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Research paper

Oxidative stress-induced premature senescence dysregulates VEGF and CFH expression in retinal pigment epithelial cells: Implications for Age-related Macular Degeneration

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ABSTRACT

Oxidative stress has a critical role in the pathogenesis of Age-related Macular Degeneration (AMD), a multifactorial disease that includes age, gene variants of complement regulatory proteins and smoking as the main risk factors. Stress-induced premature cellular senescence (SIPS) is postulated to contribute to this condition. In this study, we hypothesized that oxidative damage, promoted by endogenous or exogenous sources, could elicit a senescence response in RPE cells, which would in turn dysregulate the expression of major players in AMD pathogenic mechanisms. We showed that exposure of a human RPE cell line (ARPE-19) to a cigarette smoke concentrate (CSC), not only enhanced Reactive Oxygen Species (ROS) levels, but also induced 8-Hydroxydeoxyguanosine-immunoreactive (8-OHdG) DNA lesions and phosphorylated-Histone 2AX-immunoreactive (p-H2AX) nuclear foci. CSC-nuclear damage was followed by premature senescence as shown by positive senescence associated-β-galactosidase (SA-β-Gal) staining, and $p16^{INK4a}$ and $p21^{Waf\text{-}Cip1}$ protein upregulation. N-acetylcysteine (NAC) treatment, a ROS scavenger, decreased senescence markers, thus supporting the role of oxidative damage in CSC-induced senescence activation. ARPE-19 senescent cultures were also established by exposure to hydrogen peroxide (H_2O_2), which is an endogenous stress source produced in the retina under photo-oxidation conditions. Senescent cells upregulated the proinflammatory cytokines IL-6 and IL-8, the main markers of the senescence-associated secretory phenotype (SASP). Most important, we show for the first time that senescent ARPE-19 cells upregulated vascular endothelial growth factor (VEGF) and simultaneously downregulated complement factor H (CFH) expression. Since both phenomena are involved in AMD pathogenesis, our results support the hypothesis that SIPS could be a principal player in the induction and progression of AMD. Moreover, they would also explain the striking association of this disease with cigarette smoking.

1. Introduction

Age-related Macular Degeneration is a degenerative retinal disease that causes blindness in people 60–65 years and older [1,2]. The prevalence of any AMD is 8.69% within ages 45–85 years, leading to an estimation of 196 million affected people in 2020 [3]. Both photoreceptors and the retinal pigment epithelium show slow degenerative changes [4,5], followed by their demise and often accompanied by the development of a neovascular membrane [6]. Chronic and repetitive non-lethal RPE injury [7,8], together with an oxidative environment appear as important factors for development of this condition [9–12]. Nonetheless, there is still a gap in our understanding of the cellular mechanisms connecting oxidation-induced events to the appearance of AMD pathological changes. Among other effects, oxidants can damage DNA [13]. They can also trigger stress-induced premature cellular senescence (SIPS) [14], which might be involved in AMD [15–17].

Cellular senescence is a state characterized by an inability to proliferate despite the presence of sufficient nutrients and mitogens while maintaining cell viability and metabolic activity [18,19]. Moreover, senescent cells acquire a SASP, producing and releasing several pro-inflammatory cytokines, chemokines, proteases, growth factors, and other peptides and protein. The composition of this secretome depends on the stimuli triggering senescence and is also specific of cell type [20,21].

Several lines of evidence point to the prominence of

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inflammatory and innate immune mechanisms in AMD [22], strongly supported by the high genetic risk associated to common genetic variants of CFH and other complement proteins, such as C2/CFB, C3 and CFI [23–25]. In addition, RPE secreted cytokines such as VEGF [26] and interleukins [27–29] are involved in AMD pathogenesis and progression. Therefore, evaluation of SASP induction in stressed RPE cells could help to further understand the course of AMD.

Smoking strikingly reduces the age at the onset of this disease [30], and is firmly established as the main environmental factor in its development and progression [31–36]. Cigarette smoke-induced lesions of the RPE are well-known and have been extensively reviewed [11]. Tobacco smoke is not only a source of free radicals, but also disrupts endogenous antioxidant systems [37] Most likely, the cigarette smoke-associated risk depends on oxidative stress, a key factor for AMD development [38,39]. However, since the earliest markers of the disease appear a long time after chronic exposure to cigarette smoke, and even when smoking has been discontinued [32], AMD may actually appear after oxidative senescence induction in the RPE. Therefore, we hypothesized that an oxidative environment could elicit SIPS in RPE cells and that, in turn, senescent cells could dysregulate the expression of key factors associated with AMD. To verify these hypotheses, we established two senescent models initiated by H_2O_2 and CSC in ARPE-19 cells. We report that CSC promotes oxidative stress and oxidative DNA damage in this cell line, and we show that senescence induction by CSC is dependent on ROS. Senescent cultures upregulated the pro-inflammatory cytokines IL-6 and IL-8, the main markers of SASP. And most important, senescent cells disrupted the expression of two major players in AMD, VEGF and CFH. These results provide insight into a new pathway by which an oxidative environment, like the one caused by smoking, might contribute to RPE dysfunction. Thus, senescent RPE cells are placed in a novel context as potential contributors to chronic inflammation, complement activation, and angiogenesis in AMD.

