Table 2 Summary of patient characteristics

Fiscal year	2004		2005		2006		2007		2008		2009		2010	
	Cases	Total	Cases	Total	Cases	Total	Cases	Total	Cases	Total	Cases	Total	Cases	Total
Age (years) (IQR)	58.5 (55.5-72)	63(55–70)	65(62–76)	63(55–70)	60(59–76)	63(56–71)	61(59–63)	64(56–71)	64	64(56–71)	63(58–74)	64(56–72)	65(57–70)	65(57–72)
Gender (female:male)	3:1	3313:681	2:1	3832:818	3:0	4229:923	2:0	4448:1015	1:0	5346:1156	11:2	5895:1284	13:0	5921:1333
Disease dutaion (years) (IQR)	6(4–9)	11(5–19)	15(12–36)	10(5–19)	13(11–30)	10(5–19)	23.5 (15–32)	9(4–19)	24	10(5–19)	16(12–20)	10(5–19)	20(15–40)	10(5–19)
Arthroplastic surgery (IQR)	0(0–0)	0(0–0)	1(0–2)	0(0–0)	1(0–4)	0(0–0)	2(0–3)	0(0–0)	0%	0(0–0)	0(0–3)	0(0–0)	0(0–1)	0(0–0)
Tender joint count (IQR)	9(1–30)	3(1–6)	15(13–16)	3(1–6)	1(0–4)	2(1–6)	19(4–34)	2(0–5)	4	2(0–5)	3(1–9)	2(0–4)	2(1–4)	1(0–3)
Swollen joint count (IQR)	2(0–10)	2(0–5)	0(0–4)	2(0–5)	0(0–2)	1(0–4)	3(3–3)	1(0–3)	3	1(0–4)	2(0–6)	1(0–3)	2(0–5)	1(0–3)
MHAQ (IQR)	1.4 (0.35-1.48)	0.5(0–1)	2.3(1.3-2.9)	0.4(0–1)	1.5(0.88-2)	0.4(0–1)	1.55 (1–2.1)	0.4(0–1)	1.25	0.38(0–1)	1(0.69-1.44)	0.25 (0–0.9)	1(0.26-1.94)	0.25(0–0.9)
CRP (mg/dl) (IQR)	3.4(0.9-4.2)	0.59 (0.18-1.7)	2.7(1.75-3.35)	0.55 (0.18-1.57)	0.47 (0.05-0.71)	0.47 (0.18-1.4)	1.26 (1.02-1.5)	0.41 (0.17-1.22)	1.95	0.31 (0.13-1.03)	1.04 (0.18-2.78)	0.28 (0.11-0.9)	2.0(0.14-3.5)	0.26 (0.1-0.76)
ESR (mm/h) (IQR)	38.5 (14–66)	37(20–60)	43(17–77)	35(19–56)	23(9–46)	33(18–55)	74.5 (46–103)	31(16–51)	51	29(15–49)	37.5 (29–71.3)	28(14–47)	55.5 (5.5-84.8)	25(13–45)
DAS28-CRP (IQR)	4.15 (3.15-6.11)	3.34 (2.45-4.25)	4.71 (4.67-5.38)	3.35 (2.43-4.24)	2.98 (1.13-3.61)	3.14 (2.29-4.06)	4.93 (3.46-6.39)	2.99 (2.12-3.89)	4.19	2.85 (2.04-3.75)	3.32(2.58-4.46)	2.72 (1.94-3.6)	3.39 (2.3-4.78)	2.55 (1.83-3.46)
Stage1 (%)	0	14.5	0	15	0	15.9	0	17.7	0	16.6	8.3	18	7.7	19.6
Stage2 (%)	100	25	33.3	25.2	0	25	0	25.7	0	25.6	0	27.2	0	26.5
Stage3 (%)	0	21	33.3	20.3	0	19.9	50	20.4	0	20.7	25	19.3	15.4	19.6
Stage4 (%)	0	39.5	33.3	39.5	100	39.2	50	36.2	100	37.1	66.7	35.5	76.9	34.3
Class1 (%)	0	25	0	26.2	0	26.2	0	26.9	0	25.3	25	26	0	28.2
Class2 (%)	75	52.7	0	53.4	33.3	53.3	0	52.9	0	52	50	53.9	61.5	52.6
Class3 (%)	25	19.1	66.7	17.4	66.7	17.4	100	17.5	0	19.3	25	16.8	38.5	16.4
Class4 (%)	0	3.2	33.3	3	0	3.1	0	2.7	100	3.4	0	3.3	0	2.8
PtPainVAS (IQR)	5.5(2.3-7.0)	3.3(1.6-5.5)	6.1(4–7)	3.1(1.5-5.3)	5.5 (1.6-6.8)	3(1.4-5.2)	5.5 (2.5-8.5)	3(1.3-5.1)	4.3	2.6(1.1-5)	3.4(1.4-8.3)	2.6(1.1-5)	2.25 (0.98-4.95)	2.4(1–4.8)
PtGVAS (IQR)	5.7(3–8.4)	3.5(1.7-5.5)	6.8(2.3-8.1)	3.4(1.6-5.3)	5.9 (0.2-7.8)	3.2 (1.5-5.2)	5.5 (2.5-8.5)	3(1.4-5.1)	5.3	2.8(1.2-5)	4.7 (0.85-7.75)	2.7(1.2-5)	3.1(1.85-5.9)	1.6(0.7-2.9)
DrGVAS (IQR)	7(3.4-8.3)	2.5(1.3-4.1)	5.2(3.1-7)	2.4(1.1-4)	0.7 (0.5-3.4)	2(1–3.5)	6.15(3.3-9)	1.8(0.9-3.1)	2.6	1.6(0.6-3)	3.8(0.8-4.8)	1.8(0.8-3)	2.5(1.05-4.4)	0.25(0–0.88)

Table 2 Summary of patient characteristics *(Continued)*

Use of MTX (%)	33	48	0	53	100	53.9	50	47.3	0	51.1	53.8	64.3	0	58.2
Use of corticosteroid (%)	100	63	100	62	100	64	100	61	100	59.1	100	53	84.6	53.4
Use of biologics (%)	0	2.1	0	4.3	0	9	0	11.7	0	13.6	38.5	20	0	18.6

VAS: visual analog scale, MHAQ: Modified Health Assessment Questionnaire, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, DAS28: Disease Activity Score of 28 Joints, MTX: Methotrexate.
A comparison of the cases and total number of patients enrolled in the same fiscal year. The data are presented as the medians (IQR).

Table 3 Comparison of the background data of the cases and controls

	Cases	Controls	p value
Age (years) (IQR)	63(59–73)	63(59–69)	0.7224
Gender (female:male)	35:4	99:7	0.4614
Disease dutaion (years) (IQR)	15(12–30)	15(11–22)	0.52
Arthroplastic surgery (%)	41	31	0.1527
Tender joint count (IQR)	3(1–10)	1(0–4)	0.0087
Swollen joint count (IQR)	2(0–4)	1(0–3)	0.1389
MHAQ (IQR)	1.13(0.75-1.88)	0.5(0.1-1)	0.0001
CRP (mg/dl) (IQR)	1.5(0.26-3.05)	0.36(0.13-1.06)	0.0023
ESR (mm/h) (IQR)	46(21–70)	33(20.5-57.5)	0.1948
DAS28-CRP (IQR)	3.63(2.60-4.68)	2.81(2.01-3.43)	0.0012
Stage1 (%)	5.3	6.5	
Stage2 (%)	13.2	19.6	
Stage3 (%)	18.4	19.6	
Stage4 (%)	63.1	54.3	0.7823
Class1 (%)	7.9	20.4	
Class2 (%)	47.3	58.1	
Class3 (%)	39.5	18.3	
Class4 (%)	5.3	3.2	0.0395
PtPainVAS (IQR)	3.9(1.8-6.4)	3(1.2-5)	0.0937
PtGVAS (IQR)	4.9(2–6.8)	2.9(1.2-5)	0.0314
DrGVAS (IQR)	3.4(1.4-5.1)	1.6(1–3.1)	0.0121
Use of MTX (%)	13	40	0.0022
Use of corticosteroid (%)	90	60	0.0007
Use of biologics (%)	10	8	0.7414

The data are presented as the median (IQR). P values were calculated using the chi-square test for gender, Steinbrocker functional class and stage and arthroplastic surgery. The Mann–Whitney U test was used for the other items.

There are several possible limitations associated with this study. First, we were unable to conduct a multivariate analysis because the number of patients who underwent cervical spine surgery was too small. In addition, we were unable to obtain detailed information on the type of spinal lesions (e.g. atlantoaxial subluxation) and the surgical procedures. We also could not count the number of cases with cervical involvement because we did not take plain radiographs of the cervical spine in every patient. Therefore, we could not determine how many of the RA patients in the NinJa database had cervical involvement. Finally, the institutions participating in the NinJa database are mostly national hospitals, which may limit the generalizability of our findings. In addition, our research period was relatively short, so our results regarding the effects of biologics on the cervical spine may change in the future as more patients are treated using these agents.

Conclusions

Our findings revealed that RA patients requiring cervical spine surgery tend to have a higher disease activity than control subjects. Our results also indicate that conducting a meticulous evaluation of cervical lesions is therefore required, particularly in patients with a high disease activity in spite of the intensive use of biologics.

Competing interests

Each author certifies that they have no commercial associations that might pose a conflict of interest in connection with the submitted article.

Authors' contributions

SS, HC, JN and ST contributed to the conception and design of the study. SS, YK, HO, KT and ST contributed to the analysis, and all authors contributed to the interpretation of the results. SS drafted the article; all authors revised it critically and approved the final version submitted for publication. All authors read and approved the final manuscript.

Acknowledgements

The authors wish to acknowledge the assistance of Dr. Naoki Ishiguro. This study was funded by grants from the Ministry of Health, Labour and Welfare, Japan to Shigeto Tohma and Sakae Tanaka. The funder has played no role in the design, data collection and analysis, decision to publish or preparation of the manuscript.

