fragment that inhibits active forms of VEGF-A, the main factor responsible for CNV proliferation and vascular permeability (Ferrara et al. 2006, 2007). Benefits of ranibizumab treatment in improving best-corrected visual acuity (BCVA) have been shown in the Caucasian population (Brown et al. 2006, 2009; Rosenfeld et al. 2006; Mitchell et al. 2010). Ranibizumab is currently approved in the United States, European Union, Japan and several other countries. EXTEND-I was the first study in Japanese patients that showed the safety and efficacy of monthly ranibizumab treatment (12-month results) during multiple-injection phase in terms of BCVA gain, reduction in total area of leakage from CNV plus retinal pigment epithelium staining and foveal retinal thickness, which were consistent with the pivotal studies performed in the Caucasian population (Tano & Ohji 2010). After the patients had completed the 12-month multiple-injection phase, all patients who provided written consent and were eligible based on the inclusion and exclusion criteria of the extension phase had the opportunity to continue to receive the 'individualized flexible interval regimen' [namely, *pro re nata* (PRN), as needed] until the approval of ranibizumab in Japan. This also provided a means to assess its long-term safety and efficacy. The PRN regimen was expected to maintain the improved visual acuity (VA) with less frequent injections in the extension phase. Current treatment guidelines in Europe recommend three initial monthly dosing followed by a maintenance phase, wherein the ranibizumab administration is decided based on monthly BCVA observation (Holz et al. 2010; Mitchell et al. 2010). This recommendation is based mainly on the results of the ranibizumab pivotal randomized phase III studies, namely MARINA (Rosenfeld et al. 2006) and ANCHOR (Brown et al. 2006) with monthly ranibizumab treatment. In these studies, the improvement of the BCVA score had stabilized (almost reached a plateau) by Month 3, and further increase in BCVA was minimal during the subsequent monthly treatments. On the other hand, in another pivotal randomized Phase IIIb study, PIER, quarterly treatment regimen could not maintain the improvement in BCVA score that was obtained by the three initial monthly injections (Regillo et al. 2008). However, there were also patients who maintained their gain in BCVA score during the quarterly regimen.

The extension phase of this study was initiated, therefore, to investigate whether ranibizumab administered PRN based on monthly BCVA scores and other ophthalmic examinations at two consecutive visits could maintain the improvement in BCVA scores. The reduction in dosing frequency was expected to reduce the risk of adverse events (AEs) associated with the intravitreal injection procedure in the elderly population as well as to address the difficulties in treating AMD through monthly injection of ranibizumab in a clinical setting.

Based on the 6-month interim results of the extension phase with PRN regimen as well as the 6- and 12-month interim analyses of monthly multiple-injection phase of this study, and the results of pivotal studies in the Caucasian population, ranibizumab was approved in Japan in January 2009. This paper presents the final data on long-term efficacy (in terms of BCVA) and safety of ranibizumab with PRN regimen from whole period of the extension phase of EXTEND-I.

Methodology

Study design

EXTEND-I was an open-label, multicentre, Phase I/II study comprising three phases: a single-injection phase, a multiple-injection phase and an extension phase (Fig. 1). The single-injection phase (Group A) was designed to sequentially evaluate the safety of intravitreal injections of 0.3 and 0.5 mg ranibizumab (six patients treated with each dose). The patients who successfully completed the single-dose phase (i.e., did not experience a Grade-3 targeted AE) could enter a multiple-injection phase wherein they received the same dose for an additional 11 months. The 12-month multiple-injection phase (Groups A and B; the latter consisted of patients who did not participate in the single-injection phase)

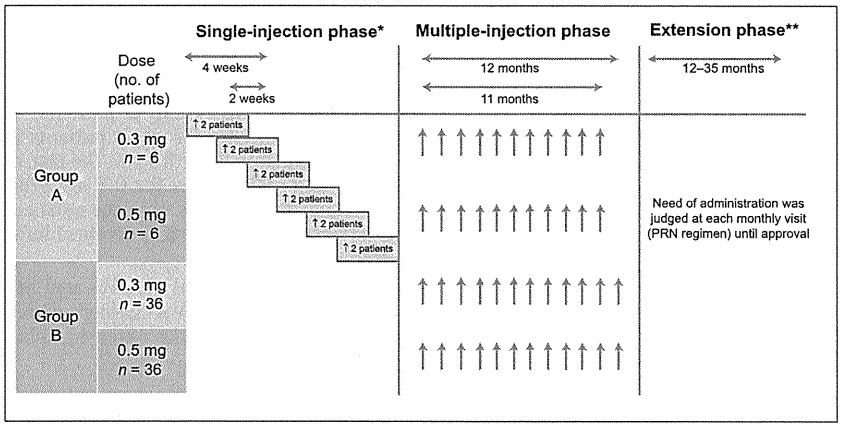

Fig. 1. EXTEND-I treatment schedule. *Upon completion of the single-dose phase, patients in Group A were eligible to enter the multiple-injection phase, which began ≥4 weeks after the final visit of the single-injection phase. Multiple injections did not begin until both doses were shown to be well tolerated in all cohorts. **Upon completion of the multiple dose phase, based on prespecified inclusion/exclusion criteria, patients could enter the extension phase. For the extension phase, treatment as per *pro re nata* regimen; dose same as core phase; retreatment at the monthly visit if loss of >5 letters in best-corrected visual acuity (BCVA) on two consecutive visits (except unscheduled visits), considering other ophthalmic examinations, such as slit-lamp examination, ophthalmoscopy, fundus photography, fluorescein angiography and optical coherence tomography, the investigator decided whether ranibizumab treatment would be performed. Similarly, if the BCVA score decreased on two consecutive visits (except unscheduled visits) by ≤5 letters using ETDRS-like visual acuity chart, a decision was taken whether treatment could be withheld. In any case, other ophthalmic examinations were taken into consideration. For the extension phase, the number of patients for Group A was 3 in the 0.3 mg group and 6 in the 0.5 mg group; the number of patients for Group B was 28 in the 0.3 mg group and 33 in the 0.5 mg group.

evaluated the safety and efficacy of both doses administered as monthly intravitreal injections in two parallel groups of 0.3 mg dose and 0.5 mg dose (Tano & Ohji 2010). The multiple-injection phase was followed by an extension phase in which the ranibizumab (0.3 or 0.5 mg) administration was on a PRN basis, but assessments were carried out on a monthly basis. If the BCVA score decreased at two consecutive visits (except unscheduled visits) by >5 letters, considering other ophthalmic examinations, such as slit-lamp examination and ophthalmoscopy for safety, fundus photography, fluorescein angiography and optical coherence tomography for efficacy, the investigator decided whether ranibizumab treatment would be administered although no specific retreatment criteria were provided for fundus photography, fluorescein angiography and optical coherence tomography and were at the discretion of the investigators. Similarly, if the BCVA score decreased at two consecutive visits (except unscheduled visits) by ≤ 5 letters, in conjunction with other ophthalmic examinations, a decision was taken whether the treatment could be withheld.

This study was conducted in accordance with the Declaration of Helsinki, International Conference on Harmonization Good Clinical Practice (GCP) guidelines and Japanese GCP. The study was approved by Institutional Review Boards at each study centre. All patients provided written informed consent before participating in the study and the extension. The trial is registered with clinicaltrials.gov (NCT00275821).

Inclusion and exclusion criteria

All patients with subfoveal CNV secondary to AMD who completed the multiple-injection phase in either of the ranibizumab groups (Groups A or B), provided written consent and met all of the inclusion criteria set at the beginning of the study (Tano & Ohji 2010) were eligible to enrol in the extension phase. Patients were allowed to participate in the extension phase regardless of the time elapsed between the exit visit of the multiple-injection phase and the participation in the extension phase.

Patients were excluded from the extension phase if they had received any anti-angiogenic drugs (bevacizumab, pegaptanib, ranibizumab, anecortave acetate, corticosteroids or protein kinase C inhibitors) or participated in any other clinical study of an investigational drug during the period from the exit visit of the multiple-injection phase to participation in the extension phase. However, as the extension phase was not started on the day of the exit visit from the multiple-injection phase, photodynamic therapy with verteporfin was allowed for the study eye during the transition period.

Efficacy assessments

The efficacy variables of the extension phase included mean change from Month 12 in BCVA score of the study eye using ETRDS chart (at a starting distance of 2 m) at the last visit of the extension phase for Group B patients only. Group A patients were not included as they were not assessed for efficacy, but only for safety throughout the study. The other efficacy variables included the proportion of patients at the last visit with a BCVA score loss <15 letters, and ≥ 30 letters, or a BCVA score gain of ≥ 15 letters in the study eye. Proportion of patients with BCVA <34 letters, approximate Snellen equivalent of 20/200 or worse, were also evaluated (ETDRS charts at a starting distance of 2 m). In the extension phase, colour fundus photography, fluorescein angiography and optical coherence tomography were performed in accordance with the routine procedures specified at each study site.

Safety assessments

All safety evaluations were based on the enrolled population (Groups A and B) of the extension phase. Safety assessments consisted of recording the frequency of the treatment collecting all AEs, serious adverse events (SAEs), with their severity, and relationship to study drug. It also included monitoring of haematology, serum chemistry, urinalysis and regular assessments of vital signs. Grade 3 targeted AEs (Tano & Ohji 2010), intraocular inflammation, myocardial infarction and stroke and AEs potentially related to systemic VEGF inhibition were analysed separately. Serum samples for the evaluation of immunoreactivity to ranibizumab (antiranibizumab antibodies) were obtained from patients prior to study administration at Month 23 and the last visit for Group A patients, and Month 24 and the last visit for Group B patients. At the last visit as well as at early termination, the assessments were performed if at least 6 months had passed since the previous measurement, on or after Month 11 for Group A patients and Month 12 for Group B patients. The last measurement in the multiple-injection phase of the study was performed at Month 11 for Group A and Month 12 for Group B.

Statistical analysis

The patient population included all enrolled patients in the extension phase. This population was used for all analyses in Groups A and B. All efficacy data presented were for observed cases without the last observation carried forward method.

Descriptive statistics of the number of injections, duration of exposure and reason of injection were presented for the enrolled population. The duration of treatment varied for each patient in the extension phase. To reduce a possible bias because of the patients who discontinued early without injection, the number of injections per year was calculated as $365.25 \times$ sum of total number of injections in the group/duration of the PRN regimen for the respective group. The number of injections per year was calculated for the respective group and not per patient. Duration of the PRN regimen was the date of the last potential treatment visit minus the date of Month 11 visit (the last treatment visit of multiple-injection phase) plus 1.

All efficacy analyses were based on the study eye. Descriptive statistics (mean, median, standard deviation, standard error, minimum and maximum) of the change from baseline (the single-injection phase of Group A and the multiple-injection phase of Group B), Month 11 and Month 12 in Group B were performed by treatment and visit. The 95% confidence intervals based on *t*-distributions and

p-values based on paired *t*-tests were determined for the change from baseline. Exact 95% confidence intervals were calculated for the proportion of patients with the specified response rates.