2. Material and methods

2.1. Reagents and cell culture

ARPE-19 cell line was obtained from American Type Culture Collection. Cells were cultured in DMEM-F12 (Life Technologies, Invitrogen, Argentina) containing 2.5 mM L-glutamine, 100 U/ml streptomycin/penicillin and 10% fetal calf serum (FCS, NATOCOR, Córdoba, Argentina). The day before beginning experimental work, FCS was reduced to 1%. Cells were seeded at 35,000 cells/cm^2 or 25,000 cells/cm^2 for viability and senescence assays respectively.

H_2O_2 (Merck Millipore) [40] and CSC (Murty Pharmaceuticals, Lexington, KY) were used at the indicated times and concentrations. Dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis MO) vehicle controls were carried out (0.25%, 0.50%, 1.00% DMSO for 100, 200 and 400 μg/ml CSC, respectively). NAC (Pharmazell, India) was freshly prepared as a 100 mM pH 7.4 solution in ddH_2O and used at a final concentration of 1 mM.

2.2. Viability assay

Cell viability was evaluated using the Acridine Orange/Ethidium Bromide (AO/EB) staining protocol [41]. After washing with PBS, pH 7.4 cells were treated with staining solution containing equal amounts of AO and EB (2 μg/ml) and evaluated with fluorescence microscopy (Nikon E600) using the FITC filter.

2.3. Detection of reactive oxygen species

The fluorogenic probe 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes, Life Technologies, Argentina) was used to evaluate intracellular levels of ROS. Cells were treated with CSC (100, 150 or 200 μg/ml) with or without 1 mM NAC for 30 min. After washing with PBS, cells were treated with 10 μM DCFH-DA during 30 min. Fluorescence intensity, was measured in a fluorometer (Hitachi F-2000, excitation 480 nm, emission 540 nm).

2.4. Detection of DNA damage

Cells were seeded in 8-well Nunc™ Lab-Tek™ Chambered Coverglasses. Following treatments, cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Fixed cells were blocked with 2% bovine serum albumin (BSA) in PBS for 1 h.

To test for the presence of DNA oxidative lesions using 8-OHdG immunoreactivity, cells were incubated with 100 μg/ml RNase A (Invitrogen) for 1 h at 37 °C, treated with 2 N HCl for 10 min and neutralized with 1M Tris–HCl pH 7.5 for 10 min [42]. After blocking, incubation with anti-8-OHdG was done overnight in 0.1% BSA. Alexa Fluor®488 rabbit anti-goat IgG (Molecular Probes; Invitrogen) in 2% BSA for 2 h at room temperature was used for detection.

Incubation with anti-p-H2AX antibody (Table 1) was done for 1 h at room temperature. Slides were washed 3 times with 0.1% Triton X-100 in PBS, and incubated with Alexa Fluor®488 goat anti-rabbit IgG (Molecular Probes; Invitrogen) for 2 h in blocking solution. After washing, cell nuclei were stained using 2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). Preparations were mounted (PBS-glicerol 1:1) and examined under a fluorescent microscope (Nikon E800).

2.5. Senescence associated β-galactosidase activity

Cells were fixed with 3% formaldehyde in PBS, pH 7.4, for 5 min and incubated at 37 °C overnight in staining solution (40 mM sodium citrate, pH 6.0, 1% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 5 mM potassium ferrocyanide, 5 mM ferricyanide, 150 mM sodium chloride, and 2 mM magnesium chloride) [43]. Cultures were examined under phase-contrast microscopy.

2.6. Protein extraction and western blot analysis

Cells were harvested in PBS and lysed with RIPA buffer with protease inhibitors (Protease Inhibitor Cocktail, Sigma-Aldrich). 50 μg protein extracts were resolved in 12% polyacrylamide gels and analyzed by immunoblotting for the indicated proteins.

Table 1
Antibodies used in these experiments.

Antigen	Abbreviation	Made in	Source
$p21^{Waf/Cip1}$		Clone SX118, Mouse	BD Biosciences (556430)
$p16^{INK4}$		Clone G175-405, Mouse	BD Biosciences (551153)
actin		Rabbit (N-terminal antibody)	Sigma-Aldrich (A2103)
GAPDH		Clone 6C5, Mouse	Santa Cruz Biotechnology (sc-32233)
8-hydroxy-guanosine	8-OHdG	Goat	ABCAM (ab10802)
Phosphorylated histone H2AX	pH2AX	rabbit	ABCAM (ab2896)
Complement factor H	CFH	Sheep	ABCAM (ab8842)

Secondary antibodies conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology (sc-2005; sc-2004). The signal was detected using enhanced chemiluminescence detection solution (Solution A: 100 mM Tris pH8.5, 2.5 mM Luminol, 0.4 mM p-Coumaric acid; Solution B: 100 mM Tris pH 8.5, 0.02% H_2O_2). The signals were visualized by exposure to light sensitive films (Amersham Hyperfilm™ ECL) and digitized.

2.7. RNA isolation and Quantitative PCR (QPCR)

Senescent cultures were established in 60 mm dishes. Total RNA was isolated by RNeasy Mini Kit (Qiagen). RNA concentration was determined spectrophotometrically. First-strand cDNA was synthesized from 1 μg of total RNA using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Primers for IL-8, VEGF, CFH and GAPDH were designed using Primer Blast and synthesized by Invitrogen (Argentina).

