J Gastroenterol (2014) 49:814–824
DOI 10.1007/s00535-013-0839-5

The Japanese Society of Gastroenterology

ORIGINAL ARTICLE—ALIMENTARY TRACT

Risk factor profiles, drug usage, and prevalence of aspirin-associated gastroduodenal injuries among high-risk cardiovascular Japanese patients: the results from the MAGIC study

Naomi Uemura · Kentaro Sugano · Hideyuki Hiraishi · Kazuyuki Shimada · Shinya Goto · Shinichiro Uchiyama · Yasushi Okada · Hideki Origasa · Yasuo Ikeda · The MAGIC Study Group

Received: 9 January 2013 / Accepted: 16 May 2013 / Published online: 12 June 2013

Abstract
Background Low-dose aspirin is widely used for the prevention of cardiovascular events. The prevalence of gastroduodenal injuries and the risk factor profile including gastroprotective drug therapy needs to be clarified in Japanese patients taking daily aspirin for cardioprotection.
Methods This Management of Aspirin-induced Gastro-Intestinal Complications (MAGIC) study was conducted with a prospective nationwide, multicenter, real-world registry of Japanese patients at high-risk of cardiovascular diseases who were taking regular aspirin (75–325 mg) for 1 month or more. All patients underwent endoscopic examination for detection of gastroduodenal ulcer and mucosal erosion. The risk factor profiles including the concurrent drug therapy were compared for those patients with gastroduodenal problems and those without.
Results Gastroduodenal ulcer and erosion were detected in 6.5, and 29.2 % of the 1,454 patients receiving aspirin, respectively. *H. pylori* infection was associated with an increased risk for ulcer: OR 1.83 (1.18–2.88 $p = 0.0082$). Risk of erosion was lower with enteric-coated aspirin than with buffered aspirin: odds ratio (OR) 0.47 (0.32–0.70, $p = 0.0002$). Patients receiving proton pump inhibitors had lower risks for both gastroduodenal ulcer and erosion: OR 0.34 (0.15–0.68, $p = 0.0050$) and 0.32 (0.22–0.46, $p < 0.0001$), respectively. However, those receiving histamine 2-receptor antagonists had reduced risks for erosion but not for ulcer: OR 0.49 (0.36–0.68, $p < 0.0001$).

The MAGIC Study Group: Management of Aspirin-induced Gastrointestinal Complications.

Trial registration: UMIN000000750.

Electronic supplementary material The online version of this article (doi:10.1007/s00535-013-0839-5) contains supplementary material, which is available to authorized users.

N. Uemura (✉)
Division of Gastroenterology, Kohnodai Hospital, National Center for Global Health and Medicine, Chiba 272-8516, Japan
e-mail: nuemura@hospk.ncgm.go.jp

K. Sugano
Division of Gasteroenterology, Department of Internal Medicine, Jichi Medical University, Shimotsuke, Japan

H. Hiraishi
Department of Gastroenterology, Dokkyo Medical University, Utsunomiya, Japan

K. Shimada
Division of Cardiology, Department of Internal Medicine, Jichi Medical University, Shimotsuke, Japan

S. Goto
Department of Medicine (Cardiology), Tokai University, Isehara, Japan

S. Uchiyama
Department of Neurology, Tokyo Women's Medical University, Tokyo, Japan

Y. Okada
Department of Cerebrovascular Disease, National Kyushu Medical Center, Fukuoka, Japan

H. Origasa
Division of Biostatistics and Clinical Epidemiology, University of Toyama, Toyama, Japan

Y. Ikeda
Faculty of Science and Engineering, Waseda University, Tokyo, Japan

Conclusion Gastroduodenal ulcer and erosion are common in Japanese patients taking low dose aspirin for cardioprotection. Proton pump inhibitors reduce the risk of gastroduodenal mucosal injury.

Keywords Low-dose aspirin · Gastroduodenal ulcer · Gastroduodenal erosion · Endoscopy · Cardiovascular patients

Introduction

Antiplatelet drug therapy reduces the risk of cardiovascular (CV) diseases in various patient populations. Aspirin use is supported with clinical evidence [1–3], but can cause adverse events, such as gastrointestinal (GI) injuries, even with a low-dose regimen [4]. According to meta-analyses, aspirin therapy increases the risk of GI bleeding by 2.7-fold as compared with results for a control arm, while it reduces the risk of major CV events by approximately 20 % [5]. These complications of GI bleeding are more complex than previously thought. Indeed, the risk of CV events increases in patients who have experienced major bleeding events within a year. Thus, GI bleeding may lead to a higher incidence of subsequent thrombotic events. The American Heart Association (AHA) recommends the use of low-dose aspirin (75–325 mg) for patients having a 10-year CV-event risk of 10 % or greater [6]. The US Preventive Services Task Force also recommends prophylactic aspirin therapy to be limited to patients with a 5-year CV risk of 3 % or greater, claiming that prophylaxis may not be beneficial for patients at low CV-event risk because the net clinical benefit is not high enough [7].

A limited amount of data is available for calculating the net clinical benefit in Japanese patients. Although it may not be directly comparable, data of the Western populations have indicated the overall relative risk of upper GI complications was 2.2 to 3.1 times higher in aspirin users than in non-aspirin users [8], whereas the odds ratio (OR) of upper GI bleeding was 5.5 in Japanese aspirin users [9]. The higher risk of GI bleeding in Japanese patients might be due to the higher prevalence of *Helicobacter pylori* infection in the elderly and those who smoke tobacco [9, 10].

We conducted the Management of Aspirin-induced Gastrointestinal Complications (MAGIC) study to determine the prevalence of endoscopic gastroduodenal ulcer and erosion in Japanese patients receiving regular aspirin for cardioprotection, and to clarify the risk factor profile including the concurrent use of gastroprotective drugs. This paper reports the baseline data obtained at the entry of this study.

Methods

Study design

This MAGIC study was conducted as an observational study in Japan. The details of the study design were published elsewhere [11]. Described briefly, the study consisted of high-risk CV patients taking low-dose aspirin for cardioprotection that were consecutively recruited from 63 nationwide institutions between April 2007 and September 2009. It was each investigator's discretion to judge "high risk of CV patients". Gastroduodenal ulcers and erosions were detected by endoscopy at enrollment. The study protocol was approved by the institutional review board in each institution. All participants signed the written informed consent. The present paper reports the baseline data of the enrollment.

Study population

The study population included patients with CV disease taking aspirin (75–330 mg daily) for at least 1 month. It included participants aged 20 years or older, and excluded those with serious hepatic, renal or pulmonary disorders, active cancer, hypersensitivity to aspirin or salicylate derivatives, pregnancy, possible pregnancy or pregnancy being planned, and prior surgical resection of esophagus, stomach, or duodenum.

Baseline demographic information

Upon the study entry, data on each patient's age, sex, underlying CV disease (e.g., coronary artery disease, cerebrovascular disease, and atrial fibrillation), comorbidities (hypertension, hyperlipidemia, diabetes mellitus, and metabolic syndrome), smoking habit, alcohol and coffee consumption, aspirin dosage and formulations (buffered or enteric coated), use of concomitant drugs, and history of upper GI ulcer were collected. All the participants were tested for the presence of *H. pylori* antibody after signing informed consent. *H. pylori* antibody in blood sample was measured using Anti-*H. pylori* IgG assay kit (SRL Inc., Tokyo, Japan). The *H. pylori* antibody was considered positive if the antibody level was $\geq$10 U/mL. The information on history of *H. pylori* eradication was collected from the patient medical records, where the eradication therapy was not well defined. Therefore, the results of eradication therapy were excluded from analysis. Antiulcer drugs included proton pump inhibitors (PPI), histamine 2-receptor antagonists (H2RA), cytoprotective antiulcer drugs, or prostaglandin analog (PGA).

Endoscopic assessment

Gastroduodenal ulcers or erosions were detected by endoscopy and the diagnosis was confirmed by the endoscopic evaluation committee (see Appendix). Gastroduodenal ulcer was defined by a mucosal break of 5 mm or greater in diameter with unequivocal depth, and erosion by mucosal change covered with white necrotic substance of less than 5 mm in diameter. The longer diameter of the lesion was measured as a standard of the length that opened biopsy forceps of 6 mm.

Study organization

The study design was formulated by the Organizing Committee (see Appendix), and data were collected through an Internet-based system.

Statistical analysis

Results were expressed as mean ± SD. Categorical variables between two groups were analyzed with Fisher's exact test, and the means of unpaired continuous variables, by Welch's t test. The prevalence and 95 % confidence interval (CI) were estimated by using the binomial distribution. The risk of gastroduodenal ulcer or erosion was estimated by the OR with 95 % CI by using univariate and multivariate logistic regression models. In the multivariate model, the odds ratio was adjusted by suspected risk factors such as age, sex, current tobacco smoking, alcohol use, diabetes mellitus, the presence of *H. pylori* antibody, and history of peptic ulcer, and uses of enteric-coated aspirin, PPI, H2RA, cytoprotective antiulcer drugs. A $p < 0.05$ was considered as statistically significant. Statistical analyses were performed by using the software R 2.14.0 (R foundation for Statistical Computing, Vienna, Austria).

Role of the funding source

The sponsor foundation had no role on the study design, selection of study institutions, selection of the committee members, data analyses, or the writing of the manuscript.

Results

Baseline characteristics of the patients

Among 1,531 patients who were consented and enrolled in the present study, 39 patients refused endoscopy and withdrew the consent, and remaining 1,492 patients received endoscopy. Data of 1,454 participants were used for analysis excluding those of 38 patients for gastric cancer, esophageal cancer, or colon cancer (Fig. 1).

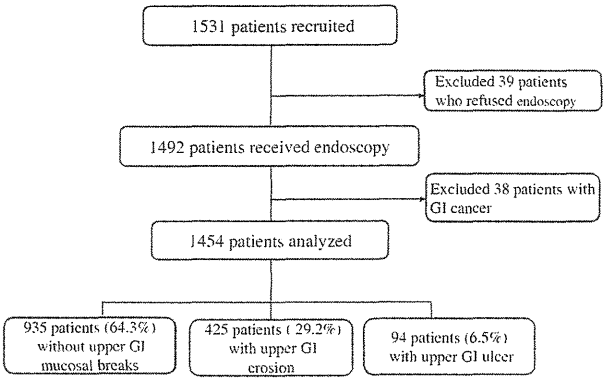

Fig. 1 Flow chart of the study patients

The mean participants' age was 68.1 ± 9.5 years, and 73.5 % of the participants were male. Aspirin was received daily for a mean duration of 4.6 ± 4.4 years (Table 1). A total of 89.4 % received enteric-coated aspirin and 10.6 %, buffered aspirin. The majority of the patients took 100 mg daily of enteric-coated aspirin (92.8 %), and 81 mg daily of buffered aspirin (96.2 %). Other NSAIDs were concomitantly used in only 6.5 %.

Baseline prevalence of gastroduodenal injury

The point prevalence of gastroduodenal ulcer was 6.5 % and erosion, 29.2 % (Table 1).

Among 94 patients with ulcer, the majority had gastric ulcer (80 cases, 85.1 %), following duodenal ulcer (10 cases, 10.6 %) and gastroduodenal ulcers (4 cases, 4.3 %).

Mean age was unexpectedly lower in the erosion (67.3 ± 9.3 years) and ulcer groups (65.1 ± 10.2 years) than in the group absent of mucosal break (AMB) (68.8 ± 9.5 years) ($p = 0.0060$ and $p = 0.0009$, respectively). In comparison with the AMB group, the ulcer group had greater proportions of male patients and current smokers ($p = 0.0103$ and $p = 0.0102$, respectively). The prevalence of diabetes mellitus was higher ($p = 0.0378$), and that of *H. pylori* antibody positive was lower only in the erosion group ($p < 0.0001$). Use of enteric-coated aspirin was significantly lower in the erosion group (84.9 %) and in the ulcer group (83.0 %) than in the AMB group (92.1 %) ($p = 0.0001$ and $p = 0.0063$, respectively).

