Neutrophil recruitment in arterial thrombus and characteristics of stroke patients with neutrophil-rich thrombus

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Neutrophil recruitment in arterial thrombus and characteristics of stroke patients with neutrophil-rich thrombus

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ABSTRACT

Neutrophil recruitment in arterial thrombus and characteristics of stroke patients with neutrophil-rich thrombus

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Purpose

Neutrophils contribute to thrombosis. However, there is limited information on the temporal course of neutrophil recruitment in thrombosis, the contribution of neutrophils to thrombus growth, and the characteristics of stroke patients with neutrophil-rich thrombi.

Materials and Methods

After inducing carotid artery thrombosis in Institute of Cancer Research mice using ferric chloride, aged thrombi were produced by ligating the distal portion of the carotid artery in mice for 0.5, 1, 2, 3, 6, or 24 h. For thrombus analysis in stroke patients, we used registry data and thrombi that were obtained during intra-arterial thrombectomy. Immunohistochemistry was performed to determine thrombus composition.

Results

In the thrombi of 70 mice, Ly6G positive cell counts (neutrophils) and histone H3-positive cell counts increased in a time-dependent manner (both $p<0.001$). Ly6G-positive cell count was strongly correlated with histone H3-positive cell counts ($r=0.910$, $p<0.001$), but not with thrombus size ($p=0.320$). In 75 stroke patients, atrial fibrillation and cardioembolism

were more frequent in the higher neutrophil group (32/37, 86.5%) than in the lower neutrophil group (19/38, 50%) (p=0.002). The median erythrocyte fraction was higher (52.0 [interquartile range 39.9−57.8] in the higher neutrophil group than in the lower neutrophil group (40.3 [interquartile range 23.5−53.2]). The fraction of neutrophils was positively correlated with that of erythrocytes (r=0.35, p=0,002).

Conclusion

Neutrophils were recruited and increased in arterial thrombosis in a time-dependent manner; however, they were not associated with the growth of formed thrombi. Neutrophil fractions in the thrombi of stroke patients appeared to be associated with atrial fibrillation and erythrocyte fraction.

Key words : neutrophil, thrombosis, stroke, neutrophil extracellular traps, intra-arterial thrombectomy

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I. INTRODUCTION

Thrombosis is a complex and dynamic process. A thrombus is composed of blood cells and plasma components, such as platelets, erythrocytes, leukocytes, and fibrin. Leukocytes are often considered bystanders or trapped components during thrombosis. However, recent studies suggest that leukocytes and inflammation contribute to thrombosis.[1,2] The formation of intravascular thrombi plays a protective role against bacterial infection by immobilizing bacteria and, thus, restricting their invasion of the tissue.[3] Therefore, thrombosis and inflammation are mutually reinforcing processes.[4]

Neutrophils are the first and most abundant leukocytes recruited to inflammation or injury sites.[5] Neutrophils also play a role in ischemic injuries and contribute to venous and arterial thrombosis.[6,7] Neutrophil depletion has been shown to lead to reduced thrombus size in deep vein thrombosis of mice.[8] Neutrophils promote thrombosis by releasing neutrophil extracellular traps (NETs), cathelicidin, and alpha defensin from their granules.[4,9,10] NETs are well-known to play a role in promoting thrombosis by providing a scaffold for thrombi and inducing platelet aggregation, thrombin activation, and fibrin clot formation.[10,11,12] Furthermore, neutrophils express tissue factor, which is a key coagulation factor in thrombosis initiation.[13] Neutrophil elastase (NE) promotes coagulation via fibrin formation and thrombus growth.[14] Previous studies suggest that neutrophils are recruited into a

thrombus to contribute to its organization. However, the time course of neutrophil recruitment to the thrombus during the early phase of arterial thrombosis remains unknown. In addition, it is unknown whether infiltrated neutrophils contribute to thrombus growth. Thrombi that are retrieved during intra-arterial mechanical thrombectomy in patients with acute stroke show heterogeneous composition.[15] Thrombus composition may somehow demonstrate the mechanism of thrombosis and the characteristics of patients with stroke.[16] In addition, the efficacy of thrombectomy and clinical outcomes may differ according to thrombus composition.[15,17] Both neutrophils and NETs are frequently and abundantly found in venous, coronary, and cerebral thrombi.[18-20] Nevertheless, previous studies have mainly focused on the role of NETs in thrombosis.[19,21] Although neutrophils are a major source of NETs and predominant leukocytes in thrombi, limited information is available on the characteristics of stroke patients with neutrophil-rich thrombi.[21]

In this study, we investigated the time course of neutrophil recruitment and infiltration in the early stages of arterial thrombosis using an $FeCl_3$-induced carotid thrombosis model in mice, as well as the relationship between neutrophil count and thrombus size. In addition, we investigated the clinical characteristics of stroke patients with high neutrophil fractions in thrombi.

II. MATERIALS AND METHODS

Preparation of experimental animals

We used 7 to 8-week-old Institute of Cancer Research mice (male and female) weighing 32-34 g. The mice were kept in a cage with soft bedding and allowed free access to food and water in a temperature-controlled animal facility under a standard 12-hour light and 12-hour dark cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine (Approval No. 2019-0023) and performed in accordance with the Association for Assessment and Accreditation of

Laboratory Animal Care guidelines.

$FeCl_3$-induced common carotid artery thrombosis

Arterial thrombosis was induced in the common carotid artery (CCA) using $FeCl_3$.[22,23] Briefly, anesthesia was induced with 5% isoflurane in a mixture of 70% N_2O and 30% O_2 and maintained with 2% isoflurane. Body temperature was continuously monitored and maintained at 37.0 ± 0.5 °C using a homeothermic blanket control unit and heating pad with a rectal probe (Harvard Apparatus, Holliston, MA, USA) during the operative procedures. Under a surgical microscope, a midline incision was made at the cervical area in the supine position, and the left CCA was exposed and isolated. Blood flow was measured in the midportion of the CCA using an ultrasonic Doppler flow probe (MA0.7PSB; Transonic Instruments, Ithaca, NY, USA) and an iWorx IX-304T data acquisition system (iWorx Systems, Inc., Dover, NH). After measurement of baseline blood flow for 5 min, arterial thrombosis was induced using $FeCl_3$. A piece of filter paper (F2877; Sigma-Aldrich Inc., St. Louis, MO, USA) was soaked in 2 μL of 50% $FeCl_3$ and placed on the midportion of the CCA for 5 min. The filter paper was then removed, the CCA was washed with normal saline, and blood flow was checked again. Subsequently, the probe was carefully positioned in the upper intact CCA, and blood flow was monitored for 10 min. The success of arterial occlusion with thrombus formation was defined as a decrease in blood flow to zero. The blood flow data were analyzed using iWorx LabScribe software (version 4.01).[23] After blood flow recording for 10 min, the CCA was washed and excised.

