Effect of cyclophosphamide-induced leukopenia on the initial stage of arterial thrombosis in $FeCl_3$-induced carotid thrombosis in mice

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Directed by Professor Ji Hoe Heo

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<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

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Background

Leukocytes have been suggested to be involved in thrombosis by releasing neutrophil extracellular traps, interacting with platelets, and engaging in coagulation. Previous studies on neutrophils and neutrophil extracellular traps have focused in their roles in venous thrombosis, and limited information is available in their role in arterial thrombosis. Furthermore, while leukocytes have been known to be associated with organizing stage of thrombus, their role in initial stage of thrombus formation is not well known. Therefore, we aimed to investigate the role of leukocyte in early stage of arterial thrombosis by inducing leukopenia using cyclophosphamide in arterial thrombosis model of mice.

Methods

This is a double-blinded randomized study that compared the effect of cyclophosphamide-induced leukopenia in early arterial thrombus formation with that of control. A total of 72 Institute of Cancer Research mice were randomly treated with 100 mg/kg of cyclophosphamide or normal saline intraperitoneally (36 male and 36 female mice, 36 mice in each treatment group). Thrombosis of carotid artery was induced by treatment of $FeCl_3$. Thrombotic occlusion was determined by monitoring of carotid bold flow using ultrasonic flow meter. The primary outcome was time to occlusion after the

$FeCl_3$ treatment. We also compared the thrombus size and histologic composition of thrombus between the groups. We evaluated correlations of counts for leukocyte subsets in the peripheral blood with the time to occlusion and thrombus size.

Results

Cyclophosphamide treatment significantly decreased counts of leukocytes by 82.8 % compared to the control group [5.81 ± 2.09 ($\times 10^9$/L) vs. 0.52 ± 0.15 ($\times 10^9$/L), P < 0.001]. The time to occlusion was significantly delayed in the cyclophosphamide group (3.31 ± 1.59 minutes) than in the control group (2.30 ± 1.14) (P = 0.003). However, the thrombus size did not differ between the groups. The immunoreactivity for Ly6G-positive cells (neutrophil), intracellular and released histone H3 (neutrophil extracellular traps) in thrombi were significantly reduced in the cyclophosphamide group than in the control group (92.8%, 50.2% and 34.3 %, respectively). The number of Ly6g-positve cells was positively correlated with that of histone H3 reactivity in thrombi of the control group (r = 0.529, p < 0.001; r = 0.425, p = 0.01), but not in thrombi of the cyclophosphamide group (r = −0.120, p = 0.485; r = 0.292, p = 0.084). Time to occlusion had a moderate negative correlation with leukocyte counts (r = −0.326, P = 0.022) in the peripheral blood of the entire group. Among subsets of leukocytes, counts for neutrophils and those for lymphocytes were significantly correlated (P = 0.023 and 0.024, respectively).

Conclusion

The cyclophosphamide-induced leukopenia attenuated thrombus formation during early stage of arterial thrombosis. Our findings suggest the potential role of leukocytes in initial stage of arterial thrombosis.

Key words: leukopenia, thrombosis, cyclophosphamide, neutrophil, neutrophil extracellular trap

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I. INTRODUCTION

Thrombosis is a pathophysiological manifestation of various diseases, including stroke, myocardial infarction, autoimmune diseases, sepsis, cancer, and haematologic diseases. The consequences of thrombosis in cerebral and coronary arteries are the leading causes of morbidity and mortality. Traditional teaching on the mechanism of thrombosis has focused on the role of the coagulation pathway, platelets, endothelium, and blood flow. However, recent studies suggest that leukocytes and red blood cells are also actively engaged in thrombus formation[1-3].

The role of leukocytes in thrombosis has gained much attention since the prothrombotic role of neutrophil extracellular traps (NETs) was suggested[4]. A NET is a meshwork of DNA fibres coated with histones and toxic granules that are released from neutrophils[5]. NETs exert their antimicrobial function by capturing microorganisms and killing them using proteinases released from granules[6]. In a pioneering study by Fuchs et al., NETs perfused with blood caused adhesion, activation, and aggregation of platelets recruited red blood cells and promoted deposition of fibrin. These features were similar to those in venous thrombosis. They also showed NETs in deep vein thrombosis in baboons[4].

The mechanism of arterial thrombosis differs from that of venous thrombosis. Arterial thrombosis is characterised by vascular wall lesions and high shear stress, whereas venous thrombosis is associated with flow stasis and excessive coagulation[7]. Since the role of NETs in thrombosis was first demonstrated in the experimental venous thrombosis model, many studies have focused on the mechanism of thrombosis mediated or enhanced by neutrophils and NETs in venous thrombosis[4,8]. As a result, leukocyte activation and NETs are regarded as important players in venous thrombosis[9,10]. Although NETs and leukocytes are found in human arterial thrombi[11], limited information is available on the role of leukocytes and NETs in arterial thrombosis.

The process of thrombus formation in the artery is dynamic. After initial adhesion of platelets to the endothelium, the thrombus grows by platelet aggregation. Activation of the coagulation pathway further promotes fibrin formation and platelet-fibrin plug construction. Leukocytes are known to infiltrate into the thrombus as it is getting organised, and this occurs during the few days of thrombus formation[12]. Neutrophil elastase promotes coagulation via fibrin formation and thrombus growth[13]. NETs are predominantly present in organising thrombi in patients with venous thromboembolism [14]. The number of neutrophils is associated with the activity of NETs in thrombi that have retracted during thrombectomy in stroke patients[11]. While leukocytes have been suggested to be associated with the organising and maturation stages of thrombi, their role in the initial stage of thrombus formation is not well known.

Cyclophosphamide, also known as cytophosphane, is primarily used for chemotherapy and is a drug that suppresses the immune system[15]. One of the major side effects of cyclophosphamide is easy bruising/bleeding, including bladder bleeding[16]. Systemic leukopenia and deficits in leukocyte activity are common effects of many diseases, such as chemotherapy-induced leukopenia and viral infections. However, little is known regarding the association between

leukopenia and thrombosis.

Thus, there are some less well-known issues related to leukocytes and thrombosis, including the role of leukocytes in the early stage of arterial thrombosis. Therefore, we investigated the effect of leukopenia induced by cyclophosphamide on the early stage of arterial thrombosis in a mouse model of arterial thrombosis.

II. MATERIALS AND METHODS

1. Study design and experimental animals

This double-blind randomized study assessed the effect of cyclophosphamide in early arterial thrombus formation. Thirty-six male weighing 28–35 g and 36 female weighing 24–29 g Institute of Cancer Research mice were used. The animals were housed in a temperature-controlled animal facility under a 12/12 h reversed light and dark cycle, placed in a plastic cage with soft bedding, and given free access to food and water. They were fed regular food pellets during the study period. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine (approval number: 2018-0261) and were performed in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care.

2. Study group, randomization, blinding, and outcomes

Animals were randomly treated with cyclophosphamide [cyclophosphamide group, 36 mice (18 males and 18 females)] or normal saline [control group, 36 mice (18 males and 18 females)]. A randomisation list with random numbers in permuted blocks of block size 4 was computer-generated by a researcher who

was not involved in the animal experiments. After preparing the study drugs and assigning a unique study number, another investigator, who was blinded to the study group, performed the surgical procedures and outcome assessments.

