco, Milano, Italy) in 1 mM MgCl2 in PBS (pH 6.0) at 37°C for 4 to 6 hours. After this, the cells were washed three times at room temperature with PBS and photographed at low magnification (×200) under a light microscope (CKX41; Olympus, Airport City, Israel). All experiments were performed at least in triplicate.

Protein Analysis

Cells were lysed in 50 mM Tris, 150 mM NaCl, and 5 mM EGTA (pH 7.5), supplemented with complete protease inhibitor cocktail, 0.75% NP-40, 2 mM Na3VO4, 50 mM NaF, and 10 mM NaPPI. Cell debris was pelleted and protein concentration in the supernatant was determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with the following primary antibodies: mouse monoclonal phospho-p38 (Thr180/Tyr182) antibodies (Sigma), rabbit polyclonal p38 antibodies (Sigma), mononclonal anti-human p53 (DO-1) antibodies (generous gift from Sara Lavi), rabbit polyclonal TAK1 antibodies (Cell Signaling, Danvers, MA, USA), and monoclonal mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (Millipore, Billerica, MA, USA). Following reaction with the appropriate secondary antibodies, peroxidase-conjugated IgG antibodies (Jackson, West Grove, PA, USA), blots were subjected to enhanced chemiluminescence (Pierce, Rockford, IL, USA) and monitored using the Molecular Imager ChemiDoc XR5+ System (Bio-Rad, Hercules, CA, USA). Specific bands were quantified by the use of Quantity One 1-D analysis software. The relative pixel intensities of the protein of interest and of the loading control protein GAPDH were calculated individually.

Immunofluorescence Staining

To determine TAK1 levels of expression (1 × 10^5 cells/well), human RPE cells were seeded on cover slips in DMEM/F12 and 10% FBS (in 6-well plates). On the following day the cells were treated with H2O2 (200 µM) for 1 hour, after which the medium was replaced with fresh human RPE medium. After this treatment, the slides were collected, washed with PBS, fixed in 4% paraformaldehyde for 10 minutes, incubated in 0.2% Triton X-100 for 5 minutes, washed twice with PBS (each time for 5 minutes), washed once with 2% BSA in PBS (5 minutes), and blocked by 50 µg/mL goat IgG in 2% BSA-PBS for 20 minutes. The slides were then incubated with TAK1 primary antibodies (Novus, Littleton, CO, USA) for 1 hour, washed twice (each time for 10 minutes) with 2% BSA-PBS, and incubated in the dark with Alexa Fluor 488-conjugated secondary antibodies (1:300; Millipore) for 30 minutes. This was followed by washing with PBS for 10 minutes and incubation for 20 minutes with senescence-associated β-galactosidase (SA-β-gal) activity, growth arrest, and cell cycle arrest in G0/G1. Accumulating evidence over recent years has shown that senescent cells have deleterious effects on the tissue microenvironment. The most significant of these effects is the acquisition of a senescence-associated secretory phenotype (SASP) that turns senescent cells into proinflammatory cells promoting chemo- kine and cytokine secretion, which may trigger angiogenesis. Transforming growth factor-β-activated kinase 1 (TAK1) is a serine/threonine kinase belonging to the family of mitogen-activated protein kinase (MAPK) kinase kinases. The TAK1 is a key regulator in the cascade of cellular responses and its activity is regulated by various cytokines, including IL-1 and TGF-β, as well as by Toll-like receptors and by CD40 and B-cell receptors. Once activated, TAK1 in turn activates the crucial intracellular kinases p38 MAPK, the c-jun N-terminal kinase, and the Ikappa B kinase complex. Ablation of TAK1 was shown to increase the levels of reactive oxygen species (ROS) in mouse keratinocytes. These findings led us to hypothesize that TAK1 may participate in the interplay between RPE cellular senescence on exposure of the RPE cells to oxidative stress. In the present study, in vitro experiments on a human RPE cell line demonstrated that TAK1 activity is essential for the normal functioning and morphology of the RPE cells. We found that TAK1 is highly expressed in RPE cells and that oxidative stress its expression is altered. Moreover, inhibition of TAK1 activity led to reduction in RPE cell proliferation, increased cell-cycle arrest at G0/G1, and SA-β-gal expression, all known features of cell senescence. Exposure to oxidative stress in combination with inhibition of the kinase activity resulted in a decline in the expression of apoptotic protein p53 and subsequently to augmented RPE cellular senescence. Finally, we found that aberrant activity of TAK1 triggers RPE cells to secrete factors that cause cellular hypertrophy and fibrotic changes in neighboring cells.
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4′,6-diamidino-2-phenylindole (DAPI) (1 mg/mL; Sigma) for nuclear staining and with phalloidin (1:2000) for actin staining. Finally, the cover slips were washed three times with PBS and glued onto the slides with mounting medium (Biomed, Hatfield, PA, USA). The photographs were de-convoluted and processed using a Leica laser confocal microscope (LSM-700; Zeiss, Oberkochen, Germany).