Risk of gastroduodenal injury

According to risk analysis (Tables 2, 3), current smoking and *H. pylori* antibody positive were significant risk factors for ulcer: OR = 1.87 (1.03–3.25, $p = 0.0321$) and

Table 1 Baseline patient characteristics

	Total (n = 1454)	AMB (n = 935) (64.3 %)	Erosion (n = 425) (29.2 %)	p value[a]	Ulcer n = 94 (6.5 %)	p value[b]
Age (year)	68.1 ± 9.5	68.8 ± 9.5	67.3 ± 9.3	0.0060	65.1 ± 10.2	0.0009
Men (%)	1068 (73.5)	669 (71.6)	320 (75.3)	0.1678	79 (84.0)	0.0103
Body weight (kg)	62.6 ± 11.0	62.0 ± 11.1	63.3 ± 10.6	0.0522	64.4 ± 12.2	0.0722
Height (cm)	161.4 ± 8.5	160.9 ± 8.5	162.3 ± 8.4	0.0047	162.4 ± 7.9	0.0689
Body mass index (kg/m^2)	23.9 ± 3.2	23.9 ± 3.2	24.0 ± 3.1	0.6021	24.3 ± 3.4	0.2780
Underlying disease						
Cerebrovascular disease (%)	626 (43.1)	395 (42.2)	192 (45.2)	0.3160	39 (41.5)	0.9132
Coronary artery disease (%)	711 (48.9)	458 (49.0)	199 (46.8)	0.4825	54 (57.4)	0.1301
Atrial fibrillation (%)	155 (10.7)	108 (11.6)	41 (9.6)	0.3489	6 (6.4)	0.1662
Comorbidity						
Hypertension (%)	1053 (72.4)	674 (72.1)	306 (72.0)	1.0000	73 (77.7)	0.2763
Hyperlipidemia (%)	830 (57.1)	522 (55.8)	253 (59.5)	0.2148	55 (58.5)	0.6635
Diabetes mellitus (%)	416 (28.6)	249 (26.6)	137 (32.2)	0.0378	30 (31.9)	0.2749
Metabolic syndrome (%)	779 (53.6)	489 (52.3)	235 (55.3)	0.3192	55 (58.5)	0.2789
H. pylori antibody positive (%)	700 (48.1)	509 (54.4)	132 (31.1)	<0.0001	59 (62.8)	0.1546
Others concurrent disease (%)	650 (44.7)	429 (45.9)	180 (42.4)	0.2395	41 (43.6)	0.7448
Previous history of peptic ulcer (%)	311 (21.4)	202 (21.6)	83 (19.5)	0.4292	26 (27.7)	0.1925
Habit						
Current tobacco smoking (%)	151 (10.4)	100 (10.7)	32 (7.5)	0.0752	19 (20.2)	0.0102
Alcohol use (%)	591 (40.6)	364 (38.9)	181 (42.6)	0.2103	46 (48.9)	0.0611
Coffee consumption (%)	767 (52.8)	482 (51.6)	233 (54.8)	0.2663	52 (55.3)	0.5169
Aspirin use						
Enteric-coated aspirin (%)	1300 (89.4)	861 (92.1)	361 (84.9)	0.0001	78 (83.0)	0.0063
Duration of aspirin use (year)	4.6 ± 4.4	4.5 ± 4.4	4.7 ± 4.4	0.4679	5.0 ± 4.7	0.2924
Concomitant drug						
Other antiplatelet (%)	355 (24.4)	228 (24.4)	107 (25.2)	0.7860	20 (21.3)	0.6128
Anticoagulant (%)	175 (12.0)	125 (13.4)	43 (10.1)	0.1092	7 (7.4)	0.1077
Other NSAID (%)	94 (6.5)	60 (6.4)	31 (7.3)	0.5593	3 (3.2)	0.2642
Antihypertensive drug (%)	1084 (74.6)	701 (75.0)	312 (73.4)	0.5464	71 (75.5)	1.0000
Angiotensin II receptor blocker	754 (51.9)	478 (51.1)	219 (51.5)	0.4390	57 (60.6)	1.0000
Lipid-lowering drug (%)	753 (51.8)	478 (51.1)	219 (51.5)	0.9069	56 (59.6)	0.1299
HMG-Co A reductase inhibitor	682 (46.9)	430 (46.0)	201 (47.3)	0.6815	51 (54.3)	0.1303
Antidiabetic drug (%)	275 (18.9)	160 (17.1)	94 (22.1)	0.0297	21 (22.3)	0.2027

A total of 1454 participants were categorized into three groups by endoscopy: the group with absence of mucosal break (AMB), the group with gastroduodenal erosion (erosion), and the group with gastroduodenal ulcer (ulcer). The proportion of participants in each demographic category was examined among the three groups. Categorical variables were tested with Fisher's exact test and continuous variables with Welch's two sample t-test

AMB absence of mucosal break

[a] p value between AMB and erosion

[b] p value between AMB and ulcer

OR = 1.83 (95 % CI 1.18–2.88, $p = 0.0082$), respectively. However, a reduced risk of erosion was found with *H. pylori* antibody positive: OR = 0.34 (0.26–0.44, $p < 0.0001$), and a reduced risk of ulcer was found in the elderly population (>65 years old): OR = 0.60 (0.39–0.94, $p = 0.0246$). The risk for erosion but not for ulcer was significantly lower in use of enteric-coated aspirin (OR = 0.47, 0.32–0.70, $p = 0.0002$) than in use of buffered aspirin (OR = 0.57, 0.32–1.05, $p = 0.0569$).

In the analysis of 690 patients not treated with antiulcer drugs, the prevalence of ulcer and erosion were significantly lower with use of enteric-coated aspirin (7.8 and 33.5 %, respectively) than with use of buffered aspirin (12.8 and 47.4 %, respectively) (Fig. 2).

Table 2 Factors associated with risk of gastroduodenal ulcer

Factor	Unadjusted OR	*p* value	Adjusted OR	*p* value
Age ≥65 years	0.58 (0.38–0.88)	0.0109	0.60 (0.39–0.94)	0.0246
Men	1.94 (1.14–3.55)	0.0212	1.45 (0.81–2.74)	0.2261
Current tobacco smoking	2.20 (1.24–3.71)	0.0047	1.87 (1.03–3.25)	0.0321
Alcohol use	1.44 (0.94–2.20)	0.0891	1.18 (0.75–1.86)	0.4736
Diabetes mellitus	1.25 (0.79–1.94)	0.3331	1.12 (0.52–2.22)	0.7526
H. pylori antibody positive	1.87 (1.21–2.91)	0.0050	1.83 (1.18–2.88)	0.0082
History of peptic ulcer	1.48 (0.91–2.34)	0.1063	1.52 (0.91–2.47)	0.0988
Enteric-coated aspirin	0.53 (0.31–0.97)	0.0285	0.57 (0.32–1.05)	0.0569
Proton pump inhibitor	0.37 (0.17–0.74)	0.0091	0.34 (0.15–0.68)	0.0050
H2-receptor antagonist	0.80 (0.45–1.35)	0.4251	0.62 (0.34–1.06)	0.0967
Cytoprotective drug	0.93 (0.51–1.61)	0.8158	0.84 (0.45–1.48)	0.5703
Angiotensin II receptor blocker	0.95 (0.62–1.46)	0.8211	0.87 (0.55–1.34)	0.5214
HMG-Co A reductase inhibitor	1.36 (0.90–2.09)	0.1489	1.38 (0.90–2.14)	0.1450
Antidiabetic drug	1.25 (0.74–2.04)	0.3801	1.20 (0.55–2.78)	0.6527

Factors associated with gastroduodenal injuries suggestive in Table 1, with significant difference and established for gastroduodenal injuries according to previous studies, were examined for risk of gastroduodenal ulcer using data of 1423 participants excluding those without *H. pylori* information. Risk of gastroduodenal ulcer was estimated by the odds ratio with 95 % confidential interval using a monovariate ("Unadjusted") or multivariate ("Adjusted", which adjusted by all listed variables) logistic regression model

Table 3 Factors associated with risk of gastroduodenal erosion

Factor	Unadjusted OR	*p* value	Adjusted OR	*p* value
Age ≥65 years	0.82 (0.64–1.05)	0.1210	0.83 (0.64–1.09)	0.1768
Men	1.23 (0.94–1.61)	0.1290	1.25 (0.93–1.70)	0.1413
Current tobacco smoking	0.69 (0.45–1.04)	0.0857	0.65 (0.41–1.01)	0.0597
Alcohol use	1.19 (0.94–1.50)	0.1497	1.14 (0.87–1.48)	0.3447
Diabetes mellitus	1.30 (1.00–1.67)	0.0465	1.06 (0.69–1.60)	0.7917
H. pylori antibody positive	0.38 (0.29–0.48)	<0.0001	0.34 (0.26–0.44)	<0.0001
History of peptic ulcer	0.94 (0.70–1.25)	0.6599	1.05 (0.77–1.43)	0.7597
Enteric-coated aspirin	0.47 (0.33–0.67)	<0.0001	0.47 (0.32–0.70)	0.0002
Proton pump inhibitor	0.44 (0.32–0.61)	<0.0001	0.32 (0.22–0.46)	<0.0001
H2-receptor antagonist	0.60 (0.44–0.81)	0.0010	0.49 (0.36–0.68)	<0.0001
Cytoprotective antiulcer drug	1.12 (0.82–1.51)	0.4776	1.01 (0.72–1.39)	0.9592
Angiotensin II receptor blocker	1.12 (0.88–1.42)	0.3496	1.21 (0.94–1.56)	0.1339
HMG-Co A reductase inhibitor	1.03 (0.81–1.30)	0.8159	1.05 (0.82–1.35)	0.6838
Antidiabetic drug	1.34 (1.00–1.78)	0.0484	1.27 (0.79–2.05)	0.3289

Factors associated with gastroduodenal injuries suggestive in Table 1, with significant difference and established for gastroduodenal injuries according to previous studies, were examined for risk of gastroduodenal erosion using data of 1330 participants excluding those without *H. pylori* information and with ulcer. Risk of gastroduodenal erosion was estimated by the odds ratio with 95 % confidential interval using a monovariate ("Unadjusted") or multivariate ("Adjusted", which adjusted by all listed variables) logistic regression model

Antiulcer drug therapy

Anti-ulcer drugs were prescribed for gastroprotection in 52.5 %. PPI, H2RA, and cytoprotective antiulcer drugs or their combination were used with similar rates, whereas use of PGA or its combination was much lower. Use of PPI alone was lower in the erosion group (10.1 %) and in the ulcer group (7.4 %) than in the AMB group (20.6 %) ($p < 0.0001$, $p = 0.0014$, respectively). However, the difference in use of H2RA was detected only in the erosion group. Moreover, use of cytoprotective antiulcer drugs was higher in the erosion group ($p = 0.0364$). In analyses, risks of both ulcer and erosion were significantly reduced with PPI therapy (OR = 0.34, 0.15–0.68, $p = 0.0050$ and OR = 0.32, 0.22–0.46, $p < 0.0001$, respectively). However, in the H2RA therapy group the risk of erosion but not of ulcer was reduced (OR = 0.49, 0.36–0.68, $p < 0.0001$). No relation was found between therapy with cytoprotective drugs and those risks (Tables 2, 3, 4).

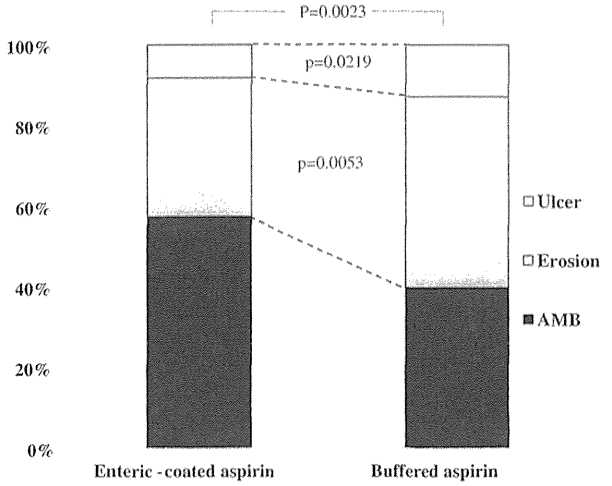

Fig. 2 Use of aspirin formulations and prevalence of gastroduodenal ulcer and erosion in patients not treated with antiulcer drugs. In 690 participants who were not treated with antiulcer drugs, prevalence of gastroduodenal erosion and ulcer were compared between patients receiving enteric-coated (88.7 %) and buffered aspirin (11.3 %). *AMB* absence of mucosal break

Upper GI cancer

Among 1,492 participants who received endoscopy, 37 participants (2.5 %, 95 % CI 1.75–3.40) had upper GI cancer, 4 patients (0.27 %, 0.07–0.68) had esophageal cancer, and 33 patients (2.21 %, 95 % CI 1.53–3.09) had gastric cancer. Additionally, colon cancer was found in one patient.

Discussion

Our study demonstrated that endoscopic gastroduodenal injuries were prevalent (35.7 %) among low-dose aspirin users in Japan, similar to Western countries. However, significant differences were found between the two regions in the methods aspirin was prescribed and the risk factors and drug treatment for gastroduodenal injuries. Use of other NSAIDs (6.5 %) with aspirin was rare in the present study, while it is frequent in Western countries. In spite of the recommendations in the AHA consensus and Japanese guidelines [12, 13], the use of PPI treatment was relatively low (19 %) and was similar to the use of H2RA or cytoprotective antiulcer agents. Cytoprotective agents are not generally used in Western countries. The recent approval (2010) of PPI for the prevention of mucosal injury in Japan may be contributing to the low PPI use.