The primary outcome was the time to occlusion after inducing arterial thrombosis using $FeCl_3$. The secondary outcome was the length and area of the thrombus.

3. Study drug and treatment

Leukopenia was induced by intraperitoneal injections of 100 mg/kg cyclophosphamide (Endoxan, Baxter Oncology GmbH, Germany) once daily for four days, and the last administration was 24 h before the induction of arterial thrombosis. The animals in the control group received the same volume of normal saline.

4. Induction of arterial thrombosis

A $FeCl_3$-induced carotid thrombosis model was used to test the effect of leukopenia[17,18]. Animals were anesthetized with 5% isoflurane in a mixture of 70% N_2O and 30% O_2. Anaesthesia was maintained with 2% isoflurane. During operative procedures, body temperature was monitored continuously with a rectal probe and maintained at 37.0 ± 0.5 ℃ using a homeothermic blanket control unit and a heating pad (Harvard Apparatus, Holliston, MA, USA). A midline cervical incision was made, and the left common carotid artery was carefully dissected under a surgical microscope. An ultrasonic Doppler flow probe (MA0.7PSB; Transonic Instruments, Ithaca, NY, USA) was placed around the midportion of the common carotid artery (CCA). Carotid blood flow was obtained using a Transonic TS420 Blood Flow Meter (Transonic Instruments, Ithaca, NY, USA) and an iWorx IX-304T data acquisition system (iWorx Systems, Inc., Dover, NH, USA). Computer-based analysis using iWorx

Labscribe 2 software (version 2.045000) was performed to minimise any bias when assessing the results. CCA baseline flow was measured for 5 min. Oxidative vascular injury with chemical stress was induced by placing a filter paper (700 μm × 500 μm) saturated with 50% $FeCl_3$ (F2877; Sigma-Aldrich Inc., St. Louis, MO, USA) on the adventitial surface of the midpoint of the exposed CCA for 5 min. After removing the filter paper, the CCA was washed with normal saline, and blood flow was recorded. Thrombus formation and arterial occlusion were determined by decreased blood flow, and complete occlusion was defined as the absence of blood flow for 10 minutes.

5. Determination of time to occlusion and measurement of thrombus size

Time to occlusion was defined as the time from removing the $FeCl_3$-saturated filter paper to the time at which blood flow first ceased. Ten minutes after complete occlusion, injured CCA segments were excised, immediately immersed in 4% paraformaldehyde for fixation, and embedded in paraffin for histological analysis. Paraffin blocks were consecutively sectioned longitudinally into 3-μm slices. Sectioned slices were mounted on glass slides and stained with haematoxylin and eosin. A light microscope (Axio Imager.D2; Carl Zeiss Microimaging, Oberkochen, Germany) was used to determine thrombus size (longitudinal length and area) for each animal, and Zeiss AxioVision software (AxioVs40 V 4.8.1.0; Carl Zeiss imaging Solution) was used for a slice representing the largest part of the thrombus.

6. Hematologic assessment

Blood was collected in 10% EDTA by cardiac puncture after complete occlusion of the CCA. Complete blood counts, with differential leukocyte counts, were obtained using an automatic cell counter (MS9-5V; Melet Schloesing Laboratories, Cergy-Pontoise, France).

7. Immunohistochemistry

Primary antibodies used for immunohistochemistry were rabbit polyclonal anti-CD41 (ab63983, 1:800; Abcam, Cambridge, UK) for platelets; rabbit polyclonal anti-fibrinogen (ab34269, 1:200; Abcam, Cambridge, UK) for fibrin/fibrinogen; rat monoclonal anti-TER-119 (116202, 1:1600; Biolengend, San Diego, CA, USA) for erythrocytes; rat monoclonal anti-Ly-6G (127602, 1:400; Biolengend, San Diego, CA, USA) for neutrophils; rabbit polyclonal anti-histone H3 (ab5103, 1:400; Abcam, Cambridge, UK) for NETs; and rabbit polyclonal anti-tissue factor (ab104513, 1:800; Abcam, Cambridge, UK) for tissue factor.

The paraffin-embedded 3-μm-thick tissue sections were deparaffinized with xylene, passed through graded ethanol for rehydration, and then subjected to heat-mediated antigen retrieval using IHC-Tek epitope retrieval solution and a steamer (IHC World, Woodstock, MD, USA), except for anti-TER-119. The sections were immersed in 100 mmol/L glycine in phosphate-buffered saline (PBS) for 10 min. To reduce nonspecific staining, the sections were washed with PBS solution and incubated with Blotto (5% hydrated non-fat dry milk containing 1% bovine serum albumin) for 20 min. The sections were incubated with primary antibodies overnight at 4℃ (CD41, fibrinogen, Ly-6G, histone H3, and tissue factor) or for 2 h at 37℃ (TER-119). After washing with PBS, the biotinylated secondary antibody was incubated for 30 min at 37℃ (Vector Laboratories, Burlingame, CA, USA). Endogenous peroxidase in the tissues was quenched by exposure to 0.3% hydrogen peroxide for 20 min. Avidin-biotin complexes were generated with streptavidin-horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA), and the peroxidase signal was developed with the use of 3,30-diaminobenzidine solution. After counterstaining with haematoxylin, the sections were dehydrated and mounted with Permount mounting medium (Fisher Scientific, Fair Lawn, NJ, USA).

8. Measurement of thrombus composition

Images of stained thrombi were acquired using an Axio Imager D2 equipped with a high-resolution camera (AxioCam HRC, Zeiss, Germany). The imaging analysis was performed semi-automatically using the colour deconvolution program in ImageJ (NIH, Bethesda, MD, USA). Pixel density was determined at a threshold value of 235 throughout all measurements. The fraction (%) of each component (platelet, erythrocyte, fibrin, and tissue factor) was calculated as the pixel-density percentage for the entire thrombus area. For neutrophils, Ly-6G-positive cells were counted. For NETs, the cellular and released forms of histone H3 were separately counted. All measurements were performed in a manner blinded to the study group.

9. Statistical analysis

Statistical analysis was performed using SPSS (version 23.0; IBM SPSS Statistics, Armonk, NY, USA). All data are expressed as mean ± standard deviation. Continuous variables were compared using the independent-sample t test and Mann–Whitney test, whereas categorical variables were compared using $\chi 2$ test, Fisher's exact test, or the Mantel–Haenszel test, as appropriate. Pearson's and Spearman's rank correlation tests were used to compare thrombus formation and peripheral blood cells. We also applied the same correlation test to compare thrombus composition and thrombus size. A two-tailed P value <0.05 was considered statistically significant.

III. RESULTS

1. Descriptive data

None of the 36 cyclophosphamide-treated and 36 control mice died during the study. Cyclophosphamide treatment significantly decreased leukocyte counts by

82.8% compared to placebo [5.8 ± 2.1 (×10^9/L) vs. 0.5 ± 0.2 (× 10^9/L), P < 0.001]. The counts of red blood cells and platelets were also slightly decreased in the cyclophosphamide group compared with the control group (Table 1).