Determination of Apoptosis

The RPE cells were trypsinized, washed once in cold binding buffer (10 mM HEPES-NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2), and resuspended at 2 × 106 cells/mL in 100 µL binding buffer. A 5-µL aliquot of annexin-V Alexa Fluor 488 was added to each tube except for the binding buffer-only and propidium iodide (PI)-only tubes, and 5 µL of 50 µg/mL PI was added to each tube except for the binding buffer-only and the annexin-V–only tubes (Mebryo, Woburn, MA, USA). Cells were incubated on ice in the dark for 15 minutes, 400 µL of cold binding buffer was added to each tube, and the samples were then returned to the ice in the dark. Cells were analyzed by flow cytometry within 1 hour of the staining (FACSCalibur; BD Biosciences, San Jose, CA, USA).

Cell-Cycle Analysis

Control and treated human RPE cells were harvested, fixed in 10 mL ice-cold 70% ethanol for 24 hours, and washed twice in ice-cold PBS (pH 7.4; Biological Industries Ltd.). Thereafter, each sample of 1 × 106 cells was pelleted and resuspended in 0.5 mL PBS containing 0.1% Triton X-100, 50 µg/mL 200 µg/mL DNase-free RNase A (Sigma), and PI (Sigma). Samples were then incubated at 37°C for 15 minutes and analyzed in flow cytometer (FACSCalibur; BD Biosciences). The distribution of cells in different phases of the cell cycle was obtained by analyzing fluorescence intensities using CellQuest software (BD Biosciences).

Gelatin Zymography

Gelatin zymography was done as described.22 Briefly, culture media were collected after treatment and subjected to SDS-PAGE in 10% polyacrylamide gels copolymerized with 0.8 mg/mL gelatin. After electrophoresis, gels were incubated in 2.5% Triton X-100 (1 hour, 37°C), followed by overnight incubation in 50 mM Tris-HCl (pH 7.8), 5 mM CaCl2, 0.02% NaN3, and 0.02% Brij. Gels were stained with 2.5% Coomassie blue R-250 (Bio-Rad) for 30 minutes, followed by destaining in deionized water with 10% acetic acid and 20% methanol. Gels were scanned and specific bands were quantified by the use of Quantity One 1-D analysis software (Bio-Rad).

Statistics

Statistics were computed using Student’s t-test, two-tailed distribution. Values of P greater than 0.05 were considered significant.

