Monocyte Activation in Patients With Age-Related Macular Degeneration

A Biomarker of Risk for Choroidal Neovascularization?

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Objective: To evaluate the activation state of macrophage function in patients with age-related macular degeneration (AMD) by quantifying the production of the proinflammatory and angiogenic factor tumor necrosis factor α (TNF-α) and by correlating its expression with dry and wet AMD.

Methods: Circulating monocytes were obtained from the blood of patients with AMD or age-matched control subjects by gradient centrifugation. The monocytes were then analyzed for TNF-α release from cultured macrophages in response to retinal pigment epithelium–derived blebs and cytokines or TNF-α messenger RNA content by reverse transcriptase–polymerase chain reaction.

Results: In human monocytes obtained from controls and AMD patients, TNF-α was expressed by freshly isolated monocytes and produced by macrophages in culture after stimulation with retinal pigment epithelium–derived blebs. However, wide variability in TNF-α expression was observed among different patients. Patients with monocytes that expressed the greatest amount of TNF-α demonstrated higher prevalence of choroidal neovascularization.

Conclusions: Both controls and AMD patients vary in the activation state (defined as TNF-α expression) of circulating monocytes. Partially active monocytes, defined as high TNF-α expression, may be a biomarker to identify patients at risk for formation of choroidal neovascularization.

Clinical Relevance: Early diagnostic testing may prove useful to detect those patients who will progress to the more severe complications of the disease.


The concept that cells of the monocyte lineage play a role in both dry and wet age-related macular degeneration (AMD) has emerged as a major mechanism in AMD pathogenesis. Monocytes and macrophages belong to the family of circulating bone marrow–derived mononuclear leukocytes. In dry AMD, macrophages and dendritic cells have been observed to insert processes into drusen or other deposits. Human and animal model studies also demonstrate that macrophages are numerous in choroidal neovascularization (CNV) and that these cells express high levels of various inflammatory and angiogenic cytokines. A reasonable assumption is that these macrophages (or other monocyte lineage cells) are recruited to the choroid from circulating monocytes in the blood, although their function (harmful or beneficial) remains unknown.

The cytokine tumor necrosis factor α (TNF-α) is a multifunctional protein synthesized by macrophages that mediates many of the inflammatory and tissue destructive functions of these cells, including angiogenesis. Tumor necrosis factor α induces proangiogenic activity in many cell types (including fibroblasts, tumor lines, and others) and can induce angiogenesis in many standard assays. Tumor necrosis factor α receptors are expressed on many cell types in the retina, including retinal pigment epithelium (RPE), Müller glial cells, and choroidal vascular cells, and TNF-α stimulation of the RPE induces many proangiogenic responses. Furthermore, TNF-α–deficient mice demonstrate reduced angiogenesis in a model of hypoxia-induced retinal neovascularization. On the basis of these observations, we sought to characterize TNF-α production by blood monocytes isolated from AMD patients and to correlate its expression with dry and wet AMD. Our results show that patients and control subjects demonstrate a wide range of expression of TNF-α cytokine in culture or messenger...
RNA (mRNA) in isolated monocytes. Furthermore, high levels of TNF-α mRNA correlated with a 5-fold risk of neovascular AMD. Analysis of monocyte TNF-α may serve as a biomarker for risk of CNV formation.

METHODS

PATIENTS

The study was conducted with the prior approval of the University of Miami School of Medicine (Miami, Fla) Institutional Review Board. Ninety-nine patients and subjects aged 21 to 90 years who were examined at the Bascom Palmer Eye Institute of the Palm Beaches, Palm Beach Gardens, Fla, were enrolled in the study. Patients underwent a complete ophthalmic examination, including fundus photography and fluorescein angiography if appropriate. Exclusion criteria included a history of human immunodeficiency virus infection, treatment for malignancy, recent acute illness that required hospitalization within 6 months, or other known immunologic condition.