Expression levels of mRNA were assessed by relative quantification (ΔΔCt method) in a cycler (Stratagene Mx 3005P System). Each reaction mix (25 μl) contained 1 unit Taq DNA polymerase (Invitrogen), 1 × PCR reaction buffer (20 mM Tris–HCl, pH 8.4, and 50 mM KCl), 1.5 mM Mg_2Cl, 200 μM of dNTPs and 0.4 μM of each specific primer (Table 2). Cycling parameters were 95 °C for 10 min, then 95 °C 30 s, 58 °C 60 s, and 72 °C 60 s for 40 cycles. Expression was normalized to GAPDH. Each reaction was run in triplicate, and dissociation curves were constructed to ensure only a single product was amplified. The relative amount of the PCR product amplified from untreated cells was set to 1. A non-template control was run in each assay.

2.8. Interleukin assays

Cells were seeded in 96 well-plates. Following damaging protocols, the medium was replaced by 50 μl of fresh culture medium with 1% FCS. Interleukins 6 and 8 were measured from frozen 24 h supernatant using the Human IL-6 and IL-8 ELISA sets (BD OptEIA™ - Human IL-8 ELISA Set, and BD OptEIA™-Human IL-6 ELISA Set, BD Biosciences). Measurements were performed using a microplate reader (Benchmark, Bio-Rad). Values were normalized to 10,000 cells.

2.9. Statistics

Quantitative data are presented as average ± SE, with an indication of the number of samples for each experiment in the text or the corresponding . Statistical tests, indicated in Results, were calculated with GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego CA; www.graphpad.com). Unless specified in the text or legends, comparisons were made using One-Way ANOVA followed by Bonferroni's multiple comparison tests. Statistical significance is shown as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

3. Results

3.1. Viability and DNA damage response after exposure to H_2O_2 and CSC

Non-lethal levels of DNA damage can activate SIPS [44]. Thus, we first determined viability of ARPE-19 cells under increasing concentrations of H_2O_2 or CSC. Cell cultures were exposed to 25–400 μM H_2O_2 during 90 min and returned to complete medium. Cell viability was evaluated by 24 h after the initiation of the experiment. Appearance of cells with an orange–yellow fluorescent nucleus after AO–EB staining depended on H_2O_2 concentration. Less than 10% of dead cells appeared at 150 μM, whereas more than 50% of the cells died after exposure to 400 μM (Fig. 1A).

ARPE-19 viability after exposure to CSC was tested within a concentration range of 50–800 μg/ml and compared to appropriate DMSO vehicle controls. Initial experiments showed that short exposures did not affect cell viability. Therefore, we increased the exposure time to 24 h. Under these conditions, less than 10% dead cells were observed after 200 μg/ml CSC, but more than 60% died following 400 μg/ml (Fig. 1A). Importantly, DMSO effects were only detected at 1%, the concentration corresponding to 400 μg/ml CSC.

Since cigarette smoke is a well-known ROS inducer [37], we tested ROS production in ARPE-19 cells exposed to three different CSC concentrations (100, 150 and 200 μg/ml). ROS were significantly increased above the DMSO controls at the 3 concentrations. In the presence of NAC, however, ROS levels were not different from those of the corresponding DMSO vehicle controls (Fig. 1B).

Therefore, further studies were conducted in cultures exposed to 150 μM H_2O_2 for 90 min, or treated with 200 μg/ml CSC for 24 h or more.

We evaluated nuclear 8-OHdG and p-H2AX immunofluorescence to assess the impact of H_2O_2 and CSC on DNA integrity. 8-OhdG content, reflecting oxidative DNA lesions, increased in exposed ARPE-19 cell cultures (Fig. 1C). These cultures also showed immunofluorescent p-H2AX nuclear foci, demonstrating the presence of an active DNA damage response (Fig. 1D).

3.2. Senescence induction

We further analyzed the ability of H_2O_2 and CSC treatments to activate a senescent response. As previously established by others [45], senescent cultures show 80% of SA-β-Gal$^+$ cells. ARPE-19 cultures exposed to 150 μM H_2O_2 and cultured in maintenance medium during 10 days exhibited this proportion. To explore senescence induction by CSC, cultures were exposed to 200 μg/ml CSC for different time periods (1–5 days) and incubated for a total of 12 days. The minimum time of CSC exposure required to obtain senescent cultures was 3 days, when SA-β-Gal$^+$ cells reached 81% (Fig. 2A and B). This exposure time was used in the following experiments.

Senescent cells are in a state of permanent cell cycle arrest that is established and maintained by the expression of cyclin-dependent kinase inhibitors (CKIs). Therefore, we used Western blotting

Table 2
Forward and reverse Primers used in QPCR.

mRNA	Forward	Reverse
IL-8	5′ GGTGCAGTTTTGCCAAGGAG 3′	5′ TTCCTTGGGGTCCAGACAGA 3′
NRF2	5′ TCTGCCAACTACTCCCAGGT 3′	5′ GGGAATGTCTGCGCCAAAAG 3′
CFH	5′TTCCAAAAGCGCAGACCAC 3′	5′TTGATTTGGAACATGTTTTGACAC 3′
GAPDH	5′ GGGGCTGCCCAGAACATCAT3′;	5′ GCCTGCTTCACCACCTTCTTG 3′