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Received: 15 March 2014 Accepted: 6 June 2014
Published: 13 June 2014

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doi:10.1186/1471-2474-15-203
Cite this article as: Sugita *et al.*: Clinical characteristics of rheumatoid arthritis patients undergoing cervical spine surgery: an analysis of National Database of Rheumatic Diseases in Japan. *BMC Musculoskeletal Disorders* 2014 **15**:203.

J Orthop Sci (2014) 19:991–996
DOI 10.1007/s00776-014-0607-2

ORIGINAL ARTICLE

Effects of fondaparinux on pulmonary embolism following hemiarthroplasty for femoral neck fracture: a retrospective observational study using the Japanese Diagnosis Procedure Combination database

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Received: 26 April 2014 / Accepted: 19 June 2014 / Published online: 18 July 2014

Abstract
Background Pulmonary embolism (PE) is recognized as an important complication in patients undergoing hip fracture surgery. However, clinical evidence demonstrating the effectiveness of pharmacological thromboprophylaxis, including fondaparinux, is limited because the occurrence of postoperative PE after hemiarthroplasty is very low. The goal of this study was to analyze the effect of fondaparinux in reducing PE following hemiarthroplasty for femoral neck fracture using large-scale retrospective data.
Methods Employing data from the Japanese Diagnosis Procedure Combination database from July 1 to December 31 between 2007 and 2010, we retrospectively identified 22,776 patients who underwent hemiarthroplasty for femoral neck fracture; we included those who received mechanical prophylaxis alone ($n = 17{,}984$) and those who received both mechanical prophylaxis and pharmacological prophylaxis with fondaparinux ($n = 4{,}792$). Logistic regression analysis was performed to compare the occurrence of postoperative PE with adjustment for sex, age, comorbidities, and type and duration of anesthesia.
Results The mean age of the patients was 79.5 ± 9.4 years. Overall, postoperative PE occurred in 189 (0.83 %) patients. The rate of postoperative PE in the fondaparinux group (0.61 %) was lower than in the control group (0.89 %), although the difference was not significant in the univariate analysis (odds ratio [OR] 0.68; $p = 0.055$). In the multivariate analysis, the fondaparinux group showed a significantly lower rate of postoperative PE than the group receiving mechanical prophylaxis alone (OR 0.67; $p = 0.047$). General anesthesia and a longer duration of anesthesia were significant risk factors for postoperative PE.
Conclusions Fondaparinux combined with mechanical prophylaxis is more effective in preventing postoperative PE following hemiarthroplasty for femoral neck fracture than mechanical prophylaxis alone.

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Introduction

Femoral neck fracture is a devastating injury, mainly caused by bone fragility due to osteoporosis [1]. In 2007, approximately 150,000 adults were hospitalized for hip fracture in Japan, and this number is expected to increase given the country's aging population [2]. Venous thromboembolism (VTE), including deep-vein thrombosis (DVT) and pulmonary embolism (PE), is now recognized a serious complication in patients with hip fracture surgery. Mortality and fatal PE rates appeared to be higher than those associated with total hip or knee arthroplasty due to degenerative disease [3].

The American College of Chest Physicians recommends that patients undergoing hip fracture surgery receive pharmacological antithrombotic prophylaxis for a minimum of 10–14 days [4]. Clinical evidence has demonstrated the effectiveness of pharmacological thromboprophylaxis (warfarin [5], low-molecular-weight heparin [6], and fondaparinux [7]) in the prevention of VTE, including asymptomatic DVT after hip fracture surgery. However, the occurrence of DVT is merely a surrogate endpoint of the occurrence of PE; asymptomatic DVT may not be an important outcome for patients [4], and therefore, we believe PE is a more important outcome to be evaluated. Nagase et al. [8] reported that fondaparinux was effective in preventing postoperative PE following total hip and knee arthroplasty. With regard to hip fracture surgery, clinical evidence demonstrating the effectiveness of fondaparinux in preventing PE is limited because the occurrence of postoperative PE after hip fracture surgery is very low.

Several reports have suggested that the occurrence of PE is lower in Asian than in Western populations; this may be due to the difference in their genetic backgrounds [9, 10]. However, recent increased awareness among physicians of postoperative PE has led to an ongoing debate about appropriate thromboprophylaxis after hip fracture surgery in Japan. In June 2007, fondaparinux was approved as pharmacological thromboprophylaxis for patients undergoing lower-limb orthopedic surgery. However, the effectiveness of fondaparinux in preventing postoperative PE after hip fracture surgery remains unclear. Using the Japanese Diagnosis Procedure Combination (DPC) administrative claims database, the present study aimed to examine the effectiveness of fondaparinux combined with mechanical prophylaxis compared with mechanical prophylaxis alone in reducing the occurrence of postoperative PE after hemiarthroplasty for femoral neck fracture.

Materials and methods

DPC database

The details of the DPC database are described elsewhere [11, 12]. Briefly, the DPC is a diagnosis-dominant case-mix system established in 2003 by the Ministry of Health, Labour and Welfare of Japan, and it is linked with a lump-sum payment system. All 82 university teaching hospitals are obliged to adopt the DPC system, whereas adoption by community hospitals is voluntary. DPC survey is conducted between July 1 and December 31 every year, where not only administrative claims data but also detailed patient data are collected for all the inpatients discharged from the participating hospitals. Data are mainly used for health policy planning by the government. This study is a secondary analysis of the anonymous database. By 2010, the number of participating hospitals had increased to 952 with 5 million patients, which represented approximately 50 % of all inpatient admissions to acute-care hospitals in Japan. The DPC database includes the following data: age and sex of patients; diagnoses, comorbidities at admission and complications after admission recorded in accordance with the International Classification of Diseases, Tenth Revision (ICD-10) codes and text data in Japanese; medical procedures coded using original Japanese codes; duration of anesthesia; drugs administered; lengths of stay (LOS); and discharge status. A single procedure code is assigned to each patient who received mechanical prophylaxis using either intermittent pneumatic compression device or compression stocking. The requirement for informed consent was waived because of the anonymous nature of the data. Study approval was obtained from the Institutional Review Board of The University of Tokyo.

Data collection

We retrospectively collected data from the DPC database of patients who were diagnosed with femoral neck fracture (ICD 10 code, S72.0) and underwent hemiarthroplasty between 2007 and 2010. We extracted data relating to the patient's sex, age, and comorbidities that could potentially affect the occurrence of PE (malignant lesions, history of cardiovascular diseases, history of cerebrovascular diseases [13], chronic renal failure [14], diabetes mellitus [15], and hyperlipidemia [16]). Data on the type of anesthesia (general or regional) and its duration (in minutes) were obtained. We also extracted data on the use of fondaparinux, enoxaparin, warfarin, aspirin, heparin, and mechanical prophylaxis for each patient. The mechanical prophylaxis included the use of an intermittent pneumatic compression device alone, compression stocking alone, or both.

Because this study aimed to analyze the independent effect of fondaparinux, we excluded patients who received any other anticoagulant for thromboprophylaxis and those who did not undergo mechanical prophylaxis. Patients who received warfarin and aspirin before surgery for treatment of other vascular, cardiac, or cerebral diseases were also excluded. Finally, we divided all the included patients into the following two groups: (1) those who received mechanical prophylaxis alone (the control group); and (2) those who received fondaparinux combined with mechanical prophylaxis (the fondaparinux group). The primary outcome was the occurrence of postoperative PE. The secondary outcome was in-hospital mortality after PE. We compared the outcomes between two groups.

Statistical analysis

The chi-square test was used to compare the proportions of the categorical data. Student's t test and the Mann–Whitney U test were used to compare the averages of the continuous variables. Multivariate logistic regression analysis was performed to analyze the effects of fondaparinux on the prevention of postoperative PE with adjustment for patient backgrounds and procedural factors. We included age, sex, and other variables whose p value was < 0.10 in the univariate analyses into the subsequent multivariate logistic regression analysis. A p value < 0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS ver. 20.0 (IBM, Armonk, NY, USA).

Results

Patient backgrounds and occurrence of postoperative PE

We identified 32,540 patients who underwent hemiarthroplasty for femoral neck fracture during the survey period. We excluded 4,268 patients who did not receive mechanical prophylaxis and 5,496 patients who were administered enoxaparin, unfractionated heparin for prophylaxis, or who preoperatively received warfarin or aspirin for the treatment of other diseases. Finally, 22,776 eligible patients were included in the analysis. These patients were divided into the following two groups: 17,984 patients in the control group; and 4,792 patients in the fondaparinux group. The average (± SD) preoperative and postoperative LOS was, respectively, 5.8 ± 6.9 and 34.3 ± 24.2 days in the control group and 5.8 ± 6.3 and 33.1 ± 22.0 days in the fondaparinux group.

Table 1 presents the patients' characteristics in total and in each group. There were 4,564 males (20.0 %). The average (± SD) age was 79.5 ± 9.4 years. The median duration of anesthesia was 127 min, and 12,408 patients (54.7 %) received general anesthesia. The fondaparinux group included a higher proportion of younger patients, those with hyperlipidemia, and those without chronic renal failure than the control group.

Table 2 shows the rate of postoperative PE, univariate odds ratio (OR), and 95 % confidence interval (CI) in each subgroup. The overall rate of postoperative PE during hospitalization was 0.83 % (189/22,776). The rate of postoperative PE in the fondaparinux group (0.61 %) was lower than in the control group (0.89 %), although the difference was not significant (OR 0.68; $p = 0.055$). The rate of postoperative PE was higher in the general anesthesia group than in the regional anesthesia group (OR 1.51; $p = 0.001$). The rate of postoperative PE was also higher in patients with ≥ 127 min of anesthesia than in those with < 127 min of anesthesia (OR 1.51; $p = 0.006$).

