Effects of Antioxidant Components of AREDS Vitamins and Zinc Ions on Endothelial Cell Activation: Implications for Macular Degeneration

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PURPOSE. To investigate whether the benefit of Age-Related Eye Disease Study (AREDS) formula multivitamins and zinc in the progression of age-related macular degeneration (AMD) may occur through inhibiting inflammatory events in the choroid.

METHODS. Mouse C166 endothelial cells (ECs) and, for some experiments, human retinal pigment epithelium (RPE)-choroid organ cultures were treated with AREDS multivitamin solution (MVS) or ZnCl₂. The cytotoxicity of MVS was evaluated using a lactate dehydrogenase colorimetric assay. Cell motility was assessed using a scratch assay. Macrophage adhesion to EC monolayers or ICAM-1 protein was determined after MVS and zinc treatment and with or without lipopolysaccharide (LPS). Quantitative reverse transcription PCR and Western blot analysis were used to determine the effects of MVS on the expression of proinflammatory molecules in treated and untreated cells.

RESULTS. AREDS MVS and zinc did not affect C166 EC viability until the 56th hour after treatment. Scratch assays showed partial inhibition of MVS and zinc on EC migration. In cell adhesion assays, MVS and zinc decreased the number of macrophages bound to EC and to ICAM-1 protein. Quantitative PCR showed that LPS increased the expression of ICAM-1 in both C166 and human RPE-choroid cultures, which was partially offset by MVS and zinc. MVS and zinc also mitigated LPS-induced ICAM-1 protein expression on Western blot analysis.

CONCLUSIONS. Treatment with AREDS MVS and zinc may affect both angiogenesis and endothelial-macrophage interactions. These results suggest that AREDS vitamins and zinc ions may slow the progression of AMD, in part through the attenuation of EC activation. (*Invest Ophthalmol Vis Sci.* 2012;53:1041-1047) DOI:10.1167/iovs.11-8531

Age-related macular degeneration (AMD) is the leading cause of untreatable blindness among the elderly, resulting in significant vision impairment for 30 to 50 million people worldwide.¹ In the United States, the estimated prevalence is 6.5%, affecting >1.75 million people, and will likely double in the next decade.^{2,3} In the past decade, nutritional supplementation was found to prevent or delay the progression of AMD in spite of no effective treatment in most cases.⁴⁻⁷ The large-

scale, randomized controlled clinical trial from the Age-Related Eye Disease Study (AREDS) group showed that nutritional supplementation was associated with reduced risk for progression to advanced AMD in patients with intermediate AMD or with advanced AMD in one eye at 5 years of follow-up.⁸ In the Blue Mountains Eye Study, a high level of zinc intake (15.8 mg/d) was significantly associated with reduced AMD progression,⁹ although dietary studies of zinc have been equivocal.¹⁰ On the basis of the results of AREDS reports, the AREDS formulation of vitamins C, E, β -carotene, and zinc with copper are routinely recommended to AMD patients.⁹ However, the mechanism of action of the AREDS formula multivitamin on cell types involved in AMD, and the physiology of the potential zinc benefit to AMD, is poorly understood.

AMD is considered to be a complex, multifactorial disease, although its etiology is still incompletely understood. Recent studies from genetic, clinical, histopathologic, and animal models have supported the notion that chronic inflammation plays an important role in the pathogenesis and progression of AMD. Vascular and inflammatory biomarkers that have been found elevated in AMD include elevated C-reactive protein^{11,12} and cytokines IL-6 and TNF- α .¹³ The presence of leukocytes in drusen and in neovascular lesions in the retina, autoantibodies relevant to drusen in patient serum, and complement activation in neovascularization and in human eyes are all supportive of the notion that inflammation plays an important role in AMD.¹⁴⁻¹⁷ Notably, choroidal leukocytes have been found to accumulate in the choroid of eyes with early and advanced macular degeneration.¹⁸⁻²¹ In view of the proposed roles of circulating leukocytes migrating to the choroid in AMD,²² molecules that mediate leukocyte-endothelial interactions are attractive candidates for understanding AMD. We hypothesized that the benefit of AREDS multivitamin solution (MVS) and zinc to AMD is gained through directly or indirectly inhibiting EC adhesion molecules and thus reducing local inflammation. In this study we sought to investigate the impact of AREDS formula and zinc ions, in conjunction with a strong inflammatory stimulus, on the expression of some proinflammatory factors, EC migration, macrophage adhesion to ECs, and intercellular adhesion molecule 1 (ICAM-1) protein expression using a mouse endothelial line (C166) as an in vitro model. We also investigated the expression of ICAM-1 in human RPE-choroid organ cultures in the presence or absence of AREDS MVS. Our data suggest that AREDS MVS may affect AMD progression through the attenuation of EC responses to inflammation, including decreased de novo expression of ICAM-1.

