Retention of tocilizumab and anti-tumour necrosis factor drugs in the treatment of rheumatoid arthritis

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Objectives: The retention of the anti-rheumatic agent tocilizumab (TCZ) has not been well documented in patients with rheumatoid arthritis (RA). We conducted an observational study to compare the retention of TCZ and anti-tumour necrosis factor (TNF) drugs in the treatment of patients with RA.

Method: We reviewed continuation rates and causes of discontinuation of biological agents (biologics) by assessing medical records of patients with RA who were administered biologics at our institute from September 1999 to April 2012, using the Osaka University Biologics for Rheumatic Diseases (BiRD) registry.

Results: A total of 401 patients were included. TCZ, infliximab (IFX), etanercept (ETN), and adalimumab (ADA) were administered to 97, 103, 143, and 58 patients, respectively. There were some differences between the baseline characteristics of the groups. The median duration (range) of TCZ, IFX, ETN, and ADA administration was 2.5 (0.1–12.6), 1.9 (0.0–7.7), 2.9 (0.0–11.3), and 1.3 (0.0–3.4) years, respectively. Continuation rates for TCZ and ETN were significantly higher than those for IFX and ADA. Multivariate analyses showed that discontinuation due to lack or loss of efficacy was significantly less common in the TCZ group than in the other groups. Discontinuation due to overall adverse events was not significantly different between treatment groups.

Conclusion: TCZ and ETN show better retention than IFX or ADA in the treatment of RA.

Many biological agents (biologics) have been developed and used for the treatment of rheumatoid arthritis (RA) (1–3). In Japan, six different biologics are currently available for the treatment of RA (4): the anti-tumour necrosis factor (TNF) drugs infliximab (IFX), etanercept (ETN), adalimumab (ADA), and golimumab; the interleukin (IL)-6 receptor blocker tocilizumab (TCZ); and the T-cell activation blocker abatacept. The retention of a drug compositely reflects efficacy, safety, and patient satisfaction with daily clinical practice. However, the retention characteristics of these biologics have not been established in clinical trials. Although continuation rates of each drug have been reported (5–7), there are few comparative studies that have evaluated their continuation in clinical practice (8–13). In particular, comparative studies that include TCZ are rare, despite its considerable efficacy in patients with RA (14–22). Thus, we conducted an observational study of daily clinical practice to assess and compare the retention of TCZ and the three anti-TNF drugs IFX, ETN, and ADA.

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Method

Study design and approval

This observational retrospective cohort study was approved by the ethics committee of Osaka University Hospital.

The Osaka University Biologics for Rheumatic Diseases (BiRD) registry

The Osaka BiRD registry includes all patients who were administered one of the six biologics TCZ, IFX, ETN, ADA, abatacept, or golimumab for the treatment of rheumatic diseases in Osaka University Hospital between September 1999 and April 2012. Patients who were initiated with biologics before entering the registry are included. In this study, we analysed patients with RA in the Osaka BiRD registry who were treated with TCZ, IFX, ETN, or ADA. Diagnosis of RA was made

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according to the RA classification criteria of the American College of Rheumatology, published in 1987 (23) and in 2010 (24). All patients had shown inadequate responses to treatment with more than one conventional disease-modifying anti-rheumatic drug (DMARD), and were initiated with one of the four biologics as the first biologic, according to the guidelines of the Japan College of Rheumatology (25, 26). To follow up patients who moved to other hospitals, we sent an inquiry in May 2012 to their current physicians. We asked whether the patients were still on the same treatment, and if not, the reason for discontinuation.

Treatments

Biologics were administered according to the package insert drug information for Japan: TCZ, 8 mg/kg as a single intravenous drip infusion administered every 4 weeks; IFX, 3 mg/kg (loading dose up to 10 mg/kg was permitted in July 2009) as a single intravenous drip infusion administered at weeks 0, 2, 6, and every 8 weeks after week 6; ETN, 25 mg or 50 mg as a single subcutaneous injection administered 1 or 2 times a week; and ADA, 40 mg as a single subcutaneous injection administered every 2 weeks. Changes in doses of biologics and other drugs were not recorded in this study. Determination of efficacy, remission, and discontinuation of biologics was dependent on each doctor.

Outcomes

The primary end-point of this study was the duration of drug administration. Specific causes of drug discontinuation were also examined, and cause-specific relative hazards of discontinuation were calculated as the secondary outcome.

Variables

We assessed variables such as sex, age, disease duration, complications (diabetes and interstitial pneumonia, IP), concomitant dosage of methotrexate (MTX) and corticosteroid (equivalent to prednisolone, PRD), the Disease Activity Score in 28 joints based on the C-reactive protein levels as assessed by three variables [DAS28-CRP(3)], and DAS28 components (27) at the initiation of the biologics.

Statistical methods

Drug continuation rates of the biologics were examined using Kaplan–Meier estimates and were compared using the log-rank test. Cases discontinued due to remission or miscellaneous reasons and missing cases were treated as censored cases. Baseline characteristics of the patients are summarized as median (range), and differences between biologics were analysed using the Kruskal–Wallis non-parametric test for continuous variables and the χ^2 test for categorical variables. Cause-specific hazard ratios (HRs) for drug discontinuation were calculated using the Cox proportional hazard model, which was adjusted for variables such as age, DAS28, existence of diabetes or IP, and concomitant dosage of MTX and PRD. We selected variables for each multivariate analysis based on the results of univariate analysis, stepwise logistic regression analysis, and the clinical importance of each variable. All analyses were performed using JMP version 9.0 (SAS Institute Inc., Cary, NC, USA), and a p-value < 0.05 was considered statistically significant. In this study, we followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement for transparent reporting (28).

Results

Study population

Of the 401 patients included in this study, 348 received the first biologic at Osaka University Hospital and 53 patients received it at other hospitals. At the end of April 2012, 322 patients were being followed at our institute, and the other 72 patients were followed through inquiries. We confirmed the duration of drug administration and causes of discontinuation in 394 patients in April 2012. Thus, the missing seven patients and those still on therapy were handled as censored cases in the Kaplan–Meier curves (Supplementary Figure 1).

Patients

TCZ, IFX, ETN, and ADA were administered as the first biologic for the treatment of RA to 97, 103, 143, and 58 patients, respectively. Demographic characteristics of the patients differed between these treatment groups (Table 1). (Where patient data points are missing, the number of patients without missing values is given at each entry.) Statistically significant differences in age, DAS28, disease duration, and concomitant use of MTX or PRD were found between treatment groups.

Drug continuation rates

Drug continuation rates were analysed using Kaplan–Meier estimates (Figure 1). At 1 year, drug continuation rates for TCZ, IFX, ETN, and ADA were 89.4, 73.1, 85.9, and 78.2%, respectively, and at 2.5 years they were 79.3, 47.2, 77.7, and 54.5%, respectively. At 5 years, the drug continuation rates for TCZ, IFX, and ETN were 66.8, 29.8, and 61.9%, respectively. Of note, treatments with TCZ and ETN showed higher continuation rates than treatments with IFX or ADA (log-rank test, $p < 0.001$). The median (range) duration of treatment with TCZ, IFX, ETN, and ADA was 2.5 (0.1–12.6), 1.9 (0.0–7.7), 2.9 (0.0–11.3), and 1.3 (0.0–3.4) years, respectively. The duration of treatment with ADA was shorter

Table 1. Baseline characteristics of the patients.

	Tocilizumab (n = 97)	Infliximab (n = 103)	Etanercept (n = 143)	Adalimumab (n = 58)	p*
Age (years)	56.6 (27.2–81.8)	54.4 (21.5–80.3)	53.0 (18.7–77.5)	60.0 (18.0–76.7)	0.0267
Male:female	17:80	22:81	18:125	10:48	0.3297
Disease duration (years)	7.71 (0.57–38.3)	5.18 (0.23–32.9)	9.60 (0.18–39.4)	6.89 (0.35–30.8)	0.0114
DAS28-CRP(3)	4.38 (2.13–7.34) (n = 79)	4.20 (1.33–6.52) (n = 61)	4.19 (1.96–6.76) (n = 117)	3.84 (1.92–6.69) (n = 48)	0.0089
PRD use (%)	82.1	89.0	73.5	80.0	0.0321
PRD (mg/day)	6.0 (0.0–17.5) (n = 95)	7.5 (0.0–25.0) (n = 91)	5.0 (0.0–20.0) (n = 132)	5.0 (0.0–20.0) (n = 55)	< 0.0001
MTX use (%)	62.1	98.9	72.0	80.4	< 0.0001
MTX (mg/week)	6.0 (0.0–16.0) (n = 95)	8.0 (0.0–12.0) (n = 93)	6.0 (0.0–10.5) (n = 132)	6.0 (0.0–12.0) (n = 56)	< 0.0001
IP (%)	15.5	7.8	10.5	10.3	0.3832
Diabetes (%)	8.3	12.6	12.6	10.3	0.7390

DAS28-CRP(3), Disease Activity Score in 28 joints based on the C-reactive protein levels as assessed by three variables; PRD, prednisolone; MTX, methotrexate; IP, interstitial pneumonia.
Values are given as median (range) or percentage.
*By the χ^2 test for categorical variables and the Kruskal–Wallis test for continuous variables.

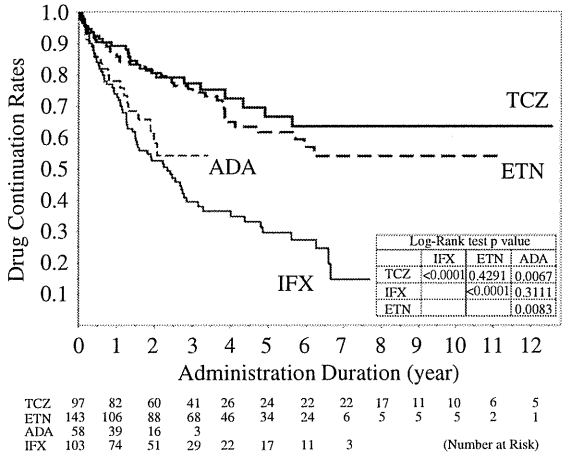

Figure 1. Kaplan–Meier curves for time to discontinuation of treatment with TCZ, IFX, ETN, and ADA. Cases discontinued due to remission or miscellaneous reasons were treated as censored cases. Drug continuation rates were compared using the log-rank test.

than that with the other biologics because ADA was not approved in Japan until June 2008.