RESULTS

Transforming Growth Factor-β-Activated Kinase 1 Inhibition Affects Apoptosis and Cell Cycle of RPE Cells

The role of TAK1 in the inflammatory response is characterized,25 but little is known about its participation in the response of RPE cells to stress. Retinal pigment epithelium cells were treated with the TAK1 inhibitor SZ-7-oxozeaenol (1 µM) for 1 hour before treatment with H2O2 (200 µM). Staining with annexin and PI showed that compared with control RPE cells, in which the number of apoptotic cells expressed as a percentage of the total number was 1.25%, the number of apoptotic cells after treatment with H2O2 alone increased over the same time period to 31%. In contrast, in cells treated with the TAK1 inhibitor before their treatment with H2O2, the number of apoptotic cells was 24% (Fig. 1A). It should be noted that the number of cells in late apoptosis (annexin and PI, upper right panel) was similar with or without TAK1 inhibitor. These results imply that TAK1 is involved in apoptosis and that its inhibition reduces this process.

To examine the effect of TAK1 inhibition on the RPE cell cycle, we treated RPE cells with TAK1 inhibitor with or without H2O2. The RPE cells are quiescent and 70% of the control RPE cells were in G0/G1 stage (Figs. 1B, 1C); however, after TAK1 inhibition, the percentage of cells at the G0/G1 stage increased to 89% of the total number. When the cells were subjected to oxidative stress, they exhibited high levels of G2/M arrest. Interestingly, this phenomenon was reduced on TAK1 inhibition before the oxidative stress (Figs. 1B, 1C). The cell-cycle arrest at G0/G1 on TAK1 inhibition was further supported by the reduction in proliferation of RPE cells observed in the presence of the TAK1 inhibitor (Fig. 1D). Control cells showed a high rate of proliferation, as reflected by their increasing optical density, which reached a peak on day 4 and followed. In contrast, RPE cells treated with TAK1 inhibitor demonstrated a slower proliferation rate that began to decrease after 3 days (Fig. 1D). These findings suggested that inhibition of TAK1 promotes cell cycle arrest and RPE-cell senescence.

Inhibition of TAK1 Increases the Expression of SA-β-gal in RPE Cells

To further characterize the effect of TAK1 inhibition on the senescence of RPE cells, we examined the effect of such inhibition on SA-β-gal expression in these cells. The number of cells expressing SA-β-gal was dramatically increased after treatment with the TAK1 inhibitor relative to the number in the control cells (Figs. 2A, 2B). This increase was further enhanced when TAK1 was inhibited and the RPE cells were exposed to oxidative stress (Figs. 2C, 2D). In cells that were exposed to oxidative stress without such pretreatment, there was extensive cell death, and by day 16 there were only a few surviving cells, with very low expression of SA-β-gal. In contrast, on days 15 and 16, SA-β-gal was strongly increased in cells that were exposed to oxidative stress after pretreatment with the TAK1 inhibitor (Figs. 2C, 2D). These findings further support the participation of TAK1 in the regulation of senescence in RPE cells.

Transforming Growth Factor-β-Activated Kinase 1 Affects p53 Expression

The p53 protein is known to play a critical role in cell-cycle regulation, DNA repair, and programmed cell death.24 In view of this knowledge, and given our finding that TAK1 inhibition reduced apoptosis, the expression of p53 in RPE cells was examined. As shown in Figure 3A, p53 expression in RPE cells under oxidative stress was strongly affected by pretreatment of the cells with the TAK1 inhibitor, seen by the inhibition of p58 phosphorylation (Figs. 3A, 3B). In control cells (without such pretreatment) the expression of p53 gradually increased, reaching a peak after 60 minutes, whereas in the pretreated cells, p53 expression peaked after 10 minutes and then declined (Fig. 3A). Over a longer
period, TAK1 inhibition reduced p53 expression to a level slightly higher than in control cells (Figs. 3C, 3D). In contrast, p53 levels in RPE cells that were exposed to oxidative stress displayed high levels of p53 after 4 days, kept increasing up to day 6, and began decreasing between days 6 and 9 (Figs. 3C, 3D). Last, we have not found long-term significant difference in the levels of TAK1 following oxidative stress (Fig. 3C).