Prevalence of gastroduodenal ulcer and erosion

The prevalence of endoscopic gastroduodenal ulcer associated with low-dose aspirin (6.5 %) was lower in our study than in previous studies. The prevalence of ulcer and erosion were 18 and 42 %, respectively, among 101 Japanese patients with ischemic heart disease in the study of Nema et al. [14], while that of upper GI ulcer was 12.4 % in 305 Japanese patients in the study of Shiotani et al. [15]. According to Yeomans et al., the point prevalence was 11 % for endoscopic gastroduodenal ulcer and 63 % for erosion in 187 patients taking aspirin for at least 24 days [4]. Factors contributing to the lower prevalence of

Table 4 Relationship between aspirin-associated gastroduodenal injuries and antiulcer drug treatment

	Total n = 1454	AMB n = 935 (64.3)	Erosion n = 425 (29.2)	p value[a]	Ulcer n = 94 (6.5)	p value[b]
No antiulcer drug (%)	690 (47.5)	390 (41.7)	242 (56.9)	<0.0001	58 (61.7)	0.0003
PPI alone (%)	243 (16.7)	193 (20.6)	43 (10.1)	<0.0001	7 (7.4)	0.0014
H2RA alone (%)	263 (18.1)	192 (20.5)	58 (13.6)	0.0025	13 (13.8)	0.1367
CAD alone (%)	171 (11.8)	98 (10.5)	62 (14.6)	0.0364	11 (11.7)	0.7246
PGA alone (%)	2 (0.1)	1 (0.1)	1 (0.2)	0.5275	0 (0.0)	1.0000
PPI + H2RA (%)	2 (0.1)	1 (0.1)	1 (0.2)	0.5275	0 (0.0)	1.0000
PPI + CAD (%)	33 (2.3)	26 (2.8)	7 (1.6)	0.2558	0 (0.0)	0.1606
PPI + PGA (%)	1 (0.1)	0 (0.0)	0 (0.0)	1.0000	1 (1.1)	0.0914
CAD + PGA (%)	1 (0.1)	0 (0.0)	1 (0.2)	0.3125	0 (0.0)	1.0000
H2RA + CAD (%)	47 (3.2)	34 (3.6)	9 (2.1)	0.1803	4 (4.3)	0.7716
PPI + H2RA + CAD (%)	1 (0.1)	0 (0.0)	1 (0.2)	0.3125	0 (0.0)	1.0000

Association of gastroduodenal injuries with concomitant use of antiulcer drug was analyzed using data of 1454 participants. The proportions of participants who received each category of antiulcer treatment were examined in the three groups of gastroduodenal conditions. Those in each treatment category were evaluated between the erosion group or the ulcer group versus the AMB group with Fisher's exact test

PPI proton pump inhibitor, *H2RA* histamine 2-receptor antagonist, *CAD* cytoprotective antiulcer drug, *PGA* prostaglandin analog

[a] p value between AMB and Erosion

[b] p value between AMB and Ulcer

ulcer or erosion in our study may be as follows: (1) a total of 41 % of the participants were treated with PPI or H2RA; (2) concomitant use of other NSAIDs was much lower; and (3) the criterion for mucosal ulcer was a mucosal break of 5 mm or greater in diameter with unequivocal depth. Nonetheless, by our estimation the prevalence of low-dose aspirin-induced endoscopic gastroduodenal ulcer in Japan is approximately 5–10 % in clinical practice.

Risk factors for gastroduodenal ulcer and erosion

Clinically important risk factors for aspirin-associated upper GI bleeding include aging, history of peptic ulcer or GI bleeding, concomitant use of anticoagulants or NSAIDs, and *H. pylori* infection in Western populations [16]. However, a limited number of studies endoscopically examined ulcer risk factors [15, 17]. In a study of Shiotani et al. [17] aging, history of peptic ulcer, and concomitant use of antithrombotic drugs and NSAIDs were associated with peptic ulcer, but regular alcohol drinking, smoking, and *H. pylori* infection were not in 425 low-dose aspirin users. In our study, a history of peptic ulcer, and the concomitant use of anticoagulants and NSAIDs had little association with endoscopic gastroduodenal ulcer and erosion. The reason may include (1) elderly patients with high risk for peptic ulcer such as those taking concomitant anticoagulants and NSAIDs might not be recruited, and (2) the number of concomitant NSAID use in this study was small, which may lead to an underestimation of the risk.

Aging was a risk factor for low-dose aspirin related gastroduodenal ulcer in many studies [4, 16, 17], whereas we observed that age >65 years old was associated a significant reduction in the risk of aspirin-associated ulcer. Furthermore in the analysis of 690 patients not treated with antiulcer drugs, the prevalence of ulcer was significantly lower in the elderly population (See the Supplementary table). The consensus of prior data is that risk of aspirin-associated ulcer increases with advancing age. This means that there may be a significant bias in our methodology or the Japanese may differ in gastric physiology from the rest of the world. In Japanese populations, the older generation has significantly reduced gastric acid secretion compared to younger generations due to atrophic gastritis [18]. Therefore, younger generations may have an inherently higher acid secretion and thus a higher risk of ulcers. However, the age-associated increase in atrophic gastritis is not specific gastritis is not a phenomenon which is specific to Japanese patients. Therefore, it is very likely to be a significant bias in our methodology that elderly patients with at high risk for peptic ulcer might not be recruited.

According to studies of Western populations, the presence of *H. pylori* infection is a significant risk for gastroduodenal ulcer [19]. Our study also demonstrated a twofold increase in ulcer risk in the presence versus the absence of *H. pylori* antibody. However, those results were conflicting with those of Shiotani et al. [15, 17] in Japanese populations where *H. pylori* infection was not associated with peptic ulcer in low-dose aspirin users. The findings may be affected by the study population and the definition of ulcer, which will be discussed in a separate section. In our study, the risk of erosion was significantly lower in the presence of *H. pylori* antibody. The cause and pathogenesis of aspirin-induced endoscopic gastroduodenal ulcer may be different from those of erosion in the presence of *H. pylori* infection.

Aspirin formulation

The prevalence of gastroduodenal injuries was significantly lower with enteric-coated aspirin than with buffered aspirin in our study. Others found that the risks of upper GI bleeding were similar among three forms of aspirin [20]. Although the prevalence of endoscopic gastroduodenal erosion was significantly lower with enteric-coated aspirin than with buffered aspirin, ulcer frequency was similar between the two formulations in the study of Nema et al. [21]. Dammann et al. [22] demonstrated that endoscopic gastroduodenal mucosal lesions were significantly less likely with enteric-coated aspirin (100 mg/day) than with plain aspirin, and the lesion score with coated aspirin was similar to that of placebo without aspirin. Further studies on the influence of aspirin formulation are needed in Japan.

Antiulcer drugs for prevention of gastroduodenal injury

Use of PPI was significantly less in the patients with ulcer or erosion, whereas use of H2RA was less in the patients with erosion, but not with ulcer. Use of cytoprotective drugs, which are widely prescribed in Japan, was higher in the patients with erosion. According to the risk analyses, only PPI presents reduced risks of both ulcer and erosion. The usefulness of PPI in the prevention of ulcers induced by low-dose aspirin is well established in Western countries and in Japan. In a comparative study by Yeomans et al. [23] the development of gastrointestinal ulcer was lower (1.6 %) with esomeprazole 20 mg/day than with placebo (5.4 %), demonstrating a reduction of 70 % in the 991 participants aged ≥60 years receiving low-dose aspirin for 26 weeks without preexisting endoscopic ulcers and without concomitant NSAIDs. Although their study design differed from ours, their findings support our study results. The effectiveness of PPI for the prevention of low-dose aspirin associated gastric or duodenal ulcers was demonstrated in a randomized comparative study by Sugano et al. [24] of a PPI, lansoprazole (15 mg/day), versus a cytoprotective antiulcer drug, gefarnate (100 mg/day), for

secondary prevention. The recurrence of ulcers was 90 % lower with lansoprazole than with gefarnate for an administration of 12 months or longer. According to Taha et al. [25] H2RA treatment with famotidine for 20 weeks reduced the risk of aspirin-induced peptic ulcer by 80 %. However, the risk of gastroduodenal erosion but not of ulcer was significantly lower with H2RA in our study. Study design and the ethnicity of the study populations may have contributed to the difference in results between the two studies.

Definition of ulcer and erosion as surrogate marker

Endoscopic gastroduodenal ulcer has been suggested to be a useful surrogate marker for potentially serious aspirin adverse event such as GI bleeding [26]. However, as described by Graham [27], ulcers are often defined by a mucosal defect of "3 mm or more" or "5 mm or more" in diameter in clinical studies, but aspirin-induced ulcer is often difficult to distinguish from erosion. No internationally recognized clear definition of "ulcer" or "a method of measuring ulcer size" has been established. Our definition of endoscopic ulcer was a mucosal defect 5 mm or more in diameter. However, when an ulcer with a 10 mm or larger diameter is defined as a "large ulcer," 25 % or more of ulcers were large ulcers in patients receiving H2RA or a cytoprotective antiulcer drug, but none of the ulcers were large ulcers in those receiving PPI in the present study (data not shown). Thus, the size of ulcers must be carefully defined for assessing effectiveness of antiulcer drugs in clinical studies that use endoscopically defined ulcers as the primary endpoint. A large cohort study is needed to clarify the risk factors of serious adverse events such as GI bleeding, and to verify endoscopically defined ulcer as a useful surrogate marker of GI bleeding in low-dose aspirin users.

Gastric cancer

This is the first study reporting the prevalence of gastric cancer diagnosed by endoscopy among aspirin users. Among 1,492 patients who received endoscopy, 37 patients had gastric cancer (2.5 %). Reports on the possible prevention of gastric cancer with aspirin have been published [28, 29], but it seems that more studies are necessary in the regions with a high prevalence of gastric cancer, such as Japan.

Limitation

We did not conduct the systematic screening in each hospital for patient recruitment. Our registry recruited patients taking preventive aspirin for high risk CV in clinical practice and gave informed consent to this study. Inclusion bias may be a potential limitation of this study.

Conclusion

Gastroduodenal ulcer and erosion are common among patients receiving low-dose aspirin for prophylaxis of CV disease in the Japanese population (35.7 %). Factors that increase risks of mucosal injuries are current tobacco smoking and the presence of *H. pylori* infection. The use of PPI is helpful to reduce the risk of ulcer and erosion. Furthermore, the association between endoscopic ulcer and serious complications such as GI bleeding should be clarified in the future.

Acknowledgments The authors are indebted to Professor David Y. Graham (of Michael E. DeBakey Veterans Affairs Medical Center at Houston, Texas, United States) for his helpful suggestions and to Koji Shimamoto, Hiroko Usami, and Yasuko Ueda for their assistance with laboratory work and statistical analyses. This study was sponsored by the Japan Cardiovascular Research Foundation.

Conflict of interest SG received research grants from Sanofi-Aventis, Eisai, Boehringer Ingelheim, Otsuka, and Daiichi-Sankyo, and received honorarium for sitting on advisory panels from Eisai, Sanofi-Aventis, Otsuka, Bayer, Novartis. AstraZeneca, Astellas, Pfizer, Medtronics-Japan, Mitsubishi Tanabe, Takeda, Daiichi-Sankyo, Mochida, and MSD. KS received grants from AstraZeneca, Takeda, Astellas and Daiichi-Sankyo and also sat on advisory panels for AstraZeneca, and Takeda. YI received honorarium for lecturing from Sanofi-Aventis, Daiichi-Sankyo and Bayer. and sat on advisory panels for AstraZeneca, Daiichi-Sankyo, and Sanofi-Aventis. All other authors declare that they have no conflicts of interest.

Appendix

Organizing Committee

Yasuo Ikeda (Chair), Shinichiro Uchiyama, Yasushi Okada, Kazuyuki Shimada, Shinya Goto, Kentaro Sugano, Hideyuki Hiraishi, Naomi Uemura, Hideki Origasa.

Endoscopic Evaluation Committee

Naomi Uemura (Chair), Takashi Kawai, Shinichi Nakamura, Chouitsu Sakamoto, Hidekazu Suzuki.

Event Ascertainment Committee

Shinichiro Uchiyama (Chair), Yasushi Okada, Kazuyuki Shimada, Shinya Goto.

Data Monitoring Committee

Saichi Hosoda (Chair), Yukito Shinohara, Toshifumi Hibi.

Data Coordinating Center

Hiroko Usami.