Causes of discontinuation

The causes of discontinuation of the biologics are shown in Table 2. The difference between lack of efficacy and loss of efficacy was not clearly defined in this study. In fact, 88.9% (16/18) of discontinuation due to lack of efficacy occurred within 6 months and 98.4% (60/61) of discontinuation due to loss of efficacy occurred after over 6 months in our registry. The three anti-TNF drugs IFX, ETN, and ADA were mainly discontinued because of lack or loss of efficacy. By contrast, the most common cause of discontinuation of TCZ was any adverse event such as a serious infection. There were five deaths during the study period: one in the IFX group (due to IP), three in the TCZ group (bacterial arthritis, brain infarction, colon cancer), and one in the ETN group (suicide).

Cause-specific cumulative discontinuation rates were assessed using Kaplan–Meier estimates (Figure 2). The cumulative discontinuation rate due to lack or loss of efficacy rose linearly in each treatment group, and the gradients of the plots were large in the IFX and ADA groups, low in the TCZ group, and intermediate in the ETN group. Cumulative discontinuation rates due to adverse events increased linearly until approximately 2 years, after which the increase slowed down in each group. Of note, discontinuation due to hypersensitivity (including systemic or injection-site acute reaction) occurred mainly before 1.5 years in the IFX group. Cumulative discontinuation rates due to remission were low, up to 11% in each group.

Multivariate analyses

We calculated the HRs of discontinuation due to each specific cause using multivariate Cox proportional hazards regression (Table 3). [Adjustment was made for sex, age, disease duration, DAS28, concomitant dosage of PRD and MTX, and complications (IP and diabetes), unless stated otherwise.] Discontinuation due to overall unfavourable causes was significantly higher in the IFX and ADA groups than in the TCZ group [IFX vs. TCZ, HR 3.23, 95% confidence interval (CI) 1.81–5.94, p < 0.0001; ADA vs. TCZ, HR 2.82, 95% CI 1.42–5.58, p = 0.0035] and was not significantly different between TCZ and ETN (ETN vs. TCZ, HR 1.53, 95% CI 0.88–2.71, p = 0.1304). HRs for discontinuation due to lack or loss of efficacy were found to be significantly higher in the anti-TNF drug groups than in the TCZ group (IFX vs. TCZ,

AQ1 Table 2. Causes of treatment discontinuation.

Causes of discontinuation	Tocilizumab	Infliximab	Etanercept	Adalimumab
Lack of efficacy*	1 (3.7)	8 (10.3)	5 (8.8)	4 (16.0)
Loss of efficacy*	4 (14.8)	30 (38.5)	18 (31.6)	9 (36.0)
All adverse events	18 (66.7)	29 (37.2)	20 (35.1)	7 (28.0)
Hypersensitivity**	1 (3.7)	15 (19.2)	4 (7.0)	1 (4.0)
Serious infection	7 (25.9)	6 (7.7)	7 (12.3)	3 (12.0)
Malignancy	3 (11.1)	5 (6.4)	5 (8.8)	0
Vascular disease	2 (7.4)	0	1 (1.8)	0
Interstitial pneumonia	1 (3.7)	3 (3.8)	0	1 (4.0)
Skin rash	1 (3.7)	0	1 (1.8)	1 (4.0)
Neutropaenia	2 (7.4)	0	1 (1.8)	0
Other adverse events	1 appendicitis	0	1 suicide	1 elevation of CK AQ2
Miscellaneous**	2 (7.4)	4 (5.1)	7 (12.3)	2 (8.0)
Remission*	2 (7.4)	7 (9.0)	7 (12.3)	3 (12.0)
Total discontinuation	27	78	57	25

Values are given as the number (percentage) of patients who discontinued biologics for each reason.
'Hypersensitivity' includes both systemic and injection-site reactions.
'Miscellaneous' include patient preferences, finances, and pregnancies.

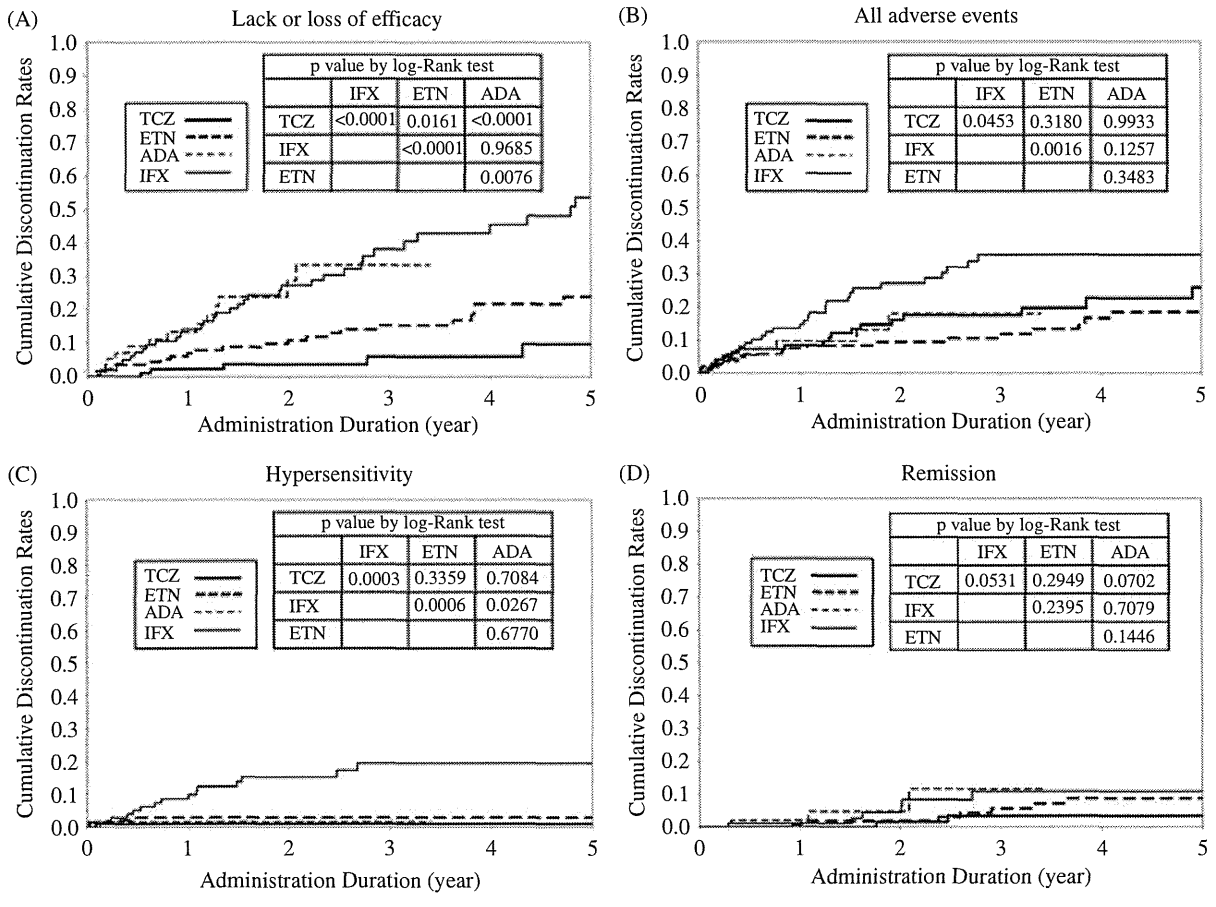

Figure 2. Cumulative discontinuation rates of the four drugs for specific reasons: (A) lack or loss of efficacy, (B) overall adverse events, (C) hypersensitivity (systemic or injection-site reaction), and (D) remission. Drug continuation rates were compared using the log-rank test.

HR 8.80, 95% CI 3.33–28.1, $p < 0.0001$; ETN vs. TCZ, HR 3.45, 95% CI 1.34–10.6, $p = 0.0088$; ADA vs. TCZ, HR 10.5, 95% CI 3.60–35.6, $p < 0.0001$).

HRs for discontinuation due to overall adverse events did not differ between treatment groups. HRs for discontinuation due to severe infection did not differ 210

Table 3. Hazard ratio (HRs) of discontinuation of the four drugs due to specific causes.

	Tocilizumab (reference)	Infliximab		Etanercept		Adalimumab		
		HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p	
All unfavourable causes	1 (ref.)	3.23 (1.81–5.94)	< 0.0001	1.53 (0.88–2.71)	0.1304	2.82 (1.41–5.57)	0.0035	*1
Lack or loss of efficacy	1 (ref.)	8.80 (3.33–28.1)	< 0.0001	3.45 (1.34–10.6)	0.0088	10.5 (3.60–35.6)	< 0.0001	*1
All adverse events	1 (ref.)	1.61 (0.73–3.55)	0.2360	0.96 (0.47–1.96)	0.9084	0.93 (0.32–2.43)	0.8921	*1
Serious infection	1 (ref.)	0.96 (0.31–2.88)	0.9375	0.74 (0.25–2.16)	0.5696	0.83 (0.18–3.06)	0.7901	*2
Hypersensitivity	1 (ref.)	23.1 (2.73–548)	0.0022	1.79 (0.22–37.4)	0.6079	2.96 (0.11–83.3)	0.4735	*3
Remission	1 (ref.)	3.00 (0.63–21.8)	0.1734	1.34 (0.26–9.99)	0.7351	3.05 (0.47–24.4)	0.2337	*1

CI, confidence interval.
The results of Cox proportional hazards model analyses are shown.
*1 Adjusted for sex, age, disease duration, DAS28, concomitant dosages of PRD and MTX, and complications (IP or diabetes).
*2 Adjusted for age.
*3 Adjusted for age, disease duration, DAS28, and concomitant dosages of MTX and PRD.
'Hypersensitivity' includes both systemic and injection-site reactions.

significantly between treatment groups after adjustment for age. HRs for discontinuation due to hypersensitivity (systemic or injection site) were significantly higher in the IFX group than in the other groups (IFX vs. TCZ, HR 23.1, 95% CI 2.73–548, p = 0.0022), after adjustment for sex, age, DAS28, disease duration, and concomitant dosage of MTX or PRD. However, HRs for discontinuation due to remission did not differ significantly between treatment groups.