FIGURE 1. Transforming growth factor-β–activated kinase 1 is involved in apoptosis and cell-cycle arrest at G0/G1 in RPE cells. (A) Double-staining (annexin and PI) assay of RPE cells. Retinal pigment epithelium cells were treated for 1 hour with the TAK1 inhibitor 5Z-7-oxozaenol (1 µM), followed by 200 µM H2O2 for 1 hour or left untreated. The cells were then washed with fresh medium and after 24 hours were analyzed. Percentages in the lower right quadrants for the cells treated with H2O2 alone and with the TAK1 inhibitor combined with H2O2 represent the increase in percentage of apoptotic cells in comparison with the 1.25% seen in control cells, and are the means of at least three independent trials. (B, C) Retinal pigment epithelium cells were pretreated with the TAK1 inhibitor with or without H2O2 as described in (A). On the following day, the cells were trypsinized, stained with PI, and subjected to flow cytometry analysis of cell-cycle distribution. The percentage of cells in each cell-cycle phase (G0/G1, S, and G2/M) was determined by its DNA content (FL2A) as reflected on the x-axis. (D) Human RPE cells were seeded in 96-well plates (5000 cells/well) in full medium and were pretreated with the TAK1 inhibitor 5Z-7-oxozaenol (1 µM) or left untreated for 1 hour. Their viability was then assayed by the XTT assay, measured in 450 nm, as specified in the Materials and Methods section. The experiment was performed in triplicate.

FIGURE 2. Transforming growth factor-β–activated kinase 1 inhibition increases SA-β-gal expression in RPE cells subjected to oxidative stress. (A) Representative photographs showing SA-β-gal staining of RPE cells treated at the indicated times with TAK1 inhibitor 5Z-7-oxozaenol or left untreated (control). (B) The histogram represents the relative amounts of cells that were stained positively with SA-β-gal (% of cells/field) in inhibitor-treated and control cells on the indicated days. (C) Representative photographs showing SA-β-gal staining in RPE cells treated with TAK1 inhibitor 5Z-7-oxozaenol (1 µM) for 1 hour and then treated with H2O2 (200 µM) for 1 hour, or treated with H2O2 (200 µM) alone. (D) Relative amounts of cells that were stained positively with SA-β-gal (% of cells/field) after treatment with H2O2 alone or with H2O2 combined with TAK1 inhibitor, on the indicated days. For each treatment, four different fields were photographed, the numbers of SA-β-gal–positive cells were counted, and the data are expressed as means ± SD. All statistics were computed using Student’s t-test. Scale bar: 100 µm.
Evaluation of TAK1 Expression During Oxidative Stress

The extent and expression pattern of TAK1 in the human RPE cells were assessed by immunofluorescence. As shown in Figure 4, TAK1 levels in control cells were stable, mainly localized within the nucleus, with no significant changes observed during the experiments. Interestingly, when the cells were exposed to oxidative stress, TAK1 expression in the nucleus was decreased, and returned to normal levels only after 48 hours. This finding implies that TAK1 expression is regulated during oxidative stress, thus demonstrating its importance in this process.

Transforming Growth Factor-β-Activated Kinase 1 Is Involved in RPE Cell Secretion