Table 1. Comparison of the body weight and complete blood cell counts between the groups.

	Control (n=36)	Cyclophosphamide (n=36)	P-value
Sex, female	18 (50)	18 (50)	1
Weight, g	29.42 ± 3.37	28.08 ± 2.18	0.051
Red blood cells, 10^{12}/L	8.95 ± 0.56	8.20 ± 0.70	<0.001
Platelet, 10^9/L	1191.83 ± 162.40	818.62 ± 161.90	<0.001
Leukocytes, 10^9/L	5.81 ± 2.09	0.52 ± 0.15	<0.001
Neutrophil, %	13.96 ± 4.90	9.47 ± 3.97	0.001
Basophil, %	0.31 ± 0.18	1.49 ± 1.38	<0.001
Monocyte, %	1.63 ± 0.77	0.45 ± 0.66	<0.001
Lymphocyte, %	77.61 ± 6.07	73.79 ± 15.28	0.249
Eosinophil, %	5.95 ± 3.88	14.58 ± 13.89	0.005

Data are expressed as mean ± SD or number (%).

All subsets of leukocytes, including neutrophils, basophils, monocytes, lymphocytes, and eosinophils, were significantly decreased in the cyclophosphamide group compared with the control group (P < 0.001) (Figure 1).

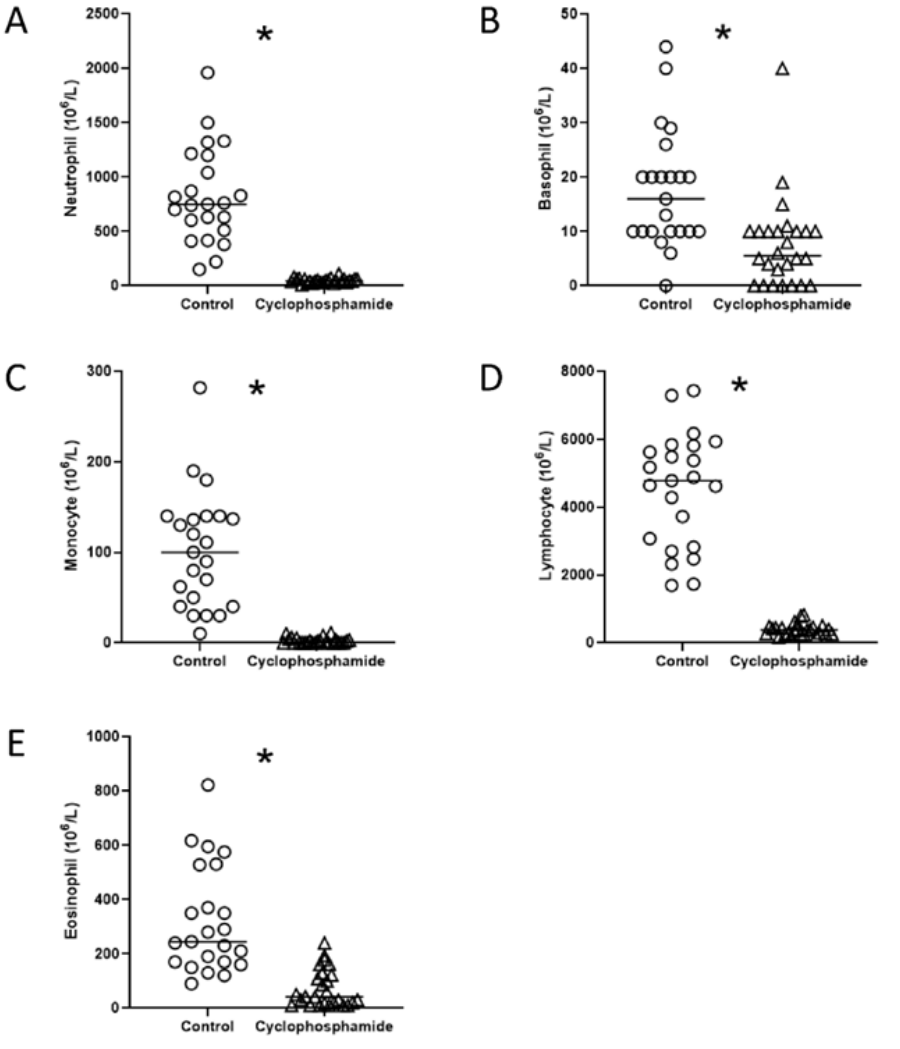

Figure 1. All subsets of leukocytes were decreased in the cyclophosphamide group. (A) Neutrophil, (B) Basophil, (C) Monocyte, (D) Lymphocyte, and (E) Eosinophil. The mean value of each group is shown as a bar. *, $P < 0.001$.

In sex-based subgroup analysis, leukocyte, red blood cell, and platelet counts in both male and female groups were decreased in the cyclophosphamide group compared to those in control group (Table 2 and 3).

Table 2. Comparison of demographic characteristics between the groups in female mice.

	Control (n=18)	Cyclophosphamide (n=18)	*P*-value
Weight, g	32.5 ± 1.4	29.7 ± 1.6	<0.001
Red blood cells, 10^{12}/L	8.9 ± 0.7	8.2 ± 0.9	0.048
Platelet, 10^{9}/L	1265.8 ± 139.0	832.7 ± 188.8	<0.001
Leukocytes, 10^{9}/L	6.9 ± 1.6	0.4 ± 0.1	<0.001
Neutrophil, %	14.4 ± 5.0	10.6 ± 4.4	0.056
Basophil, %	0.3 ± 0.1	2.0 ± 1.4	0.001
Monocyte, %	2.0 ± 0.8	0.5 ± 0.7	<0.001
Lymphocyte, %	77.7 ± 5.5	69.0 ± 16.8	0.111
Eosinophil, %	5.0 ± 3.0	17.7 ± 15.8	0.018

Data are expressed as number (%) or mean ± standard deviation.

Table 3. Comparison of demographic characteristics between the groups in male mice.

	Control (n=18)	Cyclophosphamide (n=18)	*P*-value
Weight, g	32.5 ± 1.4	29.7 ± 1.6	<0.001
Red blood cells, 10^{12}/L	8.9 ± 0.7	8.2 ± 0.9	0.048
Platelet, 10^{9}/L	1265.8 ± 139.0	832.7 ± 188.8	<0.001
Leukocytes, 10^{9}/L	6.9 ± 1.6	0.4 ± 0.1	<0.001
Neutrophil, %	14.4 ± 5.0	10.6 ± 4.4	0.056
Basophil, %	0.3 ± 0.1	2.0 ± 1.4	0.001
Monocyte, %	2.0 ± 0.8	0.5 ± 0.7	<0.001
Lymphocyte, %	77.7 ± 5.5	69.0 ± 16.8	0.111
Eosinophil, %	5.0 ± 3.0	17.7 ± 15.8	0.018

Data are expressed as number (%) or mean ± standard deviation.