Discussion

To the best of our knowledge, this is the first report to compare retention (> 3 years) of initial biologics, including TCZ, in biologic-naïve patients with RA. In this study, TCZ and ETN showed similar retention for the treatment of RA, and both were better than IFX and ADA. Multivariate analyses showed that this difference was not mainly attributable to adverse events, such as infections, but to durability of efficacy.

In general, our results are consistent with four previous observational studies reporting continuation rates for anti-TNF drugs (10–14). From the Lombardy Rheumatology Network (LOHREN) registry (n = 1064), Marchesoni et al showed that treatment continuation rates for IFX, ETN, and ADA were approximately 56, 57, and 72%, respectively, at 2.5 years. The risk of discontinuation was similar for poor efficacy and adverse events (10). In the Swiss Clinical Quality Management RA cohort (n = 2364), Du Pan et al showed that treatment continuation rates for IFX, ETN, and ADA were approximately 51, 58, and 61%, respectively, at 2.5 years (11). Lack or loss of drug efficacy represented the most common single cause of treatment discontinuation. In an analysis of the DANBIO registry (n = 2326), which is a nationwide Danish registry of rheumatology patients, Hetland et al showed that continuation rates for IFX, ETN, and ADA were approximately 44, 67, and 60%, respectively, at 2.5 years. Discontinuation due to lack of efficacy was more common than discontinuation due to adverse events (12). Analysing the Italian Study Group on Early Arthritides (GISEA) registry (n = 853), Iannone et al found that continuation rates for IFX, ETN, and ADA were approximately 52, 65, and 52%, respectively, at 2.5 years (13). These four reports agreed that IFX showed the lowest continuation rate among the three anti-TNF drugs and that lack or loss of efficacy was the main cause of discontinuation. These findings are consistent with the results from our registry.

The 1-year drug continuation rate of TCZ in real-life clinical practice was analysed in two studies (6, 7). Takeuchi et al reported a continuation rate of 71.1% for TCZ at 52 weeks in the REACTION cohort study (n = 232) (6). Similarly, Leffers et al reported a continuation rate of 64% for TCZ at 48 weeks in the DANBIO registry (n = 178) (7). The continuation rate of TCZ was reported as 88, 75, and 68% at 1, 2.5, and 5 years, respectively, in a combined analysis of six clinical studies and their extensions in Japan (n = 601) conducted by Nishimoto et al (14). The continuation rate of TCZ reported by Nishimoto et al was similar to that in our registry, which was higher than that reported in the REACTION study and DANBIO registry. This difference may be due to the fact that the former two studies included patients who participated in clinical studies whereas the latter included non-biologic-naïve patients. Of note, all these studies, including ours, found a similar tendency, indicating that TCZ is discontinued more commonly due to adverse events than to lack or loss of efficacy.

Comparisons of TCZ and anti-TNF drugs in Japanese patients with RA have been made using data from the CABUKI registry (8) and the REAL registry (9). From the CABUKI registry (n = 247, including non-biologic-naïve patients), Yoshida et al concluded that TCZ and anti-TNF drugs showed similar drug retention rates (8). However, the CABUKI registry was considered to be too small to identify drug continuation rates longer than 1 year because only seven patients were still taking TCZ at

1.5 years. From the REAL registry (n = 1022, including non-biologic-naïve patients), Sakai et al compared TCZ and anti-TNF drugs in Japanese patients with RA and showed that the continuation rates of TCZ, IFX, and ETN at 2.5 years were approximately 67, 67, and 72%, respectively, and concluded that IFX and TCZ were significantly associated with treatment discontinuation due to adverse events compared with ETN (9). The conclusion from the REAL registry differs from our results. This disparity may be explained by two differences between the studies. First, the observation period of the REAL registry was shorter than that of our registry. Second, the continuation rates of anti-TNF drugs in the REAL study were much higher than in our study and in previous reports. These differences suggest that unreported factors, such as the dosage of IFX or changes in the concomitant dosage of DMARDs and PRD, raised the continuation rates of anti-TNF drugs in that study. The continuation rate of TCZ in the REAL study was slightly lower than that in our registry and in the report of Nishimoto et al (14), probably because Nishimoto et al reported their results on the basis of clinical studies and our registry also included 29 patients (29.9%) who participated in clinical trials.

Our study has several limitations. First, our study was observational; therefore, some biases may be present, such as in the selection of patients and the choice of the biologics, although similar biases are common to all observational studies. Indeed, ADA was administered to patients who were older than those administered ETN and IFX. This bias may have lowered the observed retention rate of ADA. Concomitant use of methotrexate (MTX) was significantly higher in patients who were administered IFX than other biologics. This bias may have elevated the retention rate of IFX. Furthermore, TCZ was administered to patients who had significantly higher DAS28 scores than those administered ADA. ETN was administered to patients who had longer disease duration than those administered IFX. Concomitant glucocorticoid dosage was the highest in the IFX group, the lowest in the ETN and ADA groups, and intermediate in the TCZ group. However, the influence of these biases was unclear. Nonetheless, shorter disease duration was associated with discontinuation due to both poor efficacy and remission in our study (data not shown). Given the relatively small sample size in this study (401 patients), we limited the number of variables in the multivariate analyses and the reported 95% CIs were broadened. Nevertheless, observational cohort studies such as this one offer the only way to assess retention of biologics in real clinical settings. Importantly, TCZ clearly showed better retention than IFX and ADA, even in our small cohort. However, changes in the dosage of IFX or concomitant DMARDs and PRD were not analysed in this study, and further study is necessary to determine risk factors for discontinuation, particularly due to infection. For example, the dosage of PRD at baseline was not detected as a risk factor for discontinuation due to infections in our study (data not shown).

In conclusion, our cohort study showed that TCZ had excellent retention in the treatment of RA, and the continuation rate of TCZ was higher than that of IFX and ADA. The continuation rate of ETN was not statistically different from that of TCZ. Discontinuation due to lack or loss of efficacy was significantly less common in the TCZ-treated group. Although TCZ was discontinued mainly due to adverse events, discontinuation due to adverse events was not significantly different between TCZ and anti-TNF drugs.

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Research Article

DNA Damage in Rheumatoid Arthritis: An Age-Dependent Increase in the Lipid Peroxidation-Derived DNA Adduct, Heptanone-Etheno-2′-Deoxycytidine

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Objective. To evaluate what types of DNA damages are detected in rheumatoid arthritis (RA). *Methods.* The DNA adducts such as 8-oxo-hydroxy-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), 1,N^6-etheno-2′-deoxyadenosine (εdA), and heptanone-etheno-2′-deoxycytidine (HεdC) in genomic DNAs, derived from whole blood cells from 46 RA patients and 31 healthy controls, were analyzed by high-performance liquid chromatography tandem mass spectrometry, and their levels in RA patients and controls were compared. In addition, correlation between DNA adducts and clinical parameters of RA was analyzed. *Results.* Compared with controls, the levels of HεdC in RA were significantly higher ($P < 0.0001$) and age dependent ($r = 0.43$, $P < 0.01$), while there was no significant difference in 8-oxo-dG and εdA accumulation between RA patients and controls. HεdC levels correlated well with the number of swollen joints ($r = 0.57$, $P < 0.0001$) and weakly with the number of tender joints ($r = 0.26$, $P = 0.08$) of RA patients, while they did not show a significant association with serological markers such as C-reactive protein and matrix metalloproteinase 3. *Conclusion.* These findings indicate that HεdC may have some influence on the development of RA and/or its complications.

1. Introduction

Rheumatoid arthritis (RA) is a systemic, chronic inflammatory disease of the joints and surrounding tissues, accompanied by intense pain, irreversible joint destruction, and systemic complications [1]. Its etiology has not been fully clarified yet, but oxidative stress is one of the pathological factors, which contribute to its development [2–5]. Shao et al. recently reported detecting the presence of DNA damage and deficiency of the DNA repair enzyme, ataxia telangiectasia mutated (ATM), in RA T cells [6]. Moreover, a DNA oxidation product, 8-oxo-hydroxy-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), has been shown to be highly expressed in RA patients [2]. DNA damage induced by exposure to ionizing radiation, ultraviolet light, and exogenous or endogenous chemical mutagens causes DNA strand breakages, which are thought to be mutagenic, carcinogenic, and aging factors [2]. Additionally as new DNA damage, lipid peroxidation-derived DNA adducts have been recently reported in human [7], and lipid peroxidation has been also considered as a factor of the pathogenesis or the local inflammatory response of RA [4, 8–10]. Representative

DNA adducts are 8-oxo-dG, 1,N^6-etheno-2′-deoxyadenosine (εdA), and heptanone-etheno-2′-deoxycytidine (HεdC); the latter two of which are direct reactive oxygen species- (ROS-) derived and lipid peroxidation-derived adducts. HεdC is also deemed a 4-oxo-2(*E*-) nonenal (4-ONE-) generated product [7].

On the basis of these previous findings, in this study, in order to investigate the role of DNA adducts in the development of RA, we examined the amount of three DNA adducts, 8-oxo-dG, εdA, and HεdC, in whole blood cells from RA patients and healthy controls by means of a sensitive technique, high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS).