Production of aged thrombi in mouse

After successful thrombotic occlusion (cessation of blood flow for 10 min) by $FeCl_3$, an aged thrombus was produced by tight ligation of the distal end of the CCA for 0.5, 1, 2, 3, 6, and 24 h. After a predefined period of thrombus maturation, blood flow was recorded for 10 min to check whether the cessation of blood flow was maintained. The CCA was, thereafter, washed and excised.

Measurement of thrombus size in mouse

The excised CCA was fixed in 4% paraformaldehyde and embedded in paraffin. The CCA paraffin-embedded block was sectioned longitudinally into 4-μm slices and stained with hematoxylin and eosin. The thrombus area was measured in the slice representing the largest part of the thrombus under a light microscope (Axio Imager.D2; Zeiss, Oberkochen, Germany) and AxioVision software (AxioVs40 V 4.8.1.0; Zeiss) using Image J software.

Immunohistochemistry of mouse thrombus

The paraffinized block was sectioned into 3-μm slices. Thereafter, the sectioned slices were deparaffinized with xylene, passed through graded ethanol for rehydration, and subjected to heat-induced epitope retrieval using the IHC-Tek epitope retrieval solution and steamer (IHC World, Inc., Woodstock, MD, USA), except for TER-119. After cooling and washing with phosphate-buffered saline (PBS), the sections were immersed in 10 mM glycine in PBS for 10 min at room temperature. The sections were blocked with 1% horse serum and 5% nonfat milk in Tris-buffered saline for 20 min at room temperature and reacted with the primary antibody. The primary antibodies for mouse thrombus were anti-TER119 (BL116202; 1:1600, BioLegend, San Diego, CA) for erythrocytes; CD41 (ab63983; 1:800, Abcam, Cambridge, UK) for platelets; anti-fibrinogen (ab34269; 1:1600; Abcam) for fibrin/fibrinogen and Ly6G (BL127601; 1:400, BioLegend) for neutrophils, and citrullinated histone H3 (ab5103, 1:800; Abcam) for neutrophil extracellular traps (NETs). Primary antibodies were incubated for 2 h at 37 °C (anti-TER119) or overnight at 4 °C (anti-CD41, fibrinogen, Ly6G, and histone H3). The sections were incubated for 30 min at 37 ºC with 1:200-diluted biotin-conjugated secondary antibodies (goat anti-rabbit IgG, BA-1000, or goat anti-rat IgG, BA-9401; Vector Laboratories, Burlingame, CA, USA). Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. After reaction with horseradish peroxidase-conjugated streptavidin-biotin complex (ABC Elite Kit; Vector Laboratories), the peroxidase signal was developed using NovaRed (NovaRed Kit; Vector Laboratories). The sections were counterstained with hematoxylin, dehydrated, and

mounted using Permount mounting solution medium (Fisher Scientific, Fair Lawn, NJ, USA).

Immunofluorescence staining of mouse thrombi

For immunofluorescence staining, sectioned slices were deparaffinized, rehydrated, and subjected to antigen retrieval as described above for immunohistochemistry. Thereafter, the slices were blocked with Blotto and incubated overnight at 4 °C with primary antibodies against Ly6G (BL127601; 1:400; BioLegend) and citrullinated histone H3 (NB100-57135; 1:50, Novus Biologicals, CO). All slices were incubated for 1 h at room temperature with Alexa Fluor 488-labeled goat anti-rat IgG (ab150157; 1:200, Abcam) and Alexa Fluor 647-labeled donkey anti-rabbit IgG (ab150075; 1:200, Abcam) and mounted with Vectashield H-100 mounting medium (Vector Laboratories) containing DAPI. Images were acquired using an LSM700 laser scanning confocal microscope (Axio Imager.D2; Zeiss).

Analysis of thrombus composition in mice

Images of stained thrombi were obtained using the Stereo Investigator imaging system (MBF Bioscience, Williston, VT, USA) equipped with a light microscope (Axio Imager D2; Zeiss). After encircling the contour of the entire thrombus manually at low magnification (50x), the image was captured at a high magnification (400x) using the Virtual Slice module.[16] Analysis of the images was performed semi-automatically using the color deconvolution program in Image J (NIH, Bethesda, MD).[24] Auto threshold of pixel density was used. The fraction (%) of each component (platelets, erythrocytes, and fibrin) was calculated as the percentage of pixel density for the full thrombus area. Ly6G -positive cells and histone H3-positive cells were manually counted using a light microscope equipped with a motorized stage (MAC 6000 System, MBF Bioscience, Williston, VT). All measurements were performed in a blinded manner.

Thrombus analysis in patients with stroke

To determine the characteristics of stroke patients with neutrophil-rich thrombi, we used registry data and thrombi that were obtained during intra-arterial mechanical thrombectomy in patients with large intracerebral artery occlusion at Severance Hospital in Korea. This study was approved by the Institutional Review Board of Yonsei University College of Medicine (approval number:4-2017-0426). Written informed consent was obtained from patients or their next of kin for the use of the thrombus for research. For this study, thrombi from 75 consecutive patients whose thrombi were obtained and analyzed between December 2017 and May 2020 were used. The thrombi were immediately fixed in 4% paraformaldehyde, embedded in paraffin blocks, and stored until use. Immunohistochemistry was performed as described for mouse thrombi. The primary antibodies used for human thrombi were anti-glycophorin A (ab129024, 1:400; Abcam) for erythrocytes; anti-CD42b (ab134087, 1:100; Abcam, Cambridge, UK) for platelets; anti-fibrinogen (ab34269, 1:200; Abcam) for fibrin/fibrinogen, and NE (ab68672, 1:200; Abcam) for neutrophils. Heat-induced epitope retrieval was performed for glycophorin A. The primary antibodies were incubated overnight at 4 °C, and goat anti-rabbit IgG antibody (BA-1000, Vector Laboratories) was used as a secondary antibody for all primary antibodies. Peroxidase signal was developed using a 3,3'-diaminobenzidine solution (D5637; Sigma-Aldrich). After staining, the paraffin-embedded sections were scanned using a digital scanner (Aperio AT2; Leica Biosystems, Wetzlar, Germany).

The images were analyzed using Automated Region-of-interest based Image Analysis for automated composition analysis.[25] Auto threshold of pixel density was used. The fractions of NE, glycophorin A, CD42b, and fibrin-positive areas were calculated as the percentage pixel density of the total thrombus area.