Results

Patients

Overall, 70 patients at 11 sites participated in the extension phase from 20 March 2007 to 20 January 2009: 9 in Group A (3 and 6 in the 0.3 and 0.5 mg dose groups, respectively) and 61 in Group B (28 and 33 in the 0.3 and 0.5 mg dose groups, respectively) as shown in Table 1. In Group A, a total of seven patients were not discontinued in the extension phase. Two patients in the 0.3 mg dose group withdrew from the study, as their condition did not further require the study drug. In Group B, 22 patients in the 0.3 mg dose group and 21 patients in the 0.5 mg dose groups were not discontinued in the extension phase. Six patients in the 0.3 mg dose group and 12 patients in the 0.5 mg dose group withdrew from the extension study. The maximum number of patients discontinued as they did not require the study drug because of improvement in VA (n = 9, two in the 0.3 mg dose group and seven in the 0.5 mg dose group); other reasons being AEs (n = 4, two in each dose group), withdrawal of consent (n = 4, one in the 0.3 mg dose group and three in the 0.5 mg dose group) and protocol violation (n = 1, one in the 0.3 mg dose group). None of the AEs leading to study discontinuation was thought to be related to the study drug.

The mean duration of treatment (standard deviation, SD) during the extension phase was 1.70 (0.35) years in the 0.3 mg group and 1.93 (0.09) years in the 0.5 mg dose group in Group A (Table 1). In Group B patients, the mean duration of treatment was 1.45 (0.33) years and 1.36 (0.39) years in the 0.3 and 0.5 mg dose groups, respectively.

The baseline demographic and ocular characteristics of enrolled patients at the start of the extension phase are given in Table 2. The mean (SD) BCVA score of the study eye at the start of the extension phase was 59.1 (11.69) letters and 59.8 (15.07) letters in the 0.3 and 0.5 mg dose groups of Group B, respectively. Overall, approximate Snellen equivalent VA of almost all patients was better than 20/200 except for two patients in the 0.5 mg dose group.

Of the 61 patients in Group B, approximately 90% (25/28 and 27/33 in the 0.3 mg and the 0.5 mg dose groups, respectively, Table 3) completed Month 24 from the baseline of the multiple-injection phase of the study, i.e., these patients received treatment of ranibizumab with PRN for 12 months in the extension phase. The duration of treatment of each patient in the extension phase varied with respect to the study entry and the longest was 35 months from baseline for the 0.3 mg dose group (n = 1). For the 0.5 mg dose group, the longest was 34 months (n = 1), as shown in Fig. 2.

The exposure to ranibizumab in the extension phase of Group B is shown

Table 1. Patient disposition in the extension phase.

Disposition/patients studied	Group A Ranibizumab 0.3 mg	Group A Ranibizumab 0.5 mg	Group B Ranibizumab 0.3 mg	Group B Ranibizumab 0.5 mg
Patients (n %)				
Enrolled	3 (100.0)	6 (100.0)	28 (100.0)	33 (100.0)
Not discontinued	1 (33.3)	6 (100.0)	22 (78.6)	21 (63.6)
Discontinued	2 (66.7)	0 (0.0)	6 (21.4)	12 (36.4)
Main cause of discontinuation				
Adverse event (s)	0 (0.0)	0 (0.0)	2 (7.1)	2 (6.1)
Patient's condition does not requires study drug	2 (66.7)	0 (0.0)	2 (7.1)	7 (21.2)
Protocol violation	0 (0.0)	0 (0.0)	1 (3.6)	0 (0.0)
Patient withdrew consent	0 (0.0)	0 (0.0)	1 (3.6)	3 (9.1)
Mean duration, years, of the extension phase (SD)	1.70 (0.35)	1.93 (0.09)	1.45 (0.33)	1.36 (0.39)

Table 2. Baseline demographics of enrolled patients and ocular characteristics (study eye) at the start of the extension phase.

Characteristic	Category/statistic	Group A Ranibizumab 0.3 mg N = 3	Group A Ranibizumab 0.5 mg N = 6	Group B Ranibizumab 0.3 mg N = 28	Group B Ranibizumab 0.5 mg N = 33
Gender – n (%)	Male	3 (100.0)	5 (83.3)	19 (67.9)	28 (84.8)
	Female	0 (0.0)	1 (16.7)	9 (32.1)	5 (15.2)
Age, years	Mean (SD)	68.0 (10.15)	72.0 (4.82)	69.8 (8.72)	70.2 (7.83)
Race (%)	Asian	3 (100.0)	6 (100.0)	28 (100.0)	33 (100.0)
Best-corrected visual acuity score	Mean (SD)	72.0 (4.58)	58.5 (15.66)	59.1 (11.69)	59.8 (15.07)
	Range	68–77	42–77	39–80	36–85
Approximate Snellen equivalent n (%)	Median	40.0	70.0	71.5	63.0
	20/200 or worse	0 (0.0)	0 (0.0)	0 (0.0)	2 (6.1)
	Better than 20/200 but worse than 20/40	1 (33.3)	3 (50.0)	20 (71.4)	20 (60.6)
	20/40 or better	2 (66.7)	3 (50.0)	8 (28.6)	11 (33.3)
Intraocular pressure (mmHg)	Mean (SD)	13.3 (1.53)	14.2 (3.06)	13.5 (2.92)	13.7 (3.09)
	Range	12–15	9–18	8–20	9–23

Data of ocular characteristics are based on Month 11 visit in Group A and Month 12 visit in Group B.
N = number of enrolled patients, n = number of patients.

Table 3. Summary of patient exposure to ranibizumab for 12 months (from Month 12 to Month 24) in the extension phase (Group B, enrolled patients).

Cumulative number of injections	Ranibizumab 0.3 mg (N = 28)	Ranibizumab 0.5 mg (N = 33)
Month 24		
n	25	27
Mean (SD)	4.1 (4.12)	3.9 (4.63)
Range	0–13	0–13
0	7	9
1–2	3	8
3–6	9	2
7–9	2	3
10–12	3	4
13	1	1
Number of injections per Year	4.19	4.27

The number of injections per year is calculated as: 365.25 × total number of injections/duration of the *pro re nata* (PRN) regimen.
Number of injections per year is calculated for total group, not per patient.
Duration of the PRN regimen: date of last potential treatment visit – date of Month 11 visit + 1.
N = number of enrolled patients, n = number of patients.

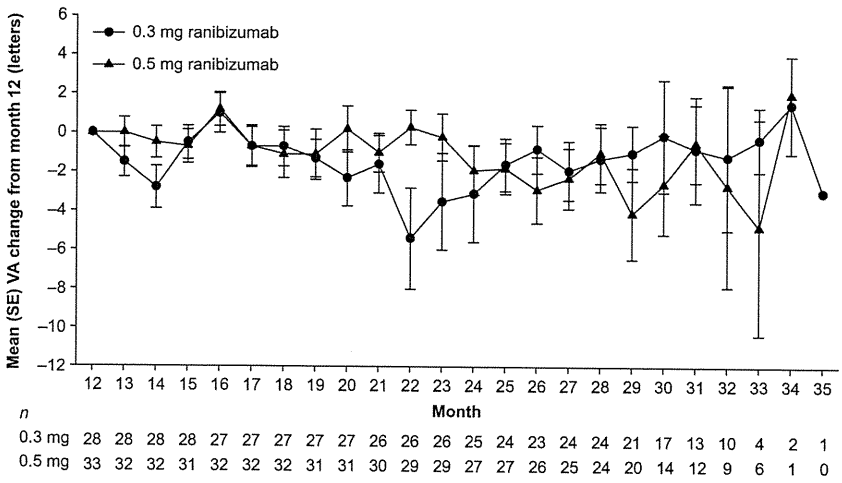

Fig. 2. Mean change from Month 12 (the start of Extension phase) in best-corrected visual acuity score (±SE) of study eye by visit during extension phase (Group B patients).

in Table 3. At Month 24, the patients had been treated with the PRN regimen for 12 months in the extension phase, and hence the maximum achievable number of injections by this visit was 13. The injection frequency of ranibizumab for individual patient varied from 0 to 13 times for this 12 months in the extension phase. The estimated number of injections per year in the extension phase was 4.19 and 4.27 in the 0.3 and 0.5 mg dose groups in Group B, respectively.

Efficacy

The mean change (SD) from Month 12 in BCVA score of the study eye to the last visit in the extension phase was −3.6 (14.82) letters in the 0.3 mg group and −2.2 (7.92) letters in the 0.5 mg group of Group B using the PRN regimen (Table 4). Furthermore, the mean change (SD) from baseline in BCVA score of the study eye to the last visit in the extension phase was 7.5 (19.12) letters in the 0.3 mg group and 7.7 (13.02) letters in the 0.5 mg group (p = 0.0475 for the 0.3 mg dose group and p = 0.0019 for the 0.5 mg dose group) (Table 4). Overall, the improvement in BCVA score at Month 12 by monthly ranibizumab injection was sustained throughout the extension phase with the PRN regimen (Fig. 2).

Table 5 shows the proportion of patients with respect to VA outcome at the last visit in the extension phase. The proportion of patients who lost <15 letters from baseline in BCVA in the study eye was 85.7% (24/28) and 97.0% (32/33) in the 0.3 and 0.5 mg dose groups, respectively. Nine patients each in the 0.3 mg (32.1%) and 0.5 mg (27.3%) dose groups gained ≥15 letters from the baseline. One patient each in the 0.3 mg (3.6%) and 0.5 mg (3.0%) dose groups lost ≥30 letters from the baseline. The proportion of patients with approximate Snellen equivalent of 20/200 or worse was 14.3% (4/28) and 6.1% (2/33) in the 0.3 and 0.5 mg groups, respectively.

The mean time to first retreatment in the extension phase since Month 11 of the multiple-injection phase (when the last monthly injection was done) in Group B was 218.5 days (range: 29–512 days) for the 0.3 mg group and 255.6 days (range: 29–571 days) for the 0.5 mg group.

Safety

In Group A patient population (n = 9), two of three (66.7%) patients in the 0.3 mg dose group and three of six (50.0%) patients in the 0.5 mg dose group experienced at least one ocular AE in the study eye during the extension phase. In Group B, 20 of 28 (71.4%) patients in the 0.3 mg dose group and 18 of 33 (54.5%) patients in the 0.5 mg dose group experienced at least one ocular AE in the study eye during the extension phase. The most common ocular AE in the study eye in Group B was conjunctival haemorrhage. Other frequent ocular AEs included retinal haemorrhage, retinal detachment and increased intraocular pressure (Table 6). Two patients in the 0.3 mg dose group of Group B experienced Grade 3 targeted AEs (intraocular inflammation, reduced VA, increased intraocular pressure, vitreous haemorrhage, retinal tear or detachment, and retinal haemorrhage). One patient experienced retinal detachment, retinal haemorrhage and vitreous haemorrhage in the study eye, and the other patient experienced retinal haemorrhage in the fellow eye.

One patient in the 0.3 mg dose group of Group B experienced iritis in the study eye among the ocular AEs defined under the group of intraocular inflammation (iritis, iridocyclitis, vitritis, uveitis, hypopyon and anterior chamber inflammation). Two kinds of ocular AEs in six patients of Group B were suspected to be study-drug related: increased intraocular pressure (two patients in the 0.3 mg dose group and three patients in the 0.5 mg dose group) and retinal haemorrhage (one patient in the 0.5 mg dose group).

Nonocular AEs were observed in four patients (44.4%) in Group A (two

Table 4. Mean change from baseline in best-corrected visual acuity score of the study eye at the last visit in the extension phase (Group B, enrolled patients).

