Diabetic macular edema (DME) is the most common cause of vision loss in diabetic retinopathy (DR).\textsuperscript{1} The disruption of blood-retinal barriers induces the influx of fluid into the retina, resulting in DME.\textsuperscript{2} Although the pathogenesis of DR is not fully elucidated, inflammatory reactions, including monocyte/macrophage recruitment and microglia activation, are considered among the causative factors of DME.\textsuperscript{3}

CD14 is a cytokine associated with the innate immune response and is expressed in microglia, monocytes, and macrophages.\textsuperscript{4} CD14 exists in two forms: membrane-bound or soluble (sCD14);\textsuperscript{5} both forms are related to the activation of the inflammatory response.\textsuperscript{6} In DME, the levels of sCD14 are elevated in the aqueous humor (AH) and vitreous humor and are associated with increased levels of VEGF.\textsuperscript{7} Microglia are resident immune cells in the retina that are involved in the inflammatory changes underlying DR. In DR, microglia proliferate, change their shape, release inflammatory cytokines, and aggregate near perivascular areas and areas of cystic retinal edema.\textsuperscript{8} Although microglia are considered the primary source of sCD14 in cerebrospinal fluid under pathologic conditions,\textsuperscript{9} knowledge regarding the association between sCD14 and microglia in patients with DR is scarce.

Hyperreflective foci (HF) on spectral-domain optical coherence tomography (SD-OCT) are markers of increased DME activity.\textsuperscript{10} The origin of HF remains controversial, and activated microglia or lipoprotein exudation are among the suspected causes.\textsuperscript{11,12}

We hypothesized that sCD14 in the AH is derived from activated microglia in the diabetic retina, and that if the level of this cytokine is correlated with the number of HF on SD-OCT in DME patients, HF could be indicative of activated microglia in DME patients.

The degree of microglia activation could vary according to the different types of DME: focal, diffuse, or combined. We speculated that the level of sCD14 and the distribution of HF could also differ depending on the type of DME because focal and diffuse edema appear to have different underlying pathophysiology; diffuse edema is thought to result from disrupted retinal vessel integrity mainly in the inner retinal layer, whereas focal edema results from microaneurysms with pathology observed primarily in the outer retina.\textsuperscript{13}

In this study, a clear correlation was observed between the level of sCD14 in the AH and either the number of HF or the level of edema in each retinal layer in DME patients. Moreover,
we also observed altered levels of sCD14 and distinct local distributions of HF based on the type of DME.

**METHODS**

**Subjects and AH Sample Collection**

AH samples were collected at the Department of Ophthalmology, Konkuk University Medical Center, Seoul, Korea. Between January 1, 2011, and March 31, 2017, 69 eyes from 51 patients with DME and 28 eyes from 28 control subjects were included in this study. All studied subjects were South Korean, a relatively homogeneous East Asian ethnicity. Eyes with the following characteristics were excluded: DME with epiretinal membrane or foveal traction, hard exudate at the macula, vitreous hemorrhage or pre- and subretinal hemorrhage, other retinal diseases, glaucoma, or a history of uveitis. Using a 30-gauge needle, AH samples from the eyes of the DME patients were obtained before an intravitreal injection of bevacizumab (IVB; 1.25 mg/0.05 mL); samples from the control eyes were obtained immediately before cataract surgery. At each visit, best corrected visual acuity (VA; logMAR), color fundus photography, and SD-OCT were evaluated. Additional injections were conducted according to pro re nata regimen with 4 to 6 weeks when intraartinal and/or subretinal fluid remained.

**sCD14 Measurement via ELISA**

The levels of sCD14 in the AH were assessed using a sandwich ELISA kit (R&D systems, Inc., Minneapolis, MN, USA). The color intensities were determined using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Duplicate samples were used in all assays.

This study followed the guidelines of the Declaration of Helsinki, and informed written consent was obtained from all patients and control subjects. The procedure for the AH collection was approved by the institutional review boards of Konkuk University Medical Center, Seoul, Korea.