Demographic data, medical history, laboratory data, and onset-to-recanalization time were collected. Onset time was defined as the last known normal time. Initial stroke severity was

assessed using the National Institutes of Health Stroke Scale. Furthermore, the etiology of stroke was determined using the Trial of ORG 10172 in Acute Stroke Treatment classification.[26] Recanalization was assessed using the modified thrombolysis in cerebral infarction (TICI) score on conventional angiography immediately after thrombectomy.[27] Functional outcomes were determined at 3 months using the modified Rankin scale (mRS). Favorable outcomes were defined as an mRS score <3.

Statistical analysis

To determine the temporal changes of neutrophil recruitment and NETs formation in arterial thrombi in mice, the Jonckheere-Terpstra test was performed. In post-hoc analysis, Ly6G-positive cell count and histone H3-positive cell count at baseline (10 min after thrombotic occlusion) were compared with their counts at different time points using the Mann–Whitney U test. Pearson's correlation test was performed to calculate the correlation between the number of Ly6G-positive cells and the fractions of erythrocytes, platelets, fibrin, and histone H3-positive cells in mouse thrombi.

To analyze the clinical characteristics of patients with neutrophil-rich thrombi, patients were categorized into higher neutrophil and lower neutrophil groups based on the median value of the neutrophil fraction. The t-test or Mann–Whitney U test was used for continuous variables, and the chi-square test or Fischer's exact test was used for categorical variables, as appropriate. The Shapiro–Wilk test was used to assess the normality of the continuous variables. Continuous variables are presented as a mean ± standard deviation or median (interquartile range [IQR]), and categorical variables are presented as numbers (percentages). Pearson's correlation test was performed to calculate the correlation between the fraction of neutrophils and the fractions of erythrocytes, platelets, and fibrin in thrombi. Multivariable logistic regression analysis was performed to determine the factors associated with favorable outcomes at 3 months (mRS <3). Age, sex, and other variables ($p<0.05$) were included in the multivariate analysis. Statistical analyses were performed

using R version 4.0.5 (http://www.R-project.org; R Core Team, Vienna, Austria). Statistical significance was set at $P < 0.05$.

III. RESULTS

Animals and aged thrombus

Seventy mice (35 males and 35 females) were used for the production and analysis of aged thrombi. Ten mice (five male and five female mice), at each time point, were used to obtain thrombi at baseline (10 min after thrombotic occlusion), 0.5, 1, 2, 3, 6, and 24 h.

Temporal changes in neutrophil infiltration and NET formation in mouse thrombi

Ly6G-positive cell (neutrophil) count was significantly increased starting from 1 h after thrombus formation in a time-dependent manner to 24 h (Figure 1A and 2A) ($p<0.001$). In addition, Histone H3-positive cell (NETs) count increased starting from 0.5 hour after thrombus formation in a time-dependent manner ($p<0.001$) (Figure 1B and 2B). Ly6G-positive cells and histone H3-positive cells increased markedly 3 h after thrombus formation (Figure 1A and 1B). Thrombus size was not increased with thrombus age ($p=0.683$) (Figure 1C).

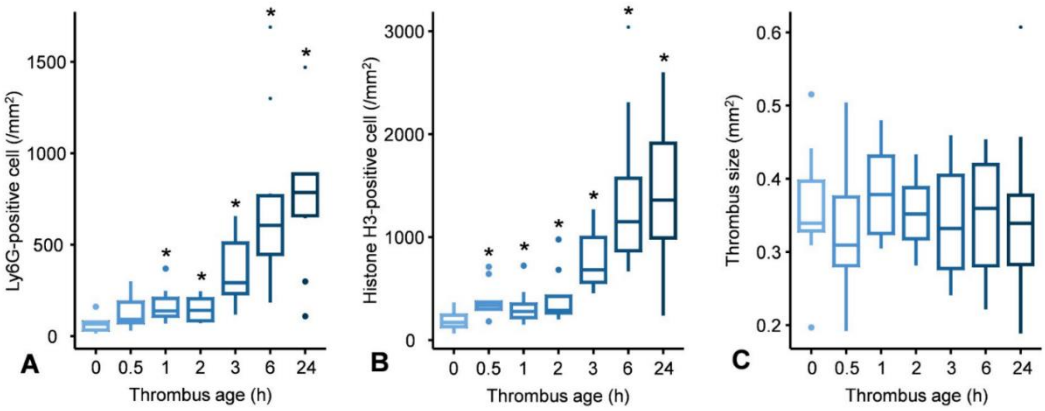

Figure 1. Temporal course of Ly6G-positive cells (A), histone H3-positive cells (B), and thrombus size (C) after inducing thrombosis in mice using $FeCl_3$. Numbers of Ly6G-positive cells and histone H3-positive cells increase in a time-dependent manner (both $p<0.01$) (Jonckheere-Terpstra test). The thrombus size was not increased with thrombus age ($p=0.683$). * $p<0.05$ in comparison with 0 h by Mann–Whitney U test