MATERIALS AND METHODS

Endothelial Cell Culture and Treatment

The C166 EC line (ATCC, Rockville, MD) was used as an EC model and cultured in Dulbecco modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) and 1%

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penicillin-streptomycin (PS; Sigma-Aldrich, St. Louis, MO). Cells were treated with buffer only, 1× or 5× AREDS MVS formula, or 20 or 60 μ M ZnCl₂ (27 and 82 mg/mL) in 1% FBS-DMEM with 1% PS at 37°C for 24 hours after cell confluence. In some experiments, lipopolysaccharide (LPS, 3 μ g/mL) was added as a proinflammatory stimulus 4 hours before cell harvest.

Dilutions of AREDS MVS were prepared from over-the-counter vitamins (PreserVision AREDS soft gel formula; Bausch & Lomb, Rochester, NY). These supplements contain vitamin A (β -carotene) (14,320 IU), vitamin C (226 mg), vitamin E (200 IU), zinc (34.8 mg), and copper (0.8 mg). Briefly, to perform these experiments, we made a number of nonempirical assumptions. We assumed 5 L blood, and we assumed that the soluble components of AREDS vitamins are dissolved and delivered to ocular tissues. To estimate the 1× AREDS MVS concentration, the soluble contents of one pill were dissolved into 50 mL Hanks balanced salt solution, and this was treated as a 100× solution. Serial dilutions were generated from this stock. The 1× and 5× AREDS MVS are approximately equivalent to 0.5-fold, and 2.5-fold concentrations of the standard AREDS formula (if patients take two caplets per day).

Human Donor Eyes, RPE-Choroid Organ Cultures, and Treatment

Human donor eyes were obtained from the Iowa Lions Eye Bank (Iowa City, IA) after consent was given by the donors' families. All experiments conformed to the Declaration of Helsinki. Human organ cultures of RPE/choroid from three donors were used as described previously.²³ Briefly, extramacular 4-mm punches of RPE and choroid were collected from eyes of donors and placed in DMEM (Invitrogen) supplemented with 10% FCS, and 1% PS. Punches were collected in a paired fashion, with pairs at an equivalent distance from the macula. All cultures were initiated within 8 hours of death. The cultures of RPE/choroid were exposed to 1× AREDS MVS and incubated for 24 hours at 37°C in 5% CO₂. In seven cultures, LPS (3 μ g/mL) was added as an inflammatory stimulus 4 hours before cell harvest and was compared to seven cultures treated with both LPS and 1× AREDS MVS. Eyes used for these experiments were unremarkable for retinal pathology.

Cell Viability Assay

To determine the extent to which AREDS MVS and ZnCl₂ induced EC death, a lactate dehydrogenase (LDH) cytotoxicity detection kit (Roche Diagnostics Corp., Indianapolis, IN) was used. Levels of LDH in the conditioned media were determined at 4, 24, and 56 hours of incubation after exposure to a range of AREDS MVS and ZnCl₂ concentrations. All samples were evaluated in triplicate. Relative cell death (RCD) was calculated as described previously.²⁴ One hundred percent RCD was represented by the maximum amount of releasable LDH enzyme activity, which was determined by lysing C166 cells with 1% Triton X-100 according to the manufacturer's recommendations.