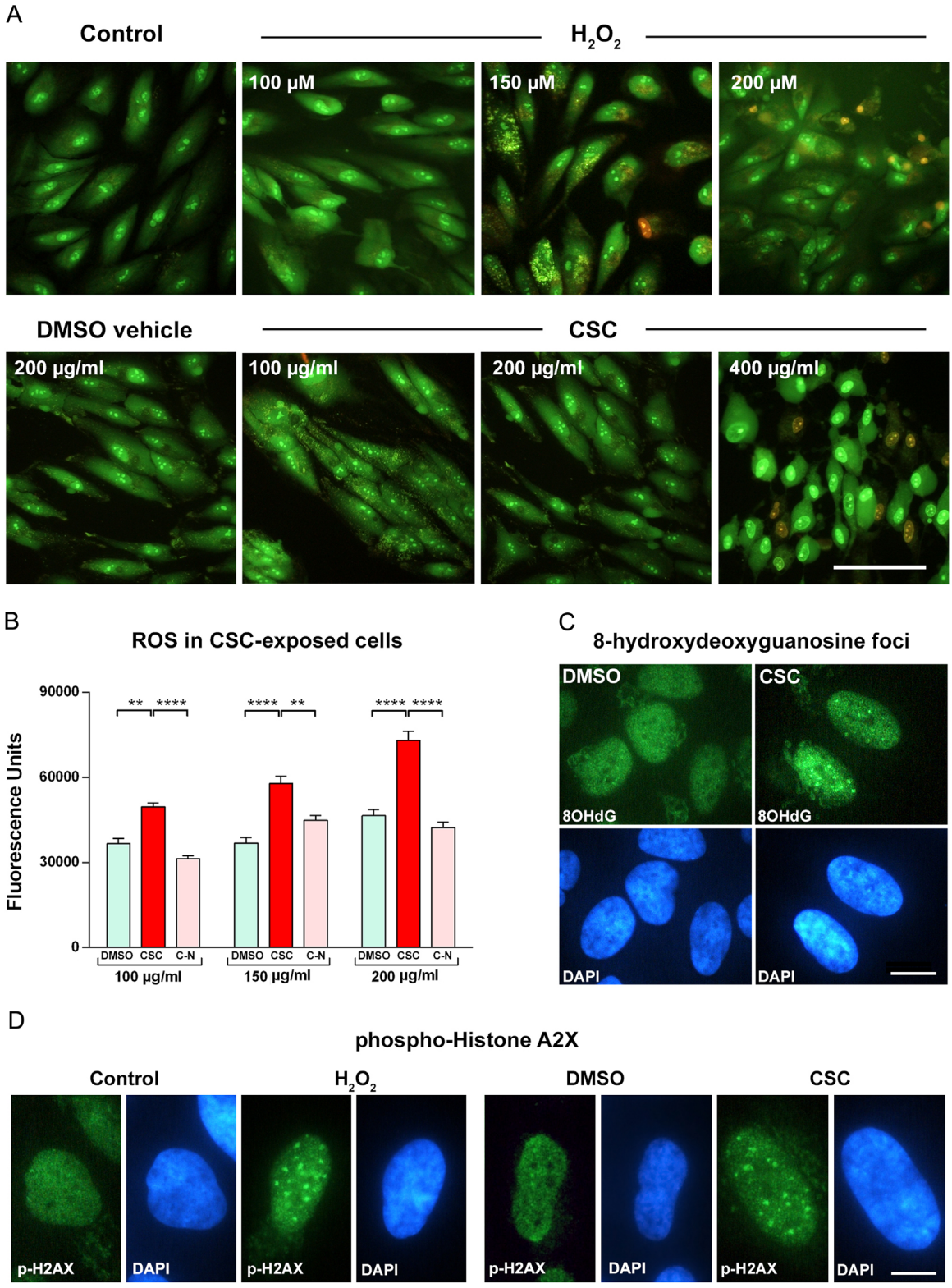

Fig. 1. Effect of H_2O_2 and CSC on cell viability and oxidative DNA damage. (A) AO/BR staining of ARPE-19 cells exposed to H_2O_2 or CSC at the indicated concentrations, during 90 min for H_2O_2 and further maintained in fresh medium for 24 h (upper panel), or during 24 h for CSC (lower panel). Bar, 50 µm. (B) Bar graphs showing the effect of different CSC concentrations on ROS levels, as shown by DCFH-DA fluorescence. Cells were treated with CSC at the indicated concentrations for 30 min in the presence of NAC when indicated (C-N). Bars represent mean ±SE of three independent experiments performed in triplicate. Statistical comparisons were made with Two-Way ANOVA followed by Bonferroni's multiple comparison test. (C) Immunofluorescence of 8-hydroxydeoxyguanosine (8OHdG) in cell nuclei, doubly stained with DAPI, in control cultures (DMSO) and cultures exposed to CSC (200 µg/ml) for 24 h. Bar, 5 µm. (D) Immunofluorescent detection of p-H2AX in cell nuclei doubly stained with DAPI. Cells were exposed to H_2O_2 (150 µM) or CSC (200 µg/ml) as indicated in (A), stained and analysed by fluorescence microscopy, Bar, 5 µm.