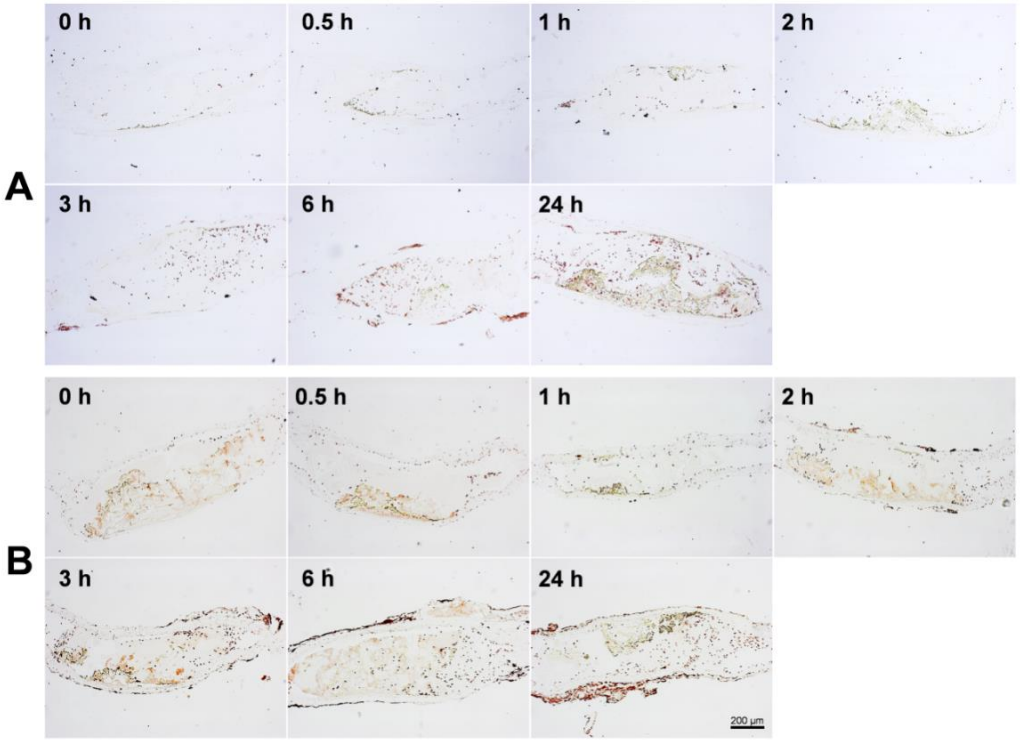

Figure 2. Representative immunohistochemistry of Ly6G-postive cells (A) and histone H3-positive cells (B) in arterial thrombi of mice.

Association of neutrophils with NETs, thrombus composition, and thrombus size

Ly6G-positive cell count was strongly correlated with the number of histone H3-positive cells ($r=0.910$, $p<0.001$) (Figure 3A). Furthermore, confocal immunofluorescence images showed that Ly6G-positive cells colocalized with histone H3-positive cells (Figure 3B). However, no significant correlation was observed between the number of Ly6G-positive cells and the fraction of platelets, fibrin, or erythrocytes (figure 4). We also determined the association between the number of Ly6G-positive cells and thrombus size. There was no significant time-dependent change in thrombus size ($p=0.683$). Additionally, no significant correlation was observed between Ly6G-positive cell count and thrombus size ($p=0.320$).

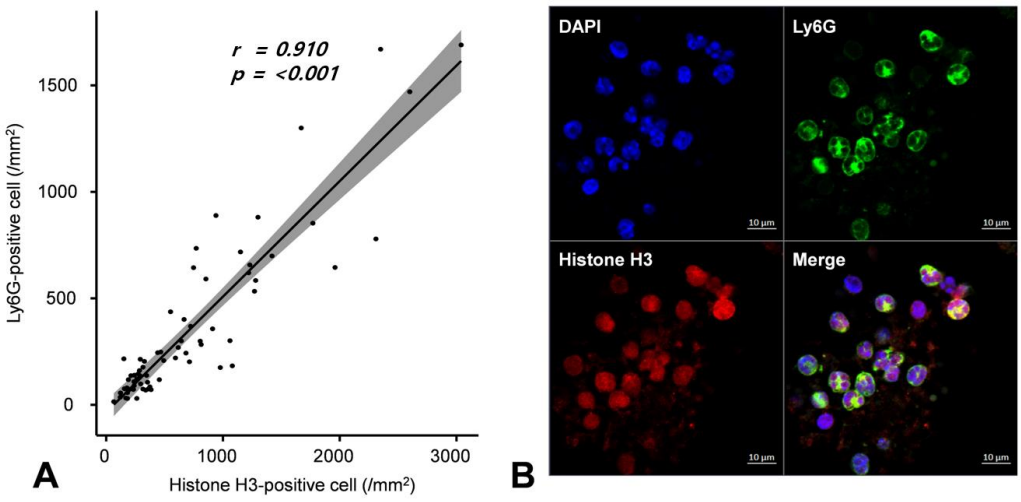

Figure 3. Correlation between counts of Ly6G-positive cells and those of histone H3-positive cells in arterial thrombi of mice. There was a strong correlation between counts of neutrophils and those of histone H3-positive cells (Pearson's correlation test) (A). Representative confocal immunofluorescence images of Ly6G-postive cells and histone H3-postive cells. Ly6G-postive cells and histone H3-postive cells are colocalized (B).

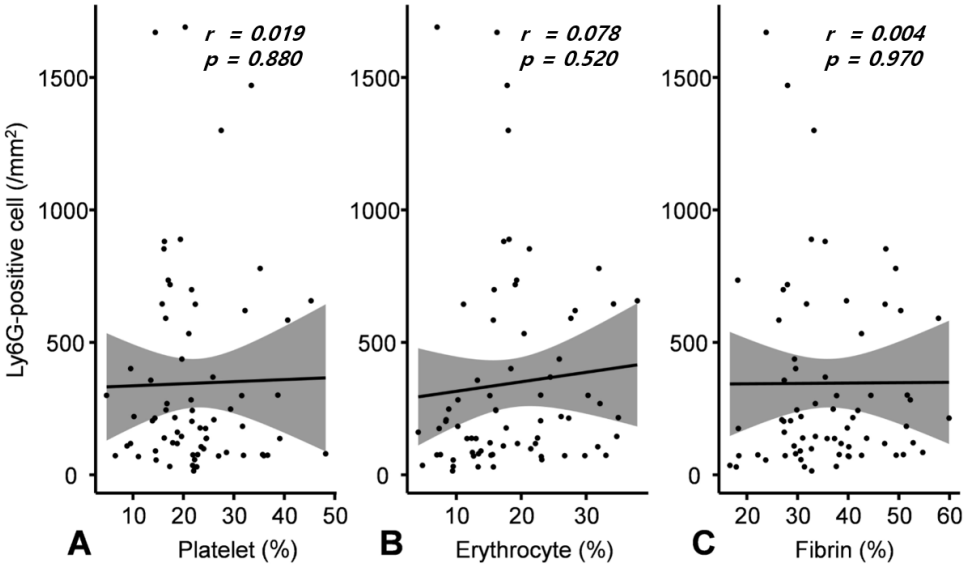

Figure. 4. Correlation of counts of Ly6G-positive cells (neutrophils) and the fractions of platelets (A), erythrocytes (B), and fibrin (C) in arterial thrombi in mice. No significant correlations were observed between Ly6G-positive cell counts and the fractions of platelets, erythrocytes, or fibrin (Pearson correlation test). The primary antibodies for immunohistochemistry were anti-Ly6G Ab for neutrophil, anti-TER119 Ab for erythrocyte, anti-CD41 Ab for platelet, and anti-fibrinogen/fibrin for fibrin.

Characteristics of stroke patients with higher neutrophil fraction in thrombi

A total of 75 patients were included in the analysis. The median fraction of neutrophils in thrombi was 28.5% (IQR 14.2–32.2). In comparison of baseline characteristics, atrial fibrillation was more frequent in the higher neutrophil group (32/37, 86.5%) than in the lower neutrophil group (19/38, 50%) (p=0.002) (Table 1). Stroke etiology was also different between the groups because cardioembolism was more frequent in the higher neutrophil group, while large artery atherosclerosis was more frequent in the lower neutrophil group (Table 1).

