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Research Paper

Oxysterol Signatures Distinguish Age-Related Macular Degeneration from Physiologic Aging

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ABSTRACT

Macrophage aging is pathogenic in numerous diseases, including age-related macular degeneration (AMD), a leading cause of blindness in older adults. Although prior studies have explored the functional consequences of macrophage aging, less is known about its cellular basis or what defines the transition from physiologic aging to disease. Here, we show that despite their frequent self-renewal, macrophages from old mice exhibited numerous signs of aging, such as impaired oxidative respiration. Transcriptomic profiling of aged murine macrophages revealed dysregulation of diverse cellular pathways, especially in cholesterol homeostasis, that manifested in altered oxysterol signatures. Although the levels of numerous oxysterols in human peripheral blood mononuclear cells and plasma exhibited age-associated changes, plasma 24-hydroxycholesterol levels were specifically associated with AMD. These novel findings demonstrate that oxysterol levels can discriminate disease from physiologic aging. Furthermore, modulation of cholesterol homeostasis may be a novel strategy for treating age-associated diseases in which macrophage aging is pathogenic.

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1. Introduction

Age-related macular degeneration (AMD) is a leading cause of blindness in adults over 50 years of age in industrialized nations [1]. Early AMD is characterized by the presence of lipoproteinaceous deposits or drusen under the retinal pigment epithelium (RPE) and/or thickening of Bruch's membrane. Although early AMD is not always associated with vision loss, it is a major risk factor for progression to one of two forms of advanced AMD: a dry form, characterized by death of RPE cells called geographic atrophy (GA) that eventually leads to death of overlying photoreceptors, or wet (neovascular) form, characterized by abnormal vascular proliferation underneath the retina called choroidal neovascularization (CNV). While both advanced dry and wet AMD can cause vision loss, wet AMD accounts for a significant fraction of the vision loss associated with AMD [2] and can often be acute and catastrophic.

The current mainstay therapies for wet AMD focus on combating abnormal angiogenesis by blocking vascular endothelial growth factor (VEGF) with targeted anti-VEGF agents. These treatments stabilize disease in a majority of patients and significantly improve visual outcomes in 30–40% of wet AMD patients [3–5]. However, these therapies often require frequent visits for repeated intraocular injections, which places a significant burden on patients and caregivers [6]. Moreover, repeated intravitreal injections are also associated with risks of their own, such as infection, scleral thinning, and long-term visual acuity loss due to continued atrophy of the RPE and photoreceptors [3, 7]. Perhaps more importantly, anti-VEGF agents do not address the pathophysiology that causes wet AMD [8]. Therefore, there is need for further research to clarify the molecular and cellular mechanisms involved in the transition from physiologic aging to AMD and to understand the pathogenesis underlying the progression from early to wet AMD, which may lead to novel strategies for targeted intervention.

In the past few decades, we and others have demonstrated that macrophages, key cells of the innate immune system, play important roles

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in the pathogenesis of wet AMD [9–12]. Moreover, it has become increasingly clear that the ability of macrophages to polarize to different activation states is an important factor affecting whether macrophages promote health or disease [13]. Depending on dynamic tissue signals and the surrounding micro-environment, macrophages can polarize to a classical pro-inflammatory (M1-like) phenotype, an alternative anti-inflammatory (M2-like) phenotype, or some intermediate between these two extremes [14]. To further complicate matters, the identity of the specific activators that cause macrophage polarization may also affect the macrophage phenotype [15].

Previously, we reported that aged macrophages tend to skew to the anti-inflammatory M2-like phenotype and are less able to inhibit abnormal angiogenesis [16]. Furthermore, aged macrophages exhibit both impairments in cholesterol efflux [17] and abnormalities in IL-10 and downstream STAT3 signaling pathways that contribute to this age-associated drift towards M2-like polarization [18]. These age-associated impairments in cholesterol efflux and other lipid-related pathways may have mechanistic consequences in disease pathogenesis [19]. This possibility is supported by the fact that polymorphisms in lipid-related genes, such as hepatic lipase (LIPC), ATP-binding cassette transporter member 1 (ABCA1), and cholesterol ester transfer protein (CETP), are associated with advanced AMD [20]. Moreover, drusen, a clinical feature of early AMD, are lipid-rich, further supporting our hypothesis that dysregulated lipid homeostasis contributes to AMD. Despite these advances, the global programmatic changes that occur during macrophage aging need further elucidation. It is also unclear what subset of these changes are associated with physiologic aging or are pathologic and contribute to age-associated disease.