Table 1 Patient demographics

	Total (n = 22,776)	MP alone (n = 17,984)	MP and fondaparinux (n = 4,792)	p value
Sex				
Male	4,564 (20.0)	3,639 (20.2)	925 (19.3)	0.152
Female	18,212 (80.0)	14,345 (79.8)	3,867 (80.7)	
Age (years)				
Mean [SD]	79.5 [±9.4]	79.6 [±9.4]	79.2 [±9.1]	0.014
≥69	3,128 (13.7)	2,475 (13.8)	653 (13.6)	0.056
70–79	6,986 (30.7)	5,449 (30.3)	1,537 (32.1)	
≥80	12,662 (55.6)	10,060 (55.9)	2,602 (54.3)	
Comorbidities				
Malignancy	1,106 (4.9)	873 (4.9)	233 (4.9)	0.908
History of cardiovascular diseases	2,139 (9.4)	1,671 (9.3)	468 (9.8)	0.317
History of cerebrovascular diseases	476 (2.1)	364 (2.0)	112 (2.3)	0.178
Chronic renal failure	814 (3.6)	748 (4.2)	66 (1.4)	<0.001
Diabetes mellitus	3,197 (14.0)	2,490 (13.8)	707 (14.8)	0.108
Hyperlipidemia	1,553 (6.8)	1,165 (6.5)	388 (8.1)	<0.001
Type of anesthesia				
Regional	10,368 (45.5)	8,149 (45.3)	2,219 (46.3)	0.220
General	12,408 (54.5)	9,835 (54.7)	2,573 (53.7)	
Duration of anesthesia (min) Median [interquartile range]	127 [102–159]	126 [102–159]	127 [102–158]	0.874

MP mechanical prophylaxis

Table 2 Rate of pulmonary embolism and univariate odds ratios in each subgroup

	Total (*n*)	PE, *n* (%)	OR	95 % CI	*p* value
All	22,776	189 (0.83)			
Prophylaxis					
MP alone	17,984	160 (0.89)	1.00		
MP and fondaparinux	4,792	29 (0.61)	0.68	0.46–1.01	0.055
Sex					
Male	4,564	31 (0.68)	1.00		
Female	18,212	158 (0.87)	1.28	0.87–1.88	0.211
Age (years)					
≥69	3,128	18 (0.58)	1.00		
70–79	6,986	55 (0.79)	1.37	0.80–2.34	0.247
≥80	12,662	116 (0.92)	1.60	0.97–2.63	0.065
Malignancy	1,106	8 (0.72)	0.87	0.43–1.76	0.689
History of cardiovascular diseases	2,139	22 (1.03)	1.27	0.82–1.99	0.288
History of cerebrovascular diseases	476	3 (0.63)	0.75	0.24–2.37	0.629
Chronic renal failure	814	8 (0.98)	1.19	0.59–2.43	0.625
Diabetes mellitus	3,197	22 (0.69)	0.81	0.52–1.26	0.342
Hyperlipidemia	1,553	12 (0.77)	0.93	0.52–1.67	0.797
Type of anesthesia					
Regional	10,368	62 (0.60)	1.00		
General	12,408	125 (1.01)	1.51	1.13–2.03	0.001
Duration of anesthesia (min)					
< 127	11,191	74 (0.66)	1.00		
≥ 127	11,224	112 (1.00)	1.51	1.13–2.03	0.006

MP mechanical prophylaxis, *PE* pulmonary embolism, *CI* confidence interval, *OR* odds ratio

Neither sex nor comorbidity was associated with the rate of postoperative PE. The overall in-hospital mortality was 1.8 % ($n = 414$). Of them, in-hospital death following PE occurred in four patients (0.083 %) in the fondaparinux group and in 21 patients (0.11 %) in the control group, but the difference was not significant ($p = 0.53$).

Multivariate logistic regression for postoperative PE

Table 3 shows the adjusted OR and 95 % CI for the occurrence of PE as determined by multivariate logistic analysis. The results show that rate of PE was significantly reduced in the fondaparinux group compared with the control group (OR 0.67; $p = 0.047$). The rate of postoperative PE was significantly higher in the general anesthesia group than in the regional anesthesia group (OR 1.49; $p = 0.013$). A longer duration of anesthesia was significantly associated with higher postoperative PE occurrence (OR 1.42; $p = 0.025$). Sex, age, and comorbidity were not associated with the occurrence of postoperative PE.

Table 3 Multivariate logistic regression for postoperative pulmonary embolism

	Adjusted OR	95 % CI	*p* value
Prophylaxis			
MP alone	1.00		
MP and fondaparinux	0.67	0.44–0.99	0.047
Sex			
Male	1.00		
Female	1.29	0.82–1.92	0.206
Age (years)			
≤ 69	1.00		
70–79	1.40	0.82–2.40	0.215
≥ 80	1.62	0.98–2.68	0.060
Type of anesthesia			
Regional	1.00		
General	1.49	1.09–2.04	0.013
Duration of anesthesia (min)			
< 127	1.00		
≥ 127	1.42	1.05–1.92	0.025

MP mechanical prophylaxis, *CI* confidence interval, *OR* odds ratio

Discussion

Recent studies in Western countries have reported the occurrence of PE to be 0.25–0.95 % following hip fracture surgery with prophylaxis [17–19]. In the present study, the overall occurrence of postoperative PE was 0.83 % among 22,776 patients who underwent hemiarthroplasty for femoral

neck fracture from 2007 to 2010; this figure is thus comparable with that of the recent Western studies. Multivariate analysis showed that there was a potentially increased benefit of fondaparinux combined with mechanical prophylaxis in preventing postoperative PE following hemiarthroplasty for femoral neck fracture compared with mechanical prophylaxis alone. Owing to data limitations, we were unable to assess postoperative bleeding caused by fondaparinux. Thus, the safety of fondaparinux remains unclear. The rate of postoperative bleeding caused by fondaparinux was 0.27 % in after-market investigation in Japan, and three major bleedings from the colon and the cerebellum were reported [20]. In addition, careful selection may be needed to administer pharmacological antithrombotic prophylaxis to patients with renal dysfunction [21]. Prophylaxis by anticoagulants might have certain risk of local and systemic bleeding after surgery. Orthopedic surgeon must think about the balance of bleeding complication risks and the effectiveness of antithrombotic prophylaxis. Because unlike DVT, PE is a fatal complication, our data on fondaparinux is important information for selecting prophylactic methods. Further study is needed to investigate the rate of bleeding complications, to balance the risk of PE and bleeding complications.

Our data indicate that general anesthesia and longer duration of anesthesia were significantly associated with postoperative PE. A higher occurrence of PE in patients who underwent general anesthesia is consistent with the findings of Khatod et al. [22]. Regional anesthesia reduces postoperative pain and has a lower risk of postoperative confusion than general anesthesia. These factors may promote patient activity and reduce the risk of DVT and PE. A greater anesthesia time generally reflects a longer operating time. Prolonged obstruction of blood flow due to kinking of the femoral veins during surgery is presumably related to the development of DVT and PE. In addition, the increase in microscopic impairment of the intimal vessel wall caused by surgical invasion leads to the release of tissue factors, which further accelerate thrombus formation [23].

This study has several limitations. First, it is a retrospective observational study: the patient allocation is nonrandomized, and our cohort of patients was not obtained through random sampling. Therefore, our results are potentially biased because of unmeasured confounders. Second, the occurrence of DVT was not assessed in this study. Asymptomatic DVT is not detected unless patients are routinely screened using enhanced computed tomography or ultrasonography of the lower limb. For similar reasons, patients with asymptomatic PE may have been underreported. The 189 patients with PE identified in this study probably included patients diagnosed as having asymptomatic PE as well as symptomatic PE. Because routine screening to detect asymptomatic PE is not generally conducted by using computed tomography or ventilation–perfusion scanning, we assume that almost all the cases of PE were symptomatic. Third, the DPC database includes information that only relates to hospitalization. We were unable to evaluate the occurrence of PE after discharge. However, the average postoperative LOS was more than 30 days for both the control and fondaparinux groups. Since the majority of postoperative PE occurs within the first month [24], we believe that most patients with postoperative PE were included in our analysis. Despite these limitations, our study presents clinical evidence that fondaparinux in combination with mechanical prophylaxis is effective in preventing PE following hemiarthroplasty for femoral neck fracture.

Acknowledgments This study was funded by grants from the Ministry of Health, Labour and Welfare, Japan (Research on Policy Planning and Evaluation grant number H22-Policy-031; Research on Intractable Diseases grant number H23-Nanchi-032); the Ministry of Education, Culture, Sports, Science and Technology, Japan (Scientific Research B, No. 22390131); and the Council for Science and Technology Policy, Japan (Funding Program for World-Leading Innovative R&D on Science and Technology FIRST program grant number 0301002001001).

Conflict of interest The authors declare that they have no conflict of interest.

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Journal of Neuroscience Research

Journal of Neuroscience Research 92:1647–1658 (2014)

Lipopolysaccharide Preconditioning Facilitates M2 Activation of Resident Microglia After Spinal Cord Injury

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The inflammatory response following spinal cord injury (SCI) has both harmful and beneficial effects; however, it can be modulated for therapeutic benefit. Endotoxin/lipopolysaccharide (LPS) preconditioning, a well-established method for modifying the immune reaction, has been shown to attenuate damage induced by stroke and brain trauma in rodent models. Although such effects likely are conveyed by tissue-repairing functions of the inflammatory response, the mechanisms that control the effects have not yet been elucidated. The present study preconditioned C57BL6/J mice with 0.05 mg/kg of LPS 48 hr before inducing contusion SCI to investigate the effect of LPS preconditioning on the activation of macrophages/microglia. We found that LPS preconditioning promotes the polarization of M1/M2 macrophages/microglia toward an M2 phenotype in the injured spinal cord on quantitative real-time polymerase chain reaction, enzyme-linked immunosorbent assay, and immunohistochemical analyses. Flow cytometric analyses reveal that LPS preconditioning facilitates M2 activation in resident microglia but not in infiltrating macrophages. Augmented M2 activation was accompanied by vascularization around the injured lesion, resulting in improvement in both tissue reorganization and functional recovery. Furthermore, we found that M2 activation induced by LPS preconditioning is regulated by interleukin-10 gene expression, which was preceded by the transcriptional activation of interferon regulatory factor (IRF)−3, as demonstrated by Western blotting and an IRF-3 binding assay. Altogether, our findings demonstrate that LPS preconditioning has a therapeutic effect on SCI through the modulation of M1/M2 polarization of resident microglia. The present study suggests that controlling M1/M2 polarization through endotoxin signal transduction could become a promising therapeutic strategy for various central nervous system diseases.