Determination of Cell Intake AREDS MVS

C166 cultures on coverslips were rinsed three times with FBS-free DMEM with 1% PS after 24-hour AREDS MVS treatment and were incubated with 10 μ M ZnAF-2F (Chemodex, Gallen, Switzerland) in FBS-free DMEM with 1% PS, a zinc-specific binding fluorescent dye, at 37°C for 20 minutes. Unbound fluorescent dye was removed by washing three times with 1.0× PBS. Cells were fixed in 4% paraformaldehyde and visualized with an inverted fluorescence microscope using an FITC filter set.

Cell Migration Assay

The motility of C166 ECs was assessed using a scratch wound assay, which measures the expansion of a cell population across a surface. The scratch wound assay was performed as previously described with modifications.²⁵ A 200- μ L suspension of C166 cells at a concentration

of 3.0×10^5 cells/mL with 5% FBS DMEM was seeded into two well culture inserts with an adhesive barrier around which the cells attached (Ibidi, Martinsried, Germany) at 37°C for 24 hours; at that point, the monolayer was nearly confluent. The barrier was then removed, and the cell monolayer with a cell-free gap was treated with MVS or ZnCl₂. LPS was added as a cell activator in some experiments. Standardized images were collected at a fixed magnification and region at 0, 12, 24, 36, 48, and 60 hours after removal of the barrier. All experiments were repeated in triplicate. Relative cell migration distance was determined with ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>), and the percentage of wound closure was calculated as described previously.²⁶

Macrophage Endothelial Cell Adhesion Assay

All animal experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were performed with permission of the Animal Care and Use Committee at the University of Iowa. C166 cells seeded on coverslips were treated with AREDS MVS or ZnCl₂ with or without LPS treatment and were then washed twice with FBS-free DMEM-1% PS after 24-hour treatment. Peritoneal macrophages from C57Bl6J mice were harvested through peritoneal lavage with 10 mL cold 1× PBS and then were isolated by centrifugation. Fresh macrophages were labeled with 4',6-diamidino-2-phenylindole (DAPI, 0.5 μ g/mL; Invitrogen, Carlsbad, CA) for 30 minutes and washed twice. Fresh macrophages labeled with DAPI (at a density of 1×10^5 cells/mL) were added to the monolayer and cocultured in the dark at room temperature for 30 minutes. Nonadherent macrophages were removed by gentle washing with 1× PBS three times after 30 minutes. The C166 cell monolayer was fixed with one-half-strength Karnovsky fixative (2% formaldehyde and 2.5% glutaraldehyde in 100 mM cacodylate buffer, pH 7.4, containing 0.025% CaCl₂) for 5 minutes. The number of binding or adherent cells was determined by counting three fields using an ocular grid and a 20× objective under a fluorescence microscope. Fields for counting adherent macrophages were randomly selected at a half-radius distance from the center of the coverslip. The mean $\pm$ SD of two repeats was calculated for each group. Control and experimental groups were compared using a *t*-test.

Adhesion of Macrophages to ICAM-1 Protein

Ninety-six well plates were coated with 10 μ g/mL ICAM-1 protein (100 μ L/well; R&D Systems, Inc., Minneapolis, MN) at 4°C overnight. Fresh peritoneal macrophages prepared from mouse were treated with AREDS MVS or ZnCl₂ at 37°C for 24 hours, then labeled with DAPI for 30 minutes. The labeled macrophages (1×10^5 /mL) were added to each well coated with protein and incubated at room temperature for 30 minutes. The nonbinding macrophages were removed through a 20-minute wash on an agitator. Macrophages were then fixed with half-strength Karnovsky fixative. The number of bound macrophages was determined by counting the fluorescent cell number in three contiguous fields in the center of the well using a 20× objective of an inverted fluorescence microscope. The mean $\pm$ SD of three repeats was calculated for each group. Control and experimental groups were compared using a *t*-test.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from cultured C166 cells or human RPE-choroid organ cultures using a commercial kit (RNeasy; Qiagen, Valencia, CA) according to the manufacturer's instructions and reverse transcribed into cDNA as described previously.²³ Quantification of specific mRNA species was performed by a real-time PCR reagent kit (SYBR Green RT-PCR Reagent Kit and ABI Prism 7700 Sequence Detection System; Applied Biosystems, Carlsbad, CA). Triplicate reactions were performed for each sample. Seven mouse genes and one human proinflammatory gene (*Icam1*, *Icam2*, *Pecam1*, *Cdh5*, *Nfkb1*, *Nfkb2*, and *Nfkbib* for mouse and *ICAM-1* for human) were investigated. To select a stably expressing reference gene in mouse C166 cells for normal-