In this study, we sought to delineate the cellular pathways involved in macrophage aging and identify potential markers that may distinguish age-associated changes that are physiologic versus those that promote age-associated disease. Our results suggest that impaired cholesterol homeostasis in macrophages is a central process perturbed during aging and that these changes lead to alterations in oxysterol signatures that can distinguish AMD from physiologic aging. These findings may allow physicians to monitor progression of disease with quantifiable serum markers and may potentially lead to novel therapeutic strategies not only for AMD but also for other age-associated diseases in which alternatively-activated macrophages are pathogenic.

2. Materials and Methods

2.1. Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the Washington University School of Medicine Animal Care and Use guidelines. We obtained old (i.e., ~18-month-old), female wild-type C57BL/6J mice from the National Institute on Aging (Bethesda, MD) and compared them to strain-matched young (i.e., ~3-month-old), female wild-type C57BL/6J controls. We harvested peritoneal macrophages five days after elicitation with a 2-ml intraperitoneal injection of 4% thioglycollate broth (Sigma-Aldrich, St. Louis, MO). We harvested splenic macrophages by performing positive magnetic cell separation with the PE selection kit (Stem Cell Technologies) and PE anti-F4/80 monoclonal antibody (clone: BM8; eBioscience, Waltham, MA), following manufacturer's instructions. We cultured peritoneal and splenic macrophages in Gibco™ RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin-streptomycin (Thermo Fisher Scientific). When indicated, we treated macrophages with 25 or 50 μg/ml of oxidized LDL (oxLDL; Alfa Aesar, Haverhill, MA) for 24 h prior to further analysis.

2.2. OCR Measurements

To perform metabolic characterization, we measured the oxygen consumption rate (OCR) of peritoneal macrophages as a surrogate marker for oxidative respiration with the XF96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA). In short, we plated peritoneal macrophages in Seahorse XF96 cell culture microplates (Seahorse Bioscience) at 100,000 cells per well. On the morning of the experiment, we washed the cells and replaced the medium with Seahorse assay medium (Seahorse Bioscience) supplemented with 25 mM glucose (Sigma-Aldrich, St. Louis, MO) and 1 mM sodium pyruvate (Thermo Fisher Scientific) and adjusted the pH to 7.4. After incubation in a non-CO_2 incubator at 37 °C for 1 h, we measured OCR at baseline and after sequential treatment with the following chemicals from the Mito Stress Test kit (Seahorse Bioscience): 3 μM oligomycin, 5 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and 1 μM rotenone/antimycin A (rot/AA). Each cycle consisted of 2 min of mixing and a 1-min pause, followed by a 5-min measurement period; we repeated each cycle 3–4 times. We normalized the background of all measurements by subtracting the average OCR of each sample after treatment with rot/AA.

2.3. Gene Expression Analysis

We extracted total RNA from peritoneal macrophages with the RNeasy Mini kit (Qiagen) and prepared cDNA with the High-Capacity Reverse Transcription kit (Applied Biosystems), following manufacturer's instructions. We performed quantitative PCR amplification of cDNA using either the TaqMan® probe-based gene expression assay for $p16^{INK4a}$ (Mm00494449_m1; Applied Biosystems) or custom TaqMan® Array Plates (Applied Biosystems) for lipid-related genes with the assays indicated in Supplemental Table S1. In all cases, we used the ΔΔCT method, normalizing to *Actb*, *18sRNA*, *Gapdh*, or the geometric mean of a combination of these endogenous controls.

2.4. Transcriptomic Profiling

We isolated total RNA from peritoneal macrophages with the mirVana kit (Ambion), performed cDNA amplification with the Ovation® Pico kit (NuGEN, San Carlos, CA), and performed target labeling with the Encore® Biotin kit (NuGEN), according to manufacturer's instructions. We then performed whole transcriptome profiling of young and aged peritoneal macrophages using Mouse Gene (MoGene) 1.0 ST arrays processed with Affymetrix Expression Console (v1.3.1.187) at standard settings (RMA background correction, median polish summarization, and quantile normalization) to generate intensity values. We assigned each probeset of the MoGene 1.0 array a detection call of 'mean + 2 × SD' of the negative controls. We performed data quality control to identify potential outliers by principal component analysis (PCA) plot and hierarchical clustering, as well as by quality control (QC) metrics (all probeset RLE means > 0.25) in Expression Console. From this QC, we omitted one sample in the young group. We then filtered data by probeset type ("main" in MoGene 1.0) and by detection call (any probeset without a "detected" call in any of the samples was removed). Any probeset without a gene symbol in the MoGene 1.0 data was also removed. 18,066 MoGene 1.0 probesets (from the total of 35,556) were kept for further analysis. We analyzed the data using the R package "limma" and generated gene lists based on *P*-values and false detection rate (FDR) q-values. We performed gene ontology (GO), pathway map, and interactome analyses with MetaCore™ (Clarivate Analytics, Philadelphia, PA). The microarray data are available at the Gene Expression Omnibus (GEO) at NCBI under accession number GSE111382.

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