Autofluorescence of the Cells in Human Subretinal Fluid

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PURPOSE. The origin of autofluorescence in the subretinal space and the autofluorescence properties of the cells were investigated in surgically collected subretinal fluid.

METHODS. Subretinal fluid was surgically collected from four eyes of patients with rhegmatogenous retinal detachment (three eyes) and Coats‘ disease (one eye). After cytocentrifuge preparation of the cells in the fluid and immunofluorescence staining, a cytologic examination was conducted by using confocal scanning laser microscopy. The autofluorescence of the cells was elucidated by measuring the fluorescence spectra with spectroscopy, to obtain different excitation laser light emission fingerprints.

RESULTS. The cells from the subretinal fluid were classified into three types: CD68-negative cells containing numerous pigmented granules, CD68-positive cells containing few pigments, and CD68-negative cells with no pigmented granules. Autofluorescence was observed in the inclusions of the cells classified into the former two types. When the cells were excited by a 458- or 488-nm laser light, emission spectra in autofluorescence showed little difference between CD68-positive and -negative cells. Peak analysis confirmed that the two types of cells showed the same emission peaks within this range of excitation light.

CONCLUSIONS. Autofluorescent inclusions appeared in the CD68-positive and -negative cells in the subretinal fluid. The macrophages in the subretinal fluid possess autofluorescence that is spectroscopically similar to lipofuscin. Autofluorescence of macrophages can be attributed to degenerated outer segments and debris from apoptotic photoreceptors. Clinicians should consider migration of macrophages, in addition to retinal pigment epithelium, as the possible source when abnormal fundus autofluorescence is observed using an ordinary set of fluorescence filters. (Invest Ophthalmol Vis Sci. 2011;52: 8534 – 8541) DOI:10.1167/iovs.11-8012

Fundus autofluorescence (FAF) mainly originates from lipofuscin in the retinal pigment epithelium. Recent studies revealed that a main constituent of lipofuscin that generates autofluorescence is pyridinium bisretinoid (A2E).1–3 The derivatives of A2E in the photoreceptor outer segments are considered to be another source of abnormal FAF. Novel imaging technology of FAF and optical coherence tomography demonstrated subretinal autofluorescent deposits in some macular diseases with serous retinal detachment, such as vitelliform macular dystrophy,1,5 central serous chorioretinopathy,6–11 choroidal melanoma,12 and Vogt-Koyanagi-Harada disease.13 Various materials or cells have been proposed as the origin of autofluorescent deposits. These include migrating retinal pigment epithelial cells, debris of degenerated photoreceptor outer segments, or macrophages that have phagocytosed the outer segments. Among them, macrophages are thought to be the main source of the subretinal autofluorescent deposits.14 Migrating macrophages in the subretinal space are often found in histopathologic specimens and surgically excised tissue from patients with several retinal diseases, such as Best‘s vitelliform macular dystrophy,15,16 age-related macular degeneration,17,18 macular holes,19 rhegmatogenous retinal detachment subretinal fluid20,21 and Coats‘ disease.21,22 However, it has not been clearly demonstrated that macrophages are the source of autofluorescence in the subretinal space. In the present study, we investigated surgically collected cells in the subretinal fluid and characterized the fluorescence properties of the cells.

METHODS

This study complied with the Declaration of Helsinki. The institutional review board at Fukushima Medical University School of Medicine approved: (1) observation using optical coherence tomography and FAF in eyes with macular and retinal disorders and the retrospective comparative analysis performed in this study and (2) biochemical or histopathologic examination of tissues and fluid collected from the eyes at the time of surgery. Written informed consent was obtained from all patients.

Subretinal fluid from four eyes of four patients who had retinal detachment was examined. All four eyes showed hyperautofluorescence in the area of retinal detachment. Three eyes with long-standing RRD and one eye with Coats‘ disease underwent a routine ophthalmic examination, including determination of best corrected visual acuity (BCVA), intraocular pressure, slit lamp biomicroscopy with a contact lens, and fundus color photography. Fundus autofluorescence was taken with a confocal laser scanning ophthalmoscope (HRA2; Heidelberg Engineering, Heidelberg, Germany) using 488-nm excitation laser and a barrier filter at 500 nm.