Visual acuity (letters)	Ranibizumab 0.3 mg $N = 28$	Ranibizumab 0.5 mg $N = 33$
Baseline		
Mean (SD)	47.9 (12.59)	50.0 (10.38)
Month 12 (start of extension phase)		
Mean (SD)	59.1 (11.69)	59.8 (15.07)
Last visit		
Mean (SD)	55.4 (17.14)	57.6 (15.36)
Change from baseline		
Mean (SD)	7.5 (19.12)	7.7 (13.02)
95% CI of the mean*	0.1, 14.9	3.0, 12.3
p-value†	0.0475	0.0019
Change from Month 12		
Mean (SD)	−3.6 (14.82)	−2.2 (7.92)
95% CI of the mean*	−9.4, 2.1	−5.0, 0.6
p-value†	0.2042	0.1186

Observed values are presented. Patients must have values at both Month 12 and last visit to be included. Baseline value is defined as the last available measurement prior to the first injection in the multiple-injection phase of the study. End of study differed between the patients and this was more evident from Month 30. Month 35 was the longest analysis point.
N = number of enrolled patients.
* Derived from t-distribution.
† Derived from paired t-test.

each in the 0.3 and 0.5 mg dose groups), 19 patients (67.9%) in the 0.3 mg group and 24 patients (72.7%) in the 0.5 mg group in Group B. Nasopharyngitis was the most common AE in Group B patients (Table 6).

Adverse events potentially related to systemic VEGF inhibition were observed in four patients (14.3%) and two patients (6.1%) in the 0.3 and 0.5 mg dose groups of Group B, respectively. One patient in each dose group experienced cerebral infarction; three patients (0.3 mg dose group) and one patient (0.5 mg dose group) experienced hypertension. In Group A, AEs potentially related to systemic VEGF inhibition were observed in two patients in the 0.3 mg dose group (blood pressure increased and haematuria in one patient and hypertension in another patient).

Nonocular AEs suspected to be related to study drug were cerebral infarction, dementia and hypertension (one patient each) in 0.3 mg group, cerebral infarction and malaise (one patient each) in 0.5 mg dose group.

There were no deaths during the extension phase. Serious adverse events were reported for one of three (33.3%) patients in the 0.3 mg dose group and one of six (16.7%) patients in the 0.5 mg dose group in Group A, four patients (14.3%) in the 0.3 mg dose group and seven patients (21.2%) in the 0.5 mg dose group of Group B. Summary of ocular and nonocular SAEs is shown in Table 7. Of the SAEs, cerebral infarction (one patient each in the 0.3 and 0.5 mg dose groups of Group B) was suspected to be related to study drug and resolved with medical treatment in both patients. Four patients (two patients each from both dose groups) in Group B discontinued from the study because of SAEs. These SAEs that led to discontinuation were, however, not suspected to be study-drug related.

During the extension phase, immunoreactivity to ranibizumab (anti-ranibizumab antibodies) was not detected in patients of Group A; however, it was detected in two patients in the 0.3 mg dose group and one patient in the 0.5 mg dose group of Group B in the extension phase. In one patient in the 0.3 mg dose group, immunoreactivity to ranibizumab was detected at Month 12 (for the first time) and at study completion visit, but not at Month 24. In another patient in the 0.3 mg dose group, immunoreactivity to ranibizumab was detected at Month 24 (for the first time) and at study completion visit. In the 0.5 mg dose group, immunoreactivity to ranibizumab was detected in one patient at Month 12 (for the first time), Month 24 and at study completion visit. Of the three patients, AEs were reported in two patients. One patient in the 0.3 mg dose group experienced mild iritis as ocular AE and moderate glaucomatocyclitic crises as ocular SAE in the study eye as well as mild back injury and fall as nonocular AE. Iritis, back injury and fall were resolved without treatment and glaucomatocyclitic crises were resolved with medical treatment. One patient in the 0.5 mg dose group experienced both of conjunctival hyperaemia and intraocular pressure increased in the study eye, and both events were mild and resolved without treatment. All these events, except for intraocular pressure increased, were not suspected to be study-drug related.

Discussion

EXTEND-I was the first study with ranibizumab in Japanese patients with primary or recurrent subfoveal CNV secondary to AMD. The 6-month results indicated that monthly ranibizumab treatment significantly improved BCVA scores at Month 6 compared with baseline; the mean change (SD) observed was of +8.1 (12.65) letters and +9.0 (9.62) letters in BCVA score in the 0.3 and 0.5 mg dose groups, respectively. The improved BCVA scores at Month 6 were maintained until Month 12 by monthly treatment; the mean change (SD) observed was of +9.5 (12.79) letters and +10.5 (11.14) letters in BCVA score in the 0.3 and 0.5 mg dose groups, respectively. Monthly intravitreal injections of ranibizumab were shown to be safe and well tolerated over 12 months in Japanese patient population (Tano & Ohji 2010).

In the extension phase, the efficacy and safety of individualized flexible interval regimen (PRN regimen) of ranibizumab was assessed. In other words, the study consecutively investigated 12 monthly injections in the multiple-injection phase followed by the extension phase with PRN regimen guided by monthly BCVA score and by other ophthalmic examinations, such as slit-lamp examination, ophthalmoscopy, fundus photography,

Table 5. Best-corrected visual acuity (BCVA) of the study eye at the last visit in Group B (Enrolled patients).

BCVA	Ranibizumab 0.3 mg (N = 28)	Ranibizumab 0.5 mg (N = 33)
Loss of < 15 letters from baseline		
n (%)	24 (85.7)	32 (97.0)
95% CI of %*	67.3, 96.0	84.2, 99.9
Gain of ≥15 letters from baseline		
n (%)	9 (32.1)	9 (27.3)
95% CI of %*	15.9, 52.4	13.3, 45.5
Loss of ≥30 letters from baseline		
n (%)	1 (3.6)	1 (3.0)
95% CI of %*	0.09, 18.3	0.08, 15.8
Visual acuity < 34 letters		
n (%)	3 (10.7)	1 (3.0)
95% CI of %*	2.27, 28.2	0.08, 15.8
Approximate Snellen equivalent of 20/200 or worse		
n (%)	4 (14.3)	2 (6.1)
95% CI of %*	4.03, 32.7	0.74, 20.2
Approximate Snellen equivalent better than 20/200 but worse than 20/40		
n (%)	18 (64.3)	20 (60.6)
95% CI of %*	44.1, 81.4	42.1, 77.1
Approximate Snellen equivalent of 20/40 or better		
n (%)	6 (21.4)	11 (33.3)
95% CI of %*	8.30, 41.0	18.0, 51.8

* Derived from the exact confidence interval. Baseline value is defined as the last available measurement prior to the first injection in the multiple dose phase of the study; N = number of enrolled patients; n = number of patients.

fluorescein angiography and optical coherence tomography.

The estimated number of ranibizumab injections per year in the extension phase was approximately four injections in both the dose groups, which is equivalent to one-third of the maximally possible number of injections per year. The actual injection interval during the extension phase was not fixed and varied among patients and even in individual subject. Consequently, the PRN regimen with monthly monitoring resulted in considerably less frequent injections than a monthly regimen in this study. This seems to suggest that fixed monthly injection of ranibizumab is not necessary for all patients to maintain the improved VA gained through the initial monthly injections.

Results from the extension phase show a slight, but not significant, decrease in BCVA score when the regimen was switched from monthly injections to the PRN regimen. Thus, based on the mean change in BCVA scores in both the multiple-injection phase and the extension phase, the monthly regimen seems to be more effective in obtaining the best treatment outcome in VA than PRN regimen. However, continuous monthly injections are not feasible for many patients because of the physical and psychological burden and risk of AEs such as eye infections associated with the invasive intravitreal injection procedure.

Based on the results of the pivotal randomized Phase III studies, MARINA, ANCHOR and PIER, a drug and disease model with good agreement with study data was developed to simulate BCVA outcomes by individualized flexible VA-guided regimen following the initial three consecutive monthly injections of ranibizumab (Holz et al. 2010). Individualized flexible VA-guided regimen (administered if BCVA decreased by > 5 letters) is suggested to sustain initial BCVA gains following the initial three consecutive monthly injections of ranibizumab. According to the model prediction, it was recommended that patients should be monitored with monthly visits and further treatment should be considered if BCVA decreased by > 5 letters.

As discussed in the modelling and simulation study and as observed in the present study, slight decrease in BCVA was noted during the PRN regimen in the extension phase unlike the monthly treatment regimen. Because the concept of the PRN regimen is to treat in case of deterioration, especially a decrease in BCVA score, a corresponding decline in the BCVA curve over time is expected, i.e., the observed decline in BCVA during the extension phase is imminent to the PRN regimen concept.

As a guidance for retreatment during the PRN regimen, in this study, BCVA decrease by > 5 letters between two consecutive scheduled visits (including the current visit) was applied, so that the decision of retreatment at the current visit was made on the basis of changes calculated between BCVA scores of the last and current scheduled visit, taking the other ophthalmic conditions into account. On the other hand, in SAILOR and SUSTAIN, although the applied retreatment criterion of BCVA was the same as adopted in this study, the starting point of calculation was any previous visit wherein the BCVA score was the highest, especially in SUSTAIN the previous visit was limited to the first three months (Mitchell et al. 2010). Therefore, the decrease of BCVA score by > 5 letters was less likely to occur in this study than in both SAILOR and SUSTAIN. From this perspective, if the retreatment criterion based on the previous highest score is applied, it is speculated that both the number of injection and the BCVA score are apt to increase in comparison with the criterion based on the two consecutive scheduled visits. In both this study and SUSTAIN, monitoring of BCVA scores and other ophthalmic examinations was performed monthly in the same manner; the decline of the BCVA score in the 0.3 mg dose group from Month 12 in this study and from Month 3 in SUSTAIN was almost the same (decrease of 2–3 letters) on an average. Furthermore, in SUSTAIN, the number of retreatments in 9 months of maintenance phase with PRN regimen after three consecutive monthly injection was 2.7 on average, which translates into approximately four times per year. This estimated number of retreatments per year in the SUSTAIN study is roughly the same as the estimated number of injections per year in the extension phase with the PRN regimen of this study. Thus, the influence of the starting point to calculate the decrease in BCVA score for

Table 6. Summary of ocular and nonocular adverse events during the extension phase.