**SD-OCT Imaging**

A volume scan comprising 25 horizontal B-scans covering 9 × 6-mm area of the macular region centered on the fovea was acquired using SD-OCT (Spectralis HRA+OCT; Heidelberg Engineering, Heidelberg, Germany).

The eyes from the DME patients were classified into two groups according to their pattern of edema as previously described.13 Focal edema was defined as the retinal fluid collection only in the outer retina (outer plexiform layer [OPL] to external limiting membrane [ELM]). In diffuse edema, the retinal fluid is located in the inner retina (internal limiting membrane [ILM] to inner nuclear layer [INL]). Combined edema, which fluid is observed in both the inner and outer retina, was also designated as diffuse edema. Thus, diffuse edema was defined as the retinal fluid located in the inner retina with or without retinal fluid in the outer retina.

Measurements of the disrupted length of the ELM and the junction between the photoreceptor inner segment and the outer segment (S/OS) were performed within a 3-mm area centered on the fovea in fovea-spanning horizontal B-scans. Central macular thickness (CMT) was measured within a 1-mm diameter circle centered on the fovea, and the volumes of the total retinal layers (from the ILM to the basement membrane), the INL, and the OPL plus ONL (OPL/ONL) were measured within a 3-mm diameter circle centered on the fovea.

The HF were manually counted within a 3-mm area using a fovea-spanning horizontal B-scan. HF were defined as discrete and well-circumscribed dots of similar reflectivity with the RPE band. The longest diameter of HF was limited to the 20- to 50-μm range. Therefore, we excluded small noise signals and large hyperreflective clumps, which are shown as hard exudates in fundus photography.

**Patient Data**

The baseline (pre-IVB) data on 69 eyes from the 51 patients with DME included demographic and clinical characteristics (i.e., age, sex, diabetes duration, presence of controlled systemic hypertension, and HbA1C) and ophthalmologic examination findings, such as best corrected VA, fluorescein angiography results, and SD-OCT results. Follow-up examinations (VA and SD-OCT) were performed in 30 eyes (11 eyes with diffuse edema and 19 eyes with focal edema) 3 months after the IVB injection (post-IVB). The VA, CMT, and the number of HF were compared to analyze the association between the level of sCD14 and visual/anatomic change. The eyes were subdivided into the following three categories according to the international clinical DR severity scale: no DR, nonproliferative DR (NPDR), and proliferative DR (PDR).

**Statistical Analysis**

The statistical analysis was performed using SPSS software version 17.0 for Windows (SPSS, Inc., Chicago, IL, USA). All values are presented as the means ± SD or numbers (%). To compare the mean values between groups, Mann-Whitney U test were performed. The χ² test was done to compare the ratio of variables between two groups. Wilcoxon signed-rank tests were performed to compare the means of the VA (logMAR), CMT, and the number of HF before and after the treatment. A multivariate regression analysis was performed using a stepwise selection of specific variables, with adjustments for age and sex. Spearman correlation coefficients were calculated to examine the bivariate relationships. P values less than 0.05 were considered statistically significant.

**RESULTS**

**Baseline Demographics, Baseline sCD14, and Findings on SD-OCT**

The baseline characteristics of the subjects are listed in Table 1. The mean sCD14 levels in the DME group were significantly higher than those in the control group (29.9 ± 41.6 vs. 8.1 ± 3.6 pg/mL, respectively; P < 0.001). The HF were distributed across all retinal layers, and the numbers ranged from 1 to 27 (mean 8.5 ± 4.8; Table 2). The number of HF in the outer retina was higher than that in the inner retina (5.3 ± 5.7 vs. 3.2 ± 2.5, respectively; P < 0.001).