ization, algorithm software (geNorm; PrimerDesign Ltd., Southampton, UK; <http://medgen.ugent.be/genorm/>) was used to evaluate the expression stability of six common reference genes (*Gapdh*, β -actin, *Hprt1*, *Ywbaz*, *Rpl19*, and *Ppia*), and expression validation was examined in two stable genes selected from the algorithm software (geNorm; PrimerDesign Ltd.). The most stable gene was chosen from two genes as the experimental reference gene. For human samples, *ICAM-2*, a homolog of ICAM-1 that is constitutively expressed²⁷ and localized to the choriocapillaris,²⁸ was selected as a reference gene for normalization. qPCR data were analyzed with data analysis software (DataAssist, version 2.0; Applied Biosystems, Carlsbad, CA). Primer sequences of target genes and references were as follows: for mouse cells—Icam-1 forward, ATTCGTTTCCGGAGAGTGTG; Icam-1 reverse, CTTTGGGATGGTAGCTGGAA; Icam-2 forward, TACCAGCCTCCAGCTCAAGT; Icam-2 reverse, ACCATTTGGTTGTCTCTGCAT; Pecam-1 forward, TCAACATAACAGAGCTGTTTCC; Pecam-1 reverse, TTCTGATACGCGACAAGACC; Cdh5 forward, GGGAAAGATCAAGTCCAACG; Cdh5 reverse, TCACACGGATGACAGAGGTC; Nfkb1 forward, 5'-AGAGCTCCGAGACGCTATCC-3'; Nfkb1 reverse, 5'-CAGTCTCTCCACCAGCTTCC-3'; Nfkb2 forward, 5'-AGACTCGTTCTGCACTTGG-3'; Nfkb2 reverse, 5'-GCTTTCAGAAGTGCCCTCAGC-3'; Nfkb3 forward, 5'-CCTGCACITGGCTGTGATT-3'; Nfkb3 reverse, 5'-ACCGGCTGCATACAACTTCT-3'; Gapdh forward, 5'-AAGGGCTCATGACACAGTC-3'; Gapdh reverse, 5'-GGTCTCTAGTGTAGCCCAAG-3'; Rpl19 forward, 5'-ATGCCAACTCCCGTCAGC-3'; Rpl19 reverse, 5'-ACCCTTCTCTTCCCTATGC-3'. Primer sequences of the other four reference genes have been described elsewhere.²⁹

For human organ cultures, the primer sequences were as follows: ICAM-1 forward, 5'-ACCTGGCAATGCCAGAC-3'; ICAM-1 reverse, 5'-GGTTGGC-TATCTTCTTGACACA-3'; ICAM-2 forward, 5'-GTCCAGGATCGGATGAGAAG-3'; ICAM-2 reverse, 5'-CGTGTCTATGGGAGATGTTTG-3'.

Western Blot Analysis

Western blot analysis was performed essentially as described previously.²⁸ Briefly, C166 cells that had been exposed to AREDS MVS, LPS, or both were lysed in protease inhibitor cocktail solution (Roche, Indianapolis, IN). Cell lysates (70 μ g) were separated on a 10% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA), transferred onto a polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA), blocked with 1% BSA/1 $\times$ PBS with 1% Tween-20, and incubated with anti-ICAM-1 (0.2 μ g/mL; R&D Systems) or anti-ICAM-2 (0.2 μ g/mL; R&D Systems). After incubation with horseradish-peroxidase-conjugated