Patients were defined as having dry AMD on basis of the presence of at least 3 drusen larger than 125 µm with or without hypertigmentary or hypopigmentary changes. Patients were classified as having neovascular AMD on the basis of standard findings by clinical examination and fluorescein angiography. No distinction was made about the type or stage of CNV (ie, classic, occult, retinal angiomatous proliferation, or disciform scar formation). Patients may have undergone treatment for their CNV. Controls included patients who were seen for routine eye examination with or without other ocular disorders but without evidence of drusen, pigmentary changes, or CNV.

After informed consent was obtained, 50 mL of blood was obtained by phlebotomy with EDTA anticoagulation, and samples were immediately placed on ice. Patients were interviewed about their use of vitamins and smoking history.

MONOCYTE RECOVERY METHOD

As a result of extensive pilot work, density gradient centrifugation was used to recover monocytes. Briefly, red blood cells were removed by centrifugation (550g for 20 minutes at room temperature). Then the buffy coat was collected in a 50-mL polypropylene conical tube, and 4 mL of OptiPrep (AXIS-SHIELD PoC AS, Oslo, Norway) solution was added, and the combination was gently mixed. A gradient solution (iodixanol–sodium chloride and HEPES) with a density of 1.088 g/mL was carefully overlaid on top of the OptiPrep–buffy coat mixture with a glass pipette. Without disturbing the layers, a second gradient solution (iodixanol–sodium chloride and HEPES) with a density of 1.078 g/mL was added on top of the 1.088-g/mL solution. Similarly, a third gradient solution (iodixanol–sodium chloride and HEPES) with a density of 1.068 g/mL was added on top of the 1.078-g/mL solution. In addition, HEPES-buffered saline was carefully added on top of the 1.068-g/mL solution. Centrifugation was performed at 200g for 10 minutes at room temperature. The top band of cells corresponding to the monocytes was collected and washed in HEPES-buffered saline. Flow cytometry using anti–CD14 or anti–CD68 immunofluorescence confirmed that purity was 85% to 95% monocytes. This technique of monocyte isolation resulted in less artifactual activation compared with other techniques tested, such as adherence, elutriation, cell sorting, magnetic beads, and other density-gradient isolation techniques. Fresh monocytes were either used for in vitro cell culture studies (in which case they were defined as macrophages) or immediately used for isolation of mRNA.

MACROPHAGE CULTURE CONDITIONS

Extensive pilot experimentation determined that the following technique was most reproducible. Monocytes were immediately plated at 2 × 10⁶ cells in triplicate onto 2% agarose-coated, 24-well plates in 10% fetal bovine serum. Agarose rather than plastic or collagen was chosen to avoid artifactual activation of cultured macrophages. Viability was approximately 85% to 90% after 24 hours. These macrophages were then stimulated with 1 of 4 conditions: medium alone, 3µM macrophe chemotactic protein 1 (MCP-1), RPE-derived cell membrane blebs, or both MCP-1 and blebs. A cytokine known to be produced by injured RPE, MCP-1 is an activation factor for macrophages. Blebs are vesicles of cell membrane and cytoplasm that form after oxidant injury and may be a component of drusen. Blebs were generated by using the spontaneously transformed adult human RPE cell line (ARPE 19), which was genetically modified by retroviral transduction with a construct containing green fluorescent protein–farnesylated r-Ras, which anchors the fluorescent marker to the inner leaflet of the plasma membrane. With the use of 10µM menadione, green fluorescent protein–modified (ie, green) blebs released into the medium were collected, washed, and concentrated by centrifugation and then characterized and quantified by flow cytometry. With the use of 3-µm fluorescent latex beads as size standards, blebs ranging in size from 0.3 to 5 µm in diameter were collected. The protocol was to add medium or MCP-1 in 1% serum overnight followed by medium or 106 blebs per milliliter the next morning. Forty-eight hours later, media were collected and TNF-α was measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, Minn). In some experiments, TNF-α or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was recovered from cultured macrophages to determine transcriptional regulation and was amplified by reverse transcriptase–polymerase chain reaction (RT-PCR) as described previously.