**Table 1.** Baseline Characteristics of Patients With DME and Control Subjects

<table>
<thead>
<tr>
<th>Parameters</th>
<th>DME</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of eyes</td>
<td>69</td>
<td>28</td>
<td>-</td>
</tr>
<tr>
<td>Age, y (SD)</td>
<td>55.4 (11.5)</td>
<td>66.6 (9.6)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>25 (36.2)</td>
<td>6 (21.4)</td>
<td>0.23†</td>
</tr>
<tr>
<td>DM duration, y (SD)</td>
<td>7.2 (5.8)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>sCD14, pg/mL (SD)</td>
<td>29.9 (41.6)</td>
<td>8.1 (3.6)</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

* Mann-Whitney U test.
† χ² test.

DM, diabetes mellitus.

* Mann-Whitney U test.
† χ² test.
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Table 2. Baseline VA and Anatomic Characteristics of Eyes From Patients With DME and Control Subjects

<table>
<thead>
<tr>
<th>Parameters</th>
<th>DME</th>
<th>Control</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of eyes</td>
<td>69</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>logMAR (SD)</td>
<td>0.53 (0.25)</td>
<td>0.41 (0.27)</td>
<td>0.53</td>
</tr>
<tr>
<td>CMT, μm (SD)</td>
<td>412.6 (112.7)</td>
<td>257.8 (20.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>INL volume, mm³ (SD)</td>
<td>1.3 (0.3)</td>
<td>1.0 (0.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>OPL/ONL volume, mm³ (SD)</td>
<td>3.5 (0.9)</td>
<td>2.4 (0.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Retinal volume, mm³ (SD)</td>
<td>10.5 (1.6)</td>
<td>8.3 (0.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No. of HF in inner retina</td>
<td>3.2 (2.3)</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of HF in outer retina</td>
<td>5.3 (3.7)</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of total HF (SD)</td>
<td>8.5 (4.8)</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Mann-Whitney U test.

Control, among 28 control subjects, SD-OCT data were available in 15 eyes of 15 subjects.

Table 3. Subgroup Analysis of sCD14 Levels According to the Type of DME, the Degree of DR, and the Presence of Hypertension

<table>
<thead>
<tr>
<th>Type of DME</th>
<th>Eyes, n</th>
<th>sCD14 Baseline, pg/mL</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffuse edema</td>
<td>23</td>
<td>50.0 (65.3)</td>
<td>0.024</td>
</tr>
<tr>
<td>Focal edema</td>
<td>46</td>
<td>19.8 (14.7)</td>
<td></td>
</tr>
</tbody>
</table>

Discussion

Microglia are considered important players in the initial inflammatory response in DR, but the cytokines secreted by microglia that are responsible for the pathogenesis of DR are not fully understood. Microglia are considered important players in the initial inflammatory response in DR, but the cytokines secreted by microglia that are responsible for the pathogenesis of DR are not fully understood. Histologic studies investigating DR in humans, perivascular microglia have been shown to be moderately increased in nonproliferative DR and hypertrophic in the inner retinal layers. In proliferative retinopathy, dilated new vessels are surrounded by microglia. Minocycline, an antibiotic that inhibits microglia, inhibits DR-induced inflammatory cytokine production and reduces the release of cytokotaxins from activated microglia. Therefore, activated microglia in DR negatively affect normal retinal cells by secreting proinflammatory and neurotoxic mediators.