Table 1. Comparison of baseline characteristics according to neutrophil composition in thrombi

	Lower neutrophil group (N=38)	Higher neutrophil group (N=37)	P-value
Demographic			
Age, years	76.5 [61.0−84.0]	78.0 [71.0−85.0]	0.256
Sex, male	15 (39.5)	14 (37.8)	>0.999
Comorbidities			
Hypertension	25 (65.8)	26 (70.3)	0.866
Diabetes mellitus	9 (23.7)	11 (29.7)	0.741
Dyslipidemia	13 (34.2)	9 (24.3)	0.492
Coronary artery disease	6 (15.8)	7 (18.9)	0.958
Atrial fibrillation	19 (50.0)	32 (86.5)	0.002
Peripheral artery disease	1 (2.6)	1 (2.7)	>0.999
Previous stroke	6 (15.8)	9 (24.3)	0.525
Active cancer	6 (15.8)	1 (2.7)	0.121
Current smoker	5 (13.2)	2 (5.4)	0.449
Laboratory findings			
Hemoglobin, g/dL	13.4 [11.8−14.4]	13.4 [12.2−14.5]	0.521
Hematocrit, %	39.0 [35.0−42.3]	39.1 [36.4−43.3]	0.289
White blood cell, $10^3/\mu L$	8.6 [7.0−10.2]	7.8 [6.6−9.2]	0.144
Platelet, $10^9/L$	200.0 [157.0−249.0]	199.0 [168.0−243.0]	0.945
ESR, mm/h	17.0 [7.0−36.0]	15.0 [7.0−36.0]	0.899
C-reactive protein, mg/L	7.8 [2.3−24.4]	7.5 [3.7−15.7]	0.763
Total cholesterol, mg/dL	145.3 ± 33.5	155.2 ± 34.9	0.218
HDL-cholesterol, mg/dL	41.5 [31.0−52.0]	46.0 [38.0−53.0]	0.337
LDL-cholesterol, mg/dL	83.7 ± 33.0	91.4 ± 27.9	0.280
Triglyceride, mg/dL	101.5 [75.0−146.0]	98.0 [57.0−121.0]	0.439

D-dimer, ng/mL	766.0 [361.0−1769.0]	758.0 [341.0−1973.0]	0.552
Fibrinogen, mg/dL	279.0 [211.0−324.0]	300.0 [168.0−335.0]	0.787
BUN, mg/dL	17.2 [12.8−21.5]	19.9 [15.8−23.2]	0.159
Creatinine, mg/dL	0.8 [0.7−1.1]	0.8 [0.7−1.0]	0.763
Albumin, mg/dL	4.0 [3.7−4.3]	3.9 [3.7−4.1]	0.489
Glucose, mg/dL	128.5 [110.0−167.0]	136.0 [121.0−150.0]	0.600
Stroke classification			0.035
Cardioembolism	23 (60.5)	32 (86.5)	
Large artery atherosclerosis	5 (13.2)	1 (2.7)	
Undetermined	10 (26.3)	4 (10.8)	
Intravenous tPA	13 (34.2)	10 (27.0)	0.672
Initial NIHSS	13.0 [10.0−18.0]	15.0 [10.0−20.0]	0.115

Data are presented as a mean ± standard deviation, median [IQR], or number (%).

ESR, erythrocyte sedimentation rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; BUN, blood urea nitrogen; tPA, tissue plasminogen activator; NIHSS, National Institute of Health Stroke Scale

In terms of thrombus composition, the fraction of platelets was significantly lower and the fraction of erythrocytes was higher in the higher neutrophil group than the lower neutrophil group (Table 2, Figure 5). The neutrophil fraction was positively correlated with that of erythrocytes (r=0.35, p=0.002) (Figure 6).

Table 2. Comparison of endovascular thrombectomy outcomes and thrombus composition

	Lower neutrophil group (N=38)	Higher neutrophil group (N=37)	P-value
Occlusion site			0.132
Basilar	4 (10.5)	1 (2.7)	
Distal ICA	4 (10.5)	8 (21.6)	
Proximal ICA	9 (23.7)	14 (37.8)	
MCA	21 (55.3)	14 37.0)	
Successful recanalization	36 (94.7)	33 (89.2)	0.646
Thrombus composition, %			
Platelet	21.6 [11.9−34.0]	14.6 [9.6−21.1]	0.032
Fibrinogen	41.1 ± 10.3	42.2 ± 10.1	0.632
Erythrocyte	40.3 [23.5−53.2]	52.0 [39.9−57.8]	0.001
Onset-to-recanalization time, min	305.5 [185.0−446.0]	383.0 [195.0−553.0]	0.491

Data are presented as a mean ± standard deviation, median [IQR], or number (%).

ICA, internal carotid artery; MCA, middle cerebral artery

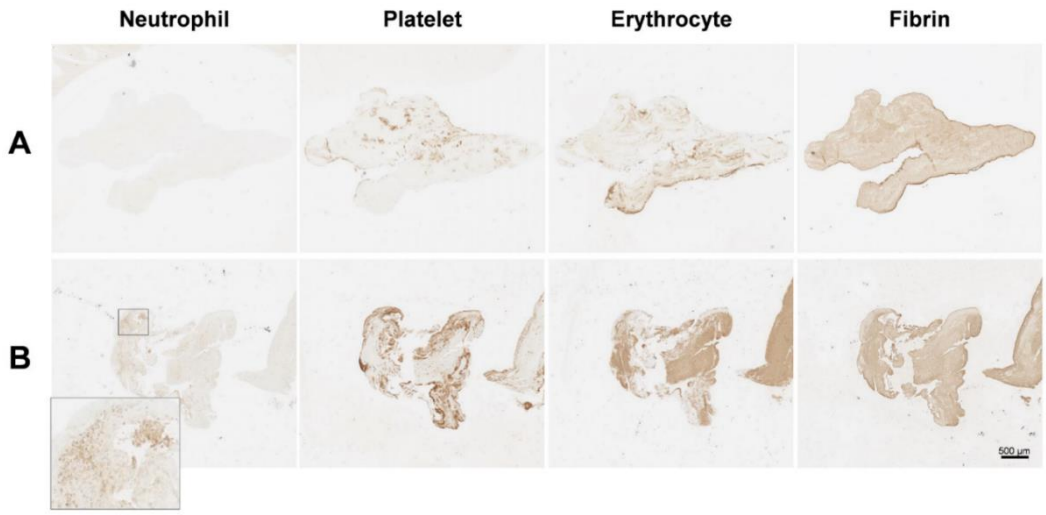

Figure 5. Representative immunohistochemistry of thrombi with lower neutrophils (A) and higher neutrophils (B) that were obtained during intra-arterial thrombectomy in patients with stroke. A portion of a neutrophil-rich area is magnified (box in B). The primary antibodies for immunohistochemistry were anti-neutrophil elastase Ab for neutrophils, anti-glycophorin A Ab for erythrocyte, anti-CD42b Ab for platelet, and anti-fibrinogen/fibrin for fibrin.