Fluid Collection

Subretinal fluid was collected during surgery for retinal detachment. All three eyes with RRD were treated with a scleral buckling procedure. The subretinal fluid was aspirated with a blunt needle attached to a sterilized syringe and introduced into the site of scleral puncture for subretinal fluid drainage. The cryopexy was always performed after subretinal fluid drainage. Specimens with blood contamination were discarded. The eye with Coats‘ disease was treated with vitrectomy. After aspiration of the crystalline lens and core vitrectomy, an inten- tional tear was made in the upper temporal quadrant by intraocular diathermy. During fluid-air exchange for retinal reattachment, the subretinal fluid was passively aspirated from the intentional tear into a sterilized syringe. Retinal photocoagulation around the intentional tear and the area of retinal vascular abnormality was made after reattachment of the retina. All four eyes were reattached after a single surgery.

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Fluorescence Spectroscopy

Collected subretinal fluid was cytocentrifuged (GP centrifuge; Beckman, Fullerton, CA) onto a glass slide coated with poly-L-lysine (P4832; Sigma-Aldrich, St. Louis, MO) at the bottom of a 24-well multiwell plate (BD Falcon, Bedford, MA) at 1500 rpm for 10 minutes. Supernatant was removed. Subsequently, the cells were incubated at 37°C in RPMI 1640 medium containing 10% FBS. After incubation for 3 hours, the cells were washed three times with PBS and fixed in acetone/methanol (2:3) for 10 minutes at −20°C. After blocking nonspecific protein in 0.1% BSA in PBS for 30 minutes at room temperature, primary antibody CD68 (ab845; Abcam, Cambridge, UK) was applied for 3 hours at room temperature, and secondary antibody (Alexa Fluor 647; Invitrogen, Carlsbad, CA) was applied for 30 minutes at room temperature. The negative control was without secondary antibody. Immunofluorescence-labeled and differential interference images were obtained (LSM510 META; Carl Zeiss Meditec, Dublin, CA). The emission fingerprint of fluorescence was also recorded using the lambda META scanning mode, in which fluorescence in a 10-nm width is recorded by a polychromatic 8-channel detector that allows fast acquisition of lambda stacks. Fluorescence was excited by an Ar⁺ laser for 458, 488, and 514 nm; an HeNe laser for 532 nm excitation; and an HeNe laser for 633-nm excitation through an appropriate dichroic mirror. To compare the emission fingerprint, the detector gain and excitation light intensity were set constant for a set of experiments. The fluorescence intensity profile was exported as text and used to analyze the peak, by using the local maximum method in the peak-finding module of the software (Origin 8; OriginLab Corp., Northampton, MA).

RESULTS

Identification of Cells

The ages of the patients with RRD were 11, 17, and 24 years (Table 1). Preoperative examination revealed yellow precipitates and corresponding granular autofluorescence on the outer surface of the retina in all three eyes with long-standing RRD. All eyes with retinal detachment had subretinal strands that showed hyperautofluorescence (Fig. 1). The eye with Coats’ disease in a 15-year-old boy showed diffuse hyperautofluorescence in the area of bullous retinal detachment. Part of hyperautofluorescence appeared as spotted hyperautofluorescent lesions between the retinal folds (Fig. 2).

The cells from the subretinal space were classified into three types. The first type was relatively large, round cells that had...
well-defined cytoplasm and nuclei covered with numerous pigments. Heavily pigmented cells were negative for CD68 staining. Autofluorescent inclusions appeared in the cytoplasm (Fig. 3). This type of cell frequently appeared in the specimens of the RRD cases. The second type was large (20–50 μm in diameter), rounded or polygonal cells with few or no pigments. Differential interference contrast microscopy showed various sizes of vacuoles in the cytoplasm. Most of the subretinal cells in the eye with Coats’ disease were positive for CD68 staining (patient 4). The contour of vacuoles was clearly observed when the cells were stained immunocytochemically. Autofluorescent inclusions appeared in the cytoplasm and in some vacuoles (Fig. 4). This type of cell appeared more frequently in the specimens from the subretinal fluid of Coats’ disease. The third type of cell is the spindle-shaped or oval cells that had few or no pigments in the cytoplasm. These cells were negative for CD68 staining and did not show autofluorescence. The density of each type of the cells was summarized in Figure 5.