FIGURE 2. Zinc from AREDS MVS is taken up by C166 ECs. Cells were stained with ZnAF-2F (a specific zinc-binding fluorescent probe). (A) Control cells stained with ZnAF-2F. (B) ECs treated with 5 $\times$ AREDS MVS stained with ZnAF-2F.

secondary antibody (GE Healthcare, Piscataway, NJ), the immunoreactive proteins were detected using a chemiluminescence kit (Immun-Star WesternC; Bio-Rad). Band intensity was measured with NIH ImageJ 1.40g software. The intensity of ICAM-1 and ICAM-2 protein was normalized to the relative intensity of β -actin. Comparison of band density between the control and treated groups was performed with a *t*-test.

RESULTS

No Effect of AREDS MVS or Zinc on C166 Endothelial Cell Viability

A colorimetric LDH assay showed no significant cytotoxicity of MVS at concentrations of 1 $\times$ and 5 $\times$ and zinc at concentrations of 20 and 60 μ M until 56 hours after treatment but a remarkable increase in the cell death rate (94.2%) at a concentration of 100 μ M at the 56th hour after treatment ($P < 0.05$) (Figs. 1A, 1B).

Endothelial Cells Internalize Zinc after AREDS MVS Treatment

Cells exposed to AREDS MVS were stained with a specific fluorescent dye (ZnAF-2F). Fluorescence was detected inside C166 cells after 24-hour AREDS MVS exposure, when a high dose (5 $\times$) of MVS was applied (Fig. 2B).

Both Zinc and AREDS MVS Delay Endothelial Cell Migration

Both MVS and zinc showed a direct inhibition on C166 cell migration ($P < 0.05$), initially observed at the 24th hour up to the 64th hour after treatment (Fig. 3). The reduction in EC migration in the presence of MVS ranged from 15% to 39% at 1 $\times$ MVS and 34% to 57% at 5 $\times$ MVS and showed a tendency to gradually decrease with time. Both tested concentrations of zinc appeared to inhibit cell migration from 58% to 87%.

AREDS MVS Inhibits LPS-Induced Macrophage Binding to Endothelial Cells and Recombinant ICAM-1 Protein

The number of macrophages that adhered to the endothelial monolayer per field was decreased by MVS at a concentration of 1 $\times$ and 5 $\times$ AREDS MVS and 60 μ M zinc ($P < 0.05$), with decreasing degrees of 46%, 36%, and 29%, respectively (Fig. 4). LPS significantly increased the number of bound macrophages to the monolayer, as previously described,³⁰ and the inclusion of MVS and zinc partially mitigated this LPS-induced increase ($P < 0.05$). The change in the number of macrophages com-

FIGURE 1. The effect of AREDS MVS and ZnCl₂ on EC (C166) viability. (A) AREDS MVS-treated cell death rate at the 4th, 24th, and 56th hours after treatment. (B) ZnCl₂-treated cell death rate at the 4th, 24th, and 56th hours after treatment. Increased cell death was observed only in EC exposed to 100 μ M ZnCl₂.

FIGURE 3. Effects of AREDS MVS and zinc on EC migration. Relative scratch closure rate at 12, 24, 36, 48, and 60 hours after scratching the EC monolayer. Two concentrations of AREDS MVS (1× and 5×) and ZnCl₂ (20 and 60 µM) were used for this experiment. The comparison of scratch closure was performed between the control (agent-free) and AREDS MVS or zinc-treated groups, at the times indicated.

pared with control conditions (agent-free without LPS) was 67.5% for 1×, 50% for 5× AREDS, 27.5% for 20 µM, and 35% for 60 µM zinc.

Zinc and AREDS MVS also inhibited macrophage binding to ICAM-1 protein at concentrations of 5× AREDS MVS and 20 µM and 60 µM zinc ($P < 0.05$). Moreover, both AREDS MVS and ZnCl₂, at all tested concentrations, partially reduced the LPS-mediated increase in binding ($P < 0.05$). Macrophages stimulated with LPS increased their binding to immobilized ICAM-1 protein by 32.3%. In the presence of ZnCl₂ and MVS, the number of bound macrophages decreased by >50% over a range of MVS and ZnCl₂ concentrations (57.9% for 1× MVS, 67.7% for 5× MVS, 69.2% for 20 µM zinc, and 52.3% for 60 µM zinc) ($P < 0.05$) (Fig. 5).