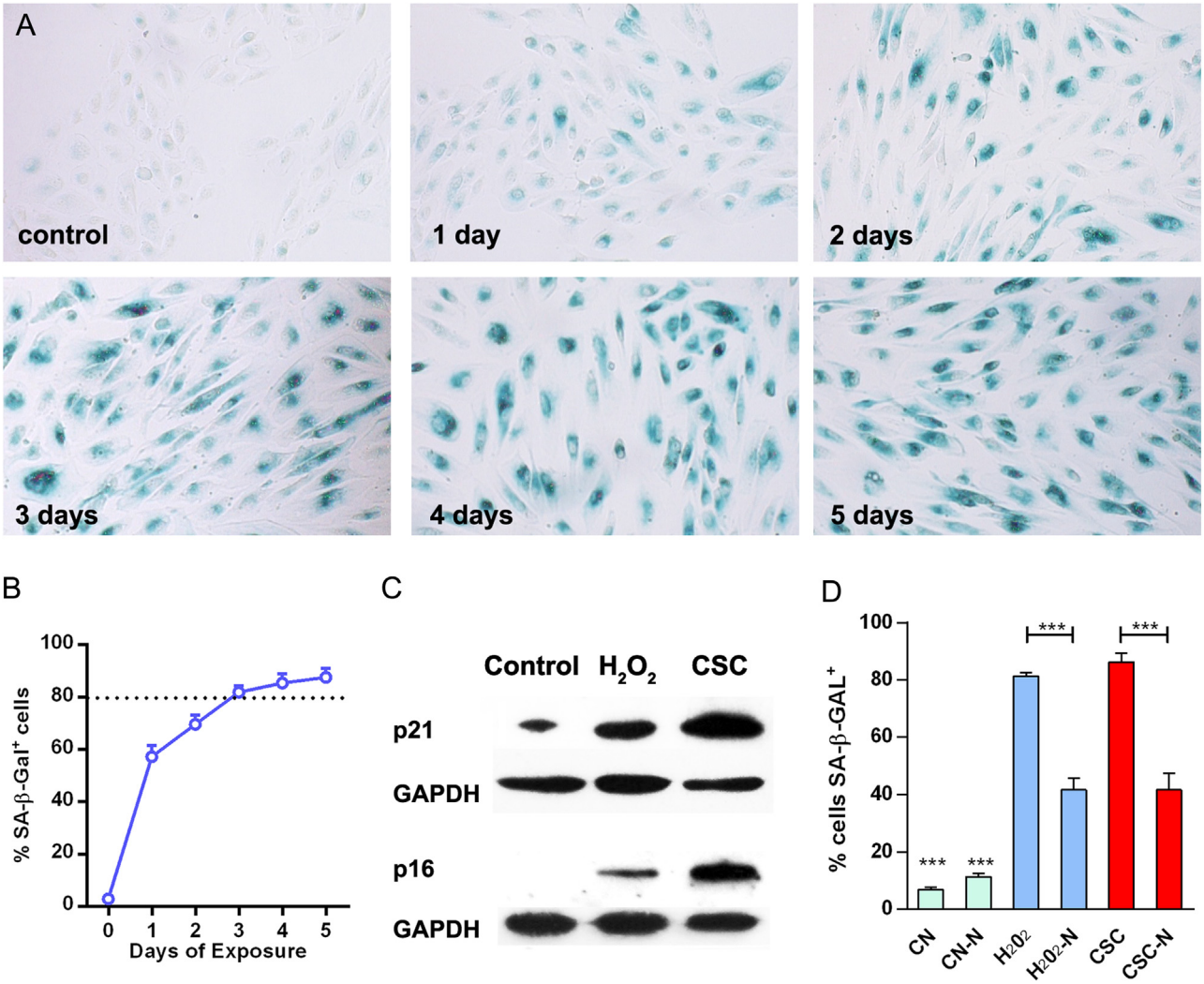

Fig. 2. Senescence markers in ARPE-19 cells exposed to H_2O_2 and CSC. (A) SA-β-Gal activity in ARPE-19 cells treated with 200 μg/ml CSC for 1–5 days, and then maintained in fresh medium for a total of 12 days. Micrographs were obtained with phase contrast microscopy. (B) Quantification of SA-β-Gal$^+$ cells cultures shown in A. SA-β-Gal$^+$ cells were scored in 3 fields of at least 250 total cells. Results are expressed as the percentage of stained cells (mean ± SE). (C) p21 and p16 expression levels in senescent cultures following exposure to H_2O_2 or CSC. Protein levels were evaluated by immunoblot, using GAPDH as a loading control. (D) Effect of NAC on SA-β-Gal activity. Bars represent the percentage of SA-β-Gal$^+$ cells in ARPE-19 monolayers exposed to H_2O_2 or CSC under senescence induction conditions, and similar cultures exposed in the presence of 1 mM NAC (CN–N, H_2O_2–N or CSC–N).

to evaluate the expression of $p21^{Waf/Cip1}$ and $p16^{INK4a}$. These proteins were barely detected in control cultures but were highly expressed in senescent ARPE-19 cell cultures (Fig. 2C). Thus, the three SIPS hallmarks, SA-β-Gal staining, and increased $p16^{INK4a}$ and $p21^{Waf/Cip1}$ expression, were present after exposure of ARPE-19 cells to H_2O_2 or CSC.

ROS are potent SIPS inducers [40]. Therefore, we tested the effect of NAC, a ROS scavenger, on the appearance of SA-β-Gal$^+$ in ARPE-19 cultures. Co-incubation with NAC during exposure to H_2O_2 or CSC, halved the proportion of SA-β-Gal$^+$ cells (Fig. 2D). These results support the involvement of ROS in the signaling pathways mediating CSC-induced senescence.