Key words: endotoxin; inflammation; microglial activation

In the acute phase of spinal cord injury (SCI), inflammatory cells such as macrophages/microglia accumulate at the injured lesion following initial mechanical trauma to the spinal cord and release a variety of cytokines (Popovich et al., 2002; Fleming et al., 2006). Although the sequence of the inflammatory responses is thought to regulate the progression of tissue damage (Bethea and Dietrich, 2002), inflammatory cells are also believed to induce tissue repair (Rapalino et al., 1998). This contradictory role would be due to the diversity in the state of macrophage/microglia activation.

Recent studies have shown that activated macrophages/microglia coexist in various states within neural and nonneural tissues (Mosser and Edwards, 2008). Two different forms of macrophages/microglia have been identified in the injured spinal cord: classically activated (M1 phenotype) and alternatively activated (M2 phenotype) macrophages/microglia (Kigerl et al., 2009). M1 cells are activated by ligands of toll-like receptor (TLR) or interferon (IFN)-γ and have a toxic effect, whereas M2 cells are activated by interleukin (IL)-4, IL-10, and IL-13 and have a regenerative effect in vitro and in vivo. Although both M1 and M2 activation are rapidly induced following SCI, M2 activation has been shown to be only transient in the injured spinal cord (Kigerl et al., 2009). Therefore, facilitating M2 activation is a promising strategy for promoting tissue repair via inflammatory responses following SCI.

Endotoxin tolerance (ET) is a classic phenomenon involving modulation of the immune system in which cells or organisms preconditioned with low

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

Contract grant sponsor: JSPS; Contract grant number: 25670660.

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Received 9 January 2014; Revised 29 May 2014; Accepted 2 June 2014

Published online 10 July 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jnr.23448

concentrations of endotoxin can tolerate further challenges with endotoxin (Biswas and Lopez-Collazo, 2009). Recent studies have revealed that TLR4 signaling is one of the principal mechanisms for ET (Fan and Cook, 2004). Lipopolysaccharide (LPS), a major endotoxin, acts as a ligand of TLR4 and mediates ET mechanisms via the LPS–TLR4 pathway (Biswas et al., 2007). Given ET mechanisms, it has been recognized that pretreatment with low doses of LPS (LPS preconditioning) induces tolerance to various models of central nervous system (CNS) inflammation. LPS preconditioning has been widely studied in stroke models since the 1990s, and attenuation of tissue damage against ischemia-induced inflammatory responses has been reported (Tasaki et al., 1997; Rosenzweig et al., 2004; Dirnagl et al., 2009; Stevens et al., 2011). More recently, LPS preconditioning has been shown to modulate macrophage/microglia phenotypes in traumatic CNS injury models, including SCI (Longhi et al., 2011; Li et al., 2013). Furthermore, preconditioning with other pharmacological approaches such as CpG (a TLR9 agonist) has been described for ischemic SCI models (Stevens et al., 2011). However, the mechanism by which macrophages/microglia induce tissue remodeling and the long-term effects of preconditioning have not been fully elucidated.

In the present study, we hypothesized that LPS preconditioning affects M1/M2 polarization during macrophage/microglia activation in the injured spinal cord. To verify this hypothesis, we preconditioned mice with LPS before inducing SCI.

MATERIALS AND METHODS

Animals

Eight-week-old female C57BL6/J mice were purchased from Charles River Laboratories (Kingston, PA). Mice were maintained in the animal facilities at the Research Institute of the National Rehabilitation Center on a 12-hr light–dark cycle and provided with food and water ad libitum. All animal protocols were approved by the ethical committee of the Research Institute of the National Rehabilitation Center.

Administration of LPS

LPS derived from *Escherichia coli* O127:B8 was purchased from Sigma-Aldrich (St. Louis, MO). For the preconditioning, mice were injected intraperitoneally with 200 μl of either LPS with 0.05 mg (25,000 endotoxin units)/kg or phosphate-buffered saline (PBS) 48 hr before being subjected to SCI.

Surgical Procedure and Postoperative Care

Mice were anesthetized with sodium pentobarbital (50 mg/kg, Somnopentyl; Kyoritsu Seiyaku, Tokyo, Japan) before the skin covering the thoracic area was shaved. The skin was then incised so that the superficial fat could be pried apart to dissect the muscle tissue and expose the thoracic laminae. A commercially available impactor (Infinite Horizons impactor; Precision Systems and Instrumentation, Fairfax, VA) was then used to induce contusion SCI with an impact force of 80 kdynes. Mice were monitored daily for general health, mobility within the cage, infections, and autophagy of the toes throughout the experiment. Bladders were manually expressed twice daily for the first week after the operation and once daily thereafter as required. Mice with surgical-site infection or disruption were excluded from the experiment.

Anti-IL-10 Therapy

Mice were injected intraperitoneally with 250 μg of anti-mouse IL-10 antibody (Biolegend, San Diego, CA) immediately before being subjected to SCI.

In Vitro Model of Preconditioning With Bone Marrow-Derived Macrophages

Bone marrow cells were obtained from the femur and tibia of 6-week-old C57BL/6 mice, and bone marrow-derived macrophages (BMDMs) were cultured in α-minimum essential medium (α-MEM; Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich) in the presence of 100 ng/ml macrophage colony-stimulating factor (M-CSF; R&D Systems, Minneapolis, MN) for 2 days. BMDMs were treated with 0.25% trypsin, plated at optimal cell density, and incubated in α-MEM containing 10% FBS in the presence of 100 ng/ml M-CSF for 2 days on the tissue culture dishes (polystyrene; BD Biosciences, Franklin Lakes, NJ). Then, we switched the medium to α-MEM containing 0.1 ng/ml LPS, and the cells were incubated for 3 hr. Forty-eight hours later, BMDMs were stimulated with α-MEM containing 10 ng/ml recombinant mouse IFN-γ (R&D Systems).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA from spinal cords ±3 mm from the epicenter lesion was isolated by using an RNeasy minikit (Qiagen, Hilden, Germany) and reverse-transcribed by using an RNA PCR kit (AMV; Takara, Shiga, Japan) according to the manufacturer's protocol. The cDNA was then used as a template in real-time polymerase chain reaction (PCR) assays based on SYBR green detection with the ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA). The PCR primers used are listed in Table I.

Enzyme-Linked Immunosorbent Assay

Spinal cords ±3 mm from the epicenter lesion were removed and lysed in T-PER tissue protein extraction reagent (ThermoFisher Scientific, Waltham, MA) containing proteases from the Halt protease inhibitor cocktail kit (Thermo Fisher Scientific). The concentrations of inducible nitric oxide synthase (iNOS) and arginase1 were determined by using enzyme-linked immunosorbent assay (ELISA) kits (Cusabio, Hubei, China) according to the manufacturer's protocols.

Flow Cytometry

Spinal cords were dissected at ±3 mm from the epicenter lesion and dissociated with neural tissue dissociation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Tissue debris was then removed via Percoll gradient centrifugation to obtain a single-cell suspension.

TABLE I. Primers

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
GAPDH	TGCACCACCAACTGCTTAGC	GGATGCAGGGATGATGTTCT
Arginase1	GAACACGGCAGTGGCTTTAAC	TGCTTAGCTCTGTCTGCTTTGC
CD206	TCTTTGCCTTTCCCAGTCTCC	TGACACCCAGCGGAATTTC
iNOS	CCCTTCAATGGTTGGTACATGG	ACATTGATCTCCGTGACAGCC
CD16	TTTGGACACCCAGATGTTTCAG	GTCTTCCTTGAGCACCTGGATC
CD86	TTGTGTGTGTTCTGGAAACGGAG	AACTTAGAGGCTGTGTTGCTGGG
VEGF	CGGAGGCAGAGAAAAGAGAAAGTG	GGGAGAGAGAGATTGGAAACACAG
IGF-1	CTGGACCAGAGACCCTTTGC	GGTGCCCTCCGAATGCT
IL-10	GCTCCAAGACCAAGGTGTCTACAA	CCGTTAGCTAAGATCCCTGGATCA
IL-4	ACTCTAGTGTTCTCATGGAGCTGC	AAAGCATGGTGGCTCAGTACTACG
IL-13	ATCTCAGCTGTGGACTCATTTTCC	CAGTTTTCTGTAGGGATGGGATGG

TABLE II. Antibodies Used for Immunohistochemistry*

Antibody	Supplier	Catalog No.	Host	Application
Primary				
Arginase1	Santa Cruz Biotechnology	sc-18354	Goat	1:100
Iba1	Wako	019-19741	Rabbit	1:500
GFAP	Millipore	MAB360	mouse	1:500
PECAM	Millipore	MAB1398Z	Hamster	1:400
CD11b	BD Pharmingen	550282	Rat	1:500
pIRF3	Cell Signaling Technology	4947	Rabbit	1:500
Secondary				
Alexa Fluor 488	Invitrogen	A-11029	Goat	1:200
		A-11055	Donkey	
		A-21206	Donkey	
Alexa Fluor 568	Invitrogen	A-11036	Goat	1:200
		A10042	Donkey	
DyLight 488	Jackson Immunoreseach	107-485-142	Goat	1:400

*Iba1, ionized calcium-binding adapter molecule 1; GFAP, glial fibrillary acidic protein; PECAM, platelet endothelial cell adhesion molecule.