Effect of AREDS Vitamin Solution and Zinc on Expression of Proinflammatory Genes

To identify a reference gene with stable expression for normalization of mRNA quantification in C166 cells, we first used algorithm software (geNorm; PrimerDesign Ltd.) to evaluate the expression stability of six reference genes (*Gapdh*, *Actβ*, *Hprt*, *Ywhaz*, *Rpl19*, and *Ppia*) and selected the two genes with the most stable expression (*Rpl19* and *Ppia*). *Rpl19* was selected as the best reference gene according to the expression

FIGURE 4. Effect of AREDS MVS or zinc on the adhesion of mouse macrophages to ECs. The mean number of adherent macrophages per field was compared between the agent-alone (AREDS or zinc-treated) and the control (agent-free) ($\dagger P < 0.05$). In addition, the mean number of adherent macrophages per field was compared between samples stimulated with LPS alone and with LPS plus AREDS-MVS or ZnCl₂ ($*P < 0.05$). Basal indicates the mean number of fluorescent profiles on the agent-free C166 monolayer without adding mouse macrophages.

FIGURE 5. Effect of AREDS MVS or zinc on the adhesion of mouse macrophages to ICAM-1 protein. The mean number of adherent macrophages per field was compared between the agent-alone (AREDS or zinc-treated) and the control (agent-free) ($\dagger P < 0.05$). In addition, the mean number of adherent macrophages per field was compared between samples stimulated with LPS-alone and with LPS plus AREDS-MVS or ZnCl₂ ($*P < 0.05$). Basal indicates the mean number of fluorescent profiles on the agent-free field without adding mouse macrophages. AREDS-MVS and ZnCl₂ modestly inhibited binding.

validation assay of these two markers ($y = 0.023x + 7.163$ in *Rpl19*).

The changes in mRNA abundance for seven genes that have some role in inflammation (*Icam1*, *Icam2*, *Pecam1*, *Cdh5*, *Nfkb1*, *Nfkb2a*, and *Nfkb2b*) was determined after AREDS MVS and zinc treatment in mouse C166 cells. Cells treated with AREDS MVS showed modest inhibition of *Nfkb2a* and *Nfkb2b* expression of 1× and 5× MVS concentrations ($P < 0.05$) but no direct inhibition of other genes. Zinc reduced the expression ICAM-1 at a concentration of 60 µM ($P < 0.05$; data not shown).

As expected, stimulating mouse C166 ECs with LPS increased the expression of all seven inflammatory genes ($P < 0.05$). When ECs were pretreated with AREDS MVS or ZnCl₂, this LPS-mediated increase was attenuated, most notably for *Icam1* expression (Fig. 6). Although the increase in expression above baseline was still notable, the elevation of *Icam1* expression was significantly decreased. For example, cells exposed to 5× AREDS MVS+LPS showed an 11.6% decrease compared

FIGURE 6. Effect of AREDS MVS and zinc on LPS-induced expression of proinflammatory genes in mouse ECs. AREDS MVS or ZnCl₂ partially inhibited LPS-induced expression of proinflammatory genes (*Icam1*, *Pecam1*, *Nfkb1*, *Nfkb2a*, and *Nfkb2b*). Comparison was performed between the agent treated with LPS induction (AREDS MVS+LPS or Zn+LPS) and the agent-free with LPS (LPS alone) normalized with *Rpl19* ($*P < 0.05$). The relative fold of the LPS-alone control was set to zero.