3.3. Senescence and the inflammatory milieu

IL-8 is a key component of the SASP. Hence, we assayed the release of IL-8 during development of H_2O_2- and CSC-induced senescence. Increases became significant after 7 days in culture (Fig. 3A). As shown by QPCR, IL-8 release was associated with IL-8 mRNA upregulation (Fig. 3B). We also assayed IL-6, another important SASP factor. This cytokine showed significant increases both after H_2O_2- and CSC-induced senescence (Fig. 3C). Much smaller increases of IL-6 and IL-8 were detected when aggressors were applied in the presence of NAC. Most likely, these differences reflect NAC-protection from oxidative stress and the consequent reduction of H_2O_2- and CSC-induced senescence (see also Fig. 2D).

3.4. VEGF and CFH expression in senescent cultures

Wet AMD is characterized by the presence of choroidal neovascularization (CNV), which affects the macula leading to photoreceptor damage and central vision loss. Since uncontrolled VEGF expression is a major player in CNV [46,47], we asked whether RPE senescence might dysregulate the expression of this angiogenic factor. We studied this possibility measuring VEGF protein and mRNA levels using Western blot and QPCR assays, respectively. Following senescence induction with H_2O_2 or CSC, cultures displayed higher VEGF protein levels compared to non-senescent ones. Furthermore, senescent ARPE-19 cells up-regulated VEGF mRNA levels more than 15-fold above control cultures (Fig. 3E).

Dysregulation of complement activation is a critical point in AMD etiopathogenesis and is a high risk factor for this pathology