Cells were blocked with FcR blocking reagent (Miltenyi Biotec), which contained a 1:10 dilution of 0.5% BSA and PBS, at 4°C for 10 min. Cells were then fixed and permeabilized for intra- and extracellular staining with a PerFix-nc kit (Beckman Coulter, Brea, CA) and incubated for 30 min at room temperature with the following fluorescent antibodies: fluorescein isothiocyanate (FITC) anti-mouse CD45 (Biolegend), PE anti-mouse iNOS (Bioss, Woburn, MA), VioGreen anti-mouse Gr-1 (Miltenyi Biotec), and arginase1 antibody (Santa Cruz Biotechnology, Dallas, TX) conjugated to biotin (biotin labeling kit; Dojindo, Kumamoto, Japan). Cells were subsequently incubated for 15 min at room temperature with antibiotin antibody conjugated to VioBlue (Miltenyi Biotec). Flow cytometry was performed, and the result was analyzed by using MACSQuant analyzers (Miltenyi Biotec).

Magnetic Cell Sorting

The tissue samples were collected, dissected, and dissociated. Tissue debris was removed by using a procedure similar to that used for flow cytometry. Cells were blocked with the FcR blocking reagent at a dilution of 1:10 in 0.5% BSA/PBS at 4°C for 10 min. Cells were then labeled with CD11b MicroBeads (Miltenyi Biotec) before they were separated by using an autoMACS Pro separator (Miltenyi Biotec). Positive fractions (CD11b-positive cells) were then collected and used to obtain total RNA by using the RNeasy minikit.

Immunohistochemistry

Mice were perfused transcardially under deep ether anesthesia with buffered 4% paraformaldehyde. The spinal cords were removed and postfixed in 4% paraformaldehyde at 4°C for 4 hr, cryoprotected in buffered 20% sucrose at 4°C for 24 hr and 30% sucrose for 24 hr, and embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA). Frozen tissues were cut into 14-μm longitudinal sections with a cryostat and mounted onto glass slides.

Sections were then blocked with 2% donkey serum in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 hr at room temperature. After this, sections were incubated overnight at room temperature with a blocking solution containing the diluted primary antibodies. Subsequently, the sections were incubated with the secondary antibodies diluted in blocking solution for 2 hr at room temperature. Slides were then mounted with ProLong Gold (Invitrogen, Carlsbad, CA), and images were captured with a DMIRE2 microscope system (Leica, Wetzlar, Germany) and an LSM5 Pascal confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany). The primary and secondary antibodies used for the immunohistochemistry are listed in Table II.

Evaluation of Vascularization

Sections that had been stained with the antiplatelet endothelial cell adhesion molecule (PECAM) antibody were examined. Four randomly selected images were obtained from the site around the injured lesion. The length of PECAM-positive blood vessels was measured by tracing the long axis of the PECAM-positive area with a pen tablet input device (Bamboo Pen; Wacom, Tokyo, Japan) and summed up in image analyzer software (WinROOF; Mitani, Tokyo, Japan).

Lesion Analysis

Tissue samples from the SCI mice were fixed, collected, postfixed, cryoprotected, embedded, cut into sections, and mounted on glass slides by using the procedure previously described under Immunohistochemistry. Myelin and myelinated axons were then stained with Luxol fast blue (LFB) solution. Briefly, the sections were hydrated with 95% ethanol, defatted with 1:1 ethanol:chloroform, hydrated with 95% ethanol, and left in a 0.1% LFB solution (solvent blue; MP Biomedicals, Santa Ana, CA) at 56°C overnight. The sections were then rinsed in 95% ethanol and distilled water, differentiated with lithium carbonate solution and 70% ethanol, rinsed with distilled water, differentiated in 95% and 100% ethanol, cleared with xylene, and mounted. The sections were examined with an optical microscope (BX51; Olympus, Tokyo, Japan), and images of the midline sections were obtained in image processing software (InStudio; Pixera, San Jose, CA). The edges of the demyelinated lesions were drawn with the pen tablet input device, and the surrounding area was automatically measured in the WinROOF image analyzer software.

Behavioral Assessments

Hind limb motor function was evaluated by using the Basso Mouse Scale (BMS) open field locomotor test, in which the scores range from zero points (no ankle movement) to nine points (complete functional recovery); the BMS has been shown to be a valid locomotor rating scale for mice (Basso et al., 2006). BMS scores were recorded 7, 14, 21, 28, 35, and 42 days after SCI. When differences in the BMS score between right and left hind limbs were detected, we took the average of the two scores. All evaluations were made by two observers blinded to experimental procedures.

Western Blotting

Nuclear protein was isolated from the injured spinal cord by using a nuclear extraction kit (Active Motif, Carlsbad, CA). Samples containing an equal amount of protein (10 μg) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Billerica, MA), and blots were then incubated overnight at 4°C in a 5% BSA TBS-T solution containing either phosphointerferon regulatory factor (IRF)-3 (Cell Signaling Technology, Danvers, MA) or lamin B (Santa Cruz Biotechnology) antibodies at a 1:1,000 dilution. Blots were then incubated for 2 hr at room temperature in a 0.5% BSA TBS-T solution containing a 1:20,000 dilution of the secondary antibodies. Immunoblots were treated with an ECL reagent (Lumigen TMA-6; Lumigen, Southfield, MI) before luminescence was detected and analyzed by using the OdysseyFc Imaging System (MS Technosystems, Osaka, Japan).

IRF-3 Activity Assay

Nuclear proteins were isolated from the injured spinal cord by using the nuclear extraction kit. IRF-3 activity was measured in 10 μg of the nuclear protein extract by using an IRF-3 activity ELISA (TransAM IRF-3; Active Motif) according to the manufacturer's protocol. The ELISA kit has an immobilized oligonucleotide containing an IRF-3 consensus binding site that could specifically bind to the active form of IRF-3 in the nuclear extract.

Statistical Analysis

Statistical analyses were performed with an unpaired two-tailed Student's *t*-test for single comparisons and a one-way ANOVA for multiple comparisons. In all statistical analyses, values were considered significant at $P < 0.05$.

RESULTS

LPS Preconditioning Facilitated M2 Activation at the Site of SCI

Mice were subjected to intraperitoneal injection of either LPS as a preconditioning treatment or saline as a control. In a preliminary study, we determined the timing and dose of LPS preconditioning as 48 hr before the induction of experimental SCI with 0.05 mg/kg LPS (Fig. 1A). To investigate the effect of LPS preconditioning on the activation of macrophages/microglia, we evaluated the M1/M2 polarization of macrophages/microglia after SCI by examining the mRNA expression of M1 markers (iNOS, CD86, and CD16) and M2 markers (arginase1 and CD206) in the injured spinal cord. The gene expression of arginase1 was significantly higher at 1 and 3 days after SCI with LPS preconditioning (Fig. 1B). We also found a tendency for higher CD206 gene expression at 7 days after SCI with LPS preconditioning. We found no difference in the gene expression of M1 markers with or without LPS preconditioning, except for CD86 gene expression, which was significantly lower at 1 day after SCI with LPS preconditioning (Fig. 1C). The protein level of arginase1 was significantly higher at 1 and 3 days after SCI with LPS preconditioning, whereas that of iNOS was equivalent with or without LPS preconditioning (Fig. 1D). In addition, we performed immunohistochemical analysis of the injured spinal cord at 3 days after SCI, when the Iba1-positive cells surrounded the injured lesion. Antibodies against arginase1 and Iba1 were used to identify the M2 phenotypic cells and macrophages/microglia, respectively. Almost all arginase1-positive cells overlapped with the Iba1-positive cells. We quantified the number of stained cells around the epicenter lesion where the Iba1-positive cells were clustered. We observed that the number of arginase1-Iba1 double-positive cells increased with LPS preconditioning. These data indicate that LPS preconditioning facilitated M2 activation of macrophages/microglia at the lesion site within 7 days after SCI.