ISOLATION OF TOTAL RNA AND REAL-TIME RT-PCR

Monocytes were recovered as described herein. Total RNA was extracted using TRI-Reagent (Sigma-Aldrich Corp, St Louis, Mo). Tumor necrosis factor α primers and probe were purchased ready to use from Applied Biosystems (Foster City, Calif). Quantitative RT-PCRs were performed using the TaqMan One-step RT-PCR Master Mix reagents kit and ABI Prism 7700 sequence detection system (Applied Biosystems) in a total volume of 50 µL of reaction mixture according to manufacturer instructions. The TaqMan ribosomal RNA control reagents kit was used to detect the 18s ribosomal RNA gene, which represented an endogenous control. Each sample was normalized to the 18s transcript content. The primer probe mixture was purchased from Applied Biosystems and used as specified by the manufacturer’s protocol. The standard curves and 18s were generated using serially diluted solutions (0.001–100 ng) of mRNA from the human monocyte line U937 stimulated with phorbol myristate acetate. The PCR assays were conducted in duplicate for each sample.

STATISTICAL ANALYSIS

Statistical analysis was performed using a Statistica software package (Statsoft, Tulsa, Okla). Comparison of patient characteristics was performed with the use of the unpaired, 2-tailed t test for independent samples. Differences in median values of the ELISA and PCR data were determined using the Mann-Whitney test. An arbitrary cutoff value was used to subdivide the ELISA data into high, low, and intermediate categories. High activity was defined as the upper 25th percentile of the spon-
taneous TNF-α production or a more than 3-fold increase after stimulation. Low activity was defined as the lower 25th percentile of the spontaneous production or a less than 2-fold increase after stimulation. For PCR data, tertiles were determined using the 33rd and 67th percentile values of the control samples, and then those cutoffs were applied to the AMD data. Odd ratios and 95% confidence intervals were calculated for comparisons of middle vs lowest tertile and highest vs lowest tertile between the patients with dry and wet AMD. The Fisher exact test was used to determine statistical significance.

### RESULTS

#### SPONTANEOUS AND INDUCED TNF-α PRODUCTION BY MACROPHAGES IN CULTURE

Table 1 gives the demographic features of the patients with AMD and the controls. Although the controls were slightly younger, none of the differences were statistically significant.

We evaluated the pattern of TNF-α production by freshly isolated blood monocytes in culture when stimulated in cell culture with RPE-derived cell membrane blebs and MCP-1. Macrophage samples from patients and controls demonstrated a similar range of quantitative concentration of cytokine produced and a qualitative pattern of TNF-α production. We found that monocyte samples from different patients demonstrated qualitatively different patterns and magnitudes of TNF-α production. Some patients demonstrated a pattern of “responsiveness to stimulation” (Figure 1A). Unstimulated cells demonstrated significant “spontaneous” production of TNF-α protein (approximately 290 pg/mL) when simply placed in nonadherent culture. However, when MCP-1 and blebs were added, macrophages demonstrated even greater “induced” production, demonstrating a more than 3-fold higher TNF-α protein secretion (921 pg/mL) and indicating transcriptional regulation of TNF-α production (Figure 1A, top).

However, we also noted that macrophages isolated from other patients demonstrated a different pattern of poor responsiveness to stimulation (Figure 1B). In this example, unstimulated macrophages produced less than 30 pg/mL. No significant increase was noted on stimulation with blebs or MCP-1 (compared with a 3-fold increase to 900 pg/mL). Also, mRNA expression was consistent with the level of protein synthesis. These cells, however, could be stimulated with the classic inflammatory activation stimuli, interferon-γ (10 ng/mL) and lipopolysaccharide (10 ng/mL), to produce TNF-α levels greater than 800 pg/mL (not shown). Repetition of the experiment on a separate day produced similar results.

A comparison of samples from AMD patients and age-matched controls demonstrated a wide range in TNF-α production for both spontaneous (stimulated by medium only) and induced (stimulated with both blebs and MCP-1) TNF-α production. The median spontaneous TNF-α production was 370 pg/mL (range, 50-3260 pg/mL) for patients with AMD and 285 pg/mL (range, 45-1380 pg/mL) for age-matched controls. The median induced TNF-α production was 460 pg/mL (range, 100-6750 pg/mL) for patients with AMD and 615 pg/mL (range, 30-1390 pg/mL) for age-matched controls. Thus, among all samples, a 70-fold range in (unstimulated) spontaneous production of TNF-α was observed (median, 365 pg/mL) and a 300-fold range in induced production (stimulated with both blebs and MCP-1) was observed (median, 625 pg/mL). The presence of high heterogeneity among controls and AMD patients indicates that differences in TNF-α production are an intrinsic function of the macrophage immune response, not the result of AMD.

