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Alcohol and nicotine consumption exacerbates choroidal neovascularization by modulating the regulation of complement system

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Abstract The objective of the present study was to investigate the effect of alcohol and nicotine consumption on the pathogenesis of choroidal neovascularization (CNV) in rats after laser-photocoagulation. Confocal microscopic analysis demonstrated an increase in CNV complex size in rats fed with alcohol (2.3-fold), nicotine (1.9-fold), and the combination of alcohol and nicotine (2.7-fold) compared with the control groups.

Immunohistochemical analysis revealed that alcohol and nicotine consumption increased MAC deposition and VEGF expression in laser spots. Expression of *CD59* by RT-PCR and Western blot was drastically reduced in the animals that were fed with alcohol, nicotine and alcohol and nicotine compared to those fed with water alone and this was associated with exacerbation of CNV.

Keywords: Choroid; Neovascularization; Macular degeneration; Alcohol; Nicotine; Complement system

1. Introduction

Age-related macular degeneration (AMD) is a complex disease that has been associated with multiple genetic and environmental risk factors [1–8]. Identification of risk factors is one of the first steps toward preventing and designing new strategies for AMD treatment. Smoking and chronic alcoholism are two environmental risk factors that have been strongly associated with human AMD severity and incidence. [7,8]. Cigarette smoking has been associated with a 2- to 3-fold increased in incidence of neovascular AMD [5–8]. Recent reports have indicated that a heavy alcohol intake may be associated with an increased risk of exudative AMD [9,10].

Choroidal neovascularization (CNV) is the creation of new blood vessels in the choroid layer of the eye, which is a common symptom of the degenerative maculopathy wet AMD. Many reports, including those from our laboratory, have demonstrated that the presence and activation of complement system is crucial for CNV development [11–14]. Several independent and unrelated studies have indicated that smoking and alcohol consumption can activate the complement system [15–20]. However, the complement system's exact role in alcohol and nicotine-induced CNV exacerbation is not known.

In our previous publications, we demonstrated that the alternative pathway of complement activation is critical for CNV development [12]. We have also established that membrane attack complex (MAC) formation and its regulation by *CD59* plays a critical role in CNV pathogenesis [13]. *CD59* is a complement regulatory protein that regulates the assembly and the activity of MAC [21].

In this study, we investigated the effect of both nicotine and alcohol consumption on laser-induced CNV in rats. Laser-induced CNV model is an accelerated model of wet type AMD and generates acute inflammation compared to subtle inflammation seen in human AMD. We have also investigated *CD59*'s role in CNV exacerbation in alcohol or nicotine-fed rats. For this study, we have only focused on *CD59* because we have already established its role [13] in the development and pathogenesis CNV or wet AMD.

2. Materials and methods

2.1. Animals

Male Brown Norway rats (4–6 weeks old) were purchased from Harlan Sprague Dawley (Indianapolis, IN). This study was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas for Medical Sciences, Little Rock, AR.

2.2. Induction of CNV

Photocoagulation, using Argon red laser (50 μm spot size, 0.05 s duration, 350 mW) induced CNV and three laser spots were placed in each eye area surrounding the optic nerve as previously described [11–13,22].

2.3. Alcohol and nicotine feeding

After laser the rats were randomly divided into five groups. Group 1 was fed alcohol (8 g/kg body weight), group 2 was fed nicotine (200 μg/ml) and group 3 was fed with combination of alcohol (8 g/kg body weight) and nicotine (200 μg/ml). Group 4, fed with water alone,

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Abbreviations: CNV, choroidal neovascularization; AMD, age related macular degeneration; VEGF, vascular endothelial growth factor; RPE, retinal pigment epithelium; FITC, fluorescein isothiocyanate; MAC, membrane attack complex; CRRY, complement receptor1-related protein y.