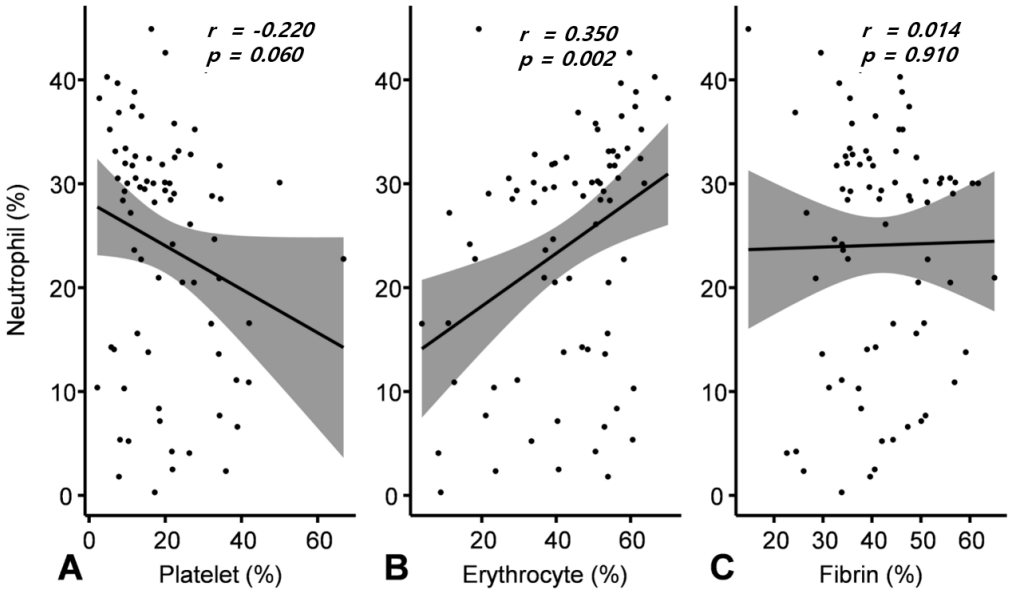

Figure 6. Correlation of the fraction of neutrophils and that of platelets (A), erythrocytes (B), and fibrin (C) in thrombi obtained from patients with stroke. The fraction of neutrophils was positively correlated with that of erythrocytes (B) (Pearson's correlation test).

Occlusion site, rate of successful recanalization (TICI 2b or 3), and onset-to-recanalization time did not differ between the groups (Table 3). mRS score at 3 months was available for 72 of the 75 patients (96%) enrolled in the study. The median neutrophil fraction was higher in patients with poor outcomes (30.0 [IQR 20.9–33.3]) than in patients with good outcomes (23.6 [IQR 10.9–30.1]) (p=0.023) (table 3). However, this difference was not significant in multivariable logistic regression analysis after adjusting for confounders (Table 4).

Table 3. Factors associated with functional outcome at 3 months after stroke

	Good outcome (N=33)	Poor outcome (N=40)	P-value
Demographic			
Age, year	72.0 [61.0–78.0]	84.0 [72.0–87.0]	<0.001
Sex, male	14 (42.4)	13 (32.5)	0.528
Comorbidities			
Hypertension	19 (57.6)	31 (77.5)	0.116
Diabetes mellitus	5 (15.2)	14 (35.0)	0.098
Dyslipidemia	11 (33.3)	10 (25.0)	0.601
Coronary artery disease	8 (24.2)	5 (12.5)	0.318
Atrial fibrillation	18 (54.5)	31 (77.5)	0.068
Peripheral artery disease	1 (3.0)	1 (2.5)	1.000
Previous stroke	5 (15.2)	10 (25.0)	0.456
Active cancer	1 (3.0)	6 (15.0)	0.184
Current smoker	4 (12.1)	3 (7.5)	0.789
Laboratory			
Hemoglobin, g/dL	13.5 [11.9–14.5]	12.6 [11.7–13.8]	0.159
Hematocrit, %	39.2 [36.4–42.9]	38.5 [35.4–40.6]	0.561
White blood cell, $10^3/\mu L$	8.1 [6.6– 9.4]	8.4 [7.0–9.3]	0.735
Platelet, $10^9/L$	199.0 [154.0–258.0]	203.0 [176.5–237.0]	0.444
ESR, mm/h	13.0 [4.0–21.0]	18.0 [8.0–39.0]	0.109
C-reactive protein, mg/L	5.7 [2.3–14.8]	8.3 [3.6–19.6]	0.506
Total cholesterol, mg/dL	146.1 ± 34.1	153.4 ± 34.4	0.362
HDL-cholesterol, mg/dL	44.0 [35.0–52.0]	44.0 [37.5–52.0]	0.756
LDL-cholesterol, mg/dL	83.4 ± 31.9	91.0 ± 29.8	0.298
Triglyceride, mg/dL	82.0 [57.0–123.0]	107.0 [75.5–152.0]	0.106
D-dimer, ng/mL	420.5 [180.5–1117.0]	1187.0 [590.0–2801.5]	0.004

Fibrinogen, mg/dL	283.0 [241.0–319.0]	280.0 [168.0–336.0]	0.688
BUN, mg/dL	16.8 [13.4–21.8]	20.5 [15.2–23.9]	0.104
Creatinine, mg/dL	0.8 [0.6-1.0]	0.8 [0.8–1.1]	0.227
Albumin, mg/dL	4.1 [3.8–4.3]	3.9 [3.7–4.1]	0.143
Glucose, mg/dL	128.0 [108.0–142.0]	138.0 [120.5–172.0]	0.038
Stroke classification			0.249
Cardioembolism	21 (63.6)	32 (80.0)	
Large artery atherosclerosis	3 (9.1)	3 (7.5)	
Undetermined	9 (27.3)	5 (12.5)	
Intravenous tPA	12 (36.4)	9 (22.5)	0.297
Initial NIHSS	10.0 [7.0–15.0]	18.0 [13.0–21.0]	<0.001
Recanalization outcome			
mTICI 2b or 3	32 (97.0)	35 (87.5)	0.299
mTICI 3	27 (81.8)	21 (52.5)	0.017
Onset-to recanalization time, min	342.0 [174.0–422.0]	388.0 [199.5–571.0]	0.338
Thrombus composition, %			
Platelet	17.2 [10.3–27.5]	17.7 [11.1–24.4]	0.864
Fibrinogen	41.9 ± 8.7	41.7 ± 11.5	0.918
Erythrocyte	46.1 [37.2–53.2]	50.8 [31.9–57.7]	0.457
Neutrophil	23.6 [10.9–30.1]	30.0 [20.9–33.3]	0.023