The most significant effect of senescence is the acquisition of an SASP.16 The SASP can convert senescent cells to proinflammatory cells that promote the secretion of chemokines,
cytokines and other proteins, including matrix metalloprotei-
nases (MMPs); those can affect the microenvironment,
including in the human retina.16,25,26 To examine the role of
TAK1 in this process, human RPE cells were treated with TAK1
inhibitor or with H2O2, alone or in combination. After 10 days,
the media was collected and subjected to zymography assay. As
can be seen in Figure 5A, the gelatin zymography assay
revealed that MMP-9 is elevated in response to oxidative stress
and to TAK1 inhibition in comparison to control cells.
Interestingly, the combined treatment of oxidative stress and
TAK1 inhibition slightly increased the levels of MMP-9 when
compared with oxidative stress or TAK1 inhibition treatments
separately (Figs. 5A, 5B). Altered secretory pattern in senescent
cells is a possible source of age-related inflammation.26 This
result suggests that TAK1-induced senescent RPE cells produce
increased concentrations of active MMP-9, which affects the
surrounding cells. To confirm the role of TAK1 in the
augmented SASP during RPE cell senescence, RPE cells were
treated with either TAK1 inhibitor, H2O2, or a combination of
both. After 2 weeks, the media was collected and centrifuged,
and the supernatants (conditioned media) were applied on
fresh RPE cells for 72 hours. As shown in Figure 5C, the cells
that received conditioned medium from control cells displayed
a normal phenotype, whereas the cells that received conditioned
medium collected from RPE cells treated with either the
TAK1 inhibitor or with H2O2 demonstrated a hypertrophic
phenotype similar to that of senescent cells. Interestingly, the
cells that received conditioned medium from cells treated with
both the TAK1 inhibitor and H2O2 demonstrated aberrant
morphology similar to that of atrophic RPE cells.

**DISCUSSION**

Aging is the most important risk factor in the development of
age-related diseases, including AMD.2 Some age-related diseases
may progress as a result of gradual accumulation of senescent
cells, whereas in older people, some diseases, although
unrelated to age, may progress at a faster rate than in younger
people owing to the presence of cellular senescence.13 Several
items of evidence suggest that senescence also contributes to
AMD.2 Senescent RPE cells exhibit altered morphology and
reach confluent growth at a lower cell-density level on
senescence inducers. Furthermore, the expression of several
genes in senescent RPE cells alters, including that of structural
genes.2 These changes might reduce the ability of the RPE layer
to form an effective barrier between the retina and the choroid,
thereby contributing to the development of AMD. Moreover, it
was recently shown that senescence is associated with drusen
formation.27 Our present data demonstrate that TAK1 is a key
player in the regulation of senescence in RPE cells. Inhibition of its kinase activity results in abundant presence of senescent RPE cells, demonstrating increased SA-β-gal and enhanced cell-cycle arrest at G0/G1.

The present study showed that TAK1 inhibition causes a reduction in the apoptotic events that occur in RPE cells on oxidative stress. Transforming growth factor-β-activated kinase 1 inhibition in combination with oxidative stress also reduced the number of cells in the G2/M phase, an event that leads to apoptosis. Similar findings have been reported in a HeLa cell line, in which cell-cycle analysis revealed a decrease in the percentage of irradiated cells in the G2/M phase due to TAK1 depletion and an increase in the S and SubG1 phases. In contrast, ablation of TAK1 causes accumulation of ROS, resulting in epithelial cell death and inflammation, as was demonstrated in other intestinal epithelial tissues.

In the RPE cells, we have found that TAK1 is being decreased on oxidative stress. It was demonstrated that on stress the level of expression of TAK1 declines, and after cells recover it returns to normal. In the long term we have not found significant difference in its expression. Even though the activity of TAK1 is mainly determined by its phosphorylation, in some processes there are significant changes in its levels of expression. During mouse embryogenesis, TAK1 displays a dynamic pattern of expression. It has been demonstrated that moderate levels of expression are present ubiquitously in the early embryo, and by embryo (E12.5) the overall levels decline and only specific tissues with high TAK1 expression are seen. These data, when combined with our results, demonstrate that TAK1 plays a role in the cell cycle and in apoptosis under stressful conditions. In addition, we demonstrated that under normal (i.e., unstressed) conditions, TAK1 inhibition resulted in a decline in the percentage of RPE cells in the S and G2/M phases and an increase in the number of cell arrested in the G0/G1 phase, a phenomenon associated with cell senescence.