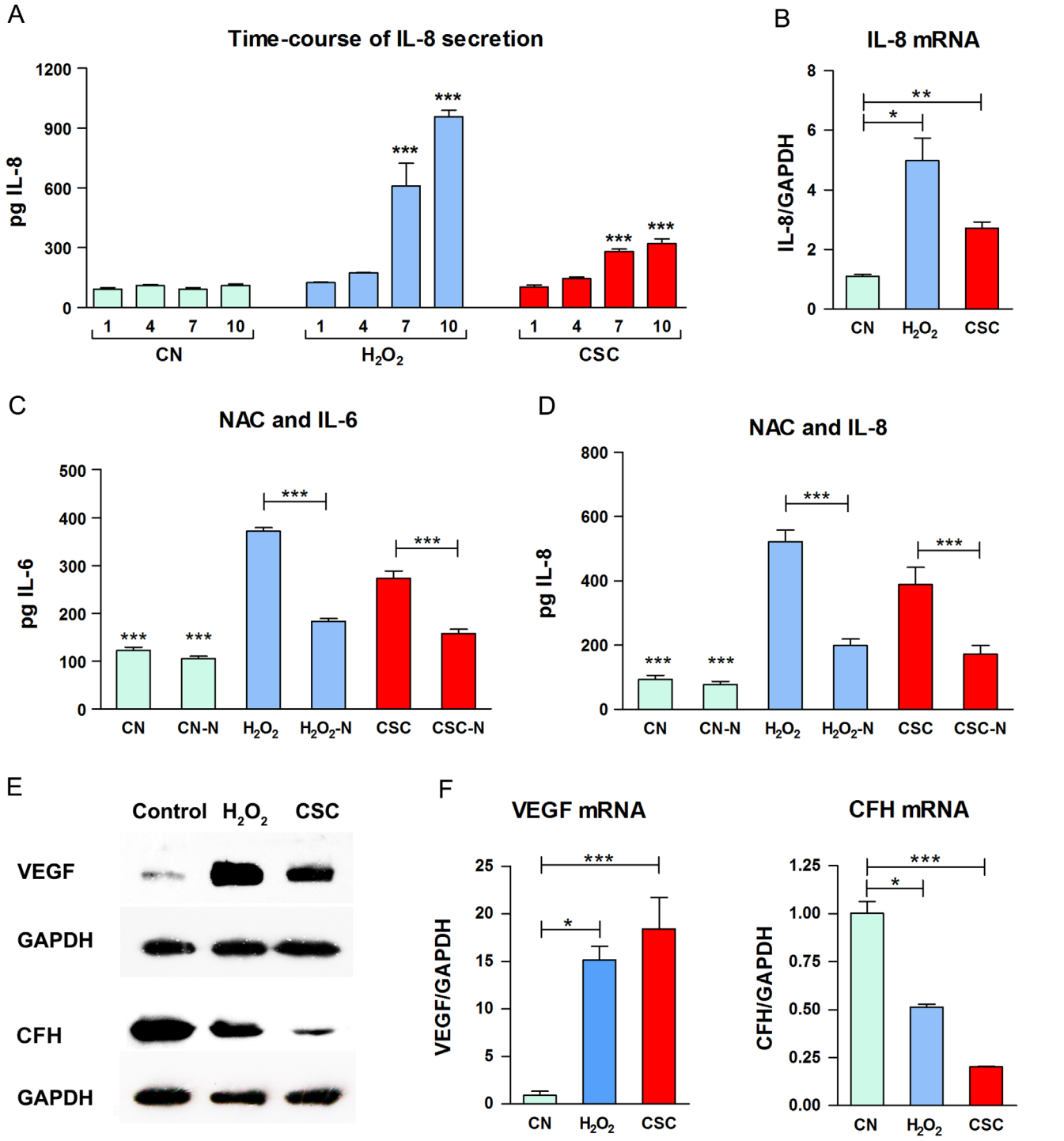

Fig. 3. Induction of SASP components in senescent cultures. (A) Cells were exposed to 150 μM H_2O_2 for 90 min or treated with 200 μg/ml CSC for 3 days and then incubated in fresh medium to complete a 10-day period. At the indicated time points, IL-8 secretion levels were measured in 24 h supernatants. Bars represent mean ±SE of three independent experiments performed in triplicate. Statistical comparisons were made with the corresponding 1-day old cultures. (B) Quantification of IL-8 mRNA levels in senescent cultures induced as described in A. IL-8 mRNA levels were quantified by real time PCR. Values correspond to fold change relative to control cultures and represent the average of 3 independent experiments (mean ±SE). GAPDH was used as a reference gene. (C) and (D) Effect of NAC treatment during senescence induction on IL-6 and IL-8 secretion. NAC was applied during exposure to H_2O_2 or CSC and maintained during 24 h following damaging treatments. IL-6 and IL-8 levels were measured in 24 h supernatants from senescent cultures by ELISA. Bars represent mean ±SE of 3 independent experiments performed in triplicate. Asterisks on top of control bars indicate that controls were different from all the senescent cultures ($p < 0.001$). (E) Analysis of VEGF and CFH expression levels in senescent cultures. ARPE-19 cells were treated with H_2O_2 or CSC as indicated in A. Immunoblots show the expression of VEGF (upper panel) and CFH (lower panel) proteins in control and senescent cultures. (F) VEGF (left panel) and CFH (right panel) mRNA levels were quantified by QPCR. The mRNA values indicate a fold change relative to control cultures (mean ±SE), and represent the average of 3 independent experiments. GAPDH was used as a reference gene.

[24,25]. Since RPE cells constitutively produce CFH [48], we asked whether senescence induction would modify CFH expression. Western blot analysis showed decreased CFH protein levels in H_2O_2- and CSC-induced ARPE-19 senescent cells. Moreover, senescent ARPE-19 cells down-regulated CFH mRNA, 2- and 5-fold for H_2O_2- and CSC-induced senescence, respectively (Fig. 3E).

4. Discussion

Cellular senescence has been hypothesized as contributing to age-associated tissue dysfunction as well as age-related diseases, like AMD, via the SASP [49]. The present study reports that two different sources of oxidative stress may switch on premature senescence in RPE cells, and that this phenomenon is accompanied by changes in the expression of key factors linked to AMD etiopathogenesis and progression. Although our evidence demonstrates the involvement of oxidative damage in senescence development, it must be emphasized that secretome is dependent on senescent engagement since changes gradually appeared several days after the oxidative insult, together with the emergence of SA-β-Gal. SIPS activation could explain the increased AMD risk associated to both former and current smoking [32,50].

4.1. Oxidative stress in the retina and senescent ARPE-19 cultures

The retina is one of the highest oxygen-consuming tissues in the human body [51]. In addition, photons impinging upon the retina not only activate the visual pigments but can also form reactive forms of oxygen, eventually affecting nearby molecules. This combination of elevated metabolic activity and exposure to visible light makes for one of the highest oxidative environments in the body [52]. Oxidative damage is restricted by the intervention of efficient antioxidant defense mechanisms. However, ROS levels increase and antioxidants decline in aging tissues, which become more susceptible to oxidative damage [10]. Current theories conceive age-related diseases as the accumulation of protein and DNA damage resulting from ROS imbalance [53]. Antioxidants decrease in light-exposed retinas, allowing the accumulation of H_2O_2 [54]. Similarly, oxidative stress could be enhanced by exposure of the retina to cigarette smoke [55]. Various components of cigarette smoke can produce superoxide anion ($O2^{\bullet -}$), followed by H_2O_2 and the reactive hydroxyl radical ($HO^{\bullet}$) [55]. Smoking also depletes micronutrients and vitamins, which act as natural oxidation inhibitors [56,57]. Exposure of ARPE-19 cells to CSC increased intracellular ROS levels and induced 8-OHdG lesions, confirming its capability to produce oxidative DNA damage. In addition, H_2O_2 and CSC exposures activated a DNA damage response, as shown by the appearance of nuclear p-H2AX foci. Although these lesions had little impact on cell viability, the proportion of SA-β-Gal^+ cells increased and, $p16^{INK4a}$ $p21^{Waf\text{-}Cip1}$ proteins were upregulated, confirming the induction of a senescent phenotype. Thus, the behavior of ARPE-19 cells resembled that reported in primary human RPE cell cultures [58,59] and other cell types [60], where non-lethal DNA damage is needed to promote SIPS. The relevance of our findings is highlighted by studies showing that a similar process may affect the RPE in vivo. RPE cells displaying senescence markers have been identified in old monkey eyes [61], AMD human donor retinas [62], and senescence experimental models in vivo [63]. Most interestingly, the increase of non-hexagonal RPE cell shapes, reminiscent of the SIPS characteristic alterations of cell shape, has been described in aging retinas [4] and AMD donor eyes [5].

4.2. Senescence, SASP and age-related pathologies

Although cell senescence is a beneficial anti-cancer mechanism, the age accumulation of senescent cells would drive aging-related pathologies [64–66]. The increase in cells expressing senescent markers has been correlated with several pathologies, such as endothelial dysfunction [67], chronic obstructive pulmonary disease, where they have been related to cigarette smoking [68], diabetic nephropathy [69], and diseased cartilage cells in arthritis [70]. Most important, direct in vivo evidence supporting a causal link between $p16^{Ink4a+}$ senescent cells and age-related pathologies has been obtained in the BubR1 progeroid mouse model, where depletion of $p16^{Ink4a+}$ cells prevents or delays the onset of major ageing phenotypes [71].