### WIDE INTERSUBJECT VARIATION IN TNF-α mRNA CONTENT IN CIRCULATING MONOCYTES FROM PATIENTS WITH AMD

We have shown herein that blood-derived macrophages from patients with AMD produce TNF-α when cultured with RPE-derived cellular debris or blebs but that high intersubject variability in TNF-α production was observed among patients. If this property reflects a physiologically important function of monocytes, then it is reasonable to assume that high or low production in culture might reflect high or low cytokine mRNA content of circulating monocytes. Thus, we sought to determine whether circulating monocytes demonstrated wide intersubject variability in expression of TNF-α mRNA content to account for the observed variation of spontaneous and induced synthesis of TNF-α in vitro.

The TNF-α mRNA content of freshly isolated monocytes was determined by real-time RT-PCR. A 200-fold range in TNF-α mRNA content was detected among AMD patients and control subjects (Figure 2). Monocytes from the total population of AMD patients demonstrated a similar range of TNF-α mRNA content as those recovered from age-matched controls. However, when AMD patients were subdivided into those with dry and wet AMD, the median value for patients with CNV was significantly greater than the value for patients with drusen (200 vs 124 arbitrary units, P=.002).

### MONOCYTE AND MACROPHAGE ACTIVATION STATUS AS A RISK FACTOR FOR CNV

We sought to test the hypothesis that high monocyte TNF-α production might be associated with CNV. We compared patients with early AMD (defined as drusen only) or neovascular AMD in terms of macrophage TNF-α production in cell culture using criteria that reflected both quantitative differences in production and the qualita-
The concept that inflammatory mechanisms play an important role in the progression of many chronic degenerative diseases has emerged as a major paradigm shift.
in our understanding of disease pathogenesis. For example, both innate and antigen-specific immune responses have been implicated in the pathogenesis of atherosclerosis, Alzheimer disease, and renal disease.\textsuperscript{22-27} Thus, similar mechanisms are likely to be involved in AMD pathogenesis. In this study, we focus on the potential role for blood-derived macrophages.

Monocytes and macrophages belong to the family of circulating bone marrow–derived mononuclear leukocytes. The monocyte (the circulating cell) and the macrophage (the tissue-infiltrating or cell culture equivalent) are important effectors in tissue repair, cell injury, inflammation, and innate immunity. In blood, the circulating monocyte can be identified by its relatively large size (12-20 µm), single-lobed indented nucleus, and the presence of cell surface markers, including CD68, CD11b/CD18, CD11c, and CD14. Monocytes can be recruited into many normal tissues by specific chemotactic factors, where they become macrophages up to 40 µm in size. In general, at least 4 different subsets of monocytes can be identified within normal tissues on the basis of function, morphologic features, and cell surface marker expression, including blood-derived macrophages (the major topic of this study), resident macrophages, microglia, and dendritic cells.\textsuperscript{28}

Cells of the monocyte lineage have been observed in histologic specimens of AMD. Macrophages have been detected along the choriocapillaris side of the Bruch membrane, underlying areas of thick drusen, or other deposits. Some investigators\textsuperscript{29} have even observed choroidal macrophages associated with sub-RPE basal laminar deposits. Processes from choroidal monocytes have been noted to insert into nodular drusen, presumably for the purpose of scavenging debris.\textsuperscript{29} The identity of these cells is uncertain, but they seem to lack typical phagocytic vacuoles and express HLA-DR, suggesting that most of the cells may represent dendritic cells.