2. Primary and secondary outcomes

After $FeCl_3$ treatment of the carotid artery, the time to occlusion was significantly longer in the cyclophosphamide group (3.3 ± 1.6 min) than in the control group (2.3 ± 1.1 min; P = 0.003) (Figure 2). There were no significant differences between the cyclophosphamide group and the control group in clot length (1.4 ± 0.5 mm vs. 1.5 ± 0.5 mm, p=0.293) and clot area (0.3 ± 0.1 mm^2 vs. 0.4 ± 0.1 mm^2).

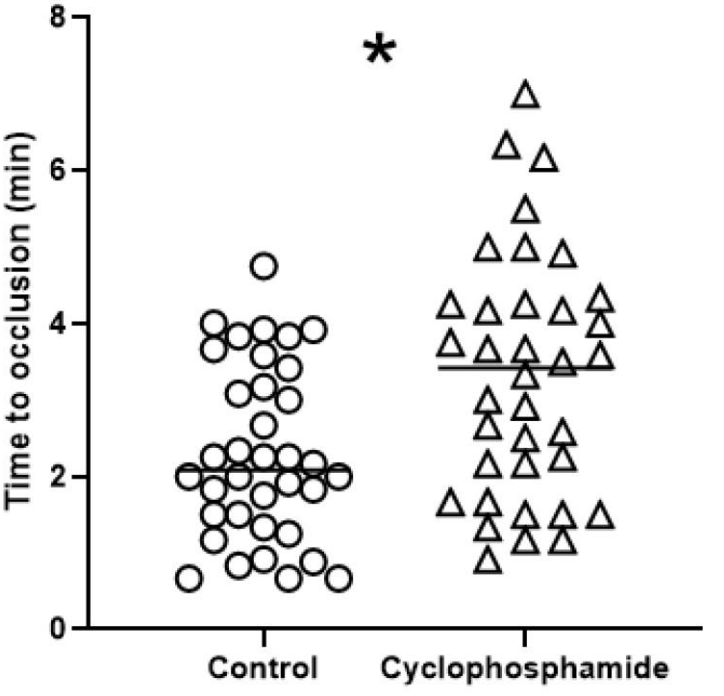

Figure 2. Comparison of time to occlusion between the control group and the cyclophosphamide group.

We looked at sex-based differences in outcomes. In female mice, time to occlusion was significantly longer in the cyclophosphamide group (3.7 ± 1.6 min) than in the control group (2.2 ± 1.2 min; P = 0.003), but not in male mice (2.9 ± 1.6 vs. 2.4 ± 1.1 min; P = 0.252) (Figure 3).

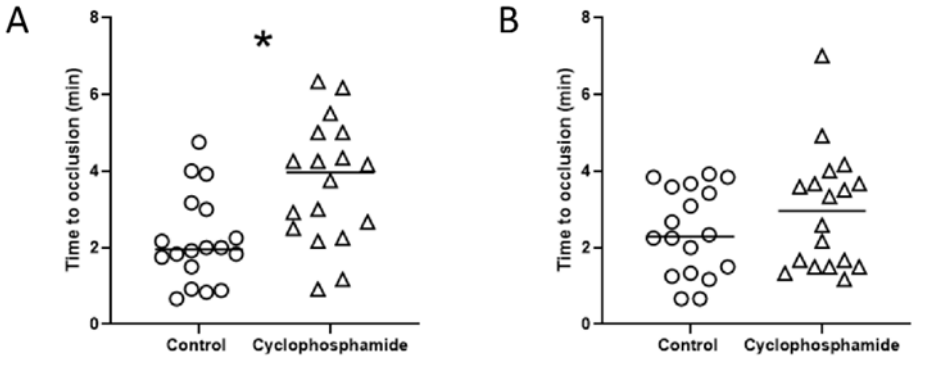

Figure 3. Comparison of time to occlusion between the control group and the cyclophosphamide group in female mice (A) and male mice (B). * P < 0.01.

There were no differences between the cyclophosphamide group and the control group in clot length (1.4 ± 0.5 mm vs. 1.6 ± 0.5 mm, P = 0.412 in female mice and 1.4 ± 0.4 mm vs. 1.5 ± 0.5 mm, p=0.529 in male mice) and clot area (0.3 ± 0.1 mm^2 vs. 0.4 ± 0.2 mm^2, P = 0.411 in female mice and 0.3 ± 0.1 mm^2 vs. 0.4 ± 0.1 mm^2, P = 0.509 in male mice) (Tables 4 and 5).

Table 4. Comparison of thrombus characteristics between the groups in female mice.

	Control (n=18)	Cyclophosphamide (n=18)	*P*-value
Time to occlusion, minutes	2.2 ± 1.2	3.7 ± 1.6	0.003
Clot length, mm	1.6 ± 0.5	1.4 ± 0.5	0.412
Clot area, mm^2	0.4 ± 0.2	0.3 ± 0.1	0.411
Immunohistochemistry			
CD 41	10.6 ± 4.2	10.9 ± 5.0	0.842
TER 119	13.8 ± 10.1	18.1 ± 13.9	0.301
Fibrinogen	19.7 ± 7.7	19.7 ± 5.7	0.977
Tissue factor	21.5 ± 7.7	19.1 ± 9.7	0.413
Ly 6G	112.6 ± 57.1	6.5 ± 6.0	<0.001
Intracellular histone H3	94.5 ± 52.5	45.2 ± 35.3	0.002
Released histone H3	213.3 ± 84.5	140.8 ± 97.8	0.023

Data are expressed as number (%) or mean ± standard deviation.
The fraction (%) of each component (CD 41, TER 119, fibrinogen, and tissue factor) was calculated as the pixel-density percentage for the entire thrombus area. Immunoreactive cells ($/mm^2$) were counted for Ly-6G, intracellular histone H3, and released histone H3.

Table 5. Comparison of thrombus characteristics between groups in male mice

	Control (n=18)	Cyclophosphamide (n=18)	*P*-value
Time to occlusion, minutes	2.4 ± 1.1	2.9 ± 1.6	0.252
Clot length, mm	1.5 ± 0.5	1.4 ± 0.4	0.529
Clot area, mm^2	0.4 ± 0.1	0.3 ± 0.1	0.509
Immunohistochemistry			
CD 41	10.2 ± 3.2	11.5 ± 7.7	0.501
TER 119	15.8 ± 7.5	21.0 ± 12.8	0.150
Fibrinogen	19.4 ± 5.5	20.9 ± 7.7	0.499
Tissue factor	17.7 ± 7.7	20.0 ± 9.8	0.440
Ly 6G	130.0 ± 98.7	10.9 ± 11.3	<0.001
Intracellular histone H3	101.6 ± 55.7	52.3 ± 54.6	0.011
Released histone H3	253.6 ± 91.2	165.9 ± 86.2	0.005

Data are expressed as number (%) or mean ± standard deviation.
The fraction (%) of each component (CD 41, TER 119, fibrinogen, and tissue factor) was calculated as the pixel-density percentage for the entire thrombus area. Immunoreactive cells (/mm^2) were counted for Ly-6G, intracellular histone H3, and released histone H3

3. Thrombus composition

The number of Ly6G-positive cells (neutrophils) was significantly lower by 92.8% in the cyclophosphamide group than in the control group (121.28 ± 79.97/mm2 vs. 8.73 ± 9.21/mm2, $p < 0.001$) (Figure 4 and 5A). The number of immunoreactive cells to histone H3 (NETs) was also lower in the cyclophosphamide group than in the control group by 50.2% for the intracellular form of histone H3 (98.0 ± 53.5 vs. 48.8 ± 45.4, $P < 0.001$) (Figure 4 and 5B) and by 34.3% for the released form of histone H3 (233.5 ± 89.1 vs. 153.4 ± 91.7, $P < 0.001$) (Figure 5C). However, there were no differences in the proportions of CD 41 (platelet), TER 119 (erythrocyte), fibrinogen, and tissue factor between the groups (Figure 5D-G).