Preferred term	Group A: Ranibizumab, 0.3 mg $N = 3$	Group A: Ranibizumab, 0.5 mg $N = 6$	Group B: Ranibizumab, 0.3 mg $N = 28$	Group B: Ranibizumab 0.5 mg $N = 33$
Ocular				
Total, *n* (%)	2 (66.7)	3 (50.0)	20 (71.4)	18 (54.5)
Asthenopia	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)
Cataract	0 (0.0)	0 (0.0)	1 (3.6)	0 (0.0)
Conjunctival haemorrhage	1 (33.3)	3 (50.0)	12 (42.9)	11 (33.3)
Conjunctival hyperaemia	0 (0.0)	0 (0.0)	2 (7.1)	1 (3.0)
Conjunctivitis	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.0)
Conjunctivitis allergic	0 (0.0)	1 (16.7)	0 (0.0)	1 (3.0)
Dry eye	1 (33.3)	0 (0.0)	0 (0.0)	1 (3.0)
Eye pain	0 (0.0)	0 (0.0)	2 (7.1)	0 (0.0)
Glaucomatocyclitic crisis	0 (0.0)	0 (0.0)	1 (3.6)	0 (0.0)
Injection site discomfort	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.0)
Intraocular pressure increased*	0 (0.0)	0 (0.0)	2 (7.1)	4 (12.1)
Iritis	0 (0.0)	0 (0.0)	1 (3.6)	0 (0.0)
Maculopathy	0 (0.0)	0 (0.0)	1 (3.6)	0 (0.0)
Myodesopsia	0 (0.0)	1 (16.7)	0 (0.0)	2 (6.1)
Ocular hypertension	0 (0.0)	0 (0.0)	1 (3.6)	0 (0.0)
Punctate keratitis	0 (0.0)	0 (0.0)	1 (3.6)	1 (3.0)
Retinal detachment[#]	0 (0.0)	1 (16.7)	3 (10.7)	4 (12.1)
Retinal haemorrhage[†]	1 (33.3)	2 (33.3)	8 (28.6)	8 (24.2)
Retinal oedema	0 (0.0)	0 (0.0)	1 (3.6)	0 (0.0)
Visual acuity reduced	0 (0.0)	0 (0.0)	1 (3.6)	0 (0.0)
Vitreous haemorrhage	0 (0.0)	0 (0.0)	1 (3.6)	0 (0.0)
Nonocular (> 5% in any group)[‡]				
Total	2 (66.7)	2 (33.3)	19 (67.9)	24 (72.7)
Colonic polyp	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)
Cough	0 (0.0)	0 (0.0)	0 (0.0)	2 (6.1)
Dental caries	0 (0.0)	0 (0.0)	1 (3.6)	2 (6.1)
Diabetes mellitus	0 (0.0)	0 (0.0)	3 (10.7)	0 (0.0)
Fall	0 (0.0)	0 (0.0)	1 (3.6)	2 (6.1)
Gastroenteritis	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)
Hypertension	0 (0.0)	0 (0.0)	3 (10.7)	1 (3.0)
Nasopharyngitis	1 (33.3)	1 (16.7)	5 (17.9)	8 (24.2)

N = number of enrolled patients; n = number of patients.
* Five incidences in Group B (2 from 0.3 mg; 3 from 0.5 mg) are suspected to be study-drug related.
[#] Serous retinal detachment in all cases.
[†] One incident in 0.5 mg (Group B) is suspected to be study-drug related.
[‡] Full list provided in Table S1.

retreatment criterion on the stabilization of BCVA score may not be so large. Apart from the difference in the starting point for the PRN regimen, the duration of consecutive monthly injection before start of PRN regimen in this study and SUSTAIN was different, i.e., 12 and 3 months, respectively, and the duration of the extension phase in this study (about one and half year) and that of maintenance phase in SUSTAIN (9 months) were also different, so that it seems to be difficult to simply compare the mean change in BCVA score between these two studies. Although the starting point to calculate the decrease of BCVA for retreatment criterion still remains to be investigated, it can be argued that a more stringent retreatment criterion may lead to better results, taking into consideration the best treatment outcome obtained by monthly injection.

Recently, based on the evidence available from prospective, multicentre studies evaluating different ranibizumab treatment schedules (ANCHOR, MARINA, PIER, PrONTO, SUSTAIN and EXCITE), it was summarized that the treatment initiation with three consecutive monthly injections of ranibizumab, followed by continued monthly injections, has provided the best VA outcomes in pivotal clinical studies (Mitchell et al. 2010). Furthermore, Mitchell et al. (2010) recommended that if continued monthly injections are not feasible after initiation, a flexible regimen may be adopted with monthly monitoring of lesion activity. The results from the extension phase with PRN regimen in EXTEND-I study are consistent with these clinical recommendations on ranibizumab treatment.

Regarding safety, the comparison between the multiple-injection phase and the extension phase is difficult as there were substantial differences between these two phases with regard to the duration, the number of patients and the number of injections. Although the mean duration of observation in the extension phase was longer than 12 months (1.45 and 1.36 years in the 0.3 and 0.5 mg dose groups, respectively), the incidence rate of ocular AEs appears to be lower than those during the 12-month multiple-injection phase (Tano & Ohji 2010). As the incidence rate of conjunctival haemorrhage, conjunctival hyperaemia and eye pain in

Table 7. Serious adverse events (SAEs) observed during the extension phase.

	Group A Ranibizumab 0.3 mg $N = 3$	Group A Ranibizumab 0.5 mg $N = 6$	Group B Ranibizumab 0.3 mg $N = 28$	Group B Ranibizumab 0.5 mg $N = 33$
Total, *n* (%)	1 (33.3)	1 (16.7)	4 (14.3)	7 (21.2)
Ocular SAE of study eye	1 (33.3)	0 (0.0)	2 (7.1)	0 (0.0)
Glaucomatocylitic crisis	0 (0.0)	0 (0.0)	1 (3.6)*	0 (0.0)
Macular degeneration	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)
Retinal detachment	0 (0.0)	0 (0.0)	1 (3.6)†	0 (0.0)
Vitreous haemorrhage	0 (0.0)	0 (0.0)	1 (3.6)	0 (0.0)
Ocular SAE of fellow eye	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.0)
Visual acuity reduced	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.0)
Nonocular SAE	0 (0.0)	1 (16.7)	2 (7.1)	6 (18.2)
Abscess neck	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.0) *
Cerebral infarction	0 (0.0)	0 (0.0)	1 (3.6)*	1 (3.0)*
Colon cancer	0 (0.0)	0 (0.0)	1 (3.6)†	0 (0.0)
Colon polyp	0 (0.0)	1 (16.7)*	0 (0.0)	0 (0.0)
Depression	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.0)†
Emphysema	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.0)
Enterocele	0 (0.0)	0 (0.0)	1 (3.6)*	0 (0.0)
Gastric cancer	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.0)†
Gastric polyps	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.0)*
Small cell lung cancer stage unspecified	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.0)†
Spondylitic myelopathy	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.0)*
SAEs causing discontinuation from study drug/study	0 (0.0)	0 (0.0)	2 (7.1)	2 (6.1)
Ocular SAE of study eye	0 (0.0)	0 (0.0)	1 (3.6)	0 (0.0)
Ocular SAE of fellow eye	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Nonocular SAE	0 (0.0)	0 (0.0)	1 (3.6)	2 (6.1)

Both of gastric cancer and small cell lung cancer stage unspecified occurred in same patient of the 0.5 mg dose group.
N = number of enrolled patients; n = number of patients.
* SAE resolved by the last visit of the study.
† SAE led to discontinuation.

the study eye appears to be lower in the extension phase than those in the multiple-injection phase, these AEs are likely to be related to the intravitreal injection of ranibizumab and subconjunctival anaesthesia. Because the estimated number of ranibizumab injections per year was reduced by about one-third because of the PRN regimen in comparison with monthly regimen, there appears to be a relationship between the lower incidence of ocular AEs and reduction of number of injections. On the other hand, the incidence rate of nonocular AEs appears to be similar to those in the multiple-injection phase.

In conclusion, given the efficacy and safety profile observed in the extension phase, an individualized flexible interval regimen (PRN regimen) of ranibizumab, guided by monthly monitoring of BCVA score and other ophthalmic examinations, appears sufficiently effective and feasible in sustaining BCVA gained by consecutive monthly treatment and helps reducing the number of injections and treatment burden. Ranibizumab administered over the extension phase in Japanese patients with subfoveal CNV secondary to AMD was safe and well tolerated.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Number (%) of patients with nonocular adverse events by preferred term in Part B (Enrolled patients).

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Effect of Cataract in Evaluation of Macular Pigment Optical Density by Autofluorescence Spectrometry

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Purpose. To assess the effect of cataract on the evaluation of macular pigment optical density (MPOD) in aged patients.

Methods. MPOD was prospectively measured using autofluorescence spectrometry before and after cataract surgery. The Lens Opacities Classification System III was used to grade the cataracts at baseline.

Results. Forty-five eyes of 41 subjects, who had no ocular disorders or fundus autofluorescence abnormalities except for age-related nuclear cataract, were included. Preoperative MPOD was 0.350 ± 0.117 density unit (DU). Regression analysis showed that a higher nuclear color score correlated with lower MPOD ($t = -2.90$, $P = 0.0063$). The preoperative MPOD prediction formula was MPOD = 0.545 − 0.069 × nuclear color score. A higher nuclear color score correlated significantly with failure to measure the MPOD ($\chi^2 = 5.08$, $P = 0.0242$). The mean postoperative MPOD was 0.600 DU (95% confidence interval [CI], 0.562–0.637), which was significantly ($P < 0.0001$) higher than the preoperative level of 0.350 DU (95% CI, 0.313–0.388). Regression analysis showed that higher preoperative MPOD correlated with higher postoperative MPOD ($t = 2.91$, $P = 0.0061$).

Conclusions. Cataract, especially its nuclear component, affects MPOD measured by autofluorescence spectrometry. Care should be taken when using this method in eyes with age-related macular maculopathy and age-related macular degeneration and in older patients who may develop these diseases. (*Invest Ophthalmol Vis Sci.* 2011;52:927–932) DOI:10.1167/iovs.10-5664

Macular pigment comprises three carotenoids (lutein, zeaxanthin, and mesozeaxanthin)[1–5] and has light-absorbing properties in the 400- to 540-nm range, with maximum absorption at approximately 460 nm.[5–8] In addition, the macular pigment itself has an antioxidative effect.[5,9–13] Thus, macular pigment helps retard some destructive processes in the retina and the retinal pigment epithelium, which may lead to macular diseases such as age-related maculopathy (ARM) and age-related macular degeneration (AMD).

Some investigators have tried to measure the macular pigment to determine its relationship with the development of ARM or AMD.[14–17] Several clinical methods of measuring macular pigment optical density (MPOD) have been used, including heterochromatic flickering photometry (HFP), motion-detection photometry, fundus reflectance spectroscopy, Raman spectrometry, and autofluorescence spectrometry.[18] To date, factors such as sex, aging, race, smoking, and cataract are postulated to affect the density of the macular pigment[15,19–21]; however, the data are not consistent among the published reports.

Cataract absorbs blue light, which damages the retina and may cause development of AMD.[22,23] In addition to visual deterioration, the blue light–absorbing property of cataracts may affect the accuracy of the MPOD measurements. Autofluorescence spectrometry, which uses a two-wavelength method to measure MPOD, acquires fundus autofluorescence (FAF) images obtained at two wavelengths, 488 and 514 nm and then the subtraction of the logarithm of these images creates an MPOD map.[24–27]

Since 488-nm is within the range of blue light, cataracts may reduce the signals in a 488-nm autofluorescence image.[28–31] Thus, when we measure the MPOD in the eyes of elderly people, we should know how cataracts affect the results.

In the present study, we used autofluorescence spectrometry to measure the MPOD in eyes by using autofluorescence spectrometry before and after surgery to determine the effects of cataract.

Methods

Study Population

We conducted a prospective interventional study at Osaka University Hospital from November 2008 to December 2009. The institutional review board approved the study.

Consecutive patients were enrolled who had no ocular disorders, including FAF abnormalities, except for age-related nuclear cataract. Subjects taking supplements containing lutein, zeaxanthin, and/or beta carotene were excluded. The patients underwent standard phacoemulsification and implantation of a yellow-tinted intraocular lens (IOL) (AcrySof SN60WF; Alcon Laboratories, Fort Worth, TX). No complications or adverse events occurred during cataract surgery or the follow-up period. In accordance with the Declaration of Helsinki, all participants provided informed consent before MPOD was measured.

Measurement and Analysis of MPOD

We used modified angiography (Heidelberg Retina Angiograph [HRA], Heidelberg Engineering, Dossenheim, Germany) to measure MPOD in all eyes before and after cataract surgery. The principle of measurement of MPOD with autofluorescence spectrometry with the two-wavelength method has been published.[18,24–27,32] All measurements were performed by two masked orthoptists, who used the same testing device and protocol. Before the study, the reliability of the measurements between these two orthoptists was confirmed as reported previously.[33] MPOD was measured within 1 week before cataract surgery and within 2 weeks after cataract surgery.