List of participating investigators

(National Center for Global Health and Medicine) Gastroenterology *Junichi Akiyama, Naomi Uemura* Cardiology *Michiaki Hiroe, Osamu Okazaki* (Hiroshima University) Clinical Neuroscience and Therapeutics *Rie Hanaoka, Hiroki Imagawa, Shinobu Imagawa, Shosuke Kitamura, Takayasu Kuwahara, Taiji Matsuo, Sayaka Oba, Toshiho Ohtsuki, Toshiko Onitake, Youji Sanomura, Takayoshi Shisido, Akemi Takamura, Masana Tatsugami, Yoshihiro Wada, Shigeto Yoshida* (Tokai University School of Medicine) Gastroenterology *Jun Koike, Masashi Matsushima, Tetsuya Mine, Takayuki Shirai, Takayoshi Suzuki, Kenichi Watanabe* Cardiology *Shinya Goto, Teruhisa Tanabe, Koichiro Yoshioka* Neurology *Shigeharu Takagi* Neurosurgery *Mitsunori Matsumae* (Tokyo Medical University Ibaraki Medical Center) Gastroenterology *Tsuyoshi Hirayama, Tadashi Ikegami, Masanori Ito, Shinichi Ito, Junichi Iwamoto* Cardiology *Masamitsu Asano, Akihiro Fukuda, Shinji Okubo* Neurology *Suguru Nojima, Kaoru Yamazaki* (Dokkyo Medical University Hospital) Gastroenterology *Takafumi Hoshino, Jun Ishikawa, Kazunari Kanke, Mitsunori Maeda, Masakazu Nakano, Rieko Ogura, Yutaka Okamoto, Yasuyuki Saifuku, Takako Sasai, Makoto Suzuki, Akihiro Tajima, Keiichi Tominaga, Mariko Uchizono, Hidetaka Watanabe, Michiko Yamagata, Yoshimitsu Yamamoto, Kenji Yoshida* Hypertension and Cardiorenal Medicine *Shigeo Horinaka, Kimihiko Ishimura, Koichi Kono, Akihisa Yabe, Hiroshi Yagi* Neurosurgery *Yasuhisa Daimon, Atsuko Ebata, Hidehiro Takekawa* (Uji Hospital) Gastroenterology *Tadashi Higaki, Kennji Mayumi, Shohei Sawada, Kaoru Shirai, Nami Takeda* (Tohoku University Graduate School of Medicine) Gastroenterology *Katsunori Iijima* Cardiology Ryoji Koshida, Hiroaki Shimoaki, Morihiko Takeda (Keio University School of Medicine) Gastroenterology *Rie Hanaoka, Hiroki Imagawa, Shinobu Imagawa, Shosuke Kitamura, Takayasu Kuwahara, Taiji Matsuo, Sayaka Oba, Toshiho Ohtsuki, Toshiko Onitake, Youji Sanomura, Takayoshi Shisido, Akemi Takamura, Masana Tatsugami, Yoshihiro Wada, Shigeto Yoshida* (Hamamatsu University School of Medicine) Clinical Research Development Center *Takahisa Furuta, Mutsuhiro Ikuma, Masafumi Nishino, Satoshi Osawa, Kenichi Yoshida* Neurosurgery *Hisaya Hiramatsu, Hiroki Namba* Clinical pharmacology *Kazuhiko Takeuti, Akiko Utsumi, Hiroshi Watanabe* (Teine Keijinkai Hospital) Cardiology *Mitsuharu Fukasawa, Akio Katanuma, Toshifumi Kin, Fukuo Komaba, Akira Kurita, Takeshi Matsui, Shinya Mitsui, Harutatsu Muto, Hiroyuki Nishimori, Masafumi Nomura, Maki Ohtsubo, Manabu Osanai, Hayato Shida, Kuniyuki, Takahashi, Tanaka Tanaka, Kei Yane* (University of Toyama) 1st Dept. Internal Medicine *Tomoki Kameyama, Naoya Kuwayama* 2nd Dept. Internal Medicine *Nozomi Fujii, Takashi Nozawa* 3rd Dept. Internal Medicine *Ayumu Hosokawa, Tohishiko Kudo, Takako Miyazaki, Tadahiro Orihara, Toshiro Sugiyama* Neurology *Akira Matsuki, Yoshiharu Taguchi, Shutaro Takashima, Kortaro Tanaka* Neurosurgery Shunro Endo, Hideo Hamada, Nakamasa Hayashi, Tadakazu Hirai (Oji General Hospital) Gastroenterology *Tadashi Doi, Akihito Fujimi, Yuji Kanisawa, Hideaki Ohta, Toshinori Okuda, Yasuhiro Sato* Cardiology *Tadashi Doi, Akihito Fujimi, Katsuhisa Ishii, Yuji Kanisawa, Nobuo Kato, Tomoaki Matsumoto, Hideaki Ohta, Hitoshi Ooiwa, Yasuhiro Sato, Daisuke Yoshida* Neurosurgery *Yoshifumi Horita, Shigeki Kashiwabawa, Takeshi Mikami* (Fujita Health University) Gastroenterology *Ichiro Hirata, Tomoyuki Shibata* Cardiology *Masatsugu Iwase, Yukio Ozaki, Masayoshi Sarai, Eiichi Watanabe* Neurology *Kunihiko Asakura, Hideo Hara, Takateru Mihara, Tatsuro Mutoh, Takako Takeuchi, Akihiro Ueda* (Gunma University Hospital) Cardiology *Masashi Arai, Yoshiaki Kaneko, Akihiko Nakano* Neurology *Koichi Okamoto* Endoscopy and Endoscopic Surgery *Hiroko Hosaka, Osamu Kawamura, Motoyasu Kusano, Yasuyuki Shimoyama* (Kokura Memorial Hospital) Cardiology *Yoshio Kazuno, Tomoharu Yoshida* (Odate Municipal General Hospital) Gastroenterology *Hitoshi Ogasawara* Neurosurgery *Masahiro Sasaki* (Nakamura Memorial Hospital) Neurosurgery *Jyoji Nakagawara, Yoshinobu Seo, Toshiiti Watanabe* (Iwate Medical University) Neurology and geriatric *Toshimi Chiba, Kuniko Watanabe, Hisashi Yonezawa* (Chiba University Graduate School of Medicine) Gastroenterology *Hitoshi Maruyama* Cardiology *Hiroshi Akazawa, Hiroshi Hasegawa, Naoki Ishio, Nakabumi Kuroda, Yoichi Kuwabara, Hideyuki Miyauchi, Taichi Murayama, Toshio Nagai, Keiichi Nakagawa, Tohru Oka, Satoshi Shindo, Ichiro Shiojima, Hiroyuki Takano, Toko Toko* (Institute of Brain and Blood Vessels Mihara Memorial Hospital) *Ban Mihara, Masaki Takao, Yutaka Tomita* Gastroenterology *Takayuki Takahashi* (Tokyo Women's Medical University) Gastroenterology *Kenji Maruyama, Yoko Masuda, Shinichi Nakamura, Yoshio Uetsuka* Cardiology *Kagari Murasaki, Sono Tooi* Neurology *Tomomi Kimura* (Nanpu Hospital)

Gastroenterology *Takako Imamura, Hiromitsu Karasumaru, Akio Matsuda, Tooru Niihara, Tatsuyuki Nioh, Syunji Shimaoka, Kotarou Tashiro* Cardiology *Kazuaki Kiyonaga, Shinichirou Toyoshima* Neurosurgery *Kazuhiro Kusumoto, Shunichi Yokoyama* (Sapporo Medical University) 1st Internal Medicine *Yoshiaki Arimura, Akira Goto, Akiyoshi Hashimoto, Masayo Hosokawa, Yoshinori Miyazaki, Hiroyuki Okuda, Kazuaki Shimamoto, Tokuma Tanuma, Nobuhiko Togashi, Kazufumi Tsuchihashi, Hiroyuki Yamamoto, Kentaro Yamashita* Neurosurgery *Masaki Saitoh* (Kamiiida Daiichi General Hospital) Gastroenterology *Kosuke Tachi* Cardiology *Satoshi Isobe* (Nippon Medical University Hospital) Cardiology *Hitoshi Takano* (Jichi Medical University) Gastroenterology *Hironari Ajibe, Tomosuke Hirasawa, Hiroyuki Osawa, Kiihi Satoh, Toru Yoshida* Cardiology *Kazuo Eguchi, Yukihiro Hojo, Satoshi Hoshide, Mitsunobu Murata, Masahisa Shimpo, Nozomu Takahashi, Shuichi Ueno, Keiji Yamamoto* (Nayoro City General Hospital) Neurosurgery *Hiroki Saito, Kazuhiro Sako.*

(Hakodate Goryokaku Hospital) Gastroenterology Kaoru Kasahara, Toshihisa Kobayashi, Nobuaki Sugawara, Ryo Suzuki, Hiroyuki Takamaru, Hidenori Yamauchi, Atsushi Yawata Cardiology *Hiroshi Oimatsu* (Nihon University School of Medicine Itabashi Hospital) Gastroenterology and hepatology *Shigeaki Mizuno, Junko Motoe* Cardiology *Masaaki Chiku, Satoshi Kunimoto, Kazumasa Miyake* (Ichinomiya Municipal Hospital) Cardiology *Chiyuki Chujyou, Youichi Iguchi, Shinichi Kanamori, Keiji Mizutani, Arihiro Nakano, Masako Oosawa, Tetsuo Shibata, Michiharu Yamada, Toshihiro Yamanaka* (Kawasaki Medical School Hospital) Gastroenterology *Akiko Shiotani* Cardiology *Yoji Neishi* (NTT Medical Center Tokyo) Gastroenterology *Nobuyuki Matsuhashi, Osamu Tagusari, Yumiko Yamaoka* (Saitama Medical Center) Gastroenterology and hepatology *Toru Aoyama, Katsuya Chinen, Shuko Isida, Kazuhito Kani, Junichi Kawashima, Naoya Miyagi, Shino Ono, Keiko Satou, Koji Yakabi, Masakatsu Yoshikawa* Neurology *Kyoichi Nomura* Gastroenterology *Madoka Hashimoto, Masayoshi Uehara* (Seiseikai Kumamoto Hospital) Cardiology *Junjiroh Koyama* Neurology *Toshiro Yonehara* (Mie University Hospital) Cardiology *Hiroshi Nakashima, Muneyoshi Tanimura, Akihiro Tsuji* Neurology *Akira Taniguchi* Endoscopy and Endoscopic Surgery *Ichiro Imoto, Kyosuke Tanaka* Clinical Research Development Center *Masakatsu Nishikawa* (Sapporo City General Hospital) Gastroenterology *Michio Nakamura, Shuji Nishikawa* Cardiology *Hiroyuki Fukuda* Neurosurgery *Masayoshi Takigami* (Yokohama City University Medical Center) Cardiovascular Center *Kiyoshi Hibi, Kazuo Kimura, Atsushi Kokawa, Kengo Tsukahara* (National Hospital Organization Kagoshima Medical Center) Cerebrovascular disease *Rikuzo Hamada, Naoko Tsubouchi* (Hokkaido University Hospital) Gastroenterology *Mototsugu Kato, Shouko Ono* Cardiology *Tomoo Furumoto, Daisuke Gotou, Naoki Ishimori, Nozomu Kawashima, Mamoru Sakakibara, Takamitsu Souma* (Yokohama Sakae Kyosai Hospital) Neurosurgery *Motohiro Nomura, Hiro Satoh, Hiroshi Shima* (Komaki City Hospital) Cardiology *Hajime Imai, Taizo Kondo, Akihiro Miyata, Itaru Ohyama* (National Hospital Organization Yokohama Medical Center) Cardiology *Kazunori Iwade, Shouzo Matsushima* (Shimane University Faculty of Medicine) Gastroenterology *Yuji Amano, Kenji Furuta, Norihisa Ishimura, Kenji Koshino, Masaharu Miki* Neurology *Hirokazu Bokura, Hiroaki Oguro, Shuhei Yamaguchi* Cardiology *Yutaka Ishibashi* (Oita University Faculty of Medicine) Gastroenterology *Tadayoshi Okimoto, Jin Tanahashi* Cardiology *Munenori Kotoku, Shigeru Naono, Takashi Sato, Akira Tamura* (National Hospital Organization Kyushu Medical Center) Gastroenterology *Naohiko Harada* Hematology *Toshiyasu Ogata, Yasushi Okada, Shinji Satoh* (Teikyo University Hospital) Gastroenterology *Takatsugu Yamamoto* Cardiology *Shuichi Ishikawa, Satoshi Koganezawa, Ken Kozuma, Yoshitaka Shiratori, Hidenori Watanabe* (Kushiro City General Hospital) Neurosurgery *Toshio Imaizumi, Kazuhiko Yonezawa* (Shinshu University Hospital) Gastroenterology *Yuichi Sato* 1st Internal Medicine *Yoshifusa Aizawa, Satoru Hirono* Neurology *Yasuhisa Akaiwa* (Niigata University Medical and Dental Hospital) Neurosurgery *Kazuhiko Nishino* (University of Yamanashi Faculty of Medicine) Neurosurgery *Kazuya Kanemaru, Hiroyuki Kinouchi, Masao Sugita* (University of Yamanashi Hospital) Gastroenterology *Tadashi Sato* (NTT Medical Center Sapporo) Gastroenterology *Shigeru Furukawa, Akihito Kobayashi, Tatsumi Koshiyama, Kimitoshi Kubo, Ken Nishi, Amane Oota, Youko Tsukuda, Akiko Yokoyama* Cardiology *Shigeru Furukawa, Tetsuro Kohya, Noriyuki Miyamoto* (Konan Kosei Hospital) Gastroenterology *Yoji Sasaki* Cardiology Shinichi Ishikawa (Ehime University Hospital) Gastroenterology *Yoshiou Ikeda, Hidehiro Murakami, Akiyoshi Ogimoto* Neurosurgery *Hideaki Watanabe* (Jichi Medical Unversity Saitama Medical Center) Gastroenterology *Satohiro Matsumoto, Noriyoshi Sagihara* Cardiology *Kenshiro Arao, Shin-Ichi Momomura, Kenichi Sakakura* (Tosei General Hospital) Gastroenterology *Takao Hayasi, Tetsuo Matsuura, Keiichi Morita, Toyohiro Sakata, Yuko Simizu* Cardiology *Takahiro Kannbara, Yusuke Uemura* (Saga University) Gastroenterology *Yasuhisa Sakata, Ryo Shimoda, Seiji Tsunada* Cardiology *Shigemasa Hashimoto, Tadashi Yamamoto* (Tsuchiya General Hospital) Gastroenterology *Yasuhiko Hayashi, Shohei Ishimaru, Masaru Shimamoto, Seiji Touge* Cardiology Tomoharu Kawase, Takehito Tokuyama (Okayama Medical Center) Gastroenterology *Hiromi Matsubara, Yoshihiro Oofuji* Cardiology

Tomohiko Mannami (National Defense Medical College Hospital) Gastroenterology *Ryota Hokari* Cardiology *Fumitaka Ohsuzu* (Toda Chuo General Hospital) Gastroenterology *Masataka Nishi* Cardiology *Tadashi Nagao, Takashi Uchiyama.*

(Yamaguchi University Hospital) Endoscopy and Endoscopic Surgery *Shingo Higaki, Jun Nishikawa* Cardiology *Toshirou Miura* (Shinshu University Hospital) Cardiology *Hiroki Kasai* Endoscopy *Taiji Akamatsu* (Research Institute for Brain and Blood Vessels-Akita) Stroke care unit *Tsuyoshi Mukoujima, Akifumi Suzuki.*