FIGURE 7. mRNA quantification of ICAM-1 in human RPE-choroid organ cultures treated with AREDS MVS. In this experiment, the numbers of human RPE-choroid organ cultures used were as follows: three organ cultures for both AREDS-free and three AREDS-treated-alone, seven for LPS-treated-alone and seven for AREDS-treated with LPS. Overall fold change is indicated, compared with agent-free control and normalized with ICAM-2. Treatment of organ cultures with LPS greatly increased ICAM-1 expression (** $P < 0.0001$). Simultaneous addition of AREDS MVS partially attenuated the effects of LPS (* $P < 0.05$). The relative fold of the agent-free control was set to zero (i.e., 0 = no change in expression).

with cells exposed to LPS in the absence of MVS; 20 μ M Zn+LPS decreased the expression 63.5% compared with LPS alone, and cells exposed to 60 μ M Zn+LPS showed a 101.2% decrease compared with MVS-free controls with LPS stimulation (i.e., the effects of LPS were completely offset by the presence of ZnCl₂) ($P < 0.05$) (Fig. 6). Lower concentrations (0.5 $\times$ and 0.1 $\times$) had a relatively small (fold change $<5\%$) but significant effect. This partial inhibition of LPS-induced expression was observed for *Pecam1* at 5 $\times$ MVS, *Nfkb1*, and *Nfkb1a* at 60 μ M zinc and *Nfkb1b* at 20 and 60 μ M zinc ($P < 0.05$) (Fig. 6).

When human RPE-choroid organ cultures were exposed to 1 $\times$ AREDS MVS, the baseline expression of human *ICAM-1* also showed no significant decrease in seven human RPE/choroid cultures ($P > 0.05$) (Fig. 7, left column). When RPE/choroid cultures were treated with LPS, *ICAM-1* expression was in-

creased by a factor of 24 ($P < 0.001$) (Fig. 7, middle column), much more than the increase induced by LPS in mouse C166 cells (data not shown), which may suggest that choroidal ECs are especially responsive to inflammatory stimuli. Simultaneous treatment of these cultures with both LPS and AREDS MVS resulted in a partial attenuation of LPS-induced expression of *ICAM-1*. Cultures receiving both LPS and 1 $\times$ MVS showed a 41% decrease in *ICAM-1* expression compared with treatment with LPS alone ($P = 0.042$) (Fig. 7, right column).

To determine whether LPS-induced *Icam1* mRNA is translated in mouse ECs, we investigated the effect of AREDS MVS at 1 $\times$ and 5 $\times$ concentrations and zinc (20 and 60 μ M) on the expression of ICAM-1 and ICAM-2 proteins. Comparison of band density between the untreated control and the treated group by Western blot analysis showed no significant impact of AREDS MVS and zinc on the endogenous expression of ICAM-1 and ICAM-2 at the protein level ($P > 0.05$), consistent with what was observed for mRNA (Figs. 8A, 8C). In LPS-stimulated cells, however, the increased expression of ICAM-1 induced by LPS was notably attenuated by the additional presence of AREDS MVS (1 $\times$ and 5 $\times$) and zinc (20 μ M and 60 μ M) (all $P < 0.05$) (Figs. 8B, 8D).

DISCUSSION

The AREDS vitamin formulation is routinely recommended in the clinic to prevent or delay the progression of AMD, based on the results of a large multicenter trial.⁸ In our cell viability assays, AREDS MVS showed little or no cytotoxicity on C166 cells, even at high concentrations (5 $\times$ AREDS MVS with ~ 187 mg/mL zinc), whereas zinc alone used at a concentration of 100 μ M (136 mg/mL) or higher (250 or 500 μ M) resulted in a significant increase in cell death. This result implies that zinc compound preparation, in the presence of other AREDS formula components, may have less cytotoxicity on cells than high dosages of zinc alone, and caution is warranted for the use of zinc alone at high dosages clinically.

Although commonly suggested for slowing the progression of AMD, the cellular and molecular basis for how AREDS vitamins may interact with AMD-associated pathways is not well understood. Suggested mechanisms include antioxidant protection of the RPE and ECs.^{31,32} Perhaps paradoxically, zinc

itself accumulates in subretinal deposits³³ and can promote the oligomerization of complement factor H.³⁴

Endothelial cell migration into Bruch's membrane and macrophage binding to vascular ECs are two important events relevant to angiogenic and inflammatory processes in the pathogenesis of AMD. We evaluated EC migration by scratch assays and determined the binding of macrophages to ECs. The results suggest that AREDS MVS and zinc ion can directly inhibit these potentially pathogenic behaviors.