Most of the senescence-associated pathology is related to the SASP [72,73]. Senescent ARPE-19 expressed and released a larger amount of the inflammatory cytokines IL-6 and IL-8 than non-senescent ones. Similarly, upregulation of IL-8 has been reported in a model of amyloid β-induced senescence in ARPE-19 cultures [74]. Expression patterns, including inflammatory cytokines, VEGF and CFH, were similar in H_2O_2 and CSC-induced senescence. Most important, they matched the expression patterns of AMD-affected retinas. IL-6 and IL-8 proinflammatory genotypes are associated to AMD pathobiology [28]. These interleukins are increased in the aqueous humor of patients with exudative AMD [75] and their concentrations have been correlated with neovascular retinal activity [76] and the volume of macular edema [77]. Furthermore, both interleukins may have toxic effects on RPE cells [78], and are significantly upregulated after senescence induction in the RPE-choroid of mice receiving subretinal amyloid-β-peptide [63].

Our data also show that VEGF is a main component of the SASP in senescent RPE cells. This growth factor, which increases endothelial permeability and proliferation, is a leading factor in AMD pathogenesis, and treatments blocking VEGF effects (such as pegaptanib, ranibizumab, and bevacizumab) constitute the main pharmacological tools against wet AMD [46,47]. Moreover, anti-VEGF agents are not only effective for the control of neovascularization, but also for the maintenance of visual acuity [79]. Interestingly, recent studies in a VEGF-hyperexpressing mouse strain suggest that this factor might also be involved in nonexudative AMD pathogenesis [80]. The mechanisms underlying VEGF upregulation in AMD are not yet completely understood; however, our present findings point to senescent RPE cells as important sources of disproportionate VEGF amounts in the aged-retina microenvironment. Increased VEGF expression has been observed in other senescent cells, such as fibroblasts, but it was not associated to a significant increase of Hypoxic-Inducible Factor 1 α (HIF-1α) [81]. Increased VEGF expression is not found in all senescent fibroblasts, but only in those where premature cell senescence depends on the engagement of the $p16^{ink4a}$/pRB pathway [81]. This seems to be the case in our experiments, since $p16^{INK4a}$ was increased in both H_2O_2- and CSC-induced senescent ARPE-19 cells.

Senescent cells downregulate a significant number of proteins [82,83]. In addition, in our model, we have shown a previously unreported decrease in the expression of CFH mRNA and protein. As described above, this complement regulatory protein is highly important in AMD pathogenesis, since some of its genetic variants are major risk factors for developing AMD [23]. Besides being secreted by the liver, CFH is locally synthesized in the RPE cells and choroid [24,25]. This factor negatively regulates the alternative complement pathway both in the fluid phase and at cell surfaces, hence limiting the formation of the membrane attack complex (MAC) which is deposited at Bruch's membrane/choriocapillaris zone [25]. Not surprisingly, CFH gene knock-down results in increased MAC deposition and activation of experimental CNV in mice [84]. Moreover, in human AMD donor eyes, immunoreactivity for CFH is reduced compared to that found in age-matched controls [85]. In line with this evidence, our finding of a reduced CFH expression in senescent ARPE-19 cells implies that SIPS may be a cause of CFH deficiency, particularly in the presence of functionally inadequate variants, enhancing complement pathway activation, and contributing to retinal damage.

The senescent phenotype appears to be mainly regulated at the transcriptional level, both by modulation of transcription factors, changes in non codifying RNAs and chromatin reorganization [20,86,87]. However, there is limited data about regulation of the

SASP. Changes underlying the secretory phenotype are independent of those producing the cell cycle arrest, as shown by SASP persistency in senescent fibroblasts reverting to a proliferative phenotype [20]. Analysis of 240 genes in tert-butylhydroperoxide (tert-BHP)-induced ARPE-19 senescent cells showed a decrease in the expression of VEGF mRNA; however, these cells showed proangiogenic properties that were associated to upregulation of Tissue Plasminogen Activator (TPA) mRNA [82]. Only an increase of p21Waf-Cip1 was observed, supporting the involvement of p16INK4a activation in senescent VEGF upregulation reported by Coppe et al. [81]. Very recent work shows that the SASP is controlled by mTOR through the regulation of NF-κB and the Ser-Thr kinase MK2, which is downstream the MAP kinase p38. Both pathways result in the strong upregulation of inflammatory cytokines, which can be prevented by rapamycin [88].

At least 4 micro-RNAs (miRNAs 9, 125b, 146a and 155) which target CFH, are under NF-κB control and are progressively upregulated in both AMD and Alzheimer's disease [89]. Although their relationship to CFH expression in senescent cells has not yet been studied, these micro-RNAs are involved in the induction of senescence [90–92] and might play a central role in the interactions between DNA damage response, cell senescence and inflam-aging [93].

5. Final conclusions

Our findings demonstrate for the first time that H_2O_2 and CSC senescent ARPE-19 cultures enhance IL-6 and IL-8 secretion, increase VEGF and downregulate CFH expression. Therefore, senescent cells as those described may trigger the three hallmarks of AMD pathogenesis: inflammation, dysregulation of the complement system, and activation of pro-angiogenic cytokines. SISP could explain how cigarette smoking increases the risk for AMD development, even in ex-smokers. A comprehensive knowledge of the senescent response in the RPE will help to understand the pathogenesis of this disease, and to establish efficient preventive and therapeutic measures.

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