**Fluorescence Spectroscopy**

Fluorescence spectroscopy was applied to the first two types of cells. The intensity of autofluorescence of inclusions in the cells with CD68-positive or -negative cells stained with anti-CD68 antibody probed by AlexaFluor-647-labeled secondary antibody was examined with various excitation lights (458, 488, 514, 543, and 633 nm). The cells were characterized by the presence of far-red to infrared fluorescence excited by a 633-nm laser (Figs. 6I, 6J). Unexpectedly, when excited by a 458- or 488-nm laser light, emission spectra of autofluorescence showed little difference, either in CD68-positive or -negative cells (Figs. 6A–D). The emission peak at 580 nm was comparable in either type of cell. Red fluorescence obtained by excitation with longer wavelengths (514 and 543 nm) was higher in CD68-positive cells (Figs. 6E–H), but the CD68-negative cells still showed significant autofluorescence. We could not find any remarkable difference in the spectra between the cells from the eyes with RRD and that from the eye with Coats’ disease. To obtain quantitative information, peak analysis was performed for each emission curve on a total of 50 autofluorescent inclusions in four to six typical cells from each of the four specimens (Table 2). Consistent with Figure 6, this analysis also indicates that almost all CD68-negative cells contain apparent autofluorescent vesicles, demonstrating that autofluorescence does not associate with CD68 expression. In the 458- and 488-nm emission spectra, the cells classified as the former two types showed the narrow peak at the same wavelength.

**DISCUSSION**

Characteristics of autofluorescence were examined in the cells collected from subretinal fluid in eyes with retinal detachment, which showed autofluorescence in the area of retinal detachment. The cells were classified morphologically and immunocytologically, with CD68 used as the marker for macrophages. We found that autofluorescent inclusions appeared in the CD68-positive cells and -negative cells. No clear difference in autofluorescence spectra was observed in the two types of cells.

Although the main origin of FAF is lipofuscin in the retinal pigment epithelium, the subretinal deposits also show autofluorescence in eyes with retinal detachment caused by RRD, central serous chorioretinopathy, Best’s disease, age-related macular degeneration, or choroidal hemangi-
It has been speculated that the autofluorescent deposits may originate from macrophages. However, little is known about autofluorescence of the macrophages in the subretinal space. In addition, although immunofluorescent staining of CD68 is frequently used as the cytomarker for macrophages in histologic study of the retina and the choroid, little information has been provided about autofluorescence of the CD68-positive cells in the eye. Thus, information about autofluorescence of the macrophages may help to interpret histologic results. In the present study, we investigated the autofluorescence properties of CD68-positive cells. AlexaFluor-647 was used as a secondary antibody dye to identify macrophages without interfering with observation of autofluorescence from the cells.

A case with subretinal precipitates in long-standing retinal detachment was reported by Vogt in 1938. In such eyes, cyto-

logic study has revealed that macrophages are the dominant cell population in the subretinal fluid. Coats’ disease is a retinal vascular disease showing yellow golden deposits in the retina and bullous retinal detachment occasionally. Numerous macrophages were confirmed in the subretinal fluid aspirated from the eyes with retinal detachment. Abnormal FAF in both diseases has not been reported. We noticed hyperautofluorescent deposits corresponding to subretinal precipitates in the eyes with RRD and spotted hyperautofluorescence in the area of retinal detachment caused by Coats’ disease. In the eyes with RRD, hyperautofluorescence appeared along subretinal strands. Previous histopathologic studies suggested that subretinal strands are composed of macrophage and retinal pigment epithelium. Macrophages may have a part in the origin of subretinal strands.

Three types of cells were morphologically identified in the subretinal fluid from the four eyes in the present study. Immuno-

FIGURE 3. A cell obtained from the eye with rhegmatogenous retinal detachment shown in Figure 1 is negative for CD68 (A). Numerous small green autofluorescent inclusions in the cytoplasm were excited at 488 nm (B). (A) and (B) are superimposed in (C). The cytoplasm of the cell was filled with numerous pigment granules (D).
fluorescent staining confirmed that most of the lightly pigmented large cells were CD68-positive macrophages. Pigmented CD68-negative cells, on the other hand, were considered likely to be derived from the retinal pigment epithelium, because of their morphologic characteristics. Since autofluorescence appeared only in these types of cells, they were possible origins of abnormal autofluorescence in the subretinal space.