To further understand which proinflammatory molecules are involved in the inhibition of AREDS MVS and zinc on cellular inflammatory behaviors, we evaluated the expression of seven genes with roles in inflammation at the mRNA level with AREDS MVS or zinc treatment.

ICAM-1 and ICAM-2 structurally belong to the immunoglobulin superfamily and have been demonstrated to play a critical role in inflammatory responses²⁷ through their interaction with integrins on the surface of monocytes, neutrophils, and other circulating leukocytes. ICAM-1 is constitutively expressed by choriocapillary ECs³⁵ and at higher levels in the macular than extramacular choriocapillaris, and it is activated by complement fragment C5a.^{23,28} Furthermore, macrophage-derived cytokines, such as TNF- α , promote the expression of ICAM-1 in the retinal pigment epithelium (RPE) and vascular ECs,³⁶ and mice with a targeted deletion in the *Icam1* gene show a substantially reduced severity of experimental choroidal neovascularization compared with wild-type mice.³⁷ In other cell types, the expression of ICAM-1 is increased by oxidative injury and inhibited by some antioxidant compounds.^{38,39} Thus, other antioxidant regimens might exert a similar effect on EC activation. In the present study, we found that AREDS vitamin solution and zinc ions partially repress de novo ICAM-1 expression induced by LPS, both in cultured ECs and in human organ cultures of choroid and RPE. Quantification of macrophage binding to ICAM-1 protein supports the concept that ICAM-1/leukocyte interactions are impaired by AREDS MVS and that this regulation can take place posttranslationally. In contrast to ICAM-1, ICAM-2 expression after the stimulation of cells with LPS was not altered by AREDS MVS. These findings suggest that AREDS vitamins and zinc may be beneficial in AMD, at least partially by the inhibition of ICAM-1 expression and ICAM-1/leukocyte interactions.

There are several limitations to this study. The human clinical studies that have characterized the impact of AREDS vitamins on the progression of AMD have been performed over the course of years, and our experiments were limited to studying the impact of AREDS MVS on acute events, such as LPS exposure. However, because answers to even the most basic questions about how AREDS vitamins affect potential inflammatory pathways in AMD are unknown, we propose that these experiments represent a reasonable starting point for understanding the impact of MVS on EC activation. Indeed, the suppression of new ICAM-1 expression in both an EC line and in organ cultures of human RPE-choroid was striking. Another limitation is that our inflammatory challenge, LPS, is not very representative of the inflammatory microenvironment in AMD, in which complement complexes, advanced glycation end products, and oxidized lipids may contribute to RPE and choriocapillaris pathology.⁴⁰⁻⁴³ Further studies will be required to determine the extent to which AREDS MVS may mitigate proinflammatory responses to the array of molecular changes that occur in the aging Bruch's membrane. Finally, our experiments in vitro required us to make several assumptions about zinc delivery that, though necessary experimentally, may not be physiological. In normal health, zinc is absorbed along the gastrointestinal tract in a mechanism dependent on a host of dietary factors⁴⁴ and is trafficked through the bloodstream by zinc-binding proteins that regulate its bioavailability.^{45,46} For these

experiments, our goal was not to accurately model the in vivo situation but to determine whether AREDS MVS had a quenching effect on inflammatory processes relevant to AMD; to this end, we used vitamin caplets that are provided to AMD patients. In vivo studies will clarify the potential roles of AREDS MVS in affecting EC activation.

In summary, these experiments provide evidence that AREDS vitamins and zinc may slow the progression of AMD through a mechanism that includes the repression of cellular proinflammatory behaviors. This effect was noted primarily for de novo expression of ICAM-1 induced by inflammatory stimuli because little effect on baseline expression was noted. These results support the hypothesis that a benefit of AREDS and zinc to AMD is the suppression of EC activation. Additional experiments will be needed to confirm this hypothesis in vivo and to further explore the mechanism by which AREDS vitamins and zinc may affect AMD risk.

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