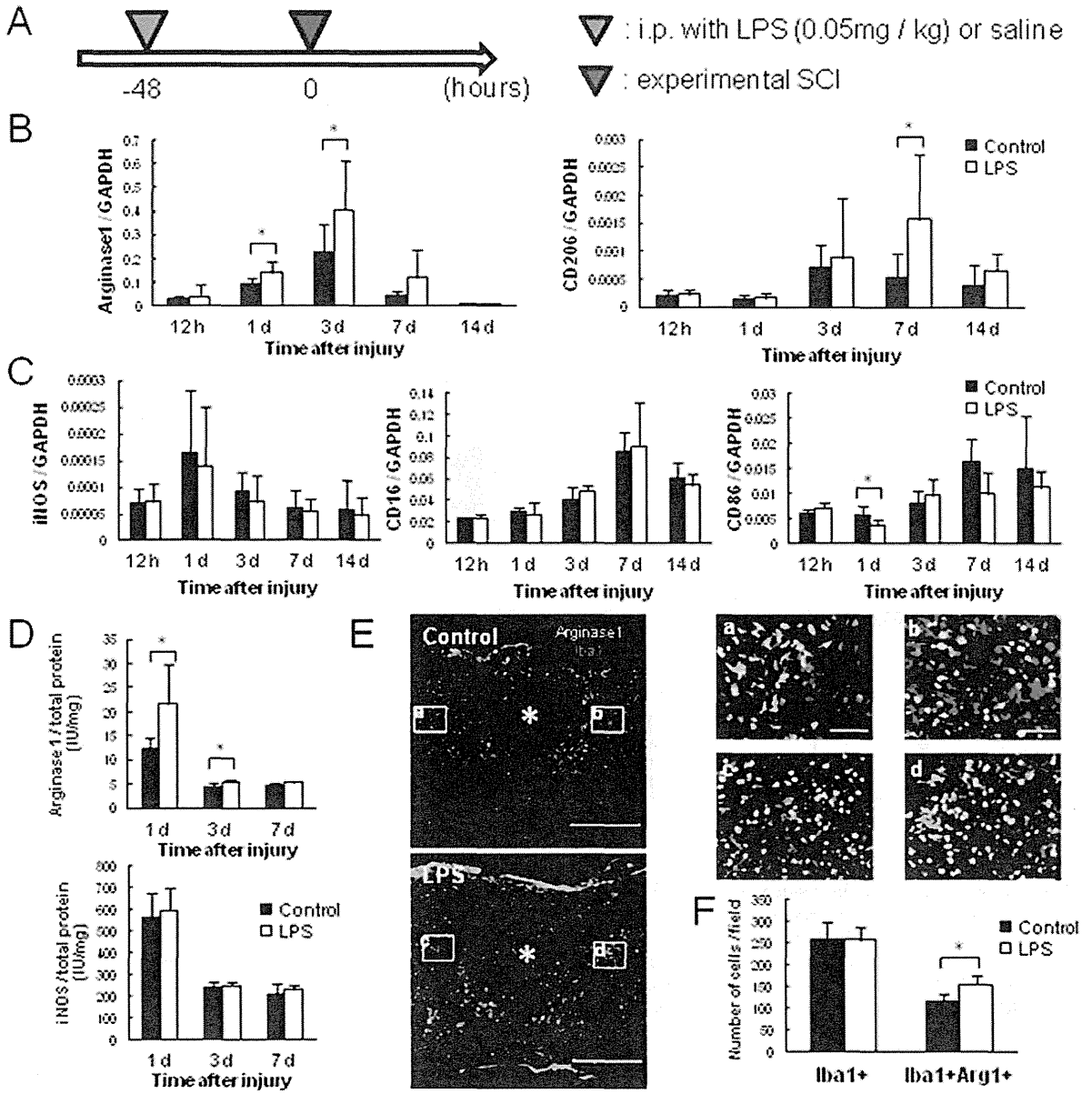

Fig. 1. LPS preconditioning facilitates M2 activation at the site of SCI. **A:** Schematic showing the protocol used for SCI and LPS preconditioning. **B,C:** M2 marker (B; arginase1, CD206) and M1 marker (C; iNOS, CD16, CD86) mRNA expression in the injured spinal cord after SCI (n = 5–8). Results are the values relative to GAPDH expression. **D:** Quantification of protein levels (iNOS, arginase1) after SCI, measured by ELISA (n = 6). Results are the values relative to the concentration of total protein. **E:** Immunohistochemical analysis of the longitudinal midline section of the spinal cord 3 days after SCI (green, arginase1; red, Iba1). The asterisks represent the epicenter of the injured lesion. **a–d:** High magnification (×40). **F:** Quantification of the number of Iba1-positive cells and Iba1 + arginase1 double-positive cells (n = 5). Results are the number of cells per field at high magnification (×20). Error bars represent SD; $*P < 0.05$. Scale bars = 500 μm in E; 50 μm in a–d.

M2 Activation Was Facilitated in Resident Microglia but Not in Macrophages

To identify the M2-activated cells, we examined the M1/M2 polarization of microglia and macrophages independently by using flow cytometry. Given the extent of CD45 expression, we distinguished between resident microglia ($CD45^{low}$) and infiltrating macrophages ($CD45^{high}$) in the injured spinal cord (Fig. 2A). We also eliminated neutrophil/monocyte (Gr-1-positive cells) from analysis (Stirling and Yong, 2008). We then

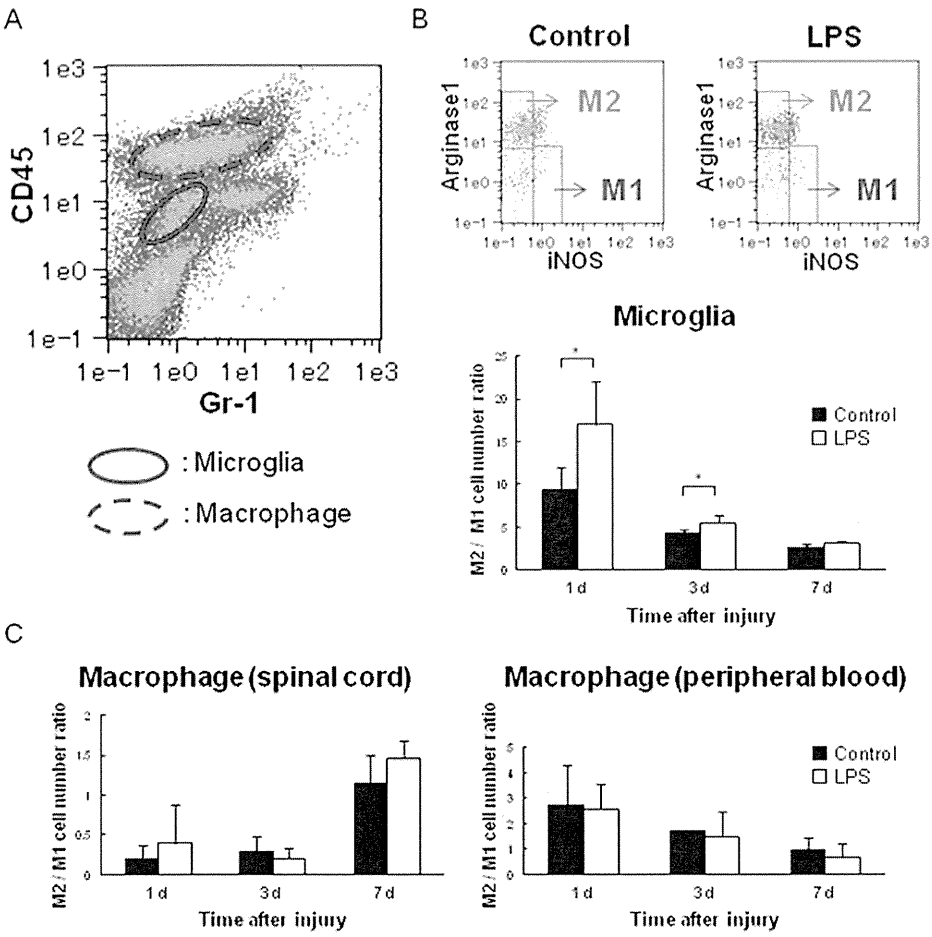

Fig. 2. M2 activation is facilitated in resident microglia but not in macrophages. **A:** Distinction between resident microglia ($CD45^{low}$) and infiltrating macrophages ($CD45^{high}$) in the injured spinal cord. The area within the solid line represents the resident microglia, and the area within the dashed line represents infiltrating macrophages. **B:** Identification of M1 cells and M2 cells by using iNOS and arginase1 expression in the resident microglia. Graph shows the quantification of the M2/M1 cell number ratio in the resident microglia identified from the injured spinal cord. **C:** Quantification of the M2/M1 cell number ratio in the infiltrating macrophages identified from the injured spinal cord and the circulating macrophages in the peripheral blood. Error bars represent SD; $*P < 0.05$.

identified M1 cells and M2 cells by using the levels of iNOS and arginase 1 expression. We found that the M2/M1 ratio in the resident microglia was significantly higher at 1 and 3 days after SCI with LPS preconditioning (Fig. 2B). In contrast, we found no difference in the M2/M1 ratio in the infiltrating macrophages identified from the injured spinal cord or the circulating macrophages from the peripheral blood (Fig. 2C). These results demonstrate that M2 activation was facilitated in resident microglia, but not with macrophages, after SCI with LPS preconditioning.

LPS Preconditioning Promoted Vascularization Around the Site of SCI

M2 macrophages have been shown to have stronger angiogenic properties than M1 macrophages in vitro and in vivo (Kodelja et al., 1997; Jetten et al., 2014). To assess the angiogenic effects of M2 activation in our model, we evaluated the vascularization around the injured lesion after SCI with immunohistochemistry. An antibody against PECAM was used to identify the vascular endothelial cells. The results showed that vascularization around the lesion had increased at 7 days after SCI with LPS preconditioning (Fig. 3A). In addition, total vascular length had increased significantly at 3 and 7 days after SCI with LPS preconditioning (Fig. 3B). No difference was found in the total vascular length for the sites that were rostral and caudal to the epicenter lesion. In addition, we examined the gene expression of two angiogenic factors, VEGF and IGF-1, in the CD11b-positive cells (macrophages/microglia) isolated from the injured spinal cord by using magnetic cell sorting. We confirmed the purity of macrophages/microglia isolated with magnetic beads to

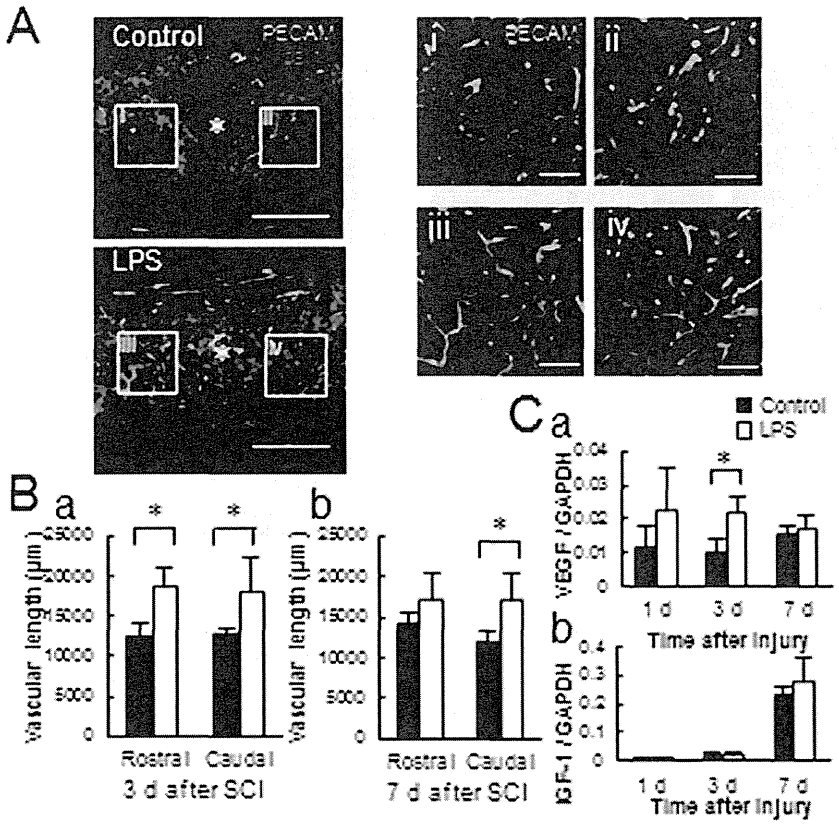

Fig. 3. LPS preconditioning promotes vascularization around the site of SCI. **A:** Immunohistochemical analysis of the longitudinal midline section of the injured spinal cord 7 days after SCI (green, PECAM; red, Iba1). The asterisks represents the epicenter of the injured lesion. **i–iv:** High magnifications (×40). **B:** Measurement of the total vascular length 3 (a) and 7 (b) days after SCI (n = 5). **C:** VEGF (a) and IGF-1 (b) mRNA expression in the CD11b-positive cells isolated from the injured spinal cord after SCI. Results are the values relative to GAPDH expression (n = 5). Error bars represent SD; $\star P < 0.05$. Scale bars = 500 μm in A; 100 μm in i–iv.

be more than 80% (80.8% ± 2.16%) by using flow cytometry and labeling with CD45. Real-time PCR showed significantly enhanced VEGF expression at 3 days after SCI with LPS preconditioning (Fig. 3C). These results demonstrate that LPS preconditioning facilitated M2 activation, which resulted in the promotion of vascularization around the site of SCI.