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Before the measurement, sufficient pupil dilation was obtained by instillation of dilating drops containing 0.5% tropicamide and 2.5% phenylephrine. Subjects sat before a table and fixated with the fellow eye on an external light source. If the fellow eye did not have adequate visual acuity (VA) for fixation, the subjects were asked to look straight as much as possible. The modified angiograph was aligned with the subject's eye, and movies were taken with the 488- and 514-nm excitation wavelengths (scan size, 30°); computed mean autofluorescence images were obtained at each wavelength, and the two images were subtracted to calculate the MPOD (expressed as the density unit [DU]). The mean MPOD, averaged along the area of an annulus with a retinal eccentricity of 0.5° (1° circle at the fovea), was selected as the value representing the MPOD. We measured the MPOD two or three times in each eye during each visit and then selected the data with the best-qualified image.[25]

The signal intensities of each 488- and 514-nm autofluorescence image were displayed in gray scale, with levels from 0 to 255. Delori et al.[24] reported the following equation for calculating MPOD, in which K is a constant, and F_p and F_f are the gray level in the perifoveal area (6° circle) and the fovea (0.5° circle), respectively.

$$\text{MPOD} = K\{\log[F_p(488\text{ nm})/F_f(488\text{ nm})]\} - \log[F_p(514\text{ nm})/F_f(514\text{ nm})]$$

To record the components while calculating the MPOD, we manually measured the F_p and F_f in the 488- and 514-nm images before and after surgery in all patients and converted the values into a logarithmic scale referring to the above formula and described the results as the mean (95% CI). The distribution profiles of gray levels of 20 × 20 pixels at the peripheral retina were also obtained, and the skew of the distribution was estimated by calculating the following equation

$$\text{Skew} = [(GL_{5\%} + GL_{95\%})/2 - GL(\text{mode})]/GL(\text{mode})$$

where $GL_{5\%}$ and $GL_{95\%}$ are the 5th and 95th percentiles of the gray level (GL), and GL(mode) is the most frequently occurring gray level. The skew, which is considered to be one of the indexes of random noise of the autofluorescence image, was also described as the mean (95% CI).

Ophthalmic Examination

The clinical examinations included best corrected VA (BCVA) determined with Landolt C charts, intraocular pressure, slit lamp biomicroscopy, and ophthalmoscopy with lens and fundus photographs taken before and after cataract surgery. The slit lamp digital images were used to assess the type and the severity of the lens opacities. Two masked ophthalmologists (YS and FG) graded the nuclear opalescence, nuclear color, and cortical and posterior subcapsular cataracts in each eye based on the Lens Opacities Classification System III (LOCS III).[34]

The nuclear opalescence and color in the observed lens were classified into standards 1 through 6 and scored from 0.1 to 6.9 (ranging from clear or colorless to very opaque or brunescent) by each observer and the average scores were chosen. Eyes with a cortical cataract were graded based on the area of the opacity. We defined eyes with a grade of more than 3.0 (area with opacity within the pupillary area exceeding 20%) as having a cortical cataract. Similarly, eyes with a posterior subcapsular cataract were graded on the basis of the size of the opacity. We defined eyes with a grade of more than 1.0 (obvious opacity at the center of the posterior lens) as having a posterior subcapsular cataract.

Statistical Analysis

Since variations in an individual patient were nearly equivalent to those between patients, all eyes in which the MPOD was measured were included in the analysis, even if both eyes of a patient underwent cataract surgeries. The BCVA was converted to the logarithm of the minimum angle of resolution (logMAR) for the statistical analysis. In comparing the mean logMAR VA and MPOD before and after cataract surgery, the 95% CI was calculated, and a paired t-test was performed.

To estimate the difference in MPOD between subgroups, we analyzed the following parameters by t-test: sex, smoking history, and presence of a cortical cataract and posterior subcapsular cataract. The correlation between the MPOD and age, nuclear opalescence score, nuclear color score, and the logMAR VA, and the correlation between nuclear opalescence or color score and logMAR VA was assessed by calculating Pearson's correlation coefficient (r). Stepwise regression analysis using the Akaike information criterion was performed to determine the variables that affected MPOD before cataract surgery and the difference between MPOD before and after cataract surgery: sex, age, smoking, nuclear opalescence and color score, cortical cataract, posterior subcapsular cataract, and preoperative logMAR VA.[35] When stepwise regression was performed to determine the variables that affected MPOD after cataract surgery, we included sex, age, smoking, postoperative logMAR VA, and preoperative MPOD as explanatory variables. Stepwise regression analysis was also conducted, to determine the variables that correlate with failure to obtain reliable MPOD data.

To estimate the difference in gray levels of the perifoveal area (F_p) and the fovea (F_f) before and after cataract surgery, we performed a paired t-test. The difference in F_p/F_f before and after surgery at 488 and 514 nm was also estimated by paired t-test. Pearson's correlation coefficient (r) was used to determine the correlation between the nuclear color score and the skew index. $P < 0.05$ was considered significant (JMP software version 8.0; SAS Institute Inc., Cary, NC).

Results

Baseline Characteristics and MPOD

A total of 45 eyes of 41 subjects (16 men, 18 eyes; 25 women, 27 eyes) were included. The baseline characteristics of all eyes are shown in Table 1. The mean age ± SD was 71.6 ± 6.7 years. Sixteen patients (18 eyes) were smokers, and 25 (27 eyes) were nonsmokers. The mean ± SD nuclear opalescence and color scores were 2.9 ± 0.9 and 3.0 ± 0.9, respectively, and they correlated very strongly with each other ($r = 0.97$, $P < 0.0001$). The logMAR BCVA (mean ± SD) was 0.39 ± 0.29. The mean preoperative MPOD measured by autofluorescence spectrometry was 0.350 ± 0.117 DU. We could not obtain reliable data (200/225 pixels) in five eyes and excluded them from the preoperative evaluation.

MPOD before cataract surgery is summarized in Table 2. It was significantly reduced, in parallel with the increases in the

Table 1. Baseline Characteristics

Parameter	
Subjects/eyes, *n*	41/45
Sex	
Men/women, *n* subjects	16/25
Men/women, *n* eyes	18/27
Age, mean y ± SD	71.6 ± 6.7
Smokers, *n*	
Yes/no, *n* subjects	16/25
Yes/no, *n* eyes	18/27
Nuclear scores by LOCS III, mean ± SD	
Opalescence	2.9 ± 0.9
Color	3.0 ± 0.9
Cortical cataract, yes/no, *n* eyes	20/25
Posterior subcapsular cataract, yes/no, *n* eyes	13/32
LogMAR VA, mean ± SD	0.394 ± 0.291
Preoperative MPOD measured by autofluorescence spectrometry, DU	0.350 ± 0.117
Subjects with unreliable data, *n*	5

TABLE 2. The MPOD Level before Cataract Surgery

Parameter	MPOD (95% CI) (DU)		P
Men/women	0.316 (0.241-0.391) (n = 16)	0.373 (0.333-0.413) (n = 24)	0.1345
Smoking, yes/no	0.322 (0.261-0.383) (n = 15)	0.367 (0.320-0.414) (n = 25)	0.2408
Cortical cataract, yes/no	0.356 (0.296-0.416) (n = 18)	0.345 (0.294-0.397) (n = 22)	0.7781
Posterior subcapsular cataract, yes/no	0.326 (0.219-0.432) (n = 9)	0.357 (0.317-0.398) (n = 31)	0.4782

	Coefficient Correlation (r)	P
Age (n = 40)	0.05	0.7570
Nuclear opalescence score by LOCS III (n = 40)	−0.37	0.0177
Nuclear color score by LOCS III (n = 40)	−0.39	0.0142
Preoperative logMAR VA (n = 40)	−0.42	0.0077

nuclear opalescence and color scores and the preoperative logMAR VA (r = −0.37, P = 0.0177; r = −0.39, P = 0.0142; and r = −0.42, P = 0.0077, respectively), although a strong correlation was found between the nuclear opalescence and color scores and the preoperative logMAR VA (r = 0.71, P < 0.0001; r = 0.70, P < 0.0001, respectively). Because these factors were considered to have some correlation with each other, we performed a stepwise regression analysis to ascertain factors that mainly affected MPOD. The analysis showed that a higher nuclear color score was the factor that correlated significantly with lower preoperative MPOD (t = −2.90, P = 0.0063) among variables that included sex, age, smoking, nuclear opalescence and color scores, cortical cataract, posterior subcapsular cataract, and preoperative logMAR VA. The analysis also showed a tendency for MPOD to be higher in the women than in the men (t = 2.01, P = 0.0515). The MPOD prediction formula was MPOD = 0.545 − 0.069 × (nuclear color score). If the nuclear color score increased 1 point, MPOD decreased 0.069 DU.

Stepwise regression analysis also was conducted to determine the factors that were correlated with failure to measure MPOD. A higher nuclear color score correlated significantly with failure to measure the MPOD (χ^2 = 5.08, P = 0.0242). A posterior subcapsular cataract also tended to correlated with failure to measure the MPOD (χ^2 = 3.25, P = 0.0713).

Postoperative MPOD

The mean postoperative logMAR VA was −0.002 (95% CI, −0.038 to 0.034), and it improved significantly compared with the preoperative logMAR VA of 0.394 (95% CI, 0.306-0.481; P < 0.0001).

We could obtain MPOD data in all eyes after cataract surgery. A representative case was shown in Figure 1. The mean postoperative MPOD was 0.600 ± 0.124 DU (95% CI, 0.562-0.637). The postoperative MPOD was significantly higher than the preoperative level of 0.350 ± 0.117 DU (95% CI, 0.313-0.388; P < 0.0001). Postoperative MPOD correlated positively with the preoperative level (r = 0.43, P = 0.0058; Fig. 2).

MPOD data after cataract surgery are summarized in Table 3. The MPOD tended to be higher in the women (0.626 [95% CI, 0.582-0.670]) than in the men (0.559 [95%

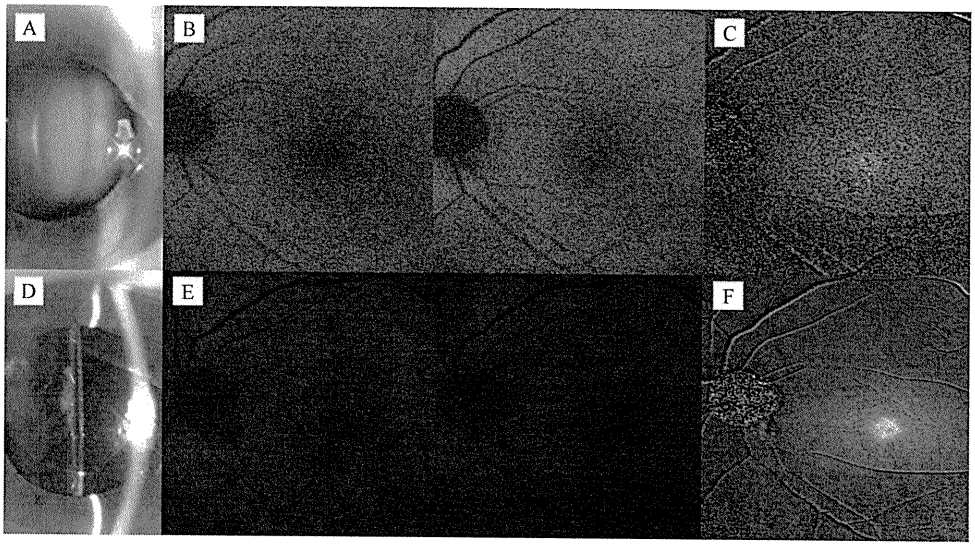

FIGURE 1. Pre- and postoperative images from an eye of a 71-year-old woman. (**A**) Preoperative color of the cataract nuclear was scored as 4.2 and the nuclear opalescence score as 4.0. (**B**) Fundus autofluorescence images captured at the 488-nm (*left*) and 514-nm (*right*) wavelengths before surgery. The sensitivity of the detector was set relatively high, and random noise was seen in both images. The macular pigment blocked more signals from the macula in the 488-nm image. (**C**) The subtraction of the logarithm of these images creates an MPOD map. Preoperative MPOD was 0.29 DU. (**D**) A yellow-tinted IOL implanted during the surgery. (**E**) Postoperative fundus autofluorescence images captured at 488-nm (*left*) and 514-nm (*right*) wavelengths. The proper setting of the sensitivity of the detector led to less random noise. (**F**) Postoperative MPOD improved to 0.66 DU.