Macrophages also contribute to the severity of CNV.\textsuperscript{26,30-32} Approximately 60% of surgically excised CNV membranes contain macrophages.\textsuperscript{3} The causal role of macrophages in regulating severity in CNV has been recently shown in a mouse model of experimental CNV in which macrophage depletion decreased severity by approximately 50%.\textsuperscript{30} Our group has also recently shown that approximately 90% of the macrophages in experimental CNV are blood derived, confirming that the analysis of circulating monocytes is relevant to AMD.\textsuperscript{31} The contribution (beneficial or harmful) of monocytes in AMD pathogenesis or progression is unknown. Theoretically, macrophages might mediate drusen resorption by scavenging and removing deposits. Conversely, macrophages may stimulate progression of drusen into CNV by releasing cytokines such as TNF-α or others into deposits and by releasing factors that regulate CNV growth and severity.

In this study, we analyzed the TNF-α expression by circulating monocytes isolated from AMD patients and observed 3 important findings. First, we demonstrate that RPE-derived blebs and MCP-1 (as surrogates for drusen) were a stimulus to induce low-grade macrophage activation. Using in vitro cell culture of monocytes from patients, we observed a 2- to 3-fold induction of TNF-α in macrophages from many but not all patients, indicating that stimuli relevant to drusen deposits might induce modest expression of cytokines by choroidal macrophages. Preliminary unpublished data for interleukin 6, a proinflammatory cytokine, gave similar results, suggesting that the observation is not restricted to TNF-α.

Second, and more important, we observed a wide range of heterogeneity of TNF-α expression in macrophages in culture or by mRNA analysis of freshly isolated circulating monocytes. The heterogeneity was present in controls and AMD patients, indicating it was a property of the macrophage immune response and not disease specific. The heterogeneity among different patients spanned a 200-fold range, a magnitude that is likely to be more physiologically important than the 3-fold induction by RPE debris in culture. On the basis of this observation, we speculate that the preexisting activation state of the monocyte (in this case, defined as the amount of TNF-α production) is more biologically relevant to AMD pathogenesis than is local induction of macrophage activation by drusen components in the eye. This is a novel finding that, if confirmed, will have implications about the regulatory mechanisms of macrophage activation beyond AMD. For example, activated macrophages may also contribute to complications of atherosclerosis, possibly contributing to the epidemiological association between AMD and vascular disease.\textsuperscript{33}

Third, our data suggest that macrophage activation state, defined as TNF-α production, might serve as a predictor of risk for progression. The presence of inflammation in chronic diseases, irrespective of the cause, has been noted to serve as a risk factor for progression. For example, in atherosclerosis, a high serum C-reactive protein level is a strong predictor of myocardial infarction and is used as a biomarker to identify high-risk patients.\textsuperscript{34} Similarly, in this study, we evaluated the possibility that the wide spectrum of intersubject variation of macrophage activation in cell culture or in mRNA levels of fresh monocytes serve as a predictor of neovascular AMD. Our results indicate that AMD patients with blood monocytes that express high TNF-α mRNA levels demonstrate an almost 5-fold increased prevalence of neovascular AMD. Tumor necrosis factor α synthesis in culture also demonstrated a suggestive trend.

Several possible weaknesses are apparent in this small pilot study. The assays are technically complex, are susceptible to experimental artifact, and must be processed on site. For example, elutriation and flow cytometry, 2 commonly used isolation techniques, induced unacceptable artificial activation. In addition, we have not extensively retested the same patients on multiple occasions to determine whether TNF-α levels are a consistent property or vary over time, but these experiments will be performed. Finally, evaluation of other macrophage-derived mediators relevant to AMD pathogenesis might be informative. Preliminary data indicate wide heterogeneity in monocyte expression of other factors relevant to neovascular AMD, including vascular endothelial growth factor.

In summary, these results suggest the hypothesis that the preexisting macrophage activation state, defined as the level of cytokine or mediator expression of the cir-
ulating monocytes, might determine the negative or positive consequence of macrophage recruitment as a disease modifier. Macrophages with high expression of relevant mediators might contribute to disease progression. Furthermore, identification of AMD patients with high levels of TNF-α or other relevant mediators may serve as a biomarker of progression. We hope that this approach can be perfected to identify high-risk patients in CNV prevention trials.

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