Data are presented as mean ± standard deviation, median [interquartile range], or number (percentage). ESR erythrocyte sedimentation rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; BUN; blood urea nitrogen; tPA, tissue plasminogen activator; NIHSS, National Institute of Health Stroke Scale; mTICI, modified thrombolysis in cerebral infarction

Table 4. Univariable and multivariable logistic analysis for factors associated with poor functional outcomes at 3 months after stroke

	Univariable		Multivariable	
	Odds ratio (95% CI)	P-value	Odds ratio (95% CI)	P-value
Demographic				
Age, year	1.07 (1.03–1.12)	0.002	1.06 (1.00–1.12)	0.038
Sex, male	0.65 (0.25–1.70)	0.383	0.59 (1.07–2.07)	0.410
Comorbidities				
Hypertension	2.54 (0.92–6.99)	0.072		
Diabetes mellitus	3.02 (0.95–9.54)	0.061		
Dyslipidemia	0.67 (0.24–1.84)	0.435		
Coronary artery disease	0.45 (0.13–1.53)	0.199		
Atrial fibrillation	2.87 (1.05–7.88)	0.041	1.50 (0.37–6.04)	0.569
Peripheral artery disease	0.82 (0.05–13.64)	0.890		
Previous stroke	1.87 (0.57–6.14)	0.304		
Active cancer	5.65 (0.64–49.49)	0.118		
Current smoker	0.59 (0.12–2.84)	0.508		
Laboratory findings				
Hemoglobin, g/dL	0.84 (0.56–1.06)	0.146		
Hematocrit, %	1.00 (0.92–1.07)	0.904		
WBC, 10^3/μL	1.01 (0.86–1.20)	0.868		
Platelet, 10^9/L	1.00 (0.99–1.01)	0.847		
ESR, mm/h	1.01 (0.99–1.04)	0.223		
CRP, mg/L	0.99 (0.9 –1.01)	0.338		
Total cholesterol, mg/dL	1.01 (0.99–1.02)	0.358		
HDL-cholesterol, mg/dL	1.00 (0.97–1.02)	0.907		
LDL-cholesterol, mg/dL	1.01 (0.99–1.02)	0.295		
Triglyceride, mg/dL	1.00 (1.00–1.00)	0.701		

D-dimer, ng/mL	1.00 (1.00–1.00)	0.059		
Fibrinogen, mg/dL	1.00 (0.99–1.06)	0.211		
BUN, mg/dL	1.07 (1.00–1.14)	0.063		
Creatinine, mg/dL	1.45 (0.32–6.63)	0.630		
Albumin, mg/dL	0.50 (0.15–1.67)	0.260		
Glucose, mg/dL	1.01 (1.00–1.03)	0.045	1.02 (1.00–1.04)	0.048
Stroke classification				
Cardioembolism	1.52 (0.28–8.28)	0.626		
Large artery atherosclerosis	Reference	Reference		
Undetermined	0.56 (0.08–3.86)	0.552		
Intravenous tPA	0.51 (0.18–1.42)	0.196		
Initial NIHSS	1.15 (1.06–1.26)	0.001	1.12 (1.01–1.24)	0.025
Recanalization outcome				
TICI 2b or 3	0.22 (0.02–1.97)	0.176		
TICI 3	0.25 (0.00–0.72)	0.011	0.20 (0.05–0.79)	0.021
Onset-to recanalization time, min	1.00 (1.00–1.00)	0.752		
Thrombus composition, %				
Platelet	1.01 (0.97–1.04)	0.797		
Fibrinogen	1.00 (0.95–1.04)	0.917		
Erythrocyte	1.00 (0.97–1.03)	0.146		
Neutrophil	1.05 (1.00–1.09)	0.032	1.03 (0.97–1.10)	0.267

CI, confidence interval; WBC, white blood cell; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; BUN, blood urea nitrogen; tPA, tissue plasminogen activator; NIHSS, National Institute of Health Stroke Scale; TICI, thrombolysis in cerebral infarction

IV. DISCUSSION

Our study showed that neutrophils are recruited to arterial thrombi in a time-dependent manner for 24 h in mice. The number of recruited neutrophils was correlated with the formation of NETs. However, thrombus size was not associated with the number of neutrophils present in the thrombus. In patients with stroke, the fraction of neutrophils in thrombi was higher in patients with atrial fibrillation or cardioembolism and positively correlated with the fraction of erythrocytes.

In this study, the number of neutrophils and histone H3-positive cells in thrombi significantly increased in a time-dependent manner after the induction of arterial thrombosis. Our findings suggest that there may be an increase in the number of neutrophils and NETs during the early stages of arterial thrombosis and play a role in the thrombotic process. We also found that the number of neutrophils was significantly correlated with that of NETs, and neutrophils and NETs were colocalized, which confirms the relationship between neutrophils and NET formation.[28] However, thrombus size did not increase over time and was not correlated with the number of neutrophils. Thrombosis is a dynamic process. Upon the initiation of the thrombotic process in pathological conditions, the thrombus grows and matures. In venous thrombosis, neutrophils play a role in the propagation of a thrombus through the release of NETs.[8] Furthermore, NETs are predominantly found in organized thrombi in patients with venous thromboembolism.[11] However, in arterial thrombosis, NETs are detected in the early stages of coronary thrombosis, but not in an organized thrombus.[29] NETs in thrombi are degraded by polarized macrophages.[30] The number of neutrophils was highest at day 1 and decreased after the induction of venous thrombosis in mice.[31] In a previous study, cyclophosphamide-induced leukopenia attenuated thrombosis in a carotid artery thrombosis model in mice, but not decreased thrombus size.[32] These findings suggest that neutrophils play a role in the early stages of arterial thrombosis; however, they appear to have a limited role in the growth of

an already formed arterial thrombus.