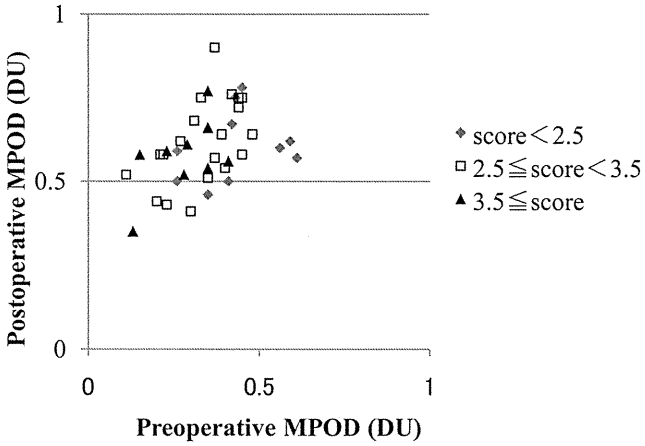

FIGURE 2. Preoperative and postoperative MPODs by the nuclear color score of the lens (n = 40). The shape of the data points represents the nuclear color scores shown at *right*. The postoperative MPOD correlated positively with the preoperative level (r = 0.43, P = 0.0058).

CI, 0.493-0.626]; P = 0.0758). In addition, it tended to be in proportion to age (r = 0.29, P = 0.0547). The postoperative level correlated positively with the preoperative one (r = 0.43, P = 0.0058). We conducted a stepwise regression analysis in which the model included sex, age, smoking, postoperative logMAR acuity, and preoperative MPOD as explanatory variables and postoperative MPOD as a response. The analysis showed that higher preoperative MPOD correlated with higher postoperative MPOD (t = 2.91, P = 0.0061).

Difference in MPOD before and after Surgery

Simple regression analysis revealed that eyes with lower preoperative MPOD and a higher nuclear color score correlated with the larger difference in MPOD before and after cataract surgery (r = −0.54, P = 0.0001 and r = 0.36, P = 0.0157, respectively). After conducting a stepwise regression analysis, we found lower preoperative MPOD and no presence of posterior subcapsular cataract correlated with a larger difference in MPOD (t = −4.51, P < 0.0001; t = −2.18, P = 0.0355, respectively).

Gray Levels and Skew before and after Surgery

Gray level at the perifoveal region (F_p) and the fovea (F_f) and their ratio (F_p/F_f) before and after cataract surgery presented in logarithmic scale are summarized in Table 4. F_p before and after surgery did not change significantly at both 488 and 514 nm (P = 0.4167, P = 0.6848, respectively). F_f at 514 nm also did not show a significant change (P = 0.3808), but F_f at 488 nm significantly decreased after surgery (P = 0.0136). In addition, the increase in F_p/F_f after surgery at 488 nm was significantly higher than that at 514 nm: 0.19 (95% CI, 0.16-0.22) and 0.07 (95% CI, 0.05-0.09), respectively (P < 0.0001).

The skew at the peripheral retina is also shown in Table 4. It was significantly higher before surgery than after in both the 488- and the 514-nm images (P = 0.0001 and P < 0.0001, respectively). Statistical analysis showed that the higher skew index at 488 nm correlated moderately with higher nuclear color score (r = 0.43, P = 0.0028), but skew at 514 nm did not correlate with the nuclear color score (r = −0.02, P = 0.8995).

Discussion

We used autofluorescence spectrometry to measure MPOD in eyes with age-related cataract, before and after cataract surgery, and observed the significant increase in MPOD after cataract surgery. Mean MPOD was 0.350 DU (95% CI, 0.313-0.388) before surgery and 0.600 DU (95% CI, 0.562-0.637) after surgery. Considering the result from the previous report that the intensity of macular pigment is stable without intervention,[36] the increased MPOD observed after cataract surgery must be artifactual. Therefore, we examined the factors that correlated with MPOD.

MPOD significantly increased after the cataractous lenses were removed and replaced with yellow IOLs, the color of which was similar to the lens color in subjects in the third decade of life. Preoperative MPOD tended to be lower when the nuclear opalescence and color scores were high, although there was no correlation between preoperative MPOD and cortical cataracts or posterior subcapsular cataracts. As expected, the nuclear opalescence score and the nuclear color score correlated very strongly (r = 0.97, P < 0.0001), but stepwise regression analysis showed that the nuclear color score was the most significant factor that affected preoperative MPOD (t = −2.90, P = 0.0063). In addition, we found in a simple regression analysis that lower preoperative MPOD and a higher nuclear color score correlated with a larger difference in MPOD. From these results, we concluded that the lower MPOD in the eyes with cataract was, to some degree, due to the yellower lenses.

As a reason for MPOD reduction by the presence of cataract, first, we hypothesized that the lens yellowing might reduce the autofluorescence level in 488-nm (blue) wavelength directly because yellow absorbs blue. However, as the lens yellowing reduced the autofluorescence at the perifoveal area and the fovea equally, it seemed not to affect the final MPOD in the equation used to calculate it.[24] Then, we examined gray levels in all images and also the skew condition. Finally, we reached the following conclusions: Excitation and emission signals are scattered by cataractous lenses, and excitation signals are partially absorbed by yellow lenses. Hence, to obtain an image bright enough to evaluate MPOD, we had to make the

TABLE 3. The MPOD Level after Cataract Surgery

	MPOD (95% CI) (DU)		P
Men/women	0.559 (0.493-0.626) (n = 18)	0.626 (0.582-0.670) (n = 27)	0.0758
Smoking, yes/no	0.573 (0.507-0.638) (n = 18)	0.617 (0.571-0.664) (n = 27)	0.2407
	Coefficient Correlation (r)		**P**
Age (n = 45)	0.29		0.0547
Postoperative logMAR VA (n = 45)	0.18		0.2313
Preoperative MPOD (n = 40)	0.43		0.0058

TABLE 4. Grey Levels before and after Cataract Surgery

	F_p	F_f	F_p/F_f	Skew (95% CI)
Before surgery				
488-nm	1.33 (1.24-1.42)	0.96 (0.86-1.07)	0.37 (0.33-0.40)	0.28 (0.19-0.36)
514-nm	1.43 (1.34-1.52)	1.22 (1.12-1.31)	0.21 (0.19-0.23)	0.26 (0.18-0.33)
After surgery				
488-nm	1.39 (1.29-1.50)	0.84 (0.72-0.96)	0.56 (0.53-0.59)	0.10 (0.06-0.14)
514-nm	1.48 (1.37-1.58)	1.19 (1.0-1.31)	0.28 (0.26-0.30)	0.10 (0.07-0.14)

Data are expressed as the gray level (95% CI).

sensitivity of the detector high in the cataractous eyes, and this adjustment resulted in higher reflectivity, as seen in Figure 1. As a result, F_p before surgery was at almost the same level after surgery. However, the skew index, which substitutes for random noise, was higher before surgery than after surgery, because high sensitivity induced high levels. This result made the F_f falsely high compared with the F_p before surgery, especially at 488 nm, because foveal signals in 488 nm are small due to the macular pigment and are easily affected by the random noise. Consequently, the increase in F_p/F_f after surgery at 488 nm was significantly higher than that at 514 nm and led to the decrease in MPOD before surgery. The statistical results, which showed a positive correlation between the skew and nuclear color score in the 488-nm wavelength images but not in the 514-nm wavelength images, may indicate that lens yellowing increases the random noise of the autofluorescence image, especially at 488 nm and, as a result, decrease the MPOD. Of course, we might consider other effects such as autofluorescence of the lens. However, as mentioned by Delori et al.,[24] its effect on the MPOD measurement should be very small.

Despite no other ocular diseases apart from cataracts, we could not obtain reliable MPOD measurements in five (11%) eyes by autofluorescence spectrometry. Such a limitation in measuring MPOD in cataractous eyes is a weak point of this method. In eyes with a higher nuclear color score or a posterior subcapsular cataract, there was a tendency toward failure to measure the MPOD. This problem may have occurred because the yellowing of the lens and the dense opacity of the posterior capsule blocked the signals from the fundus, and we could not obtain sufficient effective pixels (200/225 pixels). Also, we found that the absence of posterior subcapsular cataracts correlated with the larger difference in MPOD. The effect of posterior subcapsular cataracts on MPOD remains to be determined.

During cataract surgery, a yellow IOL, the color of which was similar to the lens color in subjects in the third decade of life, was implanted in all patients, and postoperative MPOD increased significantly compared with the preoperative level, although they correlated significantly. The postoperative MPOD may be considered to be close to the true level. Of interest, our study showed that postoperative MPOD in the women tended to be higher than that in the men. The higher MPOD in older women may be one reason that explains the lower rate of progressing AMD in Japanese women, although Caucasian women have more risk of the disease.[37,38] Increasing age is the other factor that tended to be associated with a higher postoperative MPOD, although the age range of this study population was relatively narrow. Elderly people who do not develop AMD or other fundus diseases may have higher MPOD. Because it remains controversial whether MPOD differs by sex and age,[17,21,39,40] further studies are needed.

Our findings do not replicate the results of Ciulla et al.[41] and Nolan et al.,[42] who measured MPOD before and after cataract surgery using HFP. They reported no significant differences between the two time points just before and after cataract surgery. Differences between the methods may chiefly explain this discrepancy, although there may be population-based differences contributed by race and the LOCS grade of the cataracts.

In conclusion, our data suggest that cataracts affect the measurement of MPOD by autofluorescence spectrometry, probably as a result of the high setting of the detector's sensitivity. Nuclear cataract seemed to associate with this high-sensitivity setting, producing a random noise especially in 488-nm wavelength images. Care should be taken when evaluating MPOD using this method in eyes with age-related macular maculopathy and macular degeneration and in patients who are old enough to develop these diseases. More quantitative and higher quality methods of averaging the reflectivity may yield a normalized autofluorescence image in which the effect of cataract is excluded and may allow more precise evaluation of MPOD.

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in the tear fluid did not appear to be correlated with those in the serum.