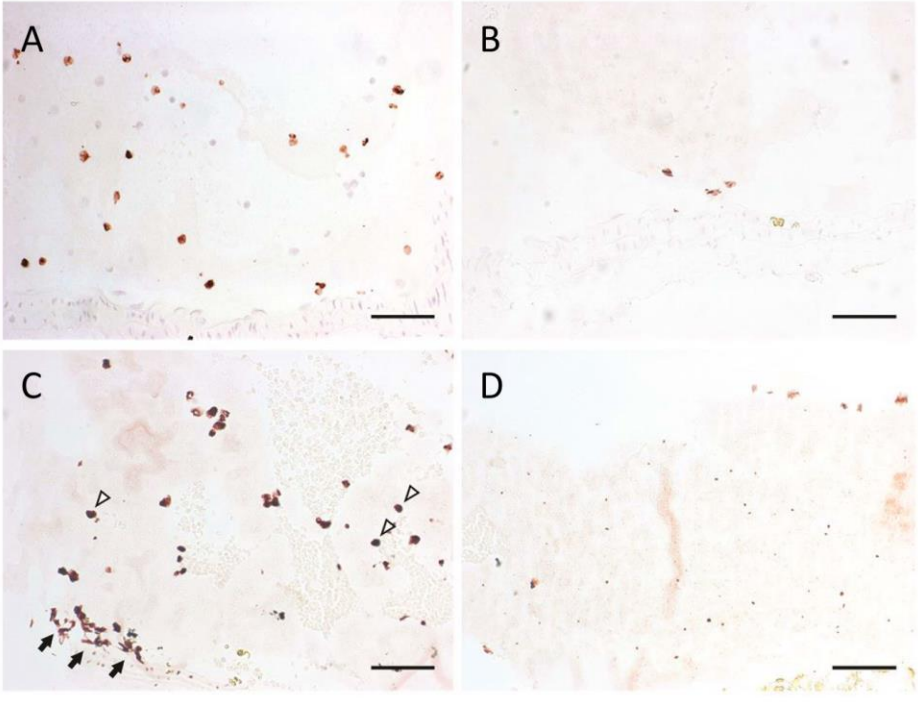

Figure 4. Representative immunohistochemical images of Ly6G (neutrophil, A and B) and histone H3 (neutrophil extracellular traps, C and D). Expression of

Ly6G and histone H3 were markedly decreased in the cyclophosphamide group (B and D) compared to the control group (A and C). Hollow arrows indicate the intracellular form of histone H3, and black arrows indicate the released form of histone H3. Scale bar = 50 μm.

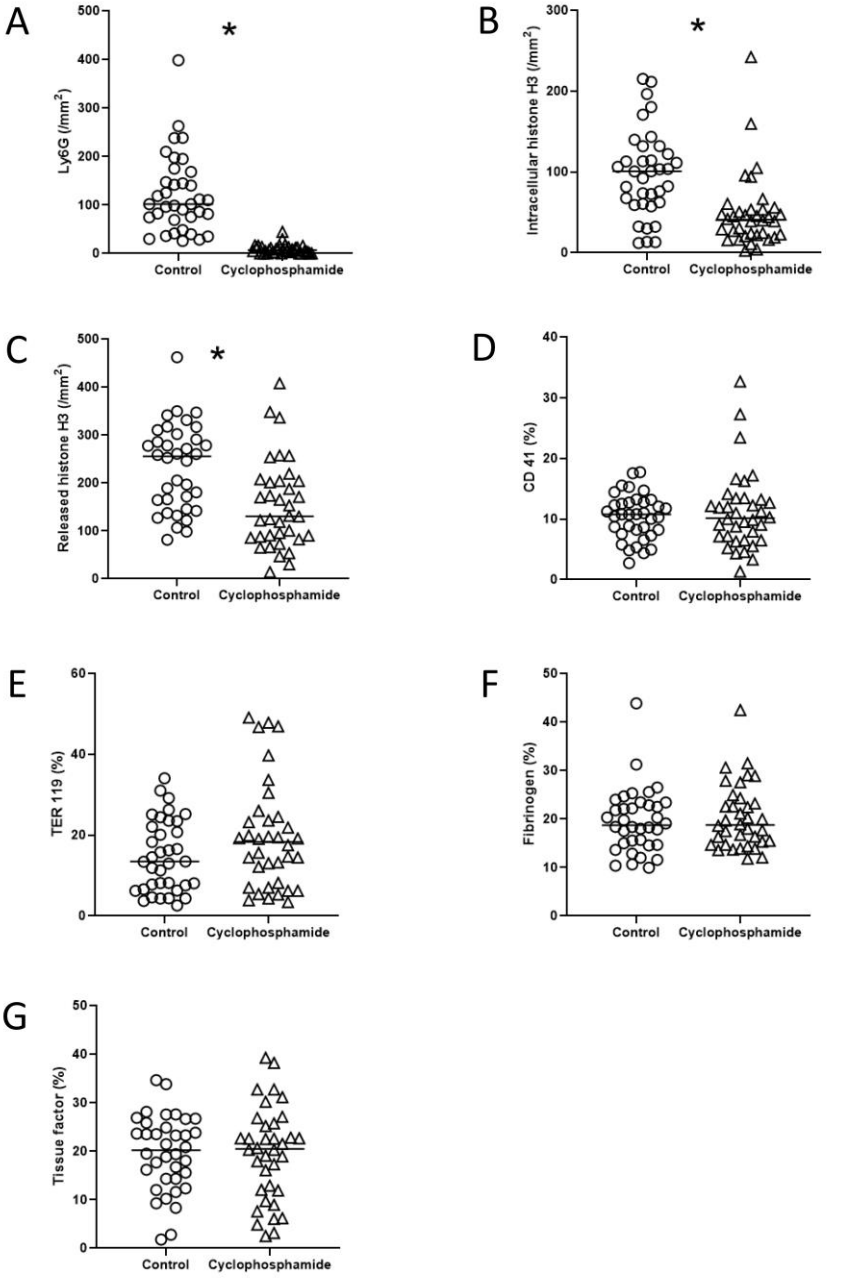

Figure 5. Comparison of thrombus composition between the groups. Cyclophosphamide led to a reduction of the number of neutrophils, the

intracellular form of neutrophil extracellular traps (NETs), and the released form of NETs. The number was counted for neutrophils (Ly6G) (A), intracellular form of histone H3 (intracellular NETs) (B), and released form of histone H3 (released NETs) (C). The proportion was measured for erythrocyte (CD 41) (D), platelet (TER 119) (E), fibrinogen (F), and tissue factor (G). The mean value of each group is represented by a horizontal bar. *, P < 0.001.