Peak analysis for 50 inclusions consistently showed similar fluorescence properties, suggesting that the inclusions in both types of cells may have similar composition of fluorescent material. Autofluorescent inclusions in both types of cells showed yellow-red peak emissions within the range of 558 to 612 nm (Fig. 6). Consistent with this observation, ex vivo experiments of the human retinal pigment epithelium showed that the peak emission of lipofuscin appeared within the range of 588 to 610 nm.\textsuperscript{36} Peak emission of lipofuscin varies by age,\textsuperscript{25,37} physical condition,\textsuperscript{38} and method of extraction. Consideration of variation of emission spectra of lipofuscin from these reports suggests that yellow-red emission originates from lipofuscin-like materials in the cells. Previously, lipofuscin was considered to be synthesized in the retinal pigment epithelium as a product of phagocytosis of the photoreceptor outer segments. Recent reports have suggested that A2E and its derivatives are synthesized in the photoreceptor outer segments before phagocytosis by the retinal pigment epithelium.\textsuperscript{39} Lipofuscin-like materials could also accumu-
Late in the macrophages. Specifically, the accumulated outer segment material in phagosomes in the macrophages may generate autofluorescence similar to that in the retinal pigment epithelium. Since the former two types of cells showed autofluorescence in the cells, they were considered to be the source of subretinal autofluorescence in part. Autofluorescence was observed in the phagosomal vacuole. Hence, autofluorescent substances may have already been synthesized in shed outer segments before phagocytosis. Recent reports have also raised this possibility. However, autofluorescence characteristics of shed substances in the subretinal space are still unknown. Further study is needed of this phenomenon.

The digested photoreceptor outer segments that may not be properly processed in the macrophages may become lipofuscin. The possibility of the photoreceptor outer segments being

**Figure 6.** Fluorescence emission fingerprints of CD68-negative and -positive cells collected from subretinal fluid in the eye with rhegmatogenous retinal detachment. (A, C, E, G, I) the CD68-negative cells; (B, D, F, H, J) the CD68-positive cells. Ex: Excitation wavelengths: 458, 488, 514, 534, and 633 nm, respectively (**top to bottom**).
processed into autofluorescent material in the subretinal macrophages could not be excluded.

CD68-positive cells showed two autofluorescence peaks within the range of 480 to 750 nm. In the present study, green emission peaks of autofluorescence, 505 nm for 457-nm excitation and 520 nm for 488-nm excitation, were observed. This emission spectrum was reported to appear in living human eyes. The flavin adenine dinucleotide (FAD) is considered to be the main source of the green emission. In the 488-nm emission spectra, the narrow peak appeared around 520 nm (Figs. 6C, 6D). A similar peak also appeared on the left shoulder of the broad peak (right), straddling 530 to 535 nm along the x-axis in the spectra induced by 458-nm light (Figs. 6A, 6B). The flavoprotein signal may be incorporated into this peak. Since the green emission was not observed in macrophages in the lung or peripheral blood, the fluorophore would be assumed to be one source of the subretinal macrophages. A minority of the retinal pigment epithelium has been reported to be CD68-positive in situ. The transformation of the retinal pigment epithelium into the macrophages is CD68-positive retinal pigment epithelial cells may look morphologically similar to macrophages. Therefore, the CD68-positive cells include the tissue macrophages and the transformed retinal pigment epithelium. To determine the contribution of the tissue macrophage to autofluorescence in the subretinal space, more specific staining is needed. However, the broad spectrum of autofluorescence and small amount of the specimens did not allow us to perform multiple staining. Further study of this issue is need.

In conclusion, we found that inclusions in the macrophages in the subretinal space emit intense autofluorescence. The spectra of the autofluorescence are very similar to that of the retinal pigment epithelium. Hence, autofluorescent properties of deposits in subretinal space raise the possibility that they are macrophages, and further critical markers are needed for clinical assessment as to their identity.

References

2. Liu J, Itagaki Y, Ben-Shabat S, Nakanishi K, Sparrow JR. The biosynthesis of A2E, a fluorophore of aging retina, involves the for...
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