2. Materials and Methods

2.1. Study Participants. The study population consisted of 46 RA (38 women and 8 men) patients and 31 healthy controls (14 women and 17 men). All RA patients, age of median (range): 52 (22–81) years, met the 1987 revised American College of Rheumatology (ACR) classification criteria for RA [11]. The median (range) number of tender joints and swollen joints were 2 (0–28) and 2 (0–12), respectively, while the median (range) serum C-reactive protein (CRP) concentration was 0.83 (0.04–6.2) mg/dL (Table 1). Patient records showed that 19 had been treated with nonsteroidal anti-inflammatory drug (NSAID) (one with celecoxib, a selective cyclooxygenase 2 (Cox-2) inhibitor and 18 with other nonselective Cox-2 inhibitors), 29 with methotrexate, 29 with prednisolone, and 5 with biologics (two with etanercept and one each with infliximab, adalimumab, and tocilizumab). The control group comprised 31 healthy volunteers without any chronic disease, with the median (range) age of 36 (22–57) years. The subjects' written consent was obtained according to the Declaration of Helsinki, and the study has been approved by the ethics committee of Osaka University Hospital. The clinical findings and laboratory data from RA patients were obtained on the same day that the peripheral blood samples were drawn, so that some laboratory test data were missing.

2.2. DNA Purification and Digestion. Genomic DNA was purified from whole blood cells with the DNA Extractor WB Kit using the Sodium Iodide method (Wako, Osaka, Japan) with the addition of deferoxamine mesylate (Sigma Aldrich Japan KK, Tokyo, Japan) to all solutions for adjustment to a final concentration of 0.1 mM in accordance with the manufacturer's protocol, after which the purified DNA was stored at −80°C. Isolated DNA was digested to nucleosides with the nuclease P1 method previously described [12]. For DNA adduct analysis, 50 μL of 30% dimethyl sulfoxide was added to each of the DNA samples and then subjected to LC-MS/MS.

2.3. DNA Adduct Standards and Stable Isotope Standards. Values for the three DNA adducts, 8-oxo-dG, εdA, and HεdC, were assessed and their chemical structures are shown in Figure 1(a). The 4-ONE DNA adduct HεdC was synthesized according to the previously published methods [13]. 8-oxo-dG and εdA were obtained from Sigma Aldrich Japan. [U-$^{15}N_5$]-8-oxo-dG was kindly provided by Dr. Shinya Shibutani, State University of New York, Stony Brook, NY, USA, and other DNA adduct stable isotope standards were synthesized according to the previously described methods [12] using [U-$^{15}N_5$]- or [U-$^{15}N_3$]-deoxynucleoside purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

2.4. LC-MS/MS Instrumentation. Chromatography was performed with a Quattro Ultima Pt triple stage quadrupole mass spectrometer (Waters-Micromass, Milford, MA, USA) equipped with the alliance 2695 separation module, and a 2487 Dual λ Absorbance Detector (Waters, Milford, MA, USA) was used. An aliquot (20 μL) of the digested DNA sample was injected and separated with the Shim-Pack XR-ODS column (3.0 mm × 75 mm, Shimadzu, Japan), eluted in a linear gradient of 5% to 30% methanol in water between 0 and 27 min, of 30% to 80% between 27 and 35 min, and then kept in 80% methanol between 35 and 40 min at a flow rate of 0.2 mL/min. The following experimental conditions were used: ion source temperature: 130°C, desolvation temperature: 380°C, cone voltage: 35 V, and collision energy: 15 eV, desolvation gas flow rate: 700 L/h, cone gas flow rate: 35 L/h, collision gas: argon. The positive ion mode was used for multireaction monitoring (MRM) analysis under the following conditions (cone voltage, collision energy, and precursor ion → product ion): [U-$^{15}N_5$]-8-oxo-dG: (40, 12, 288.8 → 172.8), [U-$^{15}N_5$]-εdA: (35, 14, 280.9 → 164.9), [U-$^{15}N_3$]-HεdC: (35, 10, 367.0 → 251.0), 8-oxo-dG: (40, 12, 283.9 → 167.9), εdA: (35, 14, 275.9 → 159.9), and HεdC: (35, 10, 364.0 → 248.0).

2.5. DNA Adduct Quantification. Each of the DNA adducts was quantified by calculating the ratio of the peak area of the target adducts to that of its isotope as follows: peak area of the potential DNA adduct/peak area of the internal standard/amount of 2′-deoxyguanosine (dG). The amount of dG in each DNA sample was estimated by monitoring the dG peak area at 254 nm with the UV-visible detector connected in series with LC-MS/MS system. QuanLynx (ver. 4.0) software (Waters-Micromass, Manchester, UK) was used to create standard curves and calculate the adduct concentrations.

2.6. Statistics. Values for 8-oxo-dG, εdA, and HεdC in RA patients and healthy controls were compared by using the mean of analysis of covariance (ANCOVA), where the model included age and sex as covariates. Before this analysis, the values were log-transformed because 8-oxo-dG, εdA, and HεdC were not normally distributed. The results were back-transformed and then expressed as adjusted geometric mean ratios and their 95% confidence interval (CI). Associations between the levels of DNA adducts and clinical or laboratory test findings observed in RA patients were analyzed with the Spearman rank correlation coefficient. *P* values less than 0.05 were considered significant. All the analyses were performed with the SAS version 9.3 for Windows (SAS Institute, Cary, NC, USA). To match the number of sex between RA patients and controls, 14 women and 7 men were randomly chosen.

Table 1: Characteristics of RA patients and controls enrolled in the study.

	RA patients	Controls
Number of subjects	46	31
Females	38 (83%)	14 (45%)
Age (years, median (range))	52 (22–81)	36 (22–57)
Disease duration (years)	10 (1–40)	
Use of NSAIDs	41%	
	(Nonselective Cox-2 inhibitor 39%, celecoxib 2%)	
Use of MTX	63%	
Dosage of MTX (mg/week)	6 (0–12)	
Use of prednisolone	63%	
Dosage of prednisolone (mg/day)	3 (0–7.5)	
Use of biologics	11%	
Number of tender joints	2 (0–28)	
Number of swollen joints	2 (0–12)	
Class	2 (1–3)	
Stage	3 (1–4)	
Positive for RF	95%	
Positive for ACPA	93%	
CRP (mg/dL)	0.83 (0.04–6.2)	

NSAIDs: nonsteroidal anti-inflammatory drugs; Cox-2: cyclooxygenase 2; MTX: methotrexate; RF: rheumatoid factor; ACPA: anticitrullinated protein antibody.

For comparison of 8-oxo-dG, εdA, and HεdC in RA patients and healthy controls, the two-sample test was conducted for log-transformed data. In addition, the bootstrap method was used to generate the 10,000 sets of sample matched by sex, and each sample was analyzed by two-sample test to assess the robustness of the conclusion from the matched analysis.

3. Results

3.1. Detection of 8-oxo-dG, εdA, and HεdC. Purified DNAs from peripheral whole blood cells from RA patients (n = 46) and controls (n = 31) were subjected to LC-MS/MS for the detection of specific DNA adducts. Several major peaks of DNA adducts were observed, and among these, the peaks corresponding to 8-oxo-dG, εdA, and HεdC and the stable isotope internal standards are shown in Figure 1(b). The volume of these DNA adducts was calculated with the method described in Section 2.

3.2. HεdC Is Elevated in Whole Blood Cells of RA Patients. The results for 8-oxo-dG and εdA are shown in Figure 2. The median (range) levels of 8-oxo-dG per 10^9 bases in RA patients and controls are 176.4 (52.5–449) and 127.1 (58.1–372), respectively, and they did not differ. Moreover, there was no significant difference in εdA between the two groups, RA: 29 (2.6–1635) (median (range)) per 10^9 bases versus control: 34.7 (0.2–121) per 10^9 bases. Four patients with RA showed an increase in εdA accumulation, but no association was detected between these values and disease activity parameters such as CRP and the number of involved joints. However, HεdC levels were significantly higher in RA patients, 10.3 (0.3–119) (median (range)) per 10^9 bases than those in controls, 0.33 (0.3–17.8) per 10^9 bases ($P < 0.0001$) (Figure 3(a)), and this significance was observed in all age-interval analyses (mean ratio at 35: $P = 0.0009$; for other age intervals: $P < 0.0001$) as shown in Figure 3(b), and the mean of level of the adduct HεdC in RA patients was approximately five times higher than that in controls. Moreover, in order to match the number of sex between RA patients and controls, the datum of 14 women and 7 men were randomly selected, and it was confirmed that HεdC (but not 8-oxo-dG and εdA) levels were significantly higher in RA patients than those in controls ($P < 0.0001$, data not shown).

3.3. Significant Positive Association between HεdC Levels and Number of Swollen Joints and Aging. Correlation analyses were performed to explore whether HεdC levels correlated with clinical or laboratory test findings including platelet count, concentrations of CRP, serum amyloid A (SAA), albumin, and matrix metalloproteinase 3 (MMP3), as well as levels of total cholesterol, triglyceride, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol in addition to the number of swollen joints and tender joints. The results are shown in Table 2. Slightly positive, but not statistically significant, correlations were observed between the levels of HεdC and the number of platelets, concentrations of CRP, SAA, and MMP3, and the number of tender joints. On the other hand, HεdC levels showed a significantly positive correlation with the number of swollen joints ($r = 0.57$, $P < 0.0001$) and the total number of swollen and tender joints ($r = 0.48$, $P = 0.0005$) (Table 2 and Figure 4) and were also age-dependent ($r = 0.43$, $P = 0.003$) (Table 2 and Figure 3(b)). Finally, HεdC levels showed no associations with disease duration, class and stage, positivity of autoantibodies such as