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served as controls and group 5, fed with glucose in water, served as pair-fed controls. All group animals were fed regular diet and were killed after 4 weeks. Alcohol and nicotine were mixed with water and the bottles were changed daily. One rat was housed per cage. The daily alcohol and nicotine consumption was measured as follows: alcohol and water mixture contained 20 ml of ethyl alcohol (100% proof, density = 0.78) and 180 ml of water. Each rat drank 31–35 ml of the mixture daily. Assuming that 10% of alcohol evaporated from the mixture daily, each rat, weighing approximately 300 g, received 8 g/kg. Dehydration was controlled by feeding the rats in alcohol group with water only for 2 h daily before the next alcohol feeding. This 4 week dose of alcohol feeding produced serum alcohol levels similar to those found in alcoholics [22]. The nicotine and water mixture contained 200 μg/ml of (−)- nicotine-hemisulfate (Sigma–Aldrich, St. Louis, MO). Each rat drank 15–20 ml of the mixture daily and consumed 3 mg/day (200 μg/ml /day). This 4 week dose of nicotine feeding produced serum nicotine levels similar to those found in chronic, moderate smokers [23,24].

2.4. Measurement of CNV and CNV lesions

Four weeks after the laser treatment, including alcohol and nicotine feeding, all animals were anesthetized (ketamine/xylazine mixture, 1:1), perfused with 1 ml of PBS containing 50 mg/ml fluorescein-labeled dextran (FITC-dextran; average molecular mass, 2×10^6; Sigma–Aldrich, St. Louis, MO) and sacrificed. Eyes were harvested and fixed in 10% phosphate-buffered formalin for 1 h, and retinal pigment epithelium (RPE)–choroid–scleral flat mounts were prepared as previously described [11–13,22]. RPE–choroid–scleral flat mounts were stained for elastin using a monoclonal antibody specific for elastin (1.0 mg/ml; 1/200 dilution; Sigma–Aldrich) followed by a Cy3-labeled secondary antibody (1.0 mg/ml; 1/200 dilution; Sigma–Aldrich). CNV incidence and size were determined by confocal microscopy (Zeiss LSM510). The CNV complex size was graded by morphometric analysis of the images (Image Pro 5.0 software; Media Cybernetics Inc., Silver Spring, MD) obtained from confocal microscopy [11–13].

2.5. Histology

Eyes were fixed with normal buffered formalin and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin. Stained section images were captured with Q Imaging GO-5 digital camera and Olympus microscope. Capillary amounts were counted and CNV area was measured using ImagePro program. Number of capillaries per 1 mm^2 of CNV area was calculated.

2.6. Immunohistochemistry

The paraffin embedded tissue sections (4 μm) were immunostained for MAC and vascular endothelial growth factor (VEGF). For MAC detection, polyclonal rabbit anti-rat C9 (1:500) was used as the primary antibody. This antibody was kindly provided by Prof. B.P. Morgan (School of Medicine, Cardiff University, Cardiff, UK). AlexaFluore 594 conjugated goat anti-rabbit IgG (invitrogen) were used as the secondary antibody. Control stains were performed with non-relevant antibodies (IgG whole molecule from rabbit serum) at concentrations similar to those of the primary antibodies. Additional controls consisted of staining by omission of the primary or secondary antibody. Sections were examined under fluorescence microscope (Olympus, Center Valley, PA). Rabbit polyclonal anti-rat $VEGF_{164}$ antibody (1:1600; R&D Systems, Minneapolis, MN), secondary goat biotinylated anti-rabbit IgG (H + L) (1:800; VectorLab, Burlingame, CA), Vectastain ABC Elite KIT (VectorLab) and Vector VIP substrate kit for peroxidase (VectorLab) were used to detect VEGF expression. Nuclei were counterstained with Vector Methyl Green (VectorLab). For negative controls, slides were incubated with 1% BSA in TBS instead of primary antibodies. After immunohistochemical labeling, sections were mounted in Permount (Fisher, Fair Lawn, NJ) and were examined under bright field microscope (Olympus). Semiquantitative scoring of positive signal was performed in the choroid. Staining intensity was graded from 0 to 3 (0 – no staining; 1 – faint; 2 – moderate; 3 – intense). Score mean value was calculated for each laser-injured area.