(Suzulan Clinic) *Masahide Wada.*

References

1. Antithrombotic Trialists' Collaboration. Collaborative meta-analysis of randomized trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high-risk patients. BMJ. 2002;324:71–86.
2. Weisman SM, Graham DY. Evaluation of the benefits and risks of low-dose aspirin in the secondary prevention of cardiovascular and cerebrovascular events. Arch Intern Med. 2002;162:2197–202.
3. Eidelman RS, Hebert PR, Weisman SM, Hennekens CH. An update on aspirin in the primary prevention of cardiovascular disease. Arch Intern Med. 2003;163:2006–10.
4. Antithrombotic Trialists' (ATT) Collaboration. Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. Lancet. 2009;373:1849–60.
5. Pearson TA, Blair SN, Daniels SR, et al. AHA Guidelines for Primary Prevention of Cardiovascular Disease and Stroke: 2002 Update: Consensus Panel Guide to Comprehensive Risk Reduction for Adult Patients Without Coronary or Other Atherosclerotic Vascular Diseases. American Heart Association Science Advisory and Coordinating Committee. Circulation. 2002;106:388–91.
6. Hayden M, Pignone M, Phillips C, Mulrow C. Aspirin for the primary prevention of cardiovascular events: a summary of the evidence for the U.S. Preventive Services Task Force. Ann Intern Med. 2002;136:161–72.
7. García Rodríguez LA, Hernández-Díaz S, de Abajo FJ. Association between aspirin and upper gastrointestinal complications: systematic review of epidemiologic studies. Br J Clin Pharmacol. 2001;52:563–71.
8. Sakamoto C, Sugano K, Ota S, et al. Case–control study on the association of upper gastrointestinal bleeding and nonsteroidal anti-inflammatory drugs in Japan. Eur J Clin Pharmacol. 2006;62:765–72.
9. Yeomans ND, Lanas AI, Talley NJ, et al. Prevalence and incidence of gastroduodenal ulcers during treatment with vascular protective doses of aspirin. Aliment Pharmacol Ther. 2005;22:795–801.
10. Chiba T, Seno H, Marusawa H, Wakatsuki Y, Okazaki K. Host factors are important in determining clinical outcomes of *Helicobacter pylori* infection. J Gastroenterol. 2006;41:1–9.
11. Origasa H, Goto S, Shimada K, MAGIC Investigators, et al. Prospective cohort study of gastrointestinal complications and vascular diseases in patients taking aspirin: rationale and design of the MAGIC Study. Cardiovasc Drugs Ther. 2011;25:551–60.
12. Bhatt DL, Scheiman J, Abraham NS, et al. American College of Cardiology Foundation Task Force on Clinical Expert Consensus Documents. ACCF/ACG/AHA 2008 expert consensus document on reducing the gastrointestinal risks of antiplatelet therapy and NSAID use: a report of the American College of Cardiology Foundation Task Force on Clinical Expert Consensus Documents. J Am Coll Cardiol. 2008;52:1502–17.
13. Guidelines for EBM Based Clinical Practice of Gastric Ulcer. 2nd ed. Team on EBM Based Clinical Practice of Gastric Ulcer, editors, Jiho; 2007. p. 101–10 (in Japanese).
14. Nema H, Kato M, Katsurada T, et al. Endoscopic survey of low-dose-aspirin-induced gastroduodenal mucosal injuries in patients with ischemic heart disease. J Gastroenterol Hepatol. 2008;23(Suppl 2):S234–6.
15. Shiotani A, Sakakibara T, Yamanaka Y, et al. Upper gastrointestinal ulcer in Japanese patients taking low-dose aspirin. J Gastroenterol. 2009;44:126–31.
16. Lanas A, Scheiman J. Low-dose aspirin and upper gastrointestinal damage: epidemiology, prevention and treatment. Curr Med Res Opin. 2007;23:163–73.
17. Shiotani A, Nishi R, Yamanaka Y, et al. Renin-angiotensin system associated with risk of upper GI mucosal injury induced by low dose aspirin: renin angiotensin system genes' polymorphism. Dig Dis Sci. 2011;56:465–71.
18. Shiotani A, Sakakibara T, Yamanaka Y, et al. The preventive factors for aspirin-induced peptic ulcer: aspirin ulcer and corpus atrophy. J Gastroenterol. 2009;44:717–25.
19. Lanas A, Fuentes J, Benito R, Serrano P, Bajador E, Sáinz R. *Helicobacter pylori* increases the risk of upper gastrointestinal bleeding in patients taking low-dose aspirin. Aliment Pharmacol Ther. 2002;16:779–86.
20. Kelly JP, Kaufman DW, Jurgelon JM, Sheehan J, Koff RS, Shapiro S. Risk of aspirin-associated major upper-gastrointestinal bleeding with enteric-coated or buffered product. Lancet. 1996;348:1413–6.
21. Nema H, Kato M, Katsurada T, et al. Investigation of gastric and duodenal mucosal defects caused by low-dose aspirin in patients with ischemic heart disease. J Clin Gastroenterol. 2009;43:130–2.
22. Dammann HG, Burkhardt F, Wolf N. Enteric costing of aspirin significantly decreases gastroduodenal mucosal lesions. Aliment Pharmacol Ther. 1999;13:1109–14.
23. Yeomans N, Lanas A, Labenz J, et al. Efficacy of esomeprazole (20 mg once daily) for reducing the risk of gastroduodenal ulcers associated with continuous use of low-dose aspirin. Am J Gastroenterol. 2008;103:2465–73.
24. Sugano K, Matsumoto Y, Itabashi T, et al. Lansoprazole for secondary prevention of gastric or duodenal ulcers associated with long-term low-dose aspirin therapy: results of a prospective, multicenter, double-blind, randomized, double-dummy, active-controlled trial. J Gastroenterol. 2011;46:724–35.
25. Taha AS, McCloskey C, Prasad R, Bezlyak V. Famotidine for the prevention of peptic ulcers and esophagitis in patients taking low-dose aspirin (FAMOUS): a phase III, randomised, double-blind, placebo-controlled trial. Lancet. 2009;374:119–25.
26. Moore A, Bjarnason I, Cryer B, et al. Evidence for endoscopic ulcers as meaningful surrogate endpoint for clinically significant upper gastrointestinal harm. Clin Gastroenterol Hepatol. 2009;7:1156–63.
27. Graham DY. Endoscopic ulcers are neither meaningful nor validated as a surrogate for clinically significant upper gastrointestinal harm. Clin Gastroenterol Hepatol. 2009;7:1147–50.
28. Rothwell PM, Fowkes FG, Belch JF, Ogawa H, Warlow CP, Meade TW. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. Lancet. 2011;377:31–41.
29. Yang P, Zhou Y, Chen B, et al. Aspirin use and the risk of gastric cancer: a meta-analysis. Dig Dis Sci. 2010;55:1533–9.

BRAIN RESEARCH 1583 (2014) 237–244

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

Brain Research

Research Report

Targeting of aquaporin 4 into lipid rafts and its biological significance

Kunihiko Asakura, Akihiro Ueda, Sayuri Shima, Tomomasa Ishikawa, Chika Hikichi, Seiko Hirota, Takao Fukui, Shinji Ito, Tatsuro Mutoh*

Department of Neurology, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan

ARTICLE INFO

Article history:
Accepted 7 August 2014
Available online 14 August 2014

Keywords:
Aquaporin 4
Lipid raft
Neuromyelitis optica
Methyl-β-cyclodextrin
Simvastatin

ABSTRACT

Neuromyelitis optica (NMO) is an inflammatory demyelinating disease of the central nervous system and is considered to be caused by the binding of NMO-IgG to aquaporin 4 (AQP4) on astrocytes, which initiates complement-dependent cytotoxicity. AQP4 has two isoforms, i.e., M1 and M23. AQP4 is considered to form heterotetramers containing both isoforms in vivo. Most of the previous studies were performed using either one of the isoforms expressing cell lines. In this study, we generated a fluorescent epitope-tagged AQP4 M1 and M23 co-expressing astrocyte cell line and examined the subcellular targeting of AQP4. In this cell line, AQP4 was targeted mostly to membrane lipid rafts fraction evidenced by sucrose density gradient ultracentrifugation followed by Western blotting with anti-AQP4 antibody. Cholesterol depletion with methyl-β-cyclodextrin or simvastatin resulted in the dislocation (relocation) of AQP4 from lipid rafts to non-rafts fraction of the membrane and AQP4 was not internalized intracellularly. This change in the localization of AQP4 on membrane significantly reduced complement-dependent cytotoxic effects of NMO-IgG obtained from patients with NMO without affecting AQP4 orthogonal arrays. Thus, these data strongly suggest that the targeting of AQP4 in the lipid rafts is closely related to the pathogenic effects of NMO-IgG.

1. Introduction

Neuromyelitis optica (NMO) is an inflammatory demyelinating disease of the central nervous system (CNS) with distinct clinical and histopathological features from multiple sclerosis (MS). NMO is characterized by optic neuritis, longitudinally extensive transverse myelitis with three or more vertebral segments in length, and NMO-IgG seropositivity (Lennon et al., 2004; Wingerchuk et al., 2006). A target antigen of NMO-IgG was identified as aquaporin-4 (AQP4), a water channel protein that is mainly expressed in the brain and spinal cord (Lennon et al., 2005). Furthermore, NMO-IgG is considered to be highly specific to NMO (Takahashi et al., 2007; Matsuoka et al., 2007; Wingerchuk et al., 2007). Recent studies have shown that NMO-IgG and complements contribute directly to disease pathogenesis (Bennett et al., 2009; Bradl et al., 2009).

Lipid rafts are originally defined biochemically as detergent-resistant membrane fractions, and are proposed to be highly

Abbreviations: AQP, aquaporin; NMO, neuromyelitis optica

*Correspondence to: 1-98 Kutsukake-cho, Toyoake, Aichi 470-1192, Japan. Fax: +81 562 93 1856.
E-mail address: kasakura@fujita-hu.ac.jp (K. Asakura).

http://dx.doi.org/10.1016/j.brainres.2014.08.014
0006-8993/

dynamic and float freely within bilayer cell membranes. Lipid rafts containing a given set of proteins can change their size and composition in response to intra- or extracellular stimuli, resulting in the activation of signaling cascade (Simons and Toomre, 2000; Mutoh, 2013). The importance of lipid rafts signaling in the pathogenesis of a variety neurological diseases, such as Alzheimer's disease (Ehehalt et al., 2003), Parkinson disease (Fortin et al., 2004), and prion disease (Hooper, 2005) has been demonstrated over recent years. It has been reported in primary astrocyte cultures that AQP4 resides in the Triton X-100 insoluble fraction (lipid rafts fraction) (Noël et al., 2009). There are two major AQP4 isoforms, i.e., M1 and M23, which have identical extracellular domain residues, but M1 has 22 more amino acids at the cytoplasmic N-terminus. Freeze-fracture electron microscopy of astrocyte end-foot membranes has revealed density packed square arrays of intramembrane proteins known as orthogonal arrays of particles (OAPs) (Landis and Reese, 1974; Wolburg, 1995). AQP4 is considered to form heterotetramers containing both isoforms in vivo. In this study, we explored the subcellular targeting of AQP4 isoforms and holo-protein by generating fluorescent epitope-tagged AQP4 M1/M23 co-expressing astrocyte cell line with single plasmid DNA transfection. Here, we show that holo-protein of AQP4 is mainly targeted to the lipid rafts fraction of the plasma membrane and a change of subcellular localization of AQP4 by the treatment with cholesterol-lowering agents significantly reduces the cytotoxic effects of NMO-IgG.

2. Results

2.1. *Expression of fluorescence epitope-tagged AQP4 M1 and M23 isoforms in AQP4 M1/M23 co-expressing OS3 cells*

The molecular size of each fluorescent epitope tag is 27 kDa. The expected sizes of AQP4 M1/RFP and M23/AcGFP are 61 kDa and 58 kDa, respectively. Fluorescence epitope-tagged AQP4 M1 and M23 were identified as a duplet band in AQP4 M1/M23 co-expressing OS3 cells by Western blotting with anti-AQP4 antibody as shown in Fig. 2A (lane 3). Each fluorescent epitope-tag was identified by Western blotting with anti-RFP or anti-GFP antibody (Fig. 2A, lanes 1 and 2). Native gel electrophoresis (BN-PAGE) of AQP4 M1/M23 co-expressing cells showed multiple bands over 242 kDa corresponding to AQP4 tetramers or orthogonal arrays of particles (OAPs) (Fig. 2B, lane 2). Under fluorescent microscope OS3 cells showed strong filamentous staining by indirect immunofluorescent staining with anti-GFAP antibody (Fig. 3A). Intrinsic AQP4 was not identified by indirect immunostaining with anti-AQP4 antibody and AQP4 mRNA was not identified by RT-PCR (data not shown). AQP4 M1/M23 co-expressing cells showed M1/RFP (Fig. 3B) and M23/GFP (Fig. 3C) cell surface autofluorescences. Sucrose density gradient ultracentrifugation followed by Western blotting with anti-AQP4 antibody showed that AQP4 was preferentially targeted in the detergent-resistant membrane fraction (fraction no. 3, lipid rafts fraction) (Fig. 3D). Lipid rafts marker Ras^{p21} was present in the lipid rafts fraction (Fig. 3E).