Although the elevated levels of sCD14 in the AH and vitreous humor have been reported to be correlated with the levels of cytokines, such as VEGF, which suggests that sCD14 could be involved in the inflammatory response in DME, information regarding the associations between sCD14 and various characteristics of DME, including the type of DME and the volumetric changes in each retinal layer, is scarce. In the present study, the level of sCD14 in the AH in the DME patients positively correlated with the INL, OPL/ONL, and total retinal volumes. The INL volume was most strongly associated with the level of sCD14, as shown by the multivariate regression analysis. In addition, the sCD14 levels were higher in diffuse edema than those in focal edema. Therefore, the level of sCD14
FIGURE. SD-OCT characteristics of DME. Segmented retinal layers of fovea-spanning B-scan (blue ~ red line: INL, red ~ pink line: OPL/ONL) are depicted. (A) Patient 1: 43-year-old man with the diffuse type of DME (fluid located in both the inner and outer retina). His best corrected VA was logMAR 0.40, and his sCD14 level was 33.6 pg/mL. HF are observed in the inner and outer retinal layers in the inverted image. Thickening of both the INL and OPL/ONL and multiple cysts in the INL and ONL are observed in the segmented retina. Diffuse thickening of the INL and OPL/ONL is evident (white-colored pixels on the heat maps). (B) Patient 2: 46-year-old man with the diffuse type of DME. His VA was logMAR 0.22, and his sCD14 level was 46.3 pg/mL. HF are observed mainly in the inner retinal layers at the center and in the outer retina in the left parafoveal lesion on the inverted image. Thickening of both the INL and OPL/ONL and multiple cysts in the INL are observed in the segmented retina. Diffuse thickening of the INL and OPL/ONL is shown as white-colored pixels on the heat maps. (C) Patient 3: 51-year-old man with the focal type of DME. His VA was logMAR 0.1, and his sCD14 level was 9.0 pg/mL. HF are dispersed mainly in the outer retinal layer on the inverted image, and the density of the HF appears sparser than that in the patients with diffuse edema. Thickening of the OPL/ONL and multiple cysts in the ONL are observed in the segmented retina. Diffuse thickening of the OPL/ONL is prominent (white-colored pixels on the heat maps). (D) Patient 4: 49-year-old man with the focal type of DME. His VA was logMAR 0.30, and his sCD14 level was 15.2 pg/mL. HF are mainly observed in the outer retinal layers on the inverted image. Diffuse thickening of the OPL/ONL is observed (white-colored pixels on the heat maps).
is associated with edema across all retinal layers in DME and is particularly associated with inner retinal changes.

Coscas et al. suggested that the origin of the HF could be activated microglia associated with the inflammatory environment in age-related macular degeneration. In DME, anti-VEGF treatment is associated with a reduction in HF, and the presence of HF has been considered a marker of active disease status. Interestingly, microglia and HF share common characteristics. Both microglia and HF are primarily found in the inner retina in DR without edema and show an extended distribution into the outer retina in DME. Vujosevic et al. reported that compared with healthy subjects, diabetic patients showed an increase in the number of HF in the inner retinal layer, suggesting that HF may represent aggregates of activated microglial cells. These authors insisted that HF are distinct in nature from lipoprotein exudates because only patients at the initial stages of DR or without DR were included in their study. Microglia have been observed in the outer retina and subretinal space as DME progresses, and this finding is consistent with our results in which the HF were distributed across all retinal layers. Moreover, following their activation, the cell bodies of microglia become larger and are therefore more likely to be detected as bright dots on SD-OCT. In addition to these common characteristics, a positive correlation was observed between the level of sCD14 and the number of HF, indicating that HF in most patients with DME, if not in all, could represent activated microglia.

After dividing the patients into two groups according to the DME type, the level of sCD14 and the total number of HF were higher in the diffuse edema group than those in the focal edema group. The number of inner retinal HF in diffuse edema was significantly higher than that in focal edema, whereas the numbers of HF in the outer retinal layer did not differ between the two DME groups. The higher levels of sCD14 and number...
of INL HF in diffuse edema might imply that there is a more severe inflammatory reaction and concomitant increase in activated microglia in the inner retina. Diffuse edema is known to be induced by the generalized breakdown of the inner blood-retinal barrier. When diffuse leakage occurs in the inner retina, fluid collects mainly in the INL, resulting in a diffuse edema pattern. Compared with focal edema, diffuse edema is associated with a more extensive area of leakage, and the activated microglia also could be more extensively distributed around areas of extensive leakage. However, in focal edema, microaneurysms are the major source of the focal fluid accumulation in the outer retina. Bolz et al. described that HF located around microaneurysm walls were likely to consist of extravasated lipoproteins. Indeed, hard exudates are commonly found around microaneurysms but are not typically observed in diffuse DME. Therefore, the increase in both the number of HF and the level of sCD14 in diffuse edema in the present study supports our hypothesis that HF in the inner retina represent activated microglia rather than lipoprotein exudates.