We further studied the consequences of TAK1 inhibition on the appearance of senescence markers: Using SA-β-gal staining showed that TAK1 inhibition increases the number of senescent RPE cells in late passage cells and in cells under oxidative stress. Oxidative stress has led to increased SA-β-gal staining in various systems. Cellular senescence identified by positive staining of SA-β-gal was also reported to occur in vitro in late-passage RPE cultures and in vivo in RPE cells in the eyes of old primates. Transforming growth factor-β-activated kinase 1 involvement in cellular senescence also has been demonstrated in other tissues, emphasizing the importance of its kinase activity in this process. This study demonstrated that in late-passage RPE cells, the levels of SA-β-gal positive are low; however, the inhibition of TAK1 dramatically increases the number of positive SA-β-gal cells. This phenomenon was further confirmed in RPEs that were treated with oxidative stress and TAK1 inhibitor, where augmented SA-β-gal-positive cells were found in comparison with RPE cells that underwent only oxidative stress. Taking all of the above findings into account, we suggest that aberrant or inhibited kinase activity of TAK1 may lead to the development of senescence in RPE cells.

The most significant effect of senescent cells on the microenvironment is the development of SASP. Using conditioned media, we showed that TAK1 inhibition has a damaging effect on neighboring RPE cells. As expected, medium collected from cells that were exposed to oxidative stress induced an inflammatory-like phenotype in RPE cells. This effect was further demonstrated by increased MMP-9 secretion from RPE cells that underwent oxidative stress or TAK1 inhibition and subsequently cell senescence. The increase in MMP-9 secretion seen in the RPE cells was supported by Cao et al., who demonstrated MMP-9 secretion as one feature of the senescence inflammatory-like phenotype.

Interestingly, when oxidative stress was combined with TAK1 inhibition, the result was increased MMP-9 secretion and a deleterious effect on the RPE cells, which resembled cell atrophy. It was recently reported that TAK1 activation is related to dysregulated cytokine and growth factor stimulation in the microenvironment of primary lymphoma cells, demonstrating TAK1 involvement in the homeostasis of the RPE and other cells in maintaining the tissue microenvironment.

Last, the results showed that TAK1 inhibition leads to aberrant activity of p53. Moreover, this study demonstrated that TAK1 inhibition causes aberrant expression of p53 over both short and long periods. A transient transfection of TAK1 in COS cells was reported to stimulate p53 reporter activity, again representing a link between TAK1 and p53. The p53 tumor suppressor is activated by numerous stressors to induce apoptosis, cell-cycle arrest, or senescence. Mice with mutant p53 exhibit early onset of phenotypes associated with aging or senescence, including reduced longevity, osteoporosis, generalized organ atrophy, and diminished stress tolerance. In view of all the above data, the importance of TAK1 in the regulation of p53 is critical in RPE cells. We therefore suggest that the proper functioning of TAK1 is essential for p53 activation and/or expression. The expression of p53 is regulated by its stabilization or its degradation. It is possible that TAK1 affects the murine double minute 2 protein (MDM2), an important negative regulator of the p53 tumor suppressor. The MDM2 functions both as an E3 ubiquitin ligase that recognizes the N-terminal transactivation domain of the p53 tumor suppressor and as an inhibitor of p53 transcriptional activation. Nevertheless, we cannot rule out a possible positive effect of TAK1 on p53 expression via regulating its transcription.

This study demonstrates, for the first time, the participation of TAK1 in the senescence of RPE cells. The results showed that TAK1 participates in the cell cycle and in apoptosis of these cells. We suggest that TAK1 plays a role in the regulation of p53, and that in its absence or inhibition, p53 expression is aberrant, and that may lead to RPE cell senescence. However, the precise mechanism of its involvement is not fully understood and needs to be further studied.

In conclusion, the results of this study identify TAK1 kinase as a key player in the senescence of RPE cells. Better understanding of the role of TAK1 in RPE cell senescence may provide new insights into the pathology of dry AMD.

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