The observed time-dependent increase in neutrophils in this study suggests that the number of neutrophils in a thrombus may be a marker of an aged thrombus. However, in the thrombi of stroke patients in this study, there was no difference in the time from onset to recanalization in the higher and lower neutrophil groups. The time from onset to thrombus formation may partly represent thrombus age. However, this information does not provide accurate timing of initial thrombus formation because stroke might develop by the embolization of a pre-existing, maturated thrombi in the heart. Besides, symptom onset time noted by the patients or their family may often be inaccurate and unknown, particularly symptoms that develop on awakening from sleep. Our findings, therefore, suggest that neutrophil count may not be an accurate indicator of thrombus age. In this study, neutrophil fraction was correlated with erythrocyte fraction in thrombi from stroke patients. In a deep venous thrombosis mouse model, neutrophils were found in erythrocyte-rich and platelet-rich areas.[33] In addition, in coronary thrombi, the number of myeloperoxidase-positive cells (mainly neutrophils) was high in the erythrocyte-rich group.[34] Activated neutrophils induce hyperaggregability of erythrocytes by secreting proteolytic enzymes and generating oxygen-free radicals.[35] These findings suggest that neutrophils may contribute to the aggregation of erythrocytes during thrombosis.

In this study, there were more patients with atrial fibrillation or cardioembolism in the higher neutrophil group. Considering that atrial fibrillation is the most common cause of cardioembolism, neutrophil dominance in the thrombus of cardioembolic stroke may be ascribed to atrial fibrillation. Inflammation contributes to thrombosis in atrial fibrillation.[36] Previous studies have failed to prove the traditional idea that atherothrombotic thrombi are platelet-rich and that cardiac thrombi are erythrocyte-rich on analyses of thrombus histology.[15] However, some studies based on chemical staining have shown leukocyte dominancy in cardioembolism.[37,38] The findings of our study, which is based on

immunohistochemistry, showed neutrophil dominancy in atrial fibrillation and cardioembolism. Almost all subsets of leukocytes, including neutrophils, monocytes, T-lymphocytes, and eosinophils, are detected in thrombi retrieved from stroke patients with cerebral artery occlusion.[21] The number of T-cells was significantly higher in thrombi from patients with atherothrombosis.[39] Assessment of different subsets of leukocytes in thrombi may provide some hints for determining the etiology of stroke.

Few studies have shown an association of neutrophil-rich thrombi with endovascular and clinical outcomes. In previous studies, an abundance of neutrophils in the thrombus was associated with poor endovascular outcomes, including lower recanalization rate, recanalization time, and a number of mechanical maneuvers needed for recanalization.[21,40] However, in this study, there was no difference in endovascular outcomes between the higher and lower neutrophil groups. Neutrophil fractions were greater in patients with poor functional outcomes at 3 months; however, they were not significant in multivariable analysis after adjusting for confounders in this study. In a recent study, although an abundance of leukocytes and NETs in thrombi was associated with poor clinical outcomes in stroke patients, they were insignificant after adjusting for possible confounders.[21] While neutrophils may be associated with thrombus stability by releasing NETs and promoting aggregation of erythrocytes, their significance on endovascular and clinical outcomes requires further study with a larger cohort and consideration of possible confounders.

This study has some limitations. We aimed to determine the temporal course of neutrophil recruitment in a thrombus during the early stage of thrombosis. As a result, the temporal course after 24 h is unknown. The sample size of stroke patients might not be large enough to determine the association between neutrophil counts in thrombi and clinical outcomes. While stroke occurs from various etiologies, including cardiac diseases and atherosclerosis, the $FeCl_3$-induced thrombosis model used in this study does not reflect the diversity of thrombosis mechanisms in stroke patients it merely represents thrombosis induced by

vascular injury. This may partly be responsible for the discrepancy of some findings between stroke patients and mice in this study. In this regard, the arterial thrombosis model in this experiment may be insufficient to fully translate the findings in stroke patients.

V. CONCLUSION

This study showed that neutrophils are recruited and increased in arterial thrombus in a time-dependent manner; however, they are not associated with the growth of formed thrombi. Neutrophils in stroke patients are associated with the etiology of atrial fibrillation and cardioembolism. The association between neutrophil fraction and erythrocyte fraction in thrombi of stroke patients suggests a possible pathophysiological relationship during thrombosis.

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ABSTRACT(IN KOREAN)

동맥혈전에서 중성구 동원과 중성구가 많은 혈전을 가진 뇌졸중 환자의 특징

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차명진

목적

중성구는 혈전증에 기여한다. 하지만 혈전증에서 시간 변화와 중성구 동원관의 관계, 혈전의 성장에 있어 중성구의 기여, 중성구가 풍부한 혈전을 가진 뇌줄종 환자의 특징에 대한 정보는 제한적이다.

방법

암 연구소의 쥐에서 염화철을 이용하여 경동맥 혈전을 유도한 후 각각 30 분, 1 시간, 2 시간, 3 시간, 6 시간, 24 시간 동안 경동맥 원위부를 묶음으로서 연령별 혈전을 만들었다. 뇌졸중 환자의 혈전 분석에서는 레지스트리 자료와 동맥내 혈전제거시 얻은 혈전을 이용하였다. 혈전의 조성을 확인하기 위해 면역조직화학을 수행하였다.

결과

70마리 쥐의 혈전에서 Ly6G 양성 세포 수(중성구)와 히스톤 H3 양성 세포 수는 시간이 증가할수록 증가하였다 (both $p<0.001$). Ly6G 양성 세포 수는

히스톤 H3 양성 세포수와 강하게 연관이 있었지만 (r=0.910, p<0.001), 혈전의 크기와는 연관성을 보이지 않았다 (p=0.320). 75명의 뇌졸중 환자들에서 심방세동과 심장성색전증은 중성구가 적은 군보다(19/38, 50%) 많은 군에서(32/37, 86.5%) 더 많은 빈도를 보였다 (p=0.002). 적혈구 분율의 중위수는 중성구가 적은 군보다 (40.3 [interquartile range 23.5–53.2]) 많은 군에서 (52.0 [interquartile range 39.9–57.8] 높았다. 중성구의 분율은 적혈구의 분율과 의미있게 연관이 있었다 (r=0.35, p=0.002).

결론

중성구는 동맥 혈전에서 시간 의존적으로 동원되고 증가하였으나 혈전의 크기 증가와는 연관이 없었다. 뇌졸중 환자들의 혈전에서 중성구 분율은 심방세동과 적혈구 분율과 연관이 있었다.

핵심되는 말: 중성구, 혈전증, 뇌졸중, 중성구 세포외 트랩,

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