This finding raises the question of the origin of HF in the outer retina. Do HF consist of migrated microglia or extravasated lipoprotein from microaneurysms? The following clues suggest that many outer retinal HF could also represent activated microglia. Compared with the control eyes, the level of sCD14 in the eyes with focal edema and cysts mainly distributed in the OPL/ONL was significantly increased. In addition, no difference was observed in the number of outer retinal HF between the edema subtypes. Hard exudates are thought to form from microaneurysms, which is the major source of leakage in focal edema. If most HF observed on OCT imaging using donor eyeballs might be needed to answer this intriguing question.

The higher baseline sCD14 levels were correlated with decreased CMT and VA improvement after the anti-VEGF treatment, which is consistent with previous reports in which anti-VEGF treatment reduced the number of microglia and their activity. The decreased microglial activity following the anti-VEGF treatment could contribute to the reduced loads of inflammatory cytokines, resulting in the reduction of CMT and the number of HF and VA improvement. Concordantly, CMT was significantly decreased in the diffuse edema group, whose baseline sCD14 was higher, while CMT in the focal edema group did not decrease. The favorable anatomic response in the diffuse edema group to the anti-VEGF treatment is consistent with previous reports. We speculate that the favorable response to the anti-VEGF treatment in the diffuse edema group might be due to the remarkable deactivation of microglia by anti-VEGF antibodies. Altogether, the level of sCD14 could be a new biomarker of functional and anatomic responses to anti-VEGF treatment or other drugs targeting the inflammatory microenvironment in DME.

This study has several limitations. First, direct evidence supporting increased microglia activation in the eyes with elevated sCD14 levels could not be obtained. Although knowledge regarding the sCD14 levels in animal models of DME is limited, correlating the level of sCD14 to visualized microglia using an immunohistologic approach might provide evidence regarding their direct relationship. Second, we could not directly show that HF on SD-OCT represent activated microglia. Thus, donor eyes from patients with DME should be scanned using SD-OCT to visualize the HF, and the HF should be colocalized with the same retinal tissue immunostained by microglial markers. Third, the association of the changes in the sCD14 levels post-IVB with other clinical parameters could not be shown in the present study due to the lack of measurement of sCD14 levels post-IVB. Thus, a future longitudinal study including the association of the changes in the sCD14 levels and HF after the treatment of DME is needed. Finally, the numbers of DME patients and control subjects were relatively small and their ages and sexes were not matched well (with younger ages and more males in the DME group than in the control group), which could affect the measurement value of sCD14. There is little information about the age and sex of INL HF in diffuse edema.
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prevalence of microglial activation or elevated sCD14 in DR or DME, although CD14 has been known to be elevated more in older individuals or males than in younger individuals or females in a neurodegenerative disease of the brain or chronic intermittent hypoxia, respectively. Further elucidation with a larger study group without bias might be needed to show direct relationships between microglial activation and HF on OCT in DME.

In conclusion, this study demonstrated that the level of sCD14 in the AH from eyes with DME is significantly higher than that in the control eyes. A positive correlation was observed between the level of sCD14 and the number of HF on OCT. Higher sCD14 levels were strongly correlated with an increased number of HF in the inner retina of patients with diffuse edema, who presented an increased INL volume. Additionally, higher sCD14 levels were markedly correlated with an increased number of HF in the inner retinas of patients with diffuse edema, who presented an increased INL volume. Further immunohistologic studies using animal models and donor eyeballs from patients with DME might be needed to obtain definitive answers as to whether HF originate from activated microglia and whether elevated sCD14 in DME is due primarily to activated microglia.

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