2.2. *Cholesterol deprivation causes alteration of AQP4 M1 and AQP4 M23 localization*

To determine whether the cholesterol deprivation alters the AQP4 distribution, AQP4 M1/M23 co-expressing cells were treated with 2.5 mM methyl-β- cyclodextrin or 20 μg/ml simvastatin for up to 48 h. Methyl-β-cyclodextrin reduced cholesterol amount

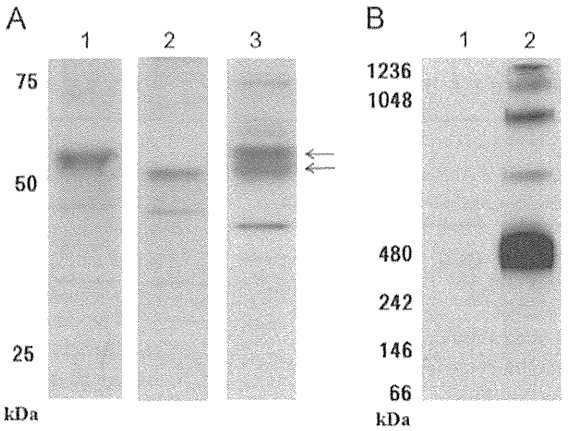

Fig. 2 – Western blotting of fluorescent epitope-tagged AQP4. (A) The lysates of AQP4 M1/M23 co-expressing OS3 cells were separated by SDS-PAGE (12% polyacrylamide gel) and AQP4s were detected by Western blotting. Lane 1: anti-RFP antibody; lane 2: anti-GFP antibody; lane 3: anti-AQP4 antibody. Each expected molecular size of the fluorescent tag was 27 kDa. The expected sizes of AQP4 M1/RFP and M23/AcGFP are 61 kDa and 58 kDa, respectively. Arrows indicate fluorescent epitope–tagged AQP4 M1 and AQP 4M23. (B) Lysate of AQP4 M1/M23 co-expressing OS3 cells was separated by Blue native-PAGE, and the membrane was blotted with anti-AQP4 antibody. Lane 1: mock-transfected OS3 cells; lane 2: AQP4 M1/M23 co-expressing OS3 cells.

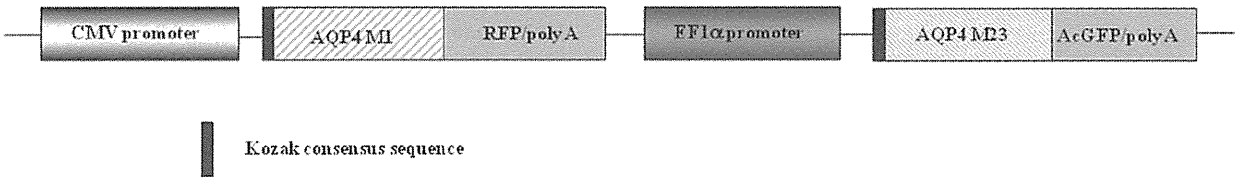

Fig. 1 – Schematic diagram of DNA construct. For AQP4 M1/M23 co-expressing cells, RFP and poly A signal were attached to the C-terminus of AQP4 M1 and AcGFP and poly A signal were attached to the C-terminus of AQP4 M23. Three fragments, i.e., AQP4 M1/RFP/poly A, EF1-α promoter, and AQP4 M23/AcGFP/poly A were inserted into a destination vector, pT-REx-DEST30 (Invitrogen), which contained the CMV promoter upstream by using MultiSite Gateway® Pro for 3-fragment recombination (Invitrogen). Kozak consensus sequence was inserted upstream of each AQP4 isozyme start codon by PCR.

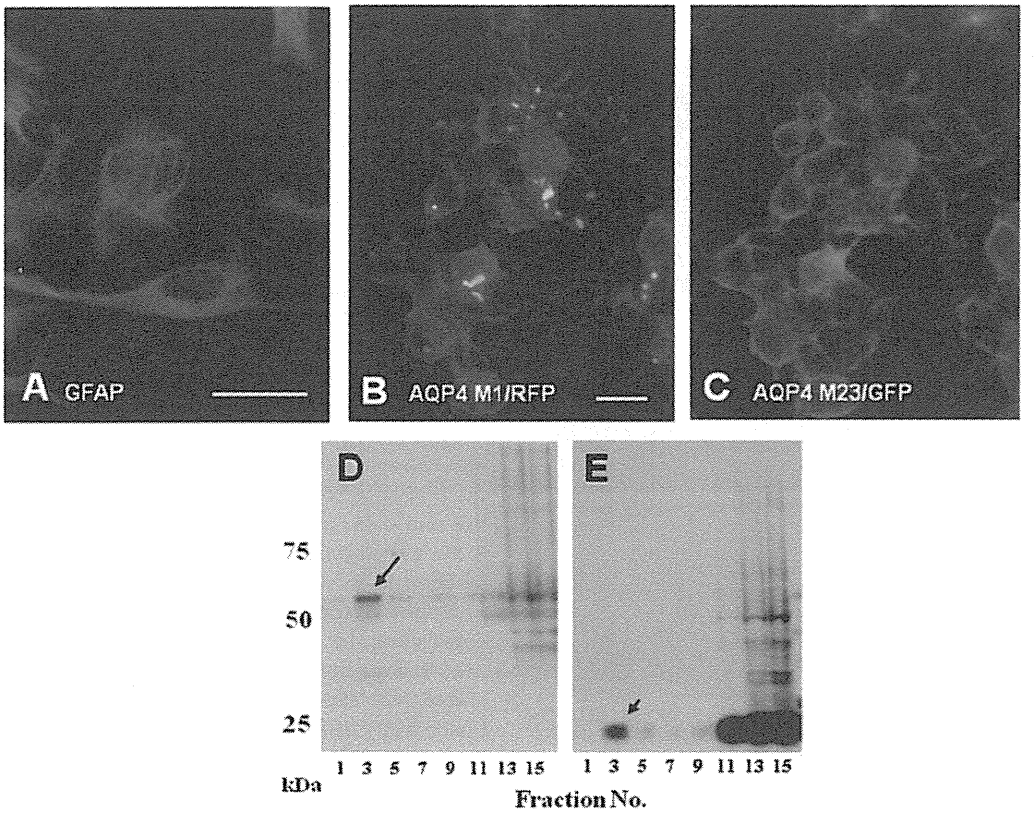

Fig. 3 – OS3 cells showed strong GFAP staining by indirect immunocytochemistry under fluorescent microscope (A). Intrinsic AQP4 was not identified by indirect immunostaining with anti-AQP4 antibody and we also confirmed that OS3 cells do not express AQP4 mRNA by RT-PCR (data not shown). AQP4 M1/M23 co-expressing OS3 cells showed M1/RFP (B) and M23/GFP (C) surface autofluorescence. Bar indicates 10 μm. Sucrose density gradient ultracentrifugation followed by Western blotting with anti-AQP4 or anti-Ras antibody. AQP4 M1/M23 co-expressing cells were subjected to the sucrose density gradient ultracentrifugation followed by Western blot analysis as described in Section 4. One ml of each fraction was collected and used for Western blot analysis probed with anti-AQP4 antibody. We applied the same protein amount of the lysates on the sucrose gradient. Fraction Nos. 1, 3, 5. 7, 9, 11, 13, and 15 were subjected to immunoblot analysis. Fraction no. 3 is the lipid rafts fraction. The membranes were immunoblotted with anti-AQP4 antibody (D) and anti-Ras antibody (E). Arrows indicate fluorescent-tagged AQP4 and small arrow indicates lipid raft marker, Ras. We repeated independent experiments three times with essentially identical results.

Table 1 – Cholesterol measurement in AQP4 M1/M23 co-expressing cells treated with cholesterol-lowering agents.

	Hours after treatment	% Reduction of cholesterol amount
A	Treatment with methyl-β-cyclodextrin	
	0	–
	24	48±3.9*
	48	56±5.1*
B	Treatment with simvastatin	
	0	–
	24	0.2±3.7
	48	20±2.9*

Data are presented as the mean±SEM. Statistical analysis by Mann–Whitney U test was performed.
* $p<0.01$.

over 50% in 48 h (Table 1A). Simvastatin reduced cholesterol amount approximately 20% in 48 h (Table 1B). After treatment with methyl-β-cyclodextrin, AQP4 was partially dislocated from the lipid rafts fraction (fraction no. 3) to the soluble fraction (fraction no. 1) (Fig. 4A and B). The amount of lipid rafts marker Ras^{p21} in the lipid rafts was also decreased and dislocated to the soluble fraction (fraction no. 1) with methyl-β-cyclodextrin treatment (Fig. 4C and D). The treatment with simvastatin showed similar results as the treatment with methyl-β-cyclodextrin (data not shown).

2.3. *Disruption of lipid rafts reduces cytotoxic effects of NMO-IgG*

We performed a CDC assay by use of a live/dead viability/cytotoxicity kit. AQP4 M1/M23 co-expressing cells were

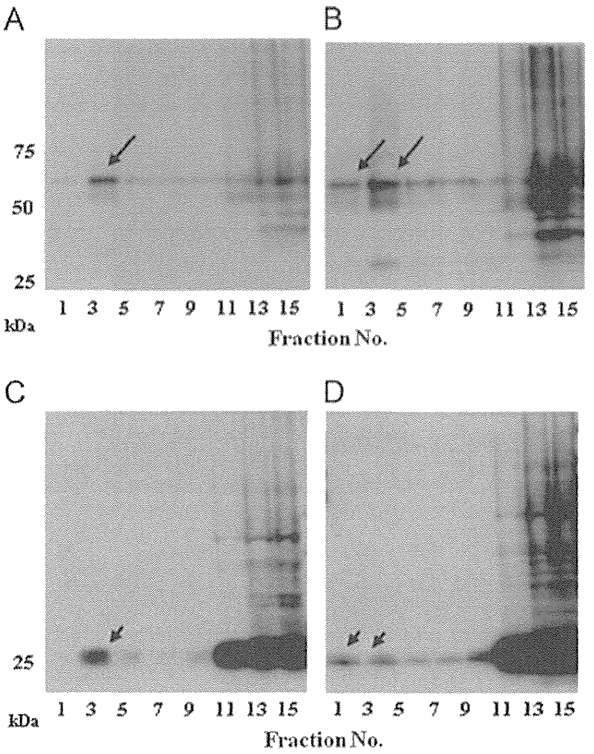

Fig. 4 – Dislocation of AQP4 isoforms by the treatment with cholesterol-lowering agents. AQP4 M1/M23 co-expressing cells (A and C) were treated with 2.5 mM methyl-β-cyclodextrin for 48 h (B and D). Sucrose density gradient ultracentrifugation was performed from each cells, and Western blotting was performed with anti-AQP4 antibody (A, B) or anti-Ras antibody (C, D). Arrows indicate fluorescent-tagged AQP4 and small arrows indicate lipid raft marker, Ras. We performed three to five independent experiments with essentially identical results.

incubated with NMO-IgG and normal human complement serum. Without prior treatment with methyl-β-cyclodextrin, the cells were susceptible to NMO-IgG and complement (red) (Fig. 5B). After treatment with methyl-β-cyclodextrin, AQP4 M1/M23 co-expressing cells were resistant to NMO-IgG and complement (Fig. 5D). To quantitate the effect of methyl-β-cyclodextrin, dead cells were counted after 60 min incubation with control or NMO-IgG (Fig. 5E) with or without 2.5 mM methyl-β-cyclodextrin. Prior treatment with 2.5 mM methyl-β-cyclodextrin for 48 h made AQP4 M1/M23 co-expressing cells significantly resistant to NMO-IgG and complement (Fig. 5E).

We then investigated whether methyl-β-cyclodextrin affected the assembly of AQP4. AQP4 M1/M23 co-expressing cells were cultured with methyl-β-cyclodextrin for up to 48 h. The lysates were separated by BN-PAGE, and Western blotting was performed with anti-AQP4 antibody (Fig. 6A). Even after 48 h treatment with methyl-β-cyclodextrin, the blotting patterns with anti-AQP4 antibody were actually unchanged, which suggests that methyl-β-cyclodextrin treatment did not affect AQP4 assembly.

Additionally, we confirmed the subcellular distribution of AQP4 before and after methyl-β-cyclodextrin treatment. AQP4 M1/M23 co-expressing cells were cultured with or without 2.5 mM methyl-β-cyclodextrin for 48 h. The subcellular fractions were obtained by ultracentifugation and each fraction was separated by SDS-PAGE, and Western blotting was performed with anti-AQP4 antibody (Fig. 6B). The AQP4 was mostly localized in membrane fraction. After cholesterol depletion AQP4 was not internalized into intracellular organelle and stayed in membrane fraction.

3. Discussion

Accumulating evidences have suggested that membrane lipid rafts play important roles in normal biology of the cells such as efficient signal transduction and protein transport (Simons and Toomre, 2000; Mutoh, 2013). In this study, we examined the precise subcellular targeting of AQP4 in an astrocyte cell line and explored the relation with the development of NMO. AQP4 is known to be expressed in ependymal cells and retinal Müller cells as well as in astrocytes, where AQP4 is localized in plasma membrane regions of glial end-feet that face blood vessels and the pia (Nielsen et al., 1997). Interestingly, active NMO lesions show a selective loss of AQP4 immunoreactivity and of GFAP expressing astrocytes (Misu et al., 2007). The two major AQP4 isoforms, M1 and M23, have identical extracellular domain residues, but M1 has 22 more amino acids at the cytoplasmic N-terminus. AQP4 is considered to form hetero-tetramers containing both isoforms in vivo. Therefore, we generated a fluorescent epitope-tagged AQP4 M1 and M23 co-expressing cell line by using single plasmid transfection system, although most of the previous studies were performed using either one of the isoforms expressing cell lines.