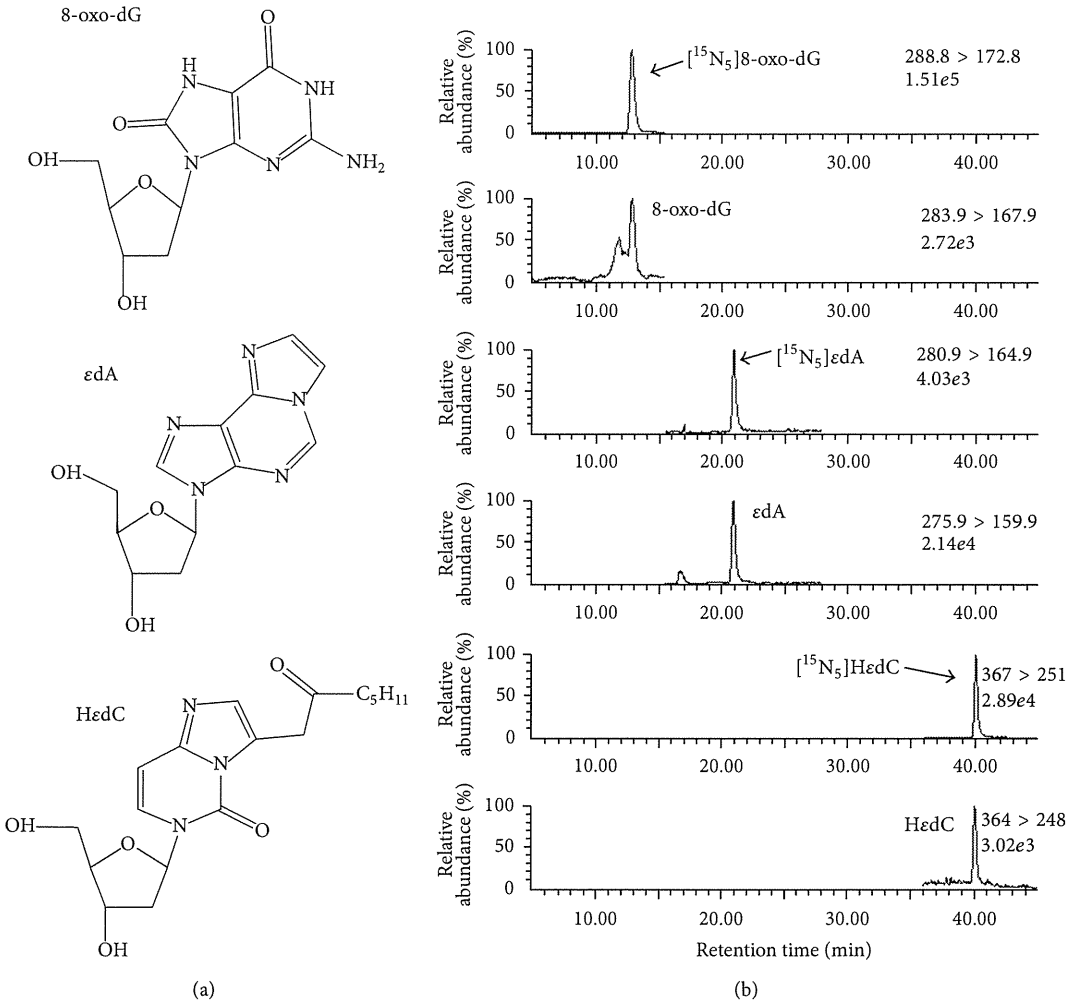

FIGURE 1: Chemical structures of DNA adducts and their corresponding peaks detected by LC-MS/MS. (a) Chemical structures of the DNA adducts 8-oxo-hydroxy-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), 1,N^6-etheno-2′-deoxyadenosine (εdA), and heptanone-etheno-2′-deoxycytidine (HεdC). (b) DNA adducts were quantified by calculating the ratio of the peak of the DNA adduct to that of its standard isotope. The respective peaks of the standard isotope and the corresponding sample are shown.

rheumatoid factor and anticitrullinated protein antibody, or use of NSAID, methotrexate, or prednisolone.

4. Discussion

The study presented here demonstrated that the levels of HεdC, a lipid peroxidation-derived DNA adduct, were significantly higher in the whole blood of RA patients than in that of controls and that this difference increased with aging. To the best of our knowledge, this is the novel finding. In addition, the strong correlation between HεdC levels and the number of swollen joints suggests that HεdC may play a pathological role in the development of RA.

The etiology of RA has not been fully clarified, but it is generally accepted that interaction between genetic predispositions and environmental factors contributes to its development [1, 2]. Several environmental factors such as smoking, infectious agents, environmental toxins, or nutrients have been found to lead to DNA modification, including the enhanced presence of DNA adducts, which affects gene activation or DNA replication [14]. These factors are also believed to have a significant influence on the development of RA. The assessment of redox status in RA has been extensively studied. In particular, a lot of oxidative stress markers, including DNA damage, have been assessed in different samples (blood, urine, and synovial fluid) of RA patients. In previous studies, the relationship between RA and oxidative stress in genomic DNA has been well documented, and ROS, produced by neutrophils infiltrating into the synovial fluid in RA, have been implicated in the pathogenesis of the disease [5, 15–17]. Oxidative products have been also found to be elevated in the lipids and proteins of RA patients [15]. Heightened DNA damage has been demonstrated through assessments by means of alkaline comet assays [5, 6] or indicated by marked elevation of 8-oxo-dG in urine, synovial fluids, and primary

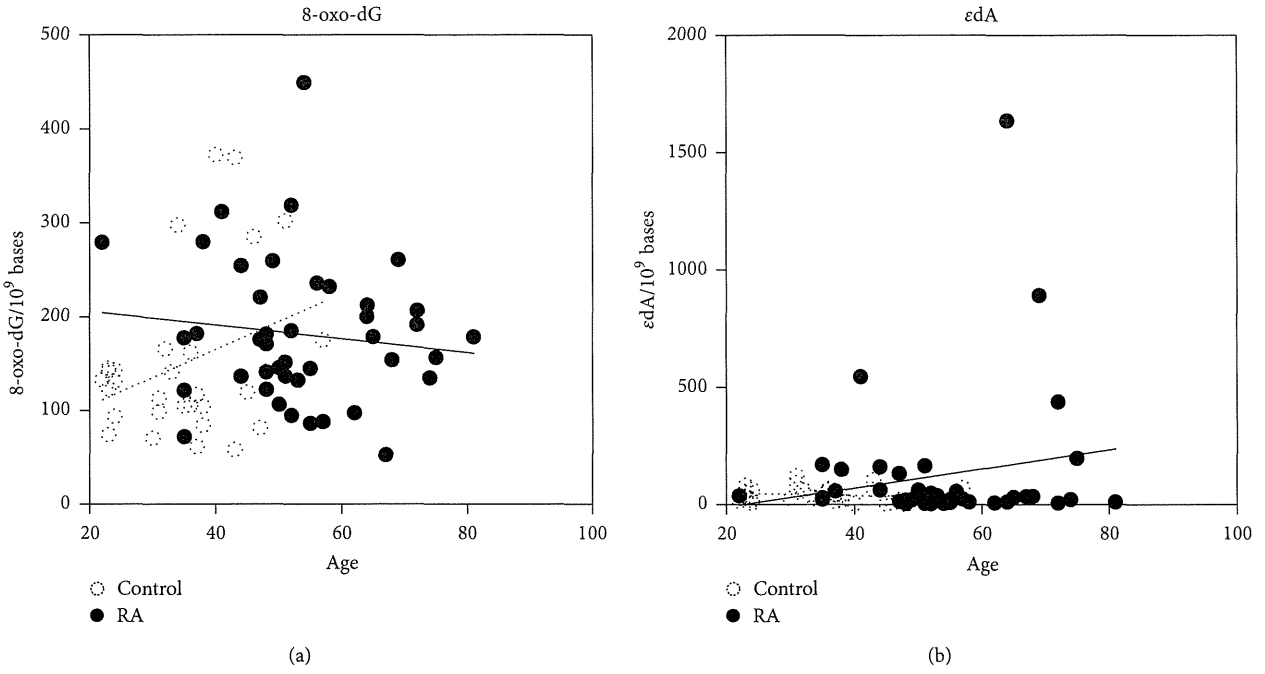

FIGURE 2: 8-oxo-dG and εdA levels in blood cells from RA patients and controls show no differences. (a) The respective median (range) values of 8-oxo-dG from RA patients and controls were 176.4 (52.5–449) and 127.1 (58.1–372) per 10^9 bases. The straight and dotted lines represent linear approximation of the values of 8-oxo-dG versus age from RA patients and controls. (b) The respective median (range) values of εdA from RA patients and controls were 29 (2.6–1635) and 34.7 (0.2–121) per 10^9 bases. The straight and dotted lines represent linear approximation of the values of εdA versus age from RA patients and controls.

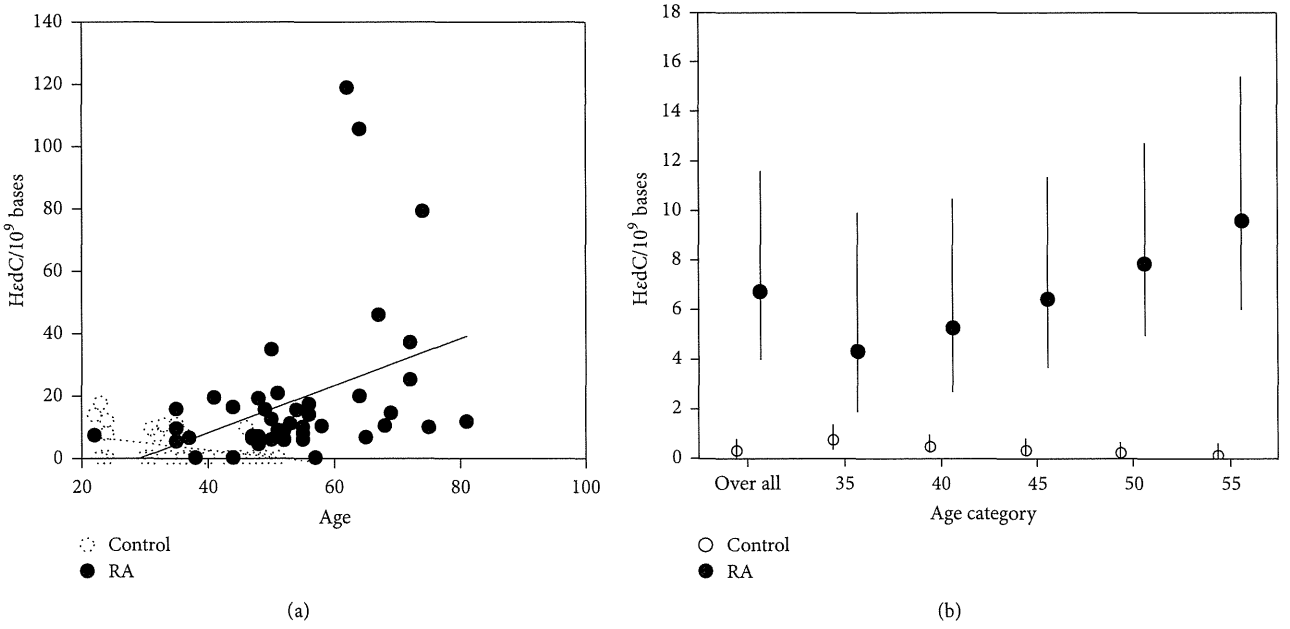

FIGURE 3: HεdC levels are significantly elevated in RA patients. (a) The respective median (range) values of HεdC from RA patients and controls were 10.3 (0.3–119) and 0.33 (0.3–17.8) per 10^9 bases. HεdC levels in RA patients were thus significantly higher than in controls ($P < 0.0001$). The straight and dotted line represent linear approximation of the values of HεdC versus age from RA patients and controls. (b) The geographic mean level of HεdC increased with aging in RA patients ($P = 0.003$).