4. Association between neutrophils and NETs in thrombi

The number of Ly6G-positive cells was positively correlated with that of histone H3 reactivity in thrombi of the control group (r = 0.529, p < 0.001; r = 0.425, p = 0.01) (Figure 6A, 6B). However, this relationship disappeared in the thrombi of the cyclophosphamide group (r = −0.120, p = 0.485; r = 0.292, p = 0.084) (Figure 6C, 6D).

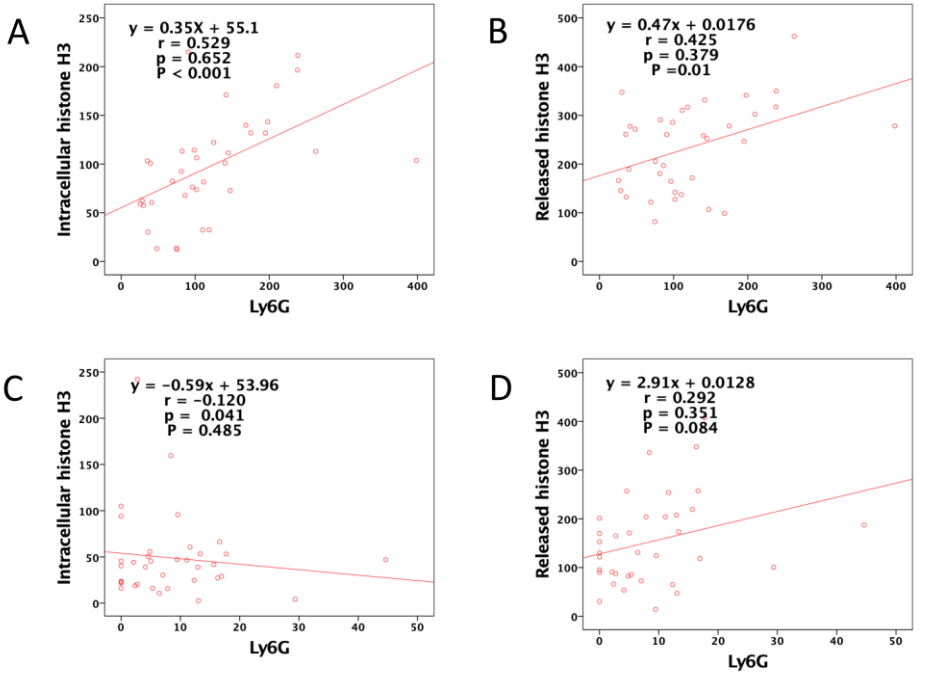

Figure 6. Correlation analyses between neutrophils (Ly6G) and neutrophil extracellular traps (NETs) in thrombi of the control group (A and B) and in the cyclophosphamide group (C and D). There was a positive correlation between neutrophils and NETs [intracellular form (A) and released form (B)] in the control group but not in the cyclophosphamide group.

5. Association between peripheral blood cells and thrombus formation

Time to occlusion had a moderate negative correlation with leukocyte counts ($r = -0.326$, $P = 0.022$) in the entire group. Among leukocyte subsets, the correlations were significant with neutrophil counts ($r = -0.324$, $p = 0.023$) and those of lymphocytes ($r = -0.322$, $P = 0.024$) but not with those of basophils, monocytes, or eosinophils. No correlations were found between time to occlusion and erythrocyte or platelet counts (Figure 7). Neither clot length nor area had significant correlations with counts of leukocytes, red blood cells, or platelets.

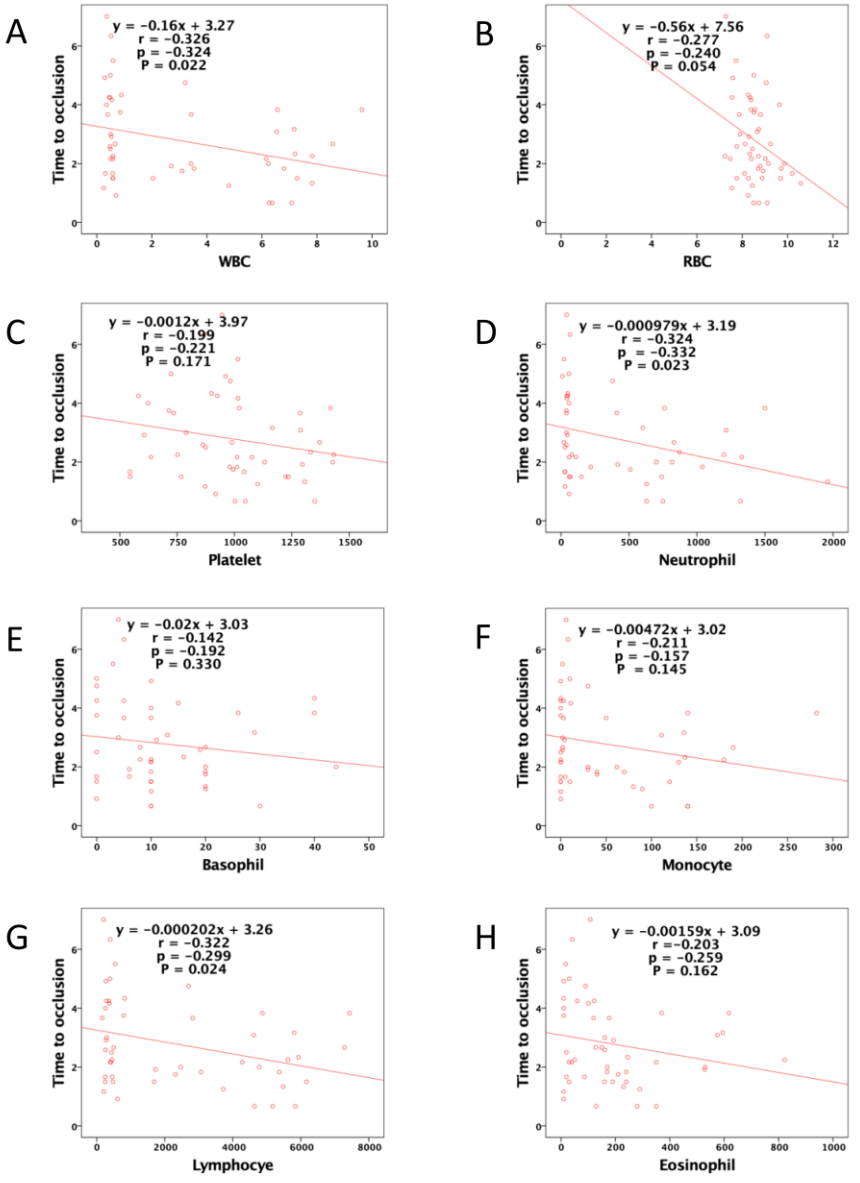

Figure 7. Correlation analyses between time to occlusion and counts for blood leukocytes (A), red blood cells (B), platelets (C), neutrophils (D), basophils (E), monocytes (F), lymphocytes (G), and eosinophils (H). Time to occlusion has negative correlations with counts for leukocytes, neutrophils, and lymphocytes in the peripheral blood.