The sucrose density gradient ultracentrifugation analysis revealed the majority of AQP4 is targeted to lipid rafts fraction of the plasma membrane in AQP4 M1/M23 co-expressing cells. Native gel electrophoresis revealed that M1/M23 formed OAPs on the cells. Previous study by Suzuki et al. showed that the two N-terminal cysteine residues of AQP4 M1 are palmitoylated (Suzuki et al., 2008), which suggests that AQP4 is the lipid rafts-resident protein, because posttranslational modification by palmitoylation on cysteine residue(s) is considered a dynamic targeting mechanism of transmembrane proteins to lipid rafts (Levental et al., 2010). In fact, AQP4 resides in the Triton X-100-insoluble fraction (lipid rafts fraction), and that perturbation of lipid rafts by the reduction of membrane cholesterol results in the alteration of AQP4 distribution in primary astrocyte culture (Noël et al., 2009). Thus, the present findings are well compatible with these previous findings. In this study, we further demonstrated that the localization of AQP4 in the lipid rafts fraction is very important for NMO-IgG to exert its cytotoxic effects to the cells, because a change in localization of AQP4 from lipid rafts fraction with cholesterol-lowering agents caused a significant reduction in cytotoxicity of NMO-IgG.

It has been reported that CDC is greatly affected by AQP4 assembly in NMO (Phuan et al., 2012). They showed that cells expressing AQP4 M1 are resistant to CDC by NMO-IgG due to the absence of OAP formation, and suggested that modification of AQP4 OAPs might greatly reduce NMO-IgG dependent CDC and NMO pathology (Phuan et al., 2012). In the present

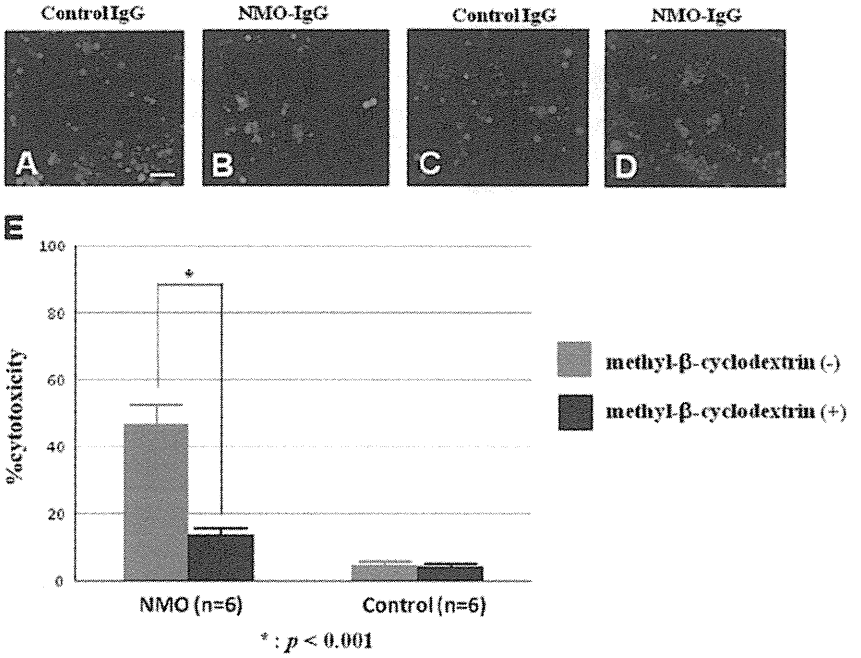

Fig. 5 – Cholesterol deprivation reduces the pathogenic effect of NMO-IgG. Representative fluorescence micrographs of AQP4 M1/M23 co-expressing cells showing live/dead (green/red) cell staining after 60 min incubation with control (A) or NMO-IgG (B). Prior treatment with 2.5 mM methyl-β-cyclodextrin for 48 h made AQP4 M1/M23 co-expressing cells significantly resistant to NMO-IgG and complement (D). Dead cells were counted after 60 min incubation with control or NMO-IgG (E) with or without 2.5 mM methyl-β-cyclodextrin. The cells were photographed under fluorescent microscope. In each experiment, 300–500 live/dead cells were counted. We examined six control sera and six NMO patient sera. Data are shown as the percentage of the live cells and are presented as the mean ± SEM. Statistical analysis by Student's *t*-test was performed, *: $p < 0.001$. Bar indicates 50 μm.

study, we demonstrated that cholesterol reduction altered the localization of AQP4 on the plasma membrane, and the pathogenic effect of NMO-IgG by CDC was significantly reduced. Interestingly, cholesterol lowering agents did not seem to affect the assembly of AQP4 OAPs, indicating that the destruction of AQP4 OAPs is not necessary for the reduction of NMO-IgG pathogenicity. The lipid rafts are now receiving much attention as a device that regulates membrane function in neurological diseases, such as Alzheimer's disease (Ehehalt et al., 2003), Parkinson disease (Fortin et al., 2004), and prion disease (Hooper, 2005). Besides these neurological disorders, in NMO the localization of AQP4 in the lipid rafts seems to be very important for the pathogenicity of this autoantibody.

4. Experimental procedures

4.1. *DNA construct for the AQP4 M1/M23 co-expressing cell line (fig.1)*

A plasmid DNA containing human AQP4 cDNA was purchased from American Type Culture Collection (ATCC, Rockville MD, MGC-22454). To obtain a cell line expressing both AQP4 M1/RFP and AQP4 M23/GFP, we utilized MultiSite Gateway® Pro for 3-fragment recombination (Invitrogen, Carlsbad, CA). The AQP4 M1 PCR product was first inserted into the pCMV6-AC- RFP vector (Origene, Rockville, MD), and the AQP4 M23 PCR product was inserted into the pEF1α-AcGFP-N1 vector (Clontech Laboratories Inc., Mountainview, CA). In addition, the Kozak consensus sequence was inserted upstream of the AQP4 M23 start codon. According to the manufacturer's protocol, we generated three entry clones. The entry clone for element 1 contained AQP4 M1/RFP and a poly A signal, element 2 contained the EF-1α promoter, and element 3 contained AQP4 M23/AcGFP and a poly A signal. These three elements were inserted into a destination vector, pT-REx-DEST30 (Invitrogen), which contained the CMV promoter upstream.

4.2. *Cell cultures*

Mouse astrocyte cell line, OS3 (Ohtani et al., 1992), which expresses an astrocyte-specific marker, glial fibrillary acidic protein (GFAP), was cultured in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Via RT-PCR, we confirmed that OS3 cells do not express AQP4 mRNA. The vectors were transfected by Lipofectamin® (Invitrogen) in OS3 cells. The transfected cells were selected by neomycin (G418). The cells were then isolated by a limiting-dilution method. The established AQP4 M1/M23 co-expressing cell lines were plated onto poly-ornithine-coated chamber slides and were observed under fluorescent microscope. For cholesterol reduction, AQP4 M1/M23 co-expressing cells were cultured with 2.5–5 mM methyl-β-cyclodextrin or 20 μg/ml simvastatin for 48 h.

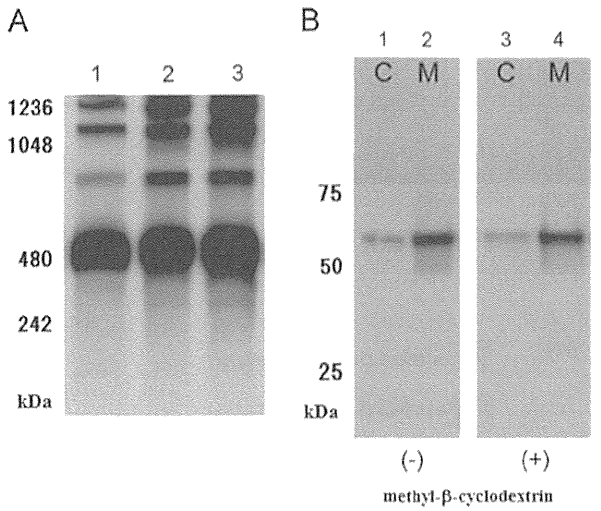

Fig. 6 – Cholestrerol deprivation does not affect AQP4 assembly and subcellular distribution. (A) AQP4 M1/M23 co-expressing cells were cultured with methyl-β-cyclodextrin for up to 48 h. The lysates were separated by BN-PAGE and Western blotting was performed with anti-AQP4 antibody (lane 1: 0 h; lane 2: 24 h; lane 3: 48 h treatment with methyl-β-cyclodextrin). Even after 48 h treatment with methyl-β-cyclodextrin, the blotting patterns with anti-AQP4 antibody were unchanged. (B) AQP4 M1/M23 co-expressing cells were cultured with methyl-β-cyclodextrin for 48 h. The cytosolic and membrane fractions were separated by ultracentrifugation and the lysates were separated by SDS-PAGE. Western Blotting was performed with anti-AQP4 antibody. C: cytosolic fraction, M: membrane fraction. Lanes 1 and 2: no treatment. Lanes 3 and 4: 48 h treatment with methyl-β-cyclodextrin.

4.3. *Immunocytochemistry*

GFAP or AQP4 staining was performed on cells fixed for 10 min at 4 °C with 2% paraformaldehyde and treated for 5 min at room temperature with 0.1% Triton X-100 in PBS, followed by blocking with 3% normal goat serum in PBS. After incubation with the rabbit polyclonal anti-GFAP antibody (DACO, Glostrup, Denmark) or rabbit anti-AQP4 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and the secondary Cy2-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch), the slides were mounted in Fluoromount (Sigma, St. Louis, MO) and were viewed with an epifluorescent microscope.

4.4. *Preparation of lipid rafts*

We prepared membrane lipid rafts from AQP4 M1/M23 co-expressing cells as described previously (Mutoh et al., 2000; Ueda et al., 2010). Briefly, the cells were cultured with or without methyl-β-cyclodextrin or simvastatin. The cells were then collected in phosphate-buffered saline containing para-nitrophenyl phosphate and homogenized using a Teflon glass homogenizer in TNE/Triton X-100 buffer (1% Triton X-100, 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA). After centrifugation at 6000 rpm at 4 °C for 5 min, the lysates were normalized for protein content and were brought to 1.5 M sucrose. A discontinuous sucrose gradient (1.2 M, 8.5 ml; 0.15 M, 2.5 ml) in TNE buffer without Triton X-100 was layered over the lysates. Gradients were centrifuged for 18 h at 38,000 rpm at 4 °C in a Beckman ultracentrifuge. One-ml fractions and the pellet were collected and used for Western blot analysis.

4.5. *Western blotting*

Extracted protein from AQP4 M1/M23 co-expressing cell line were separated by SDS-PAGE under reducing conditions on 5–20% polyacrylamide gradient gels or 12% polyacrylamide gels and transferred to PVDF membranes. The membranes were incubated with rabbit anti-AQP4 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-RFP monoclonal antibody (MBL, Nagoya, Japan) or mouse anti-GFP monoclonal antibody (Clontech) or mouse anti-pan-Ras monoclonal antibody (Calbiochem, Billerica, MA) after blocking with Tris-buffered saline containing 5% non-fat dry milk and 0.05% Tween 20 for one hour at room temperature. Bound antibody was detected by horseradish peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) using the Enhanced Chemiluminescence (ECL) system (Amersham Biosciences, Piscataway, NJ) (Asakura et al., 2007).

For native gel electrophoresis, the cells were lysed with the NativePAGE sample Prep Kit (Invitrogen), containing n-dodecyl-β-D-maltoside, for 15 min on ice. Lysates were centrifuged at 20,000*g* for 30 min at 4 °C, and the pellets were discarded. Total protein content was determined by Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). For Blue Native gel electrophoresis (BN-PAGE), the proteins mixed with Coomassie G-250 were loaded onto a NativePAGE 3–12% Bis-Tris gel along with a NativeMark™ molecular weight marker and electrophoresed with NativePAGE running buffer (Invitrogen). The proteins were blotted onto a PVDF membrane, and the native proteins were fixed by soaking the membranes for 15 min in 8% acetic acid and then destained with ethanol. Membranes were blocked with Tris-buffered saline containing 5% non-fat dry milk, and AQP4 was detected by anti-AQP4 antibody as described above.

4.6. *Patient sera and NMO antibodies*

Sera from six NMO patients fulfilled the revised diagnostic criteria (Wingerchuk et al., 2006) and six normal controls were used for experiments. IgG was purified from sera of NMO patients and normal controls using a Melon gel IgG purification kit (Thermo Fisher Scientific, Rockford, IL) and was quantitated by Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Each purified IgG was used at a concentration of 100 μg/ml.