Table 2: Correlation of HεdC with clinical and laboratory parameters.

Correlation of HεdC with	Number of subjects	Spearman rank correlation (CI)	P value
CRP	43	0.19 (−0.11, 0.47)	0.21
PLT	39	0.21 (−0.11, 0.49)	0.20
SAA	15	0.20 (−0.35, 0.64)	0.47
Albumin	15	−0.24 (−0.67, 0.31)	0.37
MMP3	26	0.14 (−0.26, 0.50)	0.50
SJC	46	0.57 (0.34, 0.74)	<0.0001
TJC	46	0.26 (−0.03, 0.51)	0.08
S&TJC	46	0.48 (0.22, 0.68)	0.0005
Age	46	0.43 (0.15, 0.64)	0.003

The Spearman rank correlation coefficient was used for statistical analyses of the associations between HεdC levels and clinical or laboratory test findings for RA patients. $P < 0.05$ was considered significant. CI: confidence interval; PLT: platelet count; SAA: serum amyloid A; MMP3: matrix metalloproteinase 3; SJC: the number of swollen joints; TJC: the number of tender joints; S&TJC: the number of swollen and tender joints.

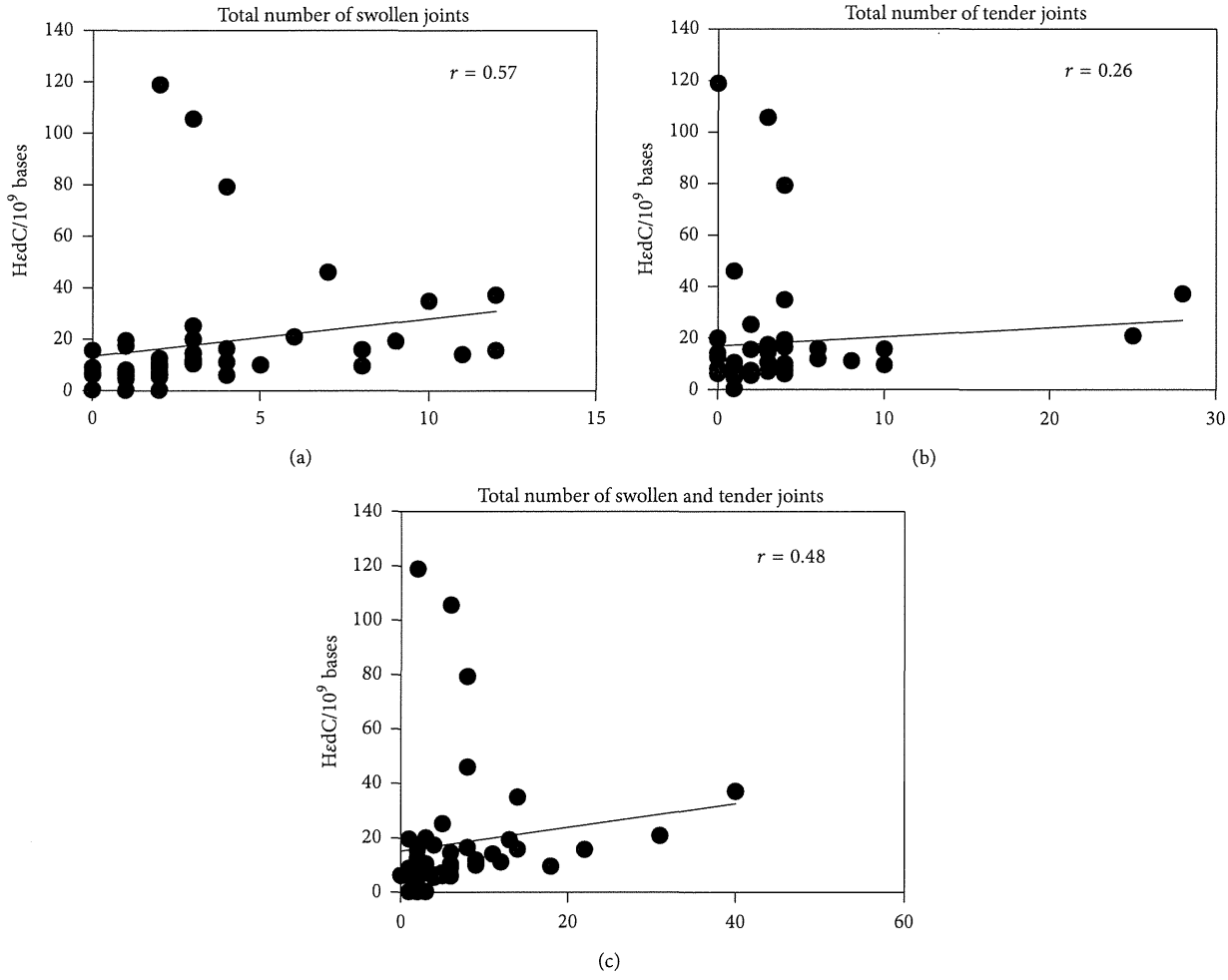

Figure 4: HεdC levels show positive correlation with the number of swollen joints and the total number of swollen plus tender joints. HεdC levels correlated weakly with the number of tender joints ($P = 0.08$) but strongly with that of swollen joints ($P < 0.0001$) and the total number of tender + swollen joints ($P = 0.0005$). The straight line represents linear approximation of the values of HεdC versus the number of involved joints in RA patients.

blood lymphocytes of RA patients [2, 15, 18]. In our assay, however, we could not detect overaccumulation of 8-oxo-dG in whole blood cells from RA patients. The reason for this discrepancy is as yet unknown, but we presume that it is due to differences in sample sources or assay methods. Previous studies have analyzed 8-oxo-dG concentrations in urine and synovial fluids by using enzyme-linked immunosorbent assay and 8-oxo-dG levels in peripheral blood lymphocytes by means of high-performance liquid chromatography, while we examined 8-oxo-dG levels in genomic DNA from whole blood cells by means of LC-MS/MS. Another more likely reason is the difference in disease activity, since that of RA patients enrolled in our study appeared to be milder since the median number of tender and swollen joints was 2 and 2, respectively, and the median CRP value was 0.83 mg/dL, values which are lower than those previously reported. However, it should be pointed out again that even in RA patients with mild disease activity, HεdC levels were elevated and correlated well with the number of involved joints, raising a possibility that HεdC may become a novel and sensitive biomarker to detect disease activity of RA.

Analysis of the chemical structure suggests that oxidative DNA damage leads to DNA strand breakages including double- and single-strand breaks and changes in the DNA quaternary structure resulting in its unwinding, and enhanced DNA unwinding has been found in the blood mononuclear cells of patients with RA [19]. The importance of lipid peroxidation-derived DNA damage in inflammatory diseases has been implied [20], and, in fact, levels of lipid peroxidation and oxidised low-density lipoprotein are highly expressed in RA patients, while a positive association between RA disease activity and lipid peroxidation has been reported [10, 15, 21]. Polyunsaturated fatty acids (PUFAs) exerting oxidative stress have been found to cause a series of α,β-unsaturated aldehydes such as acrolein, crotonaldehyde, and malondialdehyde to form lipid peroxyl radicals, which are highly DNA- and protein-reactive [7, 10, 14, 22, 23]. HεdC has an exocyclic ring and a bulky side chain formed by deoxycytidine reacting with 4-ONE and may lead to alterations in mitochondrial function [7, 14, 22–25]. The presence of HεdC has been identified in various cells and tissues [7]. Functionally, HεdC was found to block DNA synthesis and thus resulted in marked miscoding during the replication of DNA plate modified with deoxycytidine [26]. Williams et al. suggested that either arachidonic acid or linoleic acid is catalyzed by Cox-2 into 15(*S*)-hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (15(*S*)-HPETE), which then undergoes hemolytic decomposition to form the DNA-reactive bifunctional electrophile, 4-ONE [20]. Considering our findings on the correlation analysis, HεdC may be in part responsible for the pathological activities in synovium, and further studies with the use of synovium are required. It is conceivable that the high-level accumulation of HεdC on RA patients was due to imbalance of oxidative reaction over antioxidant defence system, impairment of DNA repair enzymes and/or excessive activity of 15-lipooxygenase or Cox-2, or the excess presence of 4-ONE or ω6 PUFAs in cell membranes. However, no differences were observed in the HεdC levels of RA patients even if they had been treated with NSAID or corticosteroid, which is known to suppress 15-lipoxygenase. RA patients treated with methotrexate reportedly show reduced concentrations of 8-oxo-dG [27], but we could not detect any association between HεdC and methotrexate use. Although it is important to monitor HεdC levels following treatment in order to evaluate the effect of these drugs on HεdC production, our findings also suggest that HεdC is regulated differently from 8-oxo-dG in RA patients. Further studies are required to clarify the mechanism, which accounts for excessive production of HεdC of RA patients.