Mice With LPS Preconditioning Exhibited Better Histological and Functional Improvement After SCI

We evaluated the tissue reorganization and the functional recovery in mice that received LPS preconditioning after SCI. During immunohistochemical analysis, the antibody against glial fibrillary acidic protein (GFAP) was used to identify the glial scar. We observed that the macrophages/microglia were packed in the lesion with the glial scar at 2 weeks after SCI with LPS preconditioning, whereas packing with the glial scar was incomplete without LPS preconditioning (Fig. 4A). Next, we evaluated the tissue reorganization at the site of SCI by measuring the demyelinated area with LFB staining until 6 weeks after SCI. The demyelinated area markedly decreased 1–2 weeks after SCI with LPS preconditioning. The demyelinated area became significantly smaller 2–6 weeks after SCI in mice with LPS preconditioning compared with the control group (Fig. 4B,C). For the locomotor function, we recorded a BMS score until 6 weeks after SCI and found that it was significantly higher 3–6 weeks after SCI with LPS preconditioning compared with the control group (Fig. 4D). We also found that, in mice that had undergone LPS preconditioning, the BMS score at 2 weeks after SCI was marginally higher ($P = 0.077$). Overall, mice with LPS preconditioning exhibited better tissue reorganization and functional recovery after SCI.

IL-10 Was the Main Regulator Leading to an M2 Phenotype With LPS Preconditioning

To identify the main regulator leading to an M2 phenotype with LPS preconditioning, we examined the gene expression of cytokines known to induce M2 activation by using real-time PCR. Among those cytokines,

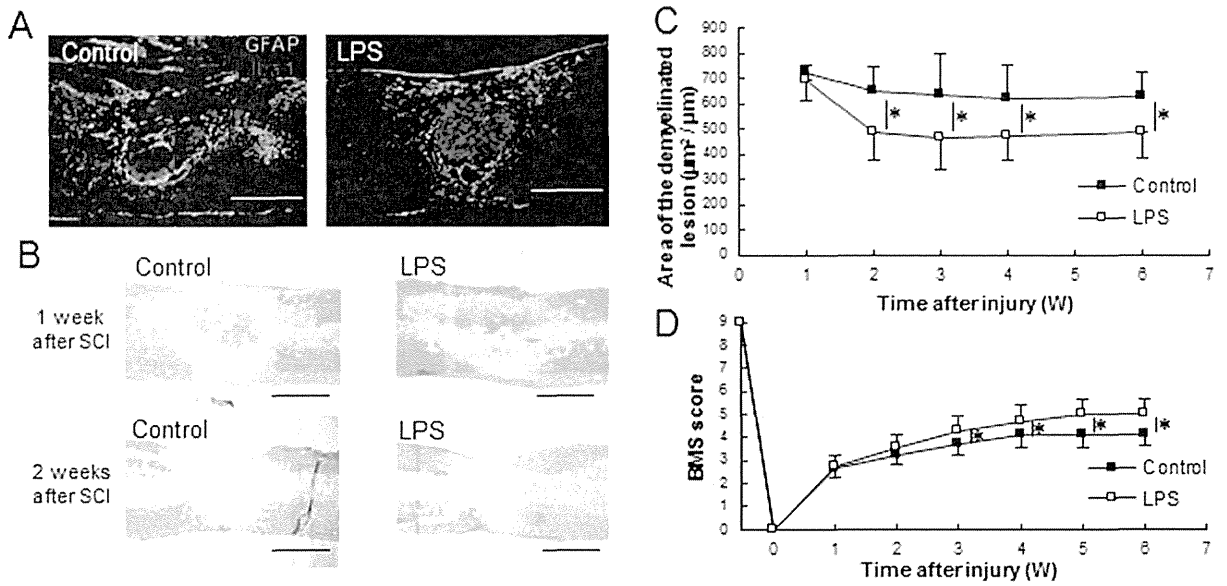

Fig. 4. Mice with LPS preconditioning exhibit better histological and functional improvement after SCI. **A:** Immunohistochemical analysis of the longitudinal midline section of the injured spinal cord at 2 weeks after SCI (left, control; right, LPS; green, GFAP; red, Iba1). **B:** LFB staining of the longitudinal midline section of the injured spinal cord 1 and 2 weeks after SCI. **C:** Quantification of the demyelinated area standardized by the diameter of the short axis of the section (n = 5). **D:** Time course of BMS scores after SCI (n = 10). Error bars represent SD; $*P < 0.05$. Scale bars = 500 μm.

IL-10 showed significantly higher gene expression at 12 hr after SCI with LPS preconditioning compared with the control group (Fig. 5 A), whereas IL-4 and IL-13 showed no difference in gene expression with or without LPS preconditioning (Fig. 5B,C). In addition, we confirmed that M2 activation after SCI in the LPS preconditioning group was significantly suppressed by administering anti- IL-10 antibody (Fig. 5D,E). We also conducted an in vitro study by using BMDMs to clarify whether IL-10 gene expression was promoted in macrophage/microglia with preconditioning. We confirmed that IL-10 gene expression levels were higher in BMDMs stimulated with LPS prior to IFN-γ stimulation than in BMDMs stimulated with IFN-γ alone (Fig. 5F). These results demonstrate that IL-10 was the main regulator of the M2 phenotype with LPS preconditioning.

LPS Preconditioning Activated IRF-3 After SCI

Finally, we investigated the activity of IRF-3, a transcription factor that is downstream of the LPS–TLR4 pathway (Honda and Taniguchi, 2006), in nuclear extracts isolated from mice with injured spinal cords. Western blots indicated that there was a significantly higher level of phosphorylated IRF-3 (pIRF-3), the activated form of IRF-3, in the nucleus at 12 hr after SCI with LPS preconditioning (Fig. 6A,B). In addition, an IRF-3 activity assay indicated that there was increased IRF-3 activity at 3 and 12 hr after SCI with LPS preconditioning (Fig. 6C). To confirm that IRF-3 is phosphorylated within the same cell where IL-10 gene expression is seen, we performed immunohistochemical analysis involving double staining with pIRF-3 and CD11b at 12 hr after SCI with LPS preconditioning. We observed that pIRF-3 was expressed mainly in CD11b-positive cells (Fig. 6D). These findings demonstrate that there was an increase in IRF-3 activity with LPS preconditioning within 12 hr of SCI, concurrent with an increase in IL-10 gene expression.

DISCUSSION

The present study indicates that LPS preconditioning facilitated M2 activation after SCI. In addition, we observed that M2 activation after SCI with LPS preconditioning contributed to the vascularization around the injured lesion, resulting in better tissue reorganization and in functional recovery. Furthermore, we found that the M2 activation after SCI with LPS preconditioning was induced by IL-10 expression as a result of the activation of IRF-3. Some studies have shown that LPS preconditioning attenuates neural damage by modulating macrophages/microglia (Lastres-Becker et al., 2006). However, the details of this modulation and its effect on macrophage/microglial activation have not yet been elucidated. To our knowledge, the current study is the first to investigate the M1/M2 polarization that occurs after SCI with LPS preconditioning.

LPS activates M1 polarization through the MyD88-NFκB pathway via TLR4 signaling (Mosser and Edwards, 2008; Kigerl et al., 2007) reported that TLR4 regulated inflammation and gliosis after SCI in a study in which