Comments

We found that the tear fluid of healthy adults contained both sCD14 and LBP. Although the concentrations of these proteins in tear fluid were substantially lower than those in serum from the same individuals, our previous in vitro experiments indicate that the amounts of sCD14 and LBP detected in tear fluid are sufficient to exert maximal effects on chemokine and adhesion molecule expression by corneal fibroblasts in the presence of LPS. The presence of these proteins in tear fluid may thus enhance the perception of LPS by corneal fibroblasts in vivo. Both sCD14 and LBP were previously detected in human reflex tears stimulated by onion vapor.[5] The concentrations of sCD14 in reflex tears were similar to those determined in the present study for basal tears, but the concentrations of LBP were lower in reflex tears than in basal tears. As the lacrimal gland contains more CD14 than LBP,[5] the difference in the LBP concentration between basal and reflex tears might suggest that LBP has additional sources such as the corneal epithelium. Our results highlight the importance of tear fluid in the defense of the cornea against microorganisms. Given that the levels of sCD14 and LBP in tear fluid may be affected by inflammation and are potential novel diagnostic markers for certain diseases, the concentrations of these proteins in tear fluid from individuals with various ocular surface disorders, including allergy, infection, and dry eye, warrant further investigation.

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Vascular Endothelial Growth Factor Concentrations in Aqueous Humor Before and After Subconjunctival Injection of Bevacizumab for Neovascular Glaucoma

Neovascular glaucoma (NVG) is a severe complication of proliferative diabetic retinopathy (PDR), central retinal vein occlusion, and other ischemic retinal vascular diseases. Vascular endothelial growth factor (VEGF) is an important component of neovascular formation.[1] Recently, the treatment of NVG with intravitreal injections of anti-VEGF antibody has been reported to cause regression of iris neovascularization[2] and reduction of VEGF concentrations in the aqueous humor.[3] At the same time, complications due to the intravitreal method of administration have also been reported.[4] We herein report subconjunctival injection of bevacizumab (Avastin; Roche, Basel, Switzerland) as an alternative, safer method of administering NVG and describe VEGF concentrations in the aqueous humor both pre- and posttreatment.

Method and Results

The present study was approved by the Institutional Review Board of the Kagawa University Hospital, Kagawa, Japan. Signed informed consent was obtained from all patients.

Subconjunctival injection of 1.25 mg (0.05 ml) bevacizumab was performed in seven eyes of seven patients with NVG secondary to PDR. The bevacizumab was injected as preoperative adjunctive therapy before trabeculectomy in all eyes. Aqueous humor samples were obtained just before the subconjunctival injection of bevacizumab and during trabeculectomy, performed 4–7 days after the injection. Free VEGF concentrations in the aqueous humor were measured with an enzyme-linked immunosorbent assay. The aqueous humor was also sampled during cataract operations from three eyes of three patients with no other ocular diseases, as a control group.

Patient characteristics and the main outcomes are summarized in Table 1. The limit of detection for VEGF was

Table 1. Patient characteristics and clinical data before and after subconjunctival injection of bevacizumab

Case no.	Age (years)	Sex	Previous surgical procedure	IOP before injection (mmHg)	VEGF concentration in aqueous humor (pg/ml)	
					Before injection	After injection (days)
1	59	Male	PRP, PEA+IOL+VIT	22	<31	<31 (7)
2	67	Male	PRP	22	93.1	<31 (5)
3	59	Female	PRP, PEA+IOL+VIT	36	478	127 (4)
4	61	Female	PRP[a]	41	4980	1178 (5)
5	50	Male	PRP	62	6650	868 (6)
6	72	Male	PRP, PEA+IOL	45	11200	1040 (5)
7	63	Female	PRP[a]	76	26400	7530 (4)

IOP, intraocular pressure; VEGF, vascular endothelial growth factor; PRP, panretinal photocoagulation; PEA, phacoemulsification aspiration; IOL, intraocular lens implantation; VIT, vitrectomy.
[a]After bevacizumab injection.

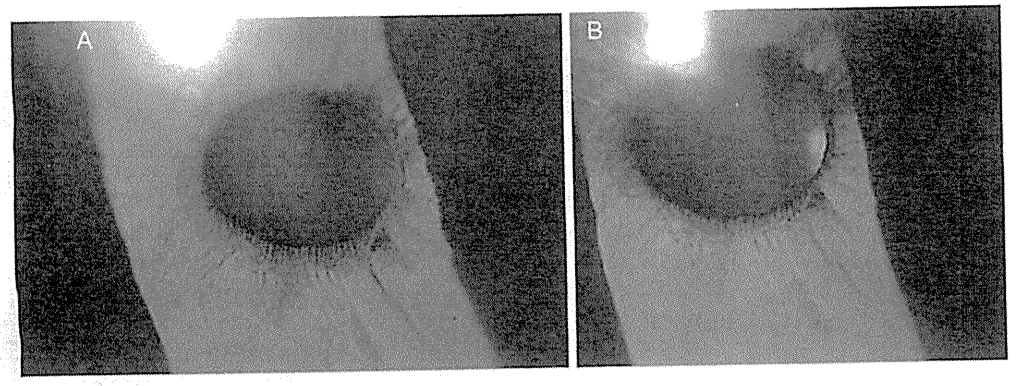

Figure 1A, B. Case 5. See also Table 1. **A** Iris neovascularization prior to subconjunctival injection. Marked neovascularization is seen between the 2 and 8 o'clock positions from the pupillary zone to the parenchyma of the iris. **B** Iris neovascularization following subconjunctival injection. The neovascularization that was seen prior to the injection has regressed.

31 pg/ml. VEGF concentrations in aqueous humor ranged from <31 pg/ml to 26 400 pg/ml before the subconjunctival injection of bevacizumab, and decreased to <31–7530 pg/ml after bevacizumab administration. Concentrations in the control group ranged from <31 pg/ml to 131 pg/ml. Regression of iris neovascularization decreased, except in two eyes, following subconjunctival injection of bevacizumab (Fig. 1). In two cases treated with PRP after a bevacizumab injection (cases 4 and 7; Table 1), the subconjunctival injection did not result in the desired regression of iris neovascularization and an additional intravitreal injection was performed.

In all eyes, bleeding during trabeculectomy was minimal and postoperative hyphema following trabeculectomy was not observed. Systemic or local complications were not observed in any of the cases.

Comment

In the present study, treatment of NVG with subconjunctival injection of bevacizumab resulted in a decreased VEGF concentration in all eyes. We also confirmed that regression of iris neovascularization could be accomplished with this treatment. However, the efficacy of subconjunctival injection was limited in patients in whom high intraocular VEGF concentrations indicated highly active NVG. VEGF concentrations in the aqueous humor of patients not receiving panretinal photocoagulation treatment may be high. Given the findings of Sawada et al.[3] regarding the efficacy of intravitreal injection of bevacizumab for the treatment of PDR, intravitreal injection appears to reduce VEGF concentrations in the aqueous humor more effectively than subconjunctival injection. In NVG cases with relatively low VEGF concentrations, however, the possibility of an overdose of bevacizumab when it is injected into the vitreous cavity cannot be ruled out.

Furthermore, although the incidence is small, severe complications from the intravitreal injection of bevacizumab, such as endophthalmitis or lens injury, have been reported.[4]

Nomoto et al.[5] reported that a minimal amount of bevacizumab could be detected in the iris and ciliary body in rabbits after subconjunctival injection of bevacizumab. Thus, this treatment is likely to result in suppression of NVG. How to select patients for whom subconjunctival injection of bevacizumab will prove helpful is a problem that requires further investigation.

Keywords: bevacizumab, neovascular glaucoma, subconjunctival injection, vascular endothelial growth factor

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Conjunctival Nevus-like Lesions Originating from a Sclerotomy Site After 23-Gauge Transconjunctival Sutureless Vitrectomy

Since transconjunctival sutureless vitrectomy (TSV) was first developed by Fujii et al.,[1] 25-gauge and 23-gauge TSV have been widely used to treat various vitreoretinal diseases because of its advantages of a shorter surgical time and faster recovery. However, questions have arisen regarding the self-sealing characteristics in some cases of postoperative hypotony.[2] We report bilateral nevus-like lesions originating from sclerotomy sites after bilateral 23-gauge TSV.

Case Report

A 51-year-old woman with diabetes mellitus presented with sudden decreased bilateral visual acuity. Best-corrected visual acuity was hand motion OD and 20/400 OS. Biomicroscopic examination results of the anterior segment was normal except for bilateral cataracts. Fundus examination showed vitreous hemorrhage OU. Thus, combined phacoemulsification and 23-gauge TSV (DORC, Zuidland, Holland) were carried out in each eye, 1 week apart. Vitreous base dissection was done under scleral depression. As sclerotomy site leakage was found during the surgery in both eyes, releasable suturing with 8-0 polyamide (Monosof, Syneture, Covidien, Mansfield, MA, USA) was performed at all sclerotomy sites.[3] All of the sutures were released on slit-lamp the day after each surgery. Each wound was evaluated for leakage by a Seidel test at the postoperative follow-up. Intraocular pressure was maintained at 10 mmHg or more, and there were no postoperative complications such as sclerotomy leakage or choroidal detachment.

At 1 month after surgery, conjunctival nevus-like lesions were found in all of the sclerotomy sites in both eyes (Fig. 1A, B).

Three months after surgery, an excisional biopsy was performed under topical anesthesia to resolve cosmetic problems and to rule out melanoma. Conjunctival excision, including of the underlying Tenon's capsule, was carried out in the inferotemporal quadrant of the right eye. As much pigment as possible was removed from the episcleral bed. Then, the amniotic membrane was laid on the episcleral bed with the basement membrane side down and sutured with 10-0 nylon interrupted sutures.

Light microscopic examination of the excised tissue showed nonkeratinizing squamous epithelium and substantia propria of the conjunctiva (Fig. 2A). Infiltration of abundant dark brownish melanophages, macrophages that have ingested melanin pigments in the substantia propria, was observed at higher magnification. Scattered, finely granular, melanin pigmentation surrounding the melanophages was also noted (Fig. 2B). Neither nevus cells nor melanoma cells were observed in the sample.

Six weeks after the conjunctival excision, a pigmented lesion remained in the right inferotemporal site.

Comments

We speculate that melanin was discharged from the sclerotomy sites. First, there was no preexisting conjunctival nevus-like lesion, and the lesion occurred exactly at the three sclerotomy sites in both eyes postoperatively. Second, the light microscopic examination revealed neither nevus cells nor melanoma cells. Smiddy et al.[4] reported similar findings after 20-gauge vitrectomy, but they presumed that the source of melanin was ruptured uveal cells caused by extensive cryotherapy, different from the present case. Vitreous base dissection is one risk factor for sclerotomy leakage requiring suture placement after 23-gauge TSV.[5] The releasable suture technique was developed to prevent incompetent wound closure during the operation and has proved effective in preventing postoperative hypotony.[3] However, it cannot prevent undetectable leakage from sclerotomy sites, in particular, the dispersion of melanin pigments in our case. To our knowledge, this complication has not been reported before and we add this case to possible complications of 23-gauge TSV in patients at risk for vitreous base dissection. Preoperative warning of the possibility of such a complication and meticulous postoperative ante-

Neuroprotection against Retinal Ischemia–Reperfusion Injury by Blocking the Angiotensin II Type 1 Receptor

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Purpose. To investigate the effects of an angiotensin-converting enzyme (ACE) inhibitor and an angiotensin II antagonist against retinal ischemia–reperfusion injury in the rat retina.