6. Correlation between thrombus composition and thrombus size

The clot length was moderately correlated with Ly6G ($r = 0.430$, $P = 0.009$), intracellular form of histone H3 ($r = 0.456$, $P = 0.005$), and released form of histone H3 ($r = 0.554$, $P < 0.001$) in the control group (Figure 8A, C, and E). The clot area also had a moderate correlation with Ly6G ($r = 0.338$, $P = 0.044$) (Figure 8B). However, in the cyclophosphamide group, no positive correlations were found between the number of Ly6G-positive cells and clot length or area ($r = -0.176$, $P=0.305$ for clot length and $r = -0.111$, $p=0.518$ for clot area) as well as between histone H3 and clot length ($r = -0.311$, $P=0.065$ in intracellular histone H3 and $r = -0.272$, $P= 0.108$ in released histone H3) or area ($r = -0.444$, $P=0.007$ in intracellular histone H3 and $r = -0.452$, $P= 0.006$ in released histone H3). Both clot length and area had no significant correlations with CD 41, TER-119, fibrinogen, or tissue factor in the control group.

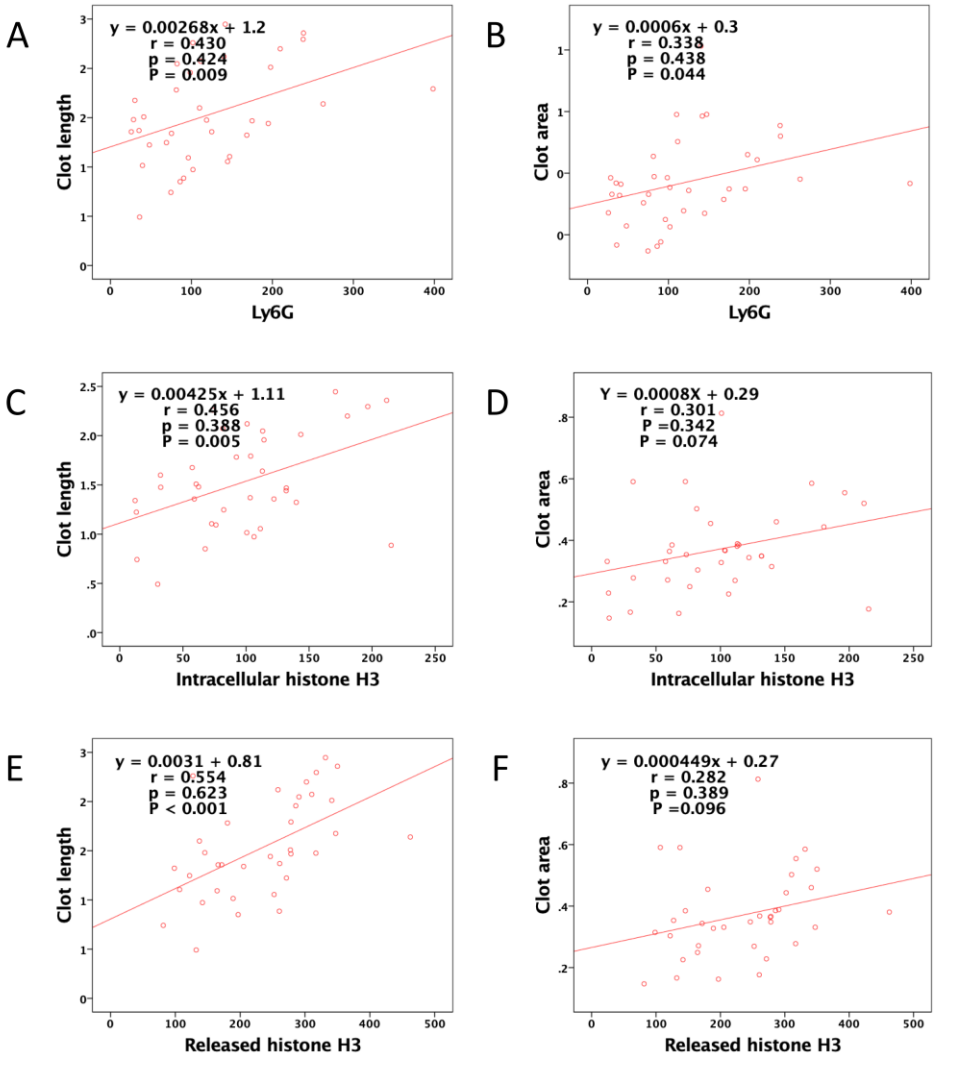

Figure 8. Correlation analyses between Ly6G and clot length (A) or clot area (B), between intracellular Histone H3 and clot length (C) or clot area (D), and between released Histone H3 and clot length (E) or clot area (F) in the control group.

IV. DISCUSSION

In this study, we demonstrated that time to initial thrombus formation was delayed in mice with cyclophosphamide-induced leukopenia. In addition, time to occlusion after $FeCl_3$ treatment was correlated with leukocyte counts in peripheral blood. Previous studies have shown that high leukocyte counts are significant risk factors for thrombosis[1]. Leukocytes interact with platelets and are associated with proteolytic cleavage of clotting factors[19-22]. Leukocytes were required for fibrin deposition during experimental thrombus formation in baboons[23].

Among leukocyte subsets, counts of neutrophils and lymphocytes were correlated with time to occlusion in this study. Various leukocyte subsets may play a role in thrombosis. Neutrophils promote clot formation through interaction with platelets, proteolytic cleavage of coagulation factors, and release of NETs[24,25]. The formation of leukocyte-platelet aggregates promotes the formation of intravascular thrombi[26,27]. Neutrophils are immediately attached to damaged blood vessels by binding to activated endothelium prior to platelets through interactions between leukocyte function-related antigens and intercellular adhesion molecule-1[28,29]. This is an important step in the activation and accumulation of platelets, and blocking this step may be an effective strategy to reduce intravascular thrombosis. Lymphocytes may also play a role in thrombosis. Tissue factor is expressed in T-lymphocytes[30]. Fingolimod reduces lymphocyte counts in peripheral blood by blocking the egress of lymphocytes from lymphoid organs. Treatment with fingolimod in the experimental stroke model reduced microvascular thrombosis[31].

In contrast to neutrophils and lymphocytes, counts for monocytes and eosinophils were not correlated with time to occlusion after $FeCl_3$ treatment in this study. However, previous studies have shown that monocytes and eosinophils also play a role in thrombosis. Monocytes were associated with

thrombosis by interaction with platelets to promote venous thrombosis in mice[32]. Activated monocytes express tissue factors and have been suggested to enhance platelet-derived clot retraction and form NETs[33,34]. Eosinophils were quickly recruited, which then interact with platelets to contribute to thrombus formation[35]. Lack of association of monocytes and eosinophils with initial stage of thrombosis in this study may be partly related to their very low counts in the mice used in this study.