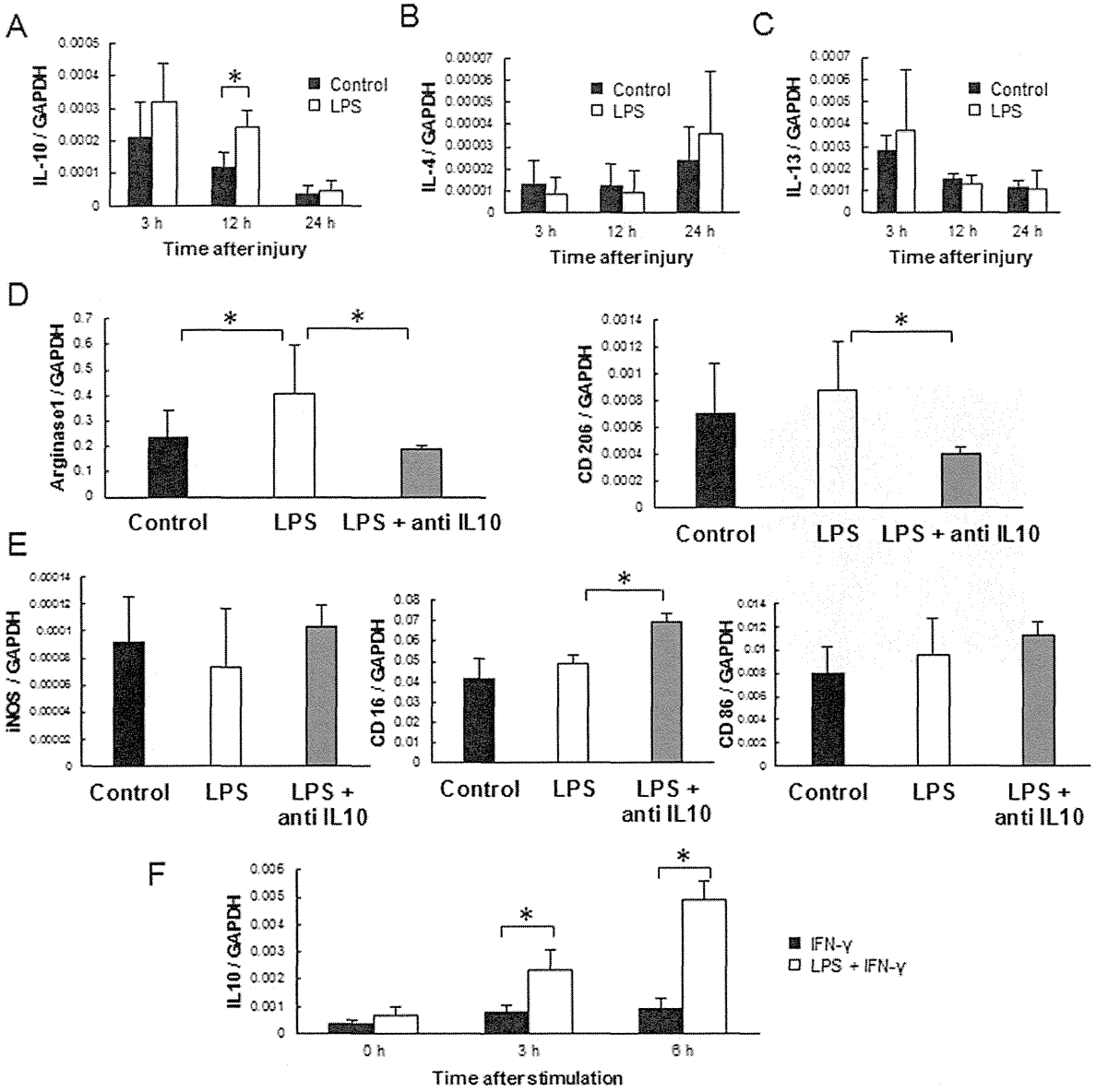

Fig. 5. IL-10 is the main regulator leading to an M2 phenotype with LPS preconditioning. **A–C:** mRNA expression of anti-inflammatory cytokines (A, IL-10; B, IL-4; C, IL-13) in the injured spinal cord (n = 6). **D,E:** M2 marker (D; arginase1, CD206) and M1 marker (E; iNOS, CD16, CD86) mRNA expression in the injured spinal cord at 3 days after SCI (n = 4). **F:** IL-10 mRNA expression in cultured BMDMs after stimulation with IFN-γ (n = 3). Results are the values relative to GAPDH expression. Error bars represent SD; $*P < 0.05$.

they used TLR4 mutant mice. In addition, TLR4 has been shown to be activated following SCI (Chen et al., 2011; Bell et al., 2013; Zhang et al., 2013). Therefore, we hypothesized that, because of LPS preconditioning, the transiently M1-polarized macrophages/microglia acquired tolerance for subsequent M1 activation with SCI and were redirected to M2 activation. In line with our hypothesis, Porta et al. (2009) reported that LPS-tolerant monocytes/macrophages express an M2 cytokine/chemokine profile in an in vitro study. It should be acknowledged that M1 activation was not found to be attenuated sufficiently when we examined the expression of M1 markers by real-time PCR and ELISA after SCI with LPS preconditioning. These data raise the possibility that the changes in the M1 population were not necessarily reflected in the M1 marker expression levels. A more precise evaluation of the M1 cell population might be required in future studies. Another possible reason why M1 marker levels were not attenuated is that the total activation of macrophages/microglia might be greater with LPS preconditioning. Although further investigations might be required, the results presented here

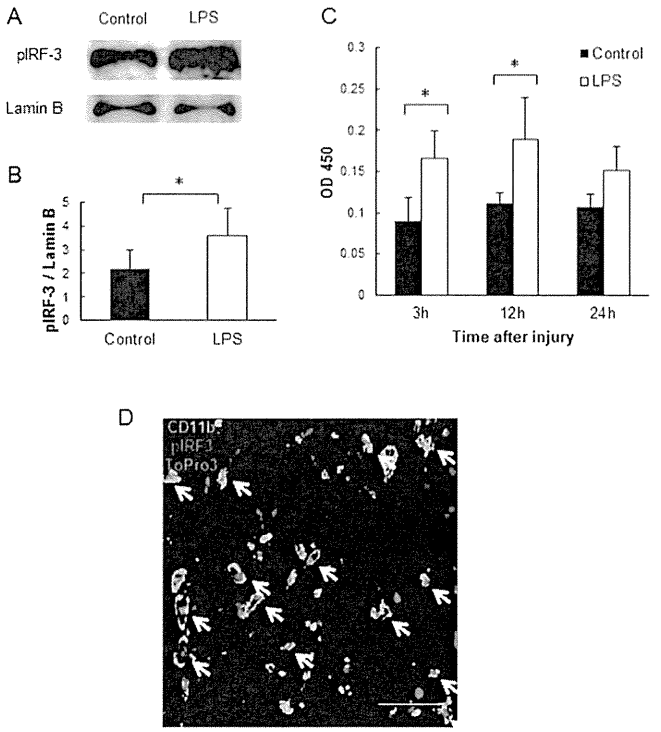

Fig. 6. LPS preconditioning activates IRF-3 concurrent with IL-10 expression after SCI. Nuclear extracts from the injured spinal cord were examined. **A:** Western blots that used an anti-pIRF3 or antilamin B (internal control) at 12 hr after SCI. **B:** Quantification of the pixel intensity of bands by Western blots (n = 5). **C:** Quantification of IRF-3 activation with an IRF-3 activity ELISA (n = 5). **D:** Immunohistochemical analysis of the longitudinal midline section of the injured spinal cord at 12 hr after SCI, at high magnification (green, CD11b; red, pIRF3; blue, To-Pro-3; arrows, CD11b + pIRF3 double-positive cells). Error bars represent SD; $*P < 0.05$. Scale bar = 50 μm.

indicate that LPS preconditioning redirected M1 activation in the spinal cord to M2 activation after SCI.

The flow cytometric results showed that M2 activation was mainly induced in microglia and not in the infiltrated macrophages, suggesting that the resident microglia were primed by the LPS. Although microglia have been observed to be activated throughout the CNS by the peripheral injection of LPS (Lin et al., 2009; Chen et al., 2012), it is unlikely that LPS directly penetrated the blood–brain barrier (Singh and Jiang, 2004). One of the plausible explanations for this is that circulating signals induced by LPS might be transmitted to the resident microglia through the endothelial cells in the CNS. This hypothesis is supported by Leow-Dyke et al. (2012), who reported that LPS stimulates TLR4, leading to subsequent endothelial activation in mixed brain cultures containing endothelial cells and microglia. Our finding that M2 activation was induced mainly in the resident microglia demonstrates that microglia are the effector cells of LPS preconditioning.

M2 macrophages have been shown to have stronger angiogenic effects than M1 macrophages in vitro and in vivo (Kodelja et al., 1997; Jetten et al., 2014). VEGF, a stimulator of angiogenesis, has been shown to increase blood vessels in the injured spinal cord (Kim et al., 2009). In a rat amyotrophic lateral sclerosis model, microglia isolated from the spinal cord have been shown to express VEGF and contribute to neural survival (Nikodemova et al., 2013). We observed that the M2 macrophages/microglia (CD11b-positive cells) promoted vascularization around the injured lesion as a result of increased expression of VEGF after SCI with LPS preconditioning. It is reasonable to assume that M2-polarized microglia induced a well-vascularized environment after SCI as a result of LPS preconditioning. In addition, we found that the macrophages/microglia were packed in the lesion with the glial scar at 2 weeks after SCI with LPS preconditioning, whereas packing of the glial scar was incomplete without LPS preconditioning, suggesting that macrophages/microglia in the injured lesion were packed with the glial scar in the earlier phase as a result of LPS preconditioning. Recent studies have shown that endothelial progenitor cells promote astrogliosis with vascularization, which is in line with our results showing that LPS preconditioning promoted gliosis (Shen et al., 2004; Kamei et al., 2012). The glial scar plays a role as a barrier for preventing the spread of toxicity and promoting phagocytic activity, although harmful effects as a result of glial scarring have also been reported (Okada et al., 2006; Rolls et al., 2009). Furthermore, we confirmed that there was better tissue reorganization, accompanied by functional recovery, up to 6 weeks after SCI as a result of LPS preconditioning. We observed that, from week 1 to week 2, the damaged area shrank after SCI as a result of the LPS preconditioning, concurrent with the earlier formation of the glial scar, suggesting that M2 activation indirectly affected tissue reorganization by promoting vascularization and accelerating glial scar formation. Gene expression of M1 and M2 markers gradually decreased from 1 week after SCI regardless of LPS preconditioning, in line with recent studies (Supp. Info. Fig. 1; Kigerl et al., 2009; Nishimura et al., 2013). Thus, we suppose that the preservation of myelin and functional recovery were not a direct effect of M2 facilitation at later time points but were due to a subsequent reaction following M2 facilitation at the acute phase after SCI. To our knowledge, this study is the first to investigate a long-term consequence of LPS preconditioning and provide evidence that immune modulation of M1/M2 polarization in the acute phase after SCI can have long-term effects on the recovery from SCI.

In our experimental model, IL-10 was identified as an M2-polarizing signal. M2 macrophages can be subdivided based on their functionality (Mantovani et al., 2002). Wound-healing macrophages are activated by IL-4 or IL-13 and belong to the M2a subset, whereas regulatory macrophages are activated by many stimuli, including IL-10, and are called M2c macrophages (David and Kroner, 2011). We believe that the M2-polarized cells