Although it is clear that HεdC levels in whole blood cells correlate well with the number of involved joints, the pathological effect of increased levels of HεdC in RA remains to be elucidated. Moreover, it is another important issue to clarify whether or not this elevation is specific for RA. Since HεdC may epigenetically alter gene accumulation and is also known to be highly mutagenic [14], this contributes to the persistent inflammation or, through p53 mutations or alternation of gene expression, may be associated with an increase in the incidence of cancer seen in RA patients [28, 29]. Further studies will be required to clarify the role of HεdC in the development of RA and its complications.

5. Conclusion

The present study shows that the lipid peroxidation-derived DNA adduct, HεdC, is highly accumulated in whole blood cells of RA and its level is age dependent. The positive association between HεdC values and the number of involved joints suggests that HεdC may become a novel biomarker to evaluate disease activity of RA. Based on the previous findings and this paper, DNA damages may play a significant role in the development of RA and/or its complications although further studies are required to elucidate the exact significance of DNA adducts in RA.

Conflict of Interests

Atsushi Ogata received a consultant fee from Chugai Pharmaceutical Co., Ltd. The other authors have no conflict of interest to declare.

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Robo4 is an effective tumor endothelial marker for antibody-drug conjugates based on the rapid isolation of the anti-Robo4 cell-internalizing antibody

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Regular Article

VASCULAR BIOLOGY

Robo4 is an effective tumor endothelial marker for antibody-drug conjugates based on the rapid isolation of the anti-Robo4 cell-internalizing antibody

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Key Points

- First therapeutic application that targets Robo4 on the tumor blood vasculature
- High-throughput screening system to isolate cell-internalizing monoclonal antibodies useful to develop effective antibody-drug conjugates

Monoclonal antibodies (mAbs) that are internalized into cells are a current focus in the development of antibody-drug conjugates (ADCs). We describe a phage display–based high-throughput screening system to rapidly isolate cell-internalizing mAbs. We simultaneously examined the cell-internalizing activities of several hundred independent mAbs and successfully isolated cell-internalizing mAbs against the tumor endothelial markers Roundabout homolog 4 (Robo4) and vascular endothelial growth factor receptor 2 (VEGFR2). Tumor accumulation of mAbs with high cell-internalizing activity was significantly higher than that of mAbs with low cell-internalizing activity. Furthermore, the antitumor effects of ADCs of mAbs with high cell-internalizing activity were significantly stronger than those of mAbs with low cell-internalizing activity. Although anti-VEGFR2 therapy caused a significant loss of body weight, anti-Robo4 therapy did not. These findings indicate that cell-internalizing activity plays an important role in the biodistribution and therapeutic effects of ADCs. Further, Robo4 can be an effective marker for tumor vascular targeting. (*Blood*. 2013;121(14):2804-2813)

Introduction

Antibody drug conjugates (ADCs), ie, monoclonal antibodies (mAbs) labeled with certain anticancer agents, are currently the focus of antibody-based drug discovery. ADCs have mAb-derived specificity and allow for targeted delivery of cytotoxic drugs to a tumor, which is expected to significantly enhance the antitumor activity of mAbs.[1] Trastuzumab ematansine (T-DM1)[2] for human epidermal growth factor receptor 2 (Her-2)–positive breast cancer and brentuximab vedotin (SGN-35)[3] for relapsed or refractory CD30-positive lymphoproliferative disorders are now in phase 3 clinical trials as effective ADCs.[4] ADCs will have an important role in overcoming some types of refractory cancers and will contribute to the field of tumor vascular targeting.[5]

An essential property of ADCs is that the mAb should be efficiently internalized into the cell where the cytotoxic effects of anticancer drugs occur.[1] The isolation of mAbs with high cell-internalizing activity (cell-internalizing mAbs) is a limiting factor in the development of ADCs. The discovery of potent cell-internalizing mAbs, however, requires labor-intensive screening of a massive number of candidates, and therefore the development of phage display–based methods to identify these candidates is highly desirable.[6,7] In the phage display–based method, a phage antibody library is added to the desired cells and then phages bound to the cell surface are removed. Only internalized phages are rescued from the intracellular compartment. Even with this method, however, the internalizing activities of individual antibody candidates must be assessed, because the concentrated phage pool comprises a "polyclonal" population. To address this issue, we used high-throughput screening methods to estimate "monoclonal" cell-internalization activities using a protein synthesis inhibitory factor (PSIF),[8] which provided a breakthrough in reducing the time-consuming screening of the cell-internalizing activity.

PSIF is a fragment of a bacterial exotoxin derived from *Pseudomonas aeruginosa*.[9] PSIF lacks its cell binding domain, and its cytotoxic portion is used in a recombinant immunotoxin.[10] Upon entry into the cell, PSIF has strong cytotoxicity by inducing ADP-ribosylation of elongation factor-2, which is essential for protein synthesis.[11] Our group previously accelerated the identification of cell-internalizing novel protein transduction domains (PTDs) by expressing PTD-PSIF fusion proteins in the supernatant of *Escherichia coli*.[8] Using this system, we successfully discovered superior HIV-Tat PTD mutants by simultaneously estimating

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the cell-internalizing activities of several hundred monoclonal PTD-PSIF fusions.[8] Therefore, we expect this method to contribute to the identification of mAbs with high cell-internalizing activity (cell-internalizing mAbs) by expressing single-chain antibody Fv (scFv)-PSIF fusion proteins to estimate the cell-internalizing activities of a very large number of antibodies.

Roundabout homolog 4 (Robo4) is a potential tumor angiogenesis marker.[12] Robo4 expression is restricted to areas of in vivo angiogenesis[13,14] and the subpopulation of hematopoietic stem cells localized in the bone marrow.[15] At angiogenic sites, Robo4 is present in the endothelial lining of blood vessels in the developing embryo,[16] placenta,[14] and tumors.[17] We previously confirmed the endothelial cell–specific expression of Robo4 using transgenic mouse lines.[18,19] Robo4 acts as a receptor that modulates vascular endothelial growth factor A (VEGF)–VEGF receptor (VEGFR) signaling.[20-23] Therefore, Robo4 is a potential marker for tumor vascular targeting because angiogenesis is only activated in tumors in the adult,[24] with the exception of some pathological states.[25,26] Another potential tumor angiogenesis marker is VEGFR2, a well-established tumor endothelial marker.[27] The VEGF-VEGFR2 signaling pathway plays a crucial role in angiogenesis, and anti-VEGF mAbs and small molecule inhibitors against VEGFR are approved for various types of cancers.[28] Anti-VEGFR2 mAbs are also used for tumor vascular targeting.[29] Although VEGFR2 is strongly expressed in active angiogenic sites, its expression is also observed in normal tissues.[30] Hypertension and proteinuria are common side effects of anti-VEGF therapy because VEGF-VEGFR signaling is also inhibited in normal tissue, including the kidney, heart, and resistance vessels.[31-33]

Here we applied the PSIF system to search for novel cell-internalizing mAbs from an immune phage antibody library. Application of this method to Robo4 and VEGFR2 led to the successful discovery of anti-Robo4 and anti-VEGFR2 cell-internalizing mAbs, as well as mAbs with low cell-internalizing activity (low-internalizing mAbs) to be used as controls. Comparing mAbs with different cell-internalizing activities revealed that higher cell-internalizing activity enhanced the tumor targeting potency of mAbs. Furthermore, comparative studies with anti-Robo4 and anti-VEGFR2 cell-internalizing mAbs in vivo indicated that Robo4 was superior to VEGFR2 in terms of the therapeutic window. This is the first report demonstrating the benefits of cell-internalizing mAbs in tumor vascular targeting. Further, these findings demonstrate the potential of Robo4 as a target for further development of novel ADCs against tumor blood vasculature.

Materials and methods

Cell culture

MS1 immortalized murine endothelial cells were cultured in Dulbecco's Modified Eagle Medium containing 5% fetal bovine serum 1% antibiotic-antimycotic mixed solution. B16BL6 murine melanoma cells were cultured in minimum essential medium containing 10% fetal bovine serum and 1% antibiotic-antimycotic mixed solution at 37°C. These cells were maintained at 37°C under a humidified 5% CO_2 atmosphere.

B16BL6 tumor-bearing mice

B16BL6 cells (1×10^6 cells/100 μL) were inoculated intracutaneously into 6-week-old female C57BL6 mice (Japan SLC Inc., Shizuoka, Japan) (day 0). Biodistribution was analyzed on the day that the tumor width reached 10 mm. The therapy experiment was started on day 3. As a validation of the model, we confirmed the expressions of VEGFR2 and Robo4 on the tumor endothelium, based on the immunofluorescence against B16BL6 tumor sections.

Antigens

Human VEGFR2 (hVEGFR2) and mouse VEGFR2 (mVEGFR2) were commercial recombinant proteins (Merck Chemicals, Inc., Darmstadt, Germany, or R&D Systems, Inc., Minneapolis, MN). Human Robo4 (hRobo4) and mouse Robo4 (mRobo4) were produced as described previously.[34]

Immune phage antibody libraries

Phage antibody libraries were constructed from the spleen and bone marrow cells of immunized mice as previously described.[35,36] Our phage antibody library comprised single-chain Fv fragment (scFv) fused with pIII phage coat protein. Four rounds of affinity panning were performed against hVEGFR2 and mVEGFR2 for the anti-VEGFR2 immune library, and against hRobo4 and mRobo4 for the anti-Robo4 immune library. Anti-FLAG panning was followed by each panning to concentrate the scFv-displaying phages, as described previously.[36]

ELISA and cytotoxicity assay using TG1 supernatant

Plasmids were extracted from TG1 cells after the fourth panning against mVEGFR2 or mRobo4. These "enriched" scFv gene libraries were cloned into a PSIF-fusion expression vector derived from pCANTAB5E.[8] Monoclonal scFv-PSIF protein was induced in the TG1 supernatant, as previously described.[8] mVEGFR2 or mRobo4 was immobilized on an immunoassay plate and blocked with 4% skim milk in phosphate-buffered saline (PBS) (4% MPBS) at 37°C for 2 hours. TG1 supernatant containing 2% MPBS was reacted with antigens at room temperature for 1 hour. Bound scFv-PSIFs were detected by anti–FLAG-horseradish peroxidase (M2, Sigma-Aldrich Corporation, St. Louis, MO). For the cytotoxicity assay, MS1 cells were seeded on a 96-well plate at 1.0×10^4 cells/well. After incubation at 37°C for 24 hours, TG1 supernatant was diluted in MS1 culture medium, and then added to the MS1 cells. After incubation at 37°C for 24 hours, cell viability was assessed using a WST-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The viability of nontreated MS1 and completely killed MS1 with 1 mM cycloheximide were defined as 100% and 0%, respectively.