In this study, we found many neutrophils and NETs within thrombi obtained very early after thrombus formation, that is, approximately 10 min after thrombotic occlusion. Most studies have focused on the role of leukocytes in thrombosis after their infiltration into the thrombus and subsequent NET formation. NETs are present in organizing thrombi in patients with venous thrombosis and are associated with thrombus maturation[14]. These processes take time and typically occur sometime after the initial thrombus formation. However, the findings in this study suggest that leukocytes are recruited very early during thrombus formation and may play a role in the initial stage of thrombosis. In addition to NET formation, leukocytes are involved in coagulation[24-31]. These findings suggest that leukocytes may contribute to thrombus formation by activating the coagulation pathway and interacting with platelets during the early stage of thrombosis.

We found that the clot area and length were correlated with the counts of Ly6G-positive cells (neutrophils) and those of histone H3 (NETs) reactivity in thrombi from the control group. These correlations were not observed in mice with cyclophosphamide-induced leukopenia. These findings support the possible association between inflammatory cells and thrombus formation. However, the clot size did not differ between the cyclophosphamide and control groups. NETs may be seen within cells, as filopodia-like features, or web-like features in human arterial thrombi[11]. The different histological features of NETs

may represent a degree of NET activity[11]. It is uncertain whether NETs within cells play a different role in thrombosis from those released. However, the role of NETs as a scaffold of thrombus may be expected when NETs are released from cells and form web-like features, and they contribute to thrombus growth. NETs in thrombi were found within cells or in a released form; however, no typical web-like features were observed in this study. This might be related to the fact that thrombi used in this study were those obtained immediately after thrombus formation; therefore, it might have been too early to form web-like features and cause thrombus growth. This may be partly responsible for the similar thrombus size between the groups.

In this study, there was a sex difference in that the effect of leukopenia on thrombosis was less obvious in male mice. Several studies have demonstrated the possible presence of sex differences in thrombosis. In a meta-analysis, women with atrial fibrillation had increased risks of thromboembolism[36] and women had a worse outcome than men after intravenous thrombolysis[37]. Pregnancy enhances NET formation[38]. Female sex was associated with a more pronounced formation of leukocyte–platelet aggregates and increased PAR-1 mediated platelet reactivity[39]. Platelets from female mice had greater reactivity to adenosine diphosphate and collagen-related peptides than those from male mice[40]. Our findings, along with previous ones, suggest that there may be some sex differences in inflammatory cell-mediated thrombosis.

The present study has some limitations. $FeCl_3$ induces thrombosis mainly via oxidative injury of the arterial wall. Therefore, the arterial thrombosis model used in this study may not fully represent thrombosis mechanisms in humans with various mechanisms. Interpretation of the sex difference in this study should be cautious because there may be species differences, and this study was performed in one mouse species. In addition, while the mice used in this study were young and non-pregnant, altering of sex hormones during pregnancy and

the postmenopausal period may have influenced thrombosis. We aimed to investigate the role of leukocytes in the early stage of thrombosis. Therefore, the role of leukocytes and NETs during thrombus growth and maturation remains unknown.

V. CONCLUSION

Cyclophosphamide-induced leukopenia attenuated thrombus formation during the early stage of arterial thrombosis. Time to occlusion was correlated with leukocyte counts in peripheral blood. Our findings suggest that leukocytes may play a role in the initial stage of arterial thrombosis.

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ABSTRACT (IN KOREAN)

Cyclophosphamide 유도 백혈구 감소증이 쥐의 $FeCl_3$ 유도 경동맥 혈전증에서 동맥 혈전증의 초기 단계에 미치는 영향

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배경

백혈구는 호중구 세포 외 트랩을 방출하고 혈소판과 상호 작용하며 응고에 관여함으로써 혈전증에 관여하는 것으로 보고되고 있다. 호중구 및 호중구 세포 외 트랩에 대한 이전 연구는 정맥 혈전증에서의 역할에 초점을 맞추어져 있으며 동맥 혈전증에서의 역할에 대하여는 알려져 있는 바가 제한적이다. 또한 백혈구는 혈전의 조직화 단계와 관련이 있는 것으로 알려져 있지만 혈전 형성의 초기 단계에서의 역할은 잘 알려져 있지 않다. 따라서 우리는 생쥐의 동맥 혈전증 모델에서 시클로포스파미드를 사용하여 백혈구 감소증을 유도하여 동맥 혈전증의 초기 단계에서 백혈구의 역할을 조사하고자 하였다.

방법

이것은 초기 동맥 혈전 형성에서 시클로포스파미드의 유도 백혈구 감소증의 효과를 대조군의 효과와 비교 한 이중 맹검 무작위 연구이다. 총 72 마리의 Institute of Cancer Research 마우스에 100 mg/kg의 시클로포스파미드 또는 생리 식염수를 복강 내 주사로 무작위로 처리하였다 (수컷 마우스 36 마리 및 암컷 마우스 36 마리,

시클로포스파미드 및 대조군 각 마우스 36 마리). 경동맥 혈전증은 $FeCl_3$ 처리에 의해 유도하였다. 혈전 성 폐색은 초음파 유량계를 사용하여 경동맥 흐름을 모니터링하여 결정하였다. 주요 결과는 $FeCl_3$ 처리 후 폐색 시간이다. 또한 혈전 크기와 혈전의 조직 학적 구성을 그룹간에 비교했다. 폐색 및 혈전 크기에 대한 시간과 말초 혈액의 백혈구 하위 집합에 대한 계수의 상관 관계를 평가하였다.

결과

시클로포스파미드 처리는 대조군에 비해 백혈구 수를 82.8 % 감소시켰다 [5.81 ± 2.09 (×109/L) vs. 0.52 ± 0.15 (× 109/L), P < 0.001].. 폐색 시간은 대조군 (2.30 ± 1.14) (P = 0.003)보다 시클로포스파미드 군 (3.31 ± 1.59 분)에서 유의하게 지연되었다. 그러나 혈전 크기는 그룹간에 차이가 없었다. 혈전에서 Ly6G 양성 세포 (호중구), 세포 내 및 방출 된 히스톤 H3 (호중구 세포 외 트랩)에 대한 면역 반응성은 대조군 (각각 92.8 %, 50.2 % 및 34.3 %)에서보다 시클로포스파미드 그룹에서 현저하게 감소했다. Ly6g 양성 세포의 수는 대조군의 혈전에서 히스톤 H3 반응성과 양의 상관 관계가 있었지만 (r = 0.529, p <0.001; r = 0.425, p = 0.01) cyclophosphamide 그룹의 혈전에서는 그렇지 않았다 (r = −0.120, p = 0.485; r = 0.292, p = 0.084). 폐색 시간은 전체 그룹의 말초 혈액에서 백혈구 수 (r = -0.326, P = 0.022)와 중간 정도의 음의 상관 관계를 가졌다. 백혈구의 하위 집합 중에서 호중구와 림프구의 수는 유의 한 상관 관계가 있었다 (각각 P = 0.023 및 0.024).

결론

시클로포스파미드에 의한 백혈구 감소증은 동맥 혈전증의 초기 단계에서 혈전 형성을 약화시켰다. 우리의 연구 결과는 동맥 혈전증의 초기 단계에서 백혈구의 잠재적인 역할을 시사한다.

핵심되는 말: 백혈구 감소증, 혈전증, 시클로포스파미드, 호중구, 호중구 세포 외 트랩

PUBLICATION LIST

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