Methods. Retinal ischemia was induced by increasing intraocular pressure to 130 mm Hg. Rats were treated with an ACE inhibitor (captopril), an angiotensin II type 1 receptor (AT1-R) antagonist (candesartan), an AT2-R antagonist (PD123319), bradykinin, or a bradykinin B2 receptor antagonist (icatibant). At 7 days after the ischemia, retinal damage was evaluated. Immunohistochemistry and image analysis were used to measure changes in the levels of reactive oxygen species (ROS) and the localization of AT1-R. Dark-adapted full-field electroretinography (ERG) was also performed.

Results. Pretreatment with captopril or candesartan significantly inhibited the ischemic injury of the inner retina. However, PD123319, bradykinin, or icatibant did not reduce the ischemic damage. In control retinas, retinal vessels were positive for AT1-R. In contrast, 12 hours after ischemia, immunohistochemical analysis detected numerous AT1-R–positive cells in the inner retina in vehicle-treated rats. After ischemia, the production of ROS was detected in retinal cells. However, pretreatment with captopril or candesartan suppressed the production of ROS. On the seventh postoperative day, the amplitudes of the ERG b-waves were significantly lower in the vehicle group than in the groups pretreated with captopril or candesartan.

Conclusions. The present findings demonstrate that ischemic damage promotes the expression of AT1-R in the inner retina. Both the ACE inhibitor and the AT1-R antagonist that were examined can block the stimulation of the AT1-R and attenuate the subsequent ischemic damage in the rat retina. (*Invest Ophthalmol Vis Sci.* 2010;51:3629–3638) DOI:10.1167/iovs.09-4107

The renin-angiotensin system is widely known as a major controller of systemic blood pressure. In this system, angiotensin II (Ang II) plays an essential role in regulating vasomotor tone and ion transport and thus can cause elevation of blood pressure. There are two Ang II receptor subtypes: Ang II type 1 receptor (AT1-R) and Ang II type 2 receptor (AT2-R).[1–4] Because major Ang II-related systemic functions are mediated by AT1-R signaling, its antagonist action is widely used for the treatment of hypertension and cardiac diseases. Chronic treatments that make use of angiotensin-converting enzyme (ACE) inhibitors or AT1-R antagonists have been reported to reduce stroke incidence and extend the lifespan in stroke-prone spontaneously hypertensive rats (SP-SHRs)[5,6] and to protect against cerebral ischemic damage in SHRs.[7,8] In the rat model of endotoxin-induced uveitis, Ang II has been shown to be a promoter of choroidal neovascularization (CNV) and retinal inflammation.[9–11] Ang II activates the NADPH-dependent oxidase complex, which is a major source of superoxide (O_2^-) and is upregulated in several pathologic conditions associated with oxidative stress.[12,13] Liu et al.[14] recently reported that administration of the AT1-R antagonist leads to a protective effect against cerebral ischemia. Moreover, recent evidence suggests that AT2-R may antagonize the action of AT1-R.[1] AT2-R is expressed in areas related to learning and control of motor activity and is found in fetal tissue. However, it is also present at low levels in adult tissue and is reexpressed in certain pathologic conditions, such as neuronal injury[15,16] and vascular injury,[17] suggesting that activation of AT2-R may play a pivotal role in the repair and regeneration of injured tissue. In addition, research is now suggesting possible therapeutic applications for AT2-R, particularly with respect to its protective effects against cerebral ischemia–induced neuronal death.[18–20]

The kallikrein system (KKS) has been implicated in various pathologic and physiological processes, including inflammation, allergies, blood coagulation, fibrinolysis, and the lowering of systemic blood pressure caused by vessel dilation and diuretic action.[21,22] Bradykinin, the central molecule of the KKS, is generated by kallikrein from kininogen, including vessel dilation and leakage.[23] There are at least two types of bradykinin receptors, B1 (B1-R) and B2 (B2-R).[24] B2-R has high affinity for the intact kinin whereas B1-R has greater affinity for the kinin metabolite but weak affinity for the intact kinin.[24,25] Most of the physiological functions of kinin are mediated by B2-R.[26]

Ischemia-induced injury to the retina, such as diabetic retinopathy and retinal vein occlusion, causes severe and long-lasting visual loss. These morbidities are hard to treat, and research is ongoing regarding possible therapeutic interventions.[27–31] In addition, many mechanisms of tissue injury-induced ischemia have been proposed.[32–35] Reactive oxygen species (ROS) trigger ischemic cell damage and lead to the hypersecretion of glutamate and aspartate.[34] An excess amount of glutamate produced under conditions of ischemia-reperfusion stimulates *N*-methyl-D-aspartate (NMDA), a subtype of the glutamate receptor,[35] and induces an influx of excess Ca^{2+} into the cell.[32,33] The purpose of the present study was to investigate the effects of the ACE inhibitor and an Ang II antagonist on neuronal death in retinal ischemia.

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Materials and Methods

Animals

Female Sprague–Dawley rats, each weighing 200 to 250 g, were obtained from Charles River Japan (Yokohama, Japan). Female rats were used because preliminary results indicated no differences between male and female rats (data not shown). Rats were permitted free access to standard rat food (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Animal care and all experiments were conducted in accordance with the approved standard guidelines for animal experimentation of the Kagawa University Faculty of Medicine and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Drugs

B2-R antagonist icatibant, AT2-R antagonist (PD123319), and bradykinin were obtained from Sigma-Aldrich (St. Louis, MO); the ACE inhibitor captopril was obtained from Wako (Osaka, Japan); and AT1-R antagonist candesartan was obtained from TRC (North York, Canada). All drugs except candesartan were dissolved in water; candesartan was dissolved in dimethyl sulfoxide (DMSO) to produce stock solutions that were then diluted to the final required concentrations. The final DMSO concentration never exceeded 5%. Drugs were administered intraperitoneally 30 minutes before the induction of ischemia with the exception of PD123319 (5 mg/kg/d), which was administered by subcutaneous osmotic minipump (Alzet model 1007D; Alza Corporation, Mountain View, CA) 1 day before ischemia. The minipumps were implanted subcutaneously into the midscapular region. As the control, animals were pretreated with intraperitoneal injections of vehicle (distilled water or 5% DMSO in PBS) 30 minutes before ischemia.

Ischemia

Rats were anesthetized with 50 mg/kg pentobarbital sodium (Abbott, Abbott Park, IL) injected intraperitoneally and 0.4% oxybuprocaine hydrochloride administered topically. The anterior chamber of the right eye was cannulated with a 27-gauge infusion needle connected to a reservoir containing normal saline. Intraocular pressure (IOP) was raised to 130 mm Hg for 45 minutes by elevating the saline reservoir. Only the right eye of each rat was subjected to ischemia. Retinal ischemia was indicated by whitening of the iris and fundus. The left eye of each rat served as the nonischemic control. Given that body temperature may influence ischemia-induced retinal ganglion cell death,[36] rectal and tympanic temperatures were maintained at approximately 37°C using a feedback-controlled heating pad (BRC, Nagoya, Japan) during the operation. After restoration of blood flow, temperature continued to be maintained at 37°C.

Histologic Examination

For the histologic examination, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) 7 days after ischemia and were perfused intracardially with phosphate-buffered saline (PBS), followed by perfusion with 4% paraformaldehyde in PBS. Anterior segments, including the lens, were removed. Posterior eyecups were embedded in paraffin, and thin sections (5-μm thickness) were cut using a microtome. Sections were carefully cut to include the full length from superior to inferior along the vertical meridian through the optic nerve head. Each eye was mounted on a silane-coated glass slide and was stained with hematoxylin and eosin. Scleral thickness was measured to confirm that the sections were not oblique.

Morphometric analysis was performed to quantify ischemic injury. Five sections were selected randomly in each eye. One person with no previous knowledge of the treatments performed all the light microscopic (magnification, 10 × 100; Olympus BX-51, Tokyo, Japan) examinations. A microscopic image of each section within 0.5 to 1 mm superior of the optic disc was scanned. In each computer image, the thickness of the inner plexiform layer (IPL) and inner nuclear layer (INL) was measured. In each eye, the thickness of the IPL + INL was obtained as the mean of four measurements. For each animal, this parameter in the right eye was normalized to that in the intact left eye and was shown as a percentage.

Retrograde Labeling of Retinal Ganglion Cells

Seven days before kill, hydroxystilbamidine (Molecular Probes Inc., Eugene, OR) was injected bilaterally into the superior colliculi of anesthetized rats. The skull was exposed and kept dry and clean. After identifying and marking the bregma, a small window was drilled in the scalp in both the right and the left hemispheres. The windows were drilled to a depth of 3.6 mm from the surface of the skull and were located 6.8 mm behind the bregma on the anteroposterior axis and 1.5 mm lateral to the midline. Using a Hamilton syringe, 1.5 μL of 2% hydroxystilbamidine was slowly injected into the bilateral superior colliculi. After the skin was sutured over the wound, antibiotic ointment was applied.

Tissue Preparation and Assessment of RGC Survival

Animals were killed with an overdose of Nembutal at 1 week after 2% hydroxystilbamidine (Molecular Probes Inc.) application. Whole flat-mounted retinas were then assayed for retinal ganglion cell density. Rat eyes were enucleated and fixed in 4% paraformaldehyde for 10 hours at room temperature. After removal of the anterior segments, the resultant posterior eyecups were left in place. Subsequently, four radial cuts were made in the periphery of the eyecup, and the retina was then carefully separated from the retinal pigment epithelium. To prepare the flat mounts, the retina was dissociated from the underlying structures, flattened by making four radial cuts, and spread on a gelatin-coated glass slide. Labeled retinal ganglion cells (RGCs) were visualized under a fluorescence microscope (BX-51/DP70; Olympus) with an ultraviolet filter (blue-violet: 395–440 nm). Fluorescence-labeled RGCs were counted in 12 microscopic fields of retinal tissue from two regions in each quadrant at two different eccentricities, 1 mm (central) and 4 mm (peripheral) away from the optic disc. Software (Image-Pro Plus, version 4.0, Media Cybernetics, Bethesda, MD) was used to count the total number of RGCs in each eye. Changes in the densities of the RGCs were expressed as the RGC survival percentage, which was based on a comparison between the surgical and the contralateral control eyes. The specimens that were compared came from different retinal regions of the same animal.

Electroretinography

ERG responses were measured after overnight dark adaptation (at least 6 hours) using a recording device (Mayo Corporation, Aichi, Japan) 7 days after ischemia. Pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride eye drops (Santen Pharmaceuticals, Osaka, Japan). All procedures were performed in dim red light, and the rats were kept warm during the procedure. A contact lens electrode was placed on the surface of the cornea. A differential electrode was placed under the skin on the forehead, and a neutral electrode was placed under the skin near the tail. Standard flash ERGs were obtained using a flash intensity of 3 cd · s/m^2 with a single flash. ERGs were obtained of both eyes for each animal. Ischemic damage to the retina was evaluated as the percentage of the a- and b-wave amplitudes from the right eyes subjected to ischemia compared with the nonischemic left eyes.

Immunohistochemistry for AT1-R

Eyes were enucleated at 6, 12, or 24 hours after 45 minutes of ischemia. Eyes were then fixed in 4% paraformaldehyde and embedded in paraffin. Retinal sections (5 μm) were rinsed in 100% ethanol twice for 5 minutes each, followed by a separate 95% ethanol and 90% ethanol rinse for 3 minutes each. The sections were then washed using PBS (pH 7.4) three times for 10 minutes each and were treated with 0.3% Triton X-100 in PBS (pH 7.4) for 1 hour After further washing