4.7. *Complement-dependent cytotoxicity (CDC) assay*

For CDC assay, the chamber slides were placed under the microscope with strong lights for several minutes prior to the experiments to fade the autofluorescence. The cells were washed with PBS and incubated at 37 °C for 60 min with

NMO-IgG in PBS containing 2% normal human complement serum (Sigma). For live/dead cell staining, cells were washed with Hanks' BSS and then incubated with 1 μM calcein-AM (live cells, green fluorescence) and 2 μM ethidium homodimer-1 (dead cells, red fluorescence) (Invitrogen) in PBS for 15 min prior to imaging. After live/dead staining, the cells were photographed under fluorescent microscope. In each experiment, 300–500 live/dead cells were counted.

4.8. *Cholesterol measurements*

AQP4 M1/M23 co-expressing cells were cultured with or without methyl-β-cyclodextrin or simvastatin for up to 48 h. Cholesterol contents were measured in cell lysates using the Amplex® Red Cholesterol Assay Kit (Invitrogen) according to the manufacturer's instruction. Cholesterol amounts were normalized by protein amounts.

4.9. *Subcellular fractionation*

To examine the subcellular distribution of AQP4, subcellular fractionation was performed as described previously (Hamano et al., 2005). AQP4 M1/M23 co-expressing cells were cultured with or without 2.5 mM methyl-β-cyclodextrin for 48 h. Then, the cells were collected and suspended in homogenization buffer (40 mM Tris HCl pH 7.4, 250 nM sucrose, 1 mM PMSF, 4 mM $CaCl_2$ and 20 mM $MgCl_2$) and homogenized with a Dounce homogenizer. The homogenates were centrifuged at 2800 rpm for 5 min at 4 °C. Resultant pellets were considered as a nuclear fraction that contains nuclei and cell debris. The supernatant was centrifuged at 75,000 rpm for 35 min in an Ultracentrifuge (Beckman Colter Optima™ MAX-XP Ultracentrifuge) at 4 °C. The resultant pellet was a membrane fraction and the supernatant as a soluble cytosolic fraction. Each fraction was subjected to SDS-PAGE followed by the immunoblotting analysis.

4.10. *Ethics statement*

Informed written consent was obtained from all NMO patients. The study was approved by the Ethics Committee of Fujita health University. We have not performed any animal experiments.

Acknowledgments

We thank Ms. R. Murai for her excellent technical assistance. This study was supported in part by MEXT-Supported Program (S1001034) for the Strategic Research Foundation at Private Universities 2010–2014 (T.M.) and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (KA: 24591279, TM: 22590945) (K.A. and T.M.), and a grant from the Ministry of Health, Labour, and Welfare of Japan (H23-Nanchi-Ippan-017) (T.M.).

The authors report no disclosure related to the present work.

REFERENCES

Asakura, K., Murayama, H., Himeda, T., Ohara, Y., 2007. Expression of L* protein of Theiler's murine encephalomyelitis virus in the chronic phase of infection. J. Gen. Virol. 88, 2268–2274.

Bennett, J.L., Lam, C., Kalluri, S.R., Saikali, P., Bautista, K., Dupree, C., Glogowska, M., Case, D., Antel, J.P., Owens, G.P., Gilden, D., Nessler, S., Stadelmann, C., Hemmer, B., 2009. Intrathecal pathogenic anti-aquaporin-4 antibodies in early neuromyelitis optica. Ann. Neurol. 66, 617–629.

Bradl, M., Misu, T., Takahashi, T., Watanabe, M., Mader, S., Reindl, M., Adzemovic, M., Bauer, J., Berger, T., Fujihara, K., Itoyama, Y., Lassmann, H., 2009. Neuromyelitis optica: pathogenicity of patient immunoglobulin in vivo. Ann. Neurol. 66, 630–643.

Ehehalt, R., Keller, P., Haass, C., Thiele, C., Simons, K., 2003. Amyloidogenic processing of the Alzheimer β-amyloid precursor protein depends on lipid rafts. J. Cell Biol. 160, 113–123.

Fortin, D.L., Troyer, M.D., Nakamura, K., Kubo, S., Anthony, M.D., 2004. Lipid rafts mediate the synaptic localization of α-synuclein. J. Neurosci. 24, 6715–6723.

Hamano, T., Mutoh, T., Tabira, T., Araki, W., Kuriyama, M., Mihara, T., Yano, S., Yamamoto, H., 2005. Abnormal intracellular trafficking of high affinity nerve growth factor receptor, Trk, in stable transfectants expressing presenilin 1 protein. Mol. Brain Res. 137, 70–76.

Hooper, N.M., 2005. Roles of proteolysis and lipid rafts in the processing of the amyloid protein and prion protein. Biochem. Soc. Trans. 33, 335–338.

Landis, D.M., Reese, T.S., 1974. Arrays of particles in freeze-fractured astrocytic membranes. J. Cell Biol. 60, 316–320.

Lennon, V.A., Wingerchuk, D.M., Kryzer, T.J., Pittock, S.J., Lucchinetti, C.F., Fujihara, K., Nakashima, I., Weinshenker, B. G., 2004. A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis. Lancet 364, 2106–2112.

Lennon, V.A., Kryzer, T.J., Pittock, S.J., Verkman, A.S., Hinson, S.R., 2005. IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel. J. Exp. Med. 202, 473–477.

Levental, I., Lingwood, D., Grzybek, M., Coskun, U., Simons, K., 2010. Palmitoylation regulates raft affinity for the majority of integral raft proteins. Proc. Natl. Acad. Sci. USA 107, 22050-22054.

Matsuoka, T., Matsushita, T., Kawano, Y., Osoegawa, M., Ochi, H., Ishizu, T., Minohara, M., Kikuchi, H., Mihara, F., Ohyagi, Y., Kira, J., 2007. Heterogeneity of aquaporin-4 autoimmunity and spinal cord lesions in multiple sclerosis in Japanese. Brain 130, 1206–1223.

Misu, T., Fujihara, K., Kakita, A., Konno, H., Nakamura, M., Watanabe, S., Takahashi, T., Nakashima, I., Takahashi, H., Itoyama, Y., 2007. Loss of aquaporin 4 in lesions of neuromyelitis optica: distinction from multiple sclerosis. Brain 130, 1224–1234.

Mutoh, T., Hamano, T., Tokuda, A., Kuriyama, M., 2000. Unglycosylated Trk protein does not co-localize nor associate with ganglioside GM1 in stable clone of PC12 cells overexpressing Trk (PCtrk cells). Glycoconj. J. 17, 233–237.

Mutoh, T., 2013. Emergence of new roles of lipid rafts in neurological disorders. J. Neurol. Transl. Neurosci. 1, 2.

Nielsen, S., Nagelhus, E.A., Amiry-Moghaddam, M., Bourque, C., Agre, P., Ottersen, O.P., 1997. Specialized membrane domains for water transport in glial cells: high-resolution immunogold cytochemistry of aquaporin-4 in rat brain. J. Neurosci. 17, 171–180.

Noël, G., Tham, D.K., Moukhles, H., 2009. Interdependence of laminin-mediated clustering of lipid rafts and the dystrophin complex in astrocytes. J. Biol. Chem. 284, 19694–19704.

Ohtani, K., Suzumura, A., Sawada, M., Marunouchi, T., Nakashima, I., Takahashi, A., 1992. Establishment of mouse oligodendrocyte/type-2 astrocyte lineage cell line by transfection with origin-defective simian virus 40 DNA. Cell Struct. Funct. 17, 325–333.

Phuan, P.W., Ratelade, J., Rossi, A., Tradtrantip, L., Verkman, A.S., 2012. Complement-dependent cytotoxicity in neuromyelitis optica requires aquaporin-4 protein assembly in orthogonal arrays. J. Biol. Chem. 287, 13829–13839.

Simons, K., Toomre, D., 2000. Lipid rafts and signal transduction. Nat. Rev. Mol. Cell. Biol. 1, 31–39.

Suzuki, H., Nishikawa, K., Hiroaki, Y., Fujiyoshi, Y., 2008. Formation of aquaporin-4 arrays is inhibited by palmitoylation of N-terminal cysteine residues. Biochim. Biophys. Acta 1778, 1181–1189.

Takahashi, T., Fujihara, K., Nakashima, I., Misu, T., Miyazawa, I., Nakamura, M., Watanabe, S., Shiga, Y., Kanaoka, C., Fujimori, J., Sato, S., Itoyama, Y., 2007. Anti-aquaporin-4 antibody is involved in the pathogenesis of NMO: a study on antibody titre. Brain 130, 1235–1243.

Ueda, A., Shima, S., Miyashita, T., Ito, S., Ueda, M., Kusunoki, S., Asakura, K., Mutoh, T., 2010. Anti-GM1 antibodies affect the integrity of lipid rafts. Mol. Cell. Neurosci. 45, 355–362.

Wingerchuk, D.M., Lennon, V.A., Pittock, S.J., Lucchinetti, C.F., Weinshenker, B.G., 2006. Revised diagnostic criteria for neuromyelitis optica. Neurology 66, 1485–1489.

Wingerchuk, D.M., Lennon, V.A., Lucchinetti, C.F., Pittock, S.J., Weinshenker, B.G., 2007. The spectrum of neuromyelitis optica. Lancet Neurol. 6, 805–815.

Wolburg, H., 1995. Orthogonal arrays of intramembranous particles: a review with special reference to astrocytes. J. Hirnforsch. 36, 239–258.

doi:10.1111/ncn3.130

CASE REPORT

Hypertrophic pachymeningitis and encephalitis in a patient with relapsing polychondritis

Ken Nakamura,[1] Keizo Sugaya,[1] Yasuhiro Nakata,[2] Sayuri Shima,[3] Tatsuro Mutoh[3] and Imaharu Nakano[1]

Departments[1] Neurology, and [2] Neuroradiology, Tokyo Metropolitan Neurological Hospital, Tokyo, and [3]Department of Neurology, Fujita Health University School of Medicine, Aichi, Japan

Key words
anti-neutral glycosphingolipid antibody, encephalitis, hypertrophic pachymeningitis, relapsing polychondritis.

Accepted for publication 21 September 2014.

Correspondence
Ken Nakamura
Department of Neurology, Tokyo Metropolitan Neurological Hospital,
2-6-1 Musashidai, Fuchu, Tokyo 183-0042, Japan. Email: m02064kn@jichi.ac.jp

Abstract

Relapsing polychondritis is a rare autoimmune multisystem disease characterized by the inflammation of cartilaginous tissues. Neurological complications with relapsing polychondritis are extremely rare. We report an 81-year-old woman with relapsing polychondritis who subsequently developed encephalitis and cognitive impairment. Brain magnetic resonance imaging showed slightly high signal intensity in the bilateral frontal dura mater using fluid-attenuated inversion recovery. T_1-weighted images with contrast enhancement showed abnormal enhancement of the dura mater, indicating hypertrophic pachymeningitis. To our knowledge, this is the first case report of relapsing polychondritis complicated by both hypertrophic pachymeningitis and encephalitis in the absence of other recognizable autoimmune diseases.

Introduction

Relapsing polychondritis (RP) is an uncommon condition of unknown etiology characterized by costochondritis throughout the body.[1] Central nervous system involvement in RP is rare; encephalitis, but not hypertrophic pachymeningitis, has been reported in some RP patients.[2] Here, we describe the first RP patient, to our knowledge, with both these conditions.

Case report

An 81-year-old woman was admitted to hospital with bilateral ear swelling and erythematous conjunctivae. Ophthalmological examination showed conjunctivitis and scleritis. Computed tomography showed tracheal wall thickening. Auricular biopsies showed cartilage and surrounding tissue inflammation, which are diagnostic criteria for RP. Her symptoms improved remarkably after treatment with oral prednisolone and azathioprine. After 1.5 years, she was readmitted with progressive cognitive impairment. Neurological examination revealed severe dementia, lack of spontaneous speech, rhythmic limb myoclonus, cogwheeling rigidity in the bilateral wrist and elbow joints, snout reflex, bilateral grasp reflex, and no meningeal sign. Blood chemistry showed an increased white blood cell count (16 500/μL) and a high C-reactive protein level (2.95 mg/dL). All serum tests were negative for antibodies, including rheumatoid factor, antinuclear antibody, anti-SSA/SSB antibodies and antineutrophil cytoplasmic antibody (ANCA), but antiglucosylceramide antibodies were detected by immunoblot analysis. The cerebrospinal fluid contained 12/mm^3 white blood cells (100% lymphocytes), 65 mg/dL protein and 80 mg/dL glucose, and was positive for oligoclonal bands. Infectious pathogen tests, including bacterial, fungal, and polymerase chain reaction assays for herpes simplex and tuberculosis, were negative. Extensive evaluations to exclude malignancy were carried out, including whole-body computed tomography and tumor markers measurements; all results were negative.

Brain T_2-weighted magnetic resonance imaging showed diffuse high signal intensity areas in the deep white matter. Fluid-attenuated inversion recovery images showed a slightly high signal intensity area in the bilateral frontal dura mater (Fig. 1a). T_1-weighted images, after intravenous contrast medium injection, showed abnormal focal and asymmetrical enhancement in the bilateral frontal dura mater and falx cerebri (Fig. 1b,c); no enhancement was observed in the pia mater. Diffusion-weighted images showed a high signal intensity area adjacent to the abnormal enhancement in the right frontal dura mater (Fig. 1d).

These findings indicated hypertrophic pachymeningitis and encephalitis associated with RP. After intravenous administration of methylprednisolone (1000 mg/day for 3 days), abnormal enhancement of the dura mater on T_1-weighted images as well as abnormal high signal intensity areas on diffusion-weighted images decreased in size (Fig. 1e–g); the patient showed no further neurological