Expression and purification of scFv, dscFv, and scFv-PSIF recombinant protein

The isolated scFv gene with 15 amino acids linker (VL-GGGGSGGG GSGGGGS-VH) was cloned into modified pET15b vector, resulting in the scFv fused by FLAG-tag and His×6 tag at the C-terminus. A scFv gene with a 5–amino acid linker (VL-GGGGS-VH) was also cloned into modified pET15b, resulting in a noncovalent scFv dimer (dscFv) fused by FLAG-tag and His×6 tag at the C-terminus. An anti-His scFv gene was also cloned but only a FLAG-tag was fused at the C-terminus. A scFv gene with a 15–amino acid linker was cloned into pYas-PSIF vectors.[37] ScFvs, dscFvs, and scFv-PSIFs were purified from inclusion bodies in *E coli* according to the previously described methods.[37] The binding affinity of each recombinant protein was assessed by surface plasmon resonance using BIAcore3000 (GE Healthcare UK Ltd., Chalfont, United Kingdom).

Expression and purification of IgG recombinant protein

IgG recombinant proteins were expressed using an OptiCHO antibody expression kit (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. IgGs were purified from cell culture supernatant with protein G column (GE Healthcare). Eluted fractions were further purified with Superdex 200 column (GE Healthcare). Anti-FLAG[IgG] (anti-FLAG M2 antibody) was purchased from Sigma-Aldrich.

Preparation of IgG-NCS

NCS was kindly provided by Kayaku Co, Ltd., Tokyo, Japan. NCS was thiolated by incubating it with 10 molar excess 2-iminothiolane (Thermo Fisher Scientific Inc., Waltham, MA) for 1 hour at room temperature. IgG recombinant proteins were reacted with 10 molar excess of SPDP

crosslinker (*N*-succinimidyl 3-[2-pyridyldithio]-propionate; Thermo Fisher) for 30 minutes on ice. SPDP-modified IgGs and thiolated NCS were separately purified using NICK columns (GE Healthcare). They were then mixed for 8 hours at room temperature. IgG-NCS were purified with a Superdex 200 column. Modification efficiency was quantified after sodium-dodecyl sulfate-polyacrylamide gel electrophoresis using a Gel DOC EZ system and Image laboratory software (Bio-Rad Laboratories, Inc., Hercules, CA).

Labeling of purified mAbs

For fluorescent labeling, mAbs were labeled using Cy5.5-NHS (GE Healthcare). For ^{125}I labeling, 100 μg mAbs in 0.4 M phosphate buffer were labeled with 0.2 mCi $Na^{125}I$ (PerkinElmer, Inc., Waltham, MA) based on the chloramine-T method.[38] For biotin labeling, mAbs were biotinylated using EZ-Link Sulfo-NHS-Biotin (Thermo Fisher). Each mAb was purified using a NICK desalting column (GE Healthcare).

Flow cytometry

Cy5.5-labeled mAb ($mAb^{Cy5.5}$; 4 μM) was incubated with 5.0×10^5 cells of MS1 cells in 6-well plates for 2 hours at 4°C. After washing three times, the cells were incubated for an additional 0.5 to 8 hours at 37°C. At each time point, cells were collected in 2-mM ethylenediaminetetraacedtic acid/PBS. Bound mAbs were digested using 0.1% trypsin/PBS at 37°C for 20 minutes (trypsinized group) or PBS (nontrypsinized group). Cellular fluorescence was measured by Gallios flow cytometer (Beckman Coulter, Inc., Miami, FL). The ratio of internalization was calculated using the following formula: internalization (%) = {internalized $mAb^{Cy5.5}$}/{total bound $mAb^{Cy5.5}$} × 100 (%) = {(MFI of $mAb)_T$ − (MFI of anti-His $[mAb])_T$}/{(MFI of $mAb)_N$ − (MFI of anti-His$[mAb])_N$} × 100 (%). MFI indicates mean fluorescence intensity. T and N indicate trypsinized and nontrypsinized groups, respectively.

In vivo biodistribution

$dscFvs^{125I}$ (0.2 nmol) was intravenously injected into B16BL6 tumor–bearing mice. At 2 hours and 24 hours after injection, the radioactivity of each organ was counted using the Wizard 2480 γ-ray counter (PerkinElmer). %ID/g tissue was calculated using following formula: %ID/g tissue = (count/g tissue)/(total injected count) × 100 (%). Two individual experiments were combined for the final data (total 11 mice/group).

Immunofluorescence of the tissue sections

B16BL6 tumor–bearing mice were administered 2 nmol of $dscFvs^{Bio}$. At 2 hours after the injection, the tumors, kidneys, and hearts were embedded in optimal cutting temperature compound (Sakura Finetek, Inc., Torrance, CA) and frozen in liquid nitrogen. Thin sections (7 μm) were prepared using a Cryostat CM1850 (Leica Microsystems GmbH, Wetzlar, Germany) and fixed in 4% paraformaldehyde. $DscFvs^{Bio}$ in the sections were stained with streptavidin phycoerythrin conjugate (eBioscience Inc., San Diego, CA) in Dako REAL Antibody Diluent (DAKO Corporation, Carpinteria, CA). CD31+ vascular endothelial cells were stained with rat anti-CD31 antibody (MEC13.3; Becton Dickinson and Company, Franklin Lakes, NJ) in Dako REAL Antibody Diluent and Alexa488 conjugated anti-rat IgG (A11006; Invitrogen). The samples were embedded with Prolong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Invitrogen) and observed with a fluorescence microscope FSX100 (Olympus Corporation, Tokyo, Japan).

In vivo therapy experiments

Activities of scFv-PSIFs and IgG-NCSes were confirmed by WST-8 assay as described before. B16BL6 cells were inoculated intracutaneously into C57BL6 mice (Japan SLC) on day 0. Mice were intravenously injected with 15 pmol scFv-PSIFs and 10 pmol IgG-NCSs on days 3, 5, 7, 9, and 11 (7 mice/group). The volume of the tumor was calculated according to the following formula: tumor volume (mm^3) = {major axis of tumor (mm)} × {minor axis of tumor (mm)}^{2} × 0.4.

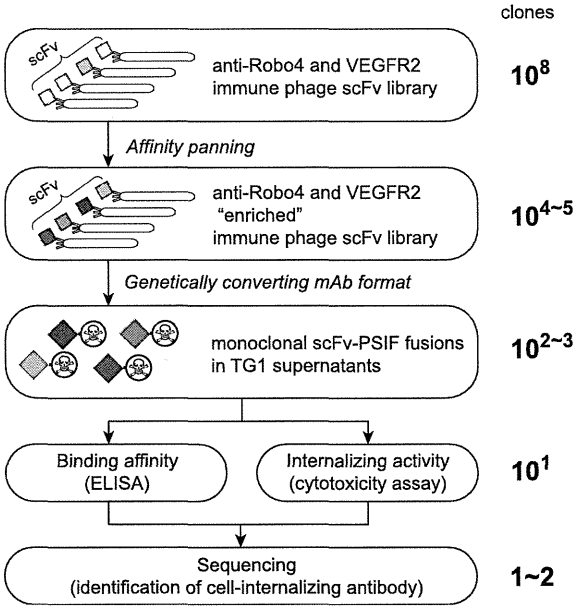

Figure 1. Phage display-based method to search for cell-internalizing mAbs. The phage antibody library was "enriched" by affinity panning against the desired antigens. Plasmids encoding scFvs were collected from TG1 *E coli* strains infected by "enriched" phage libraries. Genes of scFvs were digested out and ligated into a PSIF fusion protein expression vector. These plasmids were then transformed to TG1, and then single colonies were picked up. From these individual colonies, monoclonal scFv-PSIF fusions were induced in TG1 supernatants. Using these fusion proteins, binding affinities and internalizing activities of several hundreds of scFv-PSIFs were easily estimated by ELISA and cytotoxicity assays, respectively. Finally, genes of positive scFvs were collected from TG1, and cell-internalizing scFvs were identified by sequencing. In this report, we used anti-Robo4 and anti-VEGFR2 immune phage scFv libraries as the phage antibody libraries, and mRobo4 and mVEGFR2 as the desired antigens.

Results

High-throughput screening for cell-internalizing mAbs

To identify cell-internalizing mAbs, we applied the phage immune scFv library to high-throughput screening of cell-internalizing molecules based on the PSIF system[8] (Figure 1). Our anti-Robo4 or anti-VEGFR2 phage library comprised approximately 3.0×10^8 or 5.0×10^8 independent scFvs, which was validated by sequence analysis. To assess the qualities of the libraries, affinity panning was performed against each antigen. During the panning, output phages were increased, suggesting that the desired scFvs were enriched in the library (supplemental Figure 1A-B,E-F). After the fourth panning, >40% of monoclonal scFvs showed specific binding in enzyme-linked immunosorbent assay (ELISA) (supplemental Figure 1C-D,G-H).

To validate the efficacy of cell-internalizing mAbs in a mouse model, we selected libraries enriched against murine antigens (mRobo4 and mVEGFR2) and chose MS1 murine endothelial cells for the screening of cell-internalizing mAbs because we confirmed the expressions of both mRobo4 and mVEGFR2 in MS1 cells using reverse transcriptase–polymerase chain reactioin. For the screening, scFv genes obtained after the fourth round of panning were cloned into the PSIF expression vector. Monoclonal scFv-PSIFs were expressed in TG1 supernatants (315 clones per library). For anti-Robo4s, 178 of 315 clones bound to mRobo4 in ELISA and 20 of these 178 binders were cytotoxic against MS1 cells