2.7. RT-PCR analysis

After 30 days , animals from each group (n = 5/group) were killed; RPE–choroid–scleral tissues harvested from the enucleated eyes were pooled separately for each group and total RNA was prepared using the RNA Isolation kit (Qiagen, Germantown, MD). Equal amounts of the total RNA (0.1 μg) were converted to cDNA, and then used to detect the mRNA levels of β-actin, and *CD59* by semiquantitative RT-PCR using reagents (Applied Biosystems, Foster City, CA). Sense and antisense oligonucleotide primers were synthesized at Integrated DNA Technologies, and 30 cycles were used for PCR. Negative controls consisted of RNA omission or reverse transcriptase from the reaction mixture. Primer sequences including the predicted sizes of amplified cDNA are as follows: β-actin (318 bp) forward: 5′-GTTTGAGACCTTCAACACC-3′, reverse: 5′-GTGGCCATCTCTTGCTCGAAGTC-3′; CD59 (302 bp) forward: 5′-CTGCTTCTGGCTGTCCTCTG-3′, reverse: 5′-ACGCTGTCTTCCCCAATAGG-3′.

PCR products analyzed on a 1% agarose gel were examined by using Quantity One (Bio-Rad, Hercules, CA). All reactions were normalized to β-actin expression. Experiments were repeated three times with similar results.

2.8. Western blot analysis

RPE–choroid–scleral tissues harvested from all animal groups (as described above for RT-PCR) were pooled separately. Pooled tissue was homogenized and solubilized in ice-cold PBS containing protease inhibitors and total protein concentration was determined [11–13]. After SDS–PAGE on 12% linear slab gel, separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. Blots were incubated with mouse anti-rat *CD59* (AbD Serotec, Raleigh, NC), or monoclonal anti-β-actin (mouse IgG1; Sigma–Aldrich). After washing and incubation with HRP-conjugated secondary antibody, blots were developed using the ECL Western blotting detection system "ECL Plus" (Amersham Biosciences, Piscataway, NJ). Quantitation of *CD59* and β-actin was determined by analyzing band intensity using Quantity One 4.2.0 (Bio-Rad). Experiments were repeated three times with similar results.

2.9. Statistical analysis

Data were expressed as the means ± S.D. and were analyzed and compared using Student's t-test, and differences were considered statistically significant with $P < 0.05$.

3. Results

3.1. Effect of alcohol and nicotine feeding on CNV

All animals were divided into five groups and CNV was induced by laser-photocoagulation as described in Section 2. After laser treatment, group 1 was fed alcohol (8 g/kg body weight), group 2 was fed nicotine (200 μg/ml) and group 3 was fed a combination of alcohol (8 g/kg body weight) and nicotine (200 μg/ml). Water-fed animals were placed in group 4 (no nicotine or alcohol) and served as controls. Group 5 animals were fed with glucose mixed in water and served as pair-fed control. All animals were monitored daily for alcohol/nicotine consumption and change in body weight. After initial weight loss, rate of weight gain in all groups was similar to that in controls (data not shown).

Animals were killed after 4 weeks of treatments, perfused with FITC-dextran, and eyes were enucleated. RPE–choroid–scleral flat mounts were stained with goat anti-elastin antibody (primary antibody). CNV incidence and size was determined by confocal microcopy. Fig. 1 shows representative confocal micrographs of the RPE–choroid–scleral flat mounts from each group. Red color in the micrographs represents the exposed Bruch's membrane due to laser treatment and green fluorescence represents the new vessels formed after the laser treatment. CNV complex size (i.e. area) was measured using Image Pro-Plus software in micron unit. Confocal analyses demonstrated a significant increase in CNV complex size in alcohol-fed rats (Fig. 1C), nicotine-fed rats (Fig. 1D) and combination of alcohol and nicotine-fed rats (Fig. 1E) compared to