Monocyte recruitment in $FeCl_3$-induced carotid thrombosis model in mice and characteristics of stroke patients according to thrombus composition

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Directed by Professor Ji Hoe Heo

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ABSTRACT

Monocyte recruitment in $FeCl_3$-induced carotid thrombosis model in mice and characteristics of stroke patients according to thrombus composition

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Purpose

Inflammatory cells have been suggested to play a role in thrombus formation. However, the role of monocyte in arterial thrombosis and clinical characteristics of patients with monocyte-rich thrombus are less well understood. We investigated the recruitment of monocytes to arterial thrombus over time using the $FeCl_3$-induced carotid thrombosis model in mice and characteristics of stroke patients with higher monocyte composition in thrombus.

Materials and Methods

After inducing carotid artery thrombosis in Institute of Cancer Research mice using $FeCl_3$, aged thrombus was produced by ligating the distal portion of the carotid artery in mice for 0.5, 1, 2, 3, 6, 24, 48 or 72 hours. For thrombus analysis in stroke patients, we used registry data and thrombi that were obtained during endovascular thrombectomy. Immunohistochemistry was performed to determine thrombus composition, and immunofluorescence was performed to evaluate the expression of extracellular traps and monocyte. Clinical characteristics and outcomes were compared between the higher and the lower monocyte groups, which were categorized based on its median value of monocyte fraction in thrombus.

Results

In the thrombi of 90 mice, CD68 (monocyte) counts increased from 3 hours and histone H3-positive cell (extracelluar traps) counts increased from 0.5 hour. The counts of both CD68 and histone H3-positive cells increased in a time-dependent manner, and then decreased from 48 hours (both $p<0.001$). The fraction of monocyte was positively correlated with that of extracellular traps in mouse ($r=0.705$, $p<0.001$) and those in stroke patients ($r=0.366$, $p<0.001$). In human thrombus, monocyte and extracellular traps were colocalized in confocal immunofluorescence image. In 102 stroke patients, the higher monocyte group showed higher blood platelet counts (median 228×10^9/L, interquartile range [177 – 267] vs. median 186×10^9/L, interquartile range [164 – 225], $p=0.036$), less frequent parenchymal hematoma (8.0% vs. 28.8%, $p=0.007$), and more frequent functional independence (54.0% vs. 32.7%, $p=0.030$) than the lower monocyte group. In multivariable logistic regression analysis, the higher monocyte group was associated with functional independence (odds ratio 4.954, 95% confidence interval 1.467-16.724, $p=0.010$).

Conclusion

Monocytes and extracellular traps were increasingly recruited early hours and then decreased from 48 hours in arterial thrombosis of mouse model. In both mouse model and human, the fraction of monocytes and extracellular trips showed a positive correlation. Less frequent parenchymal hematoma and more frequent functional independence were observed in the higher monocyte group. Higher monocyte composition in thrombus of stroke patients was an independent predictor of functional independence.

Key words: monocyte, thrombosis, stroke, extracellular traps, endovascular thrombectomy, functional outcome

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I. INTRODUCTION

As the term "immunothrombosis" suggests, various inflammatory cells interact each other and exchange their important signals during thrombus formation.[1,2] Recent studies have shown that leukocytes such as neutrophils and monocytes play important roles in the formation of arterial thrombosis in acute stroke, together with platelets and fibrin.[2] Neutrophil extracellular traps (NETs) is a web-like chromatic structure which can entangle pathogens and remove it.[3,4] NETs have gained attention due to its special and not yet clear roles in thrombosis. Both neutrophils and NETs are frequently and plentifully found in various thrombotic conditions, such as venous,[2] coronary,[5] and cerebral arterial thrombus.[6] Previous studies have mainly focused on the role of NETs in promoting thrombosis by providing a scaffold for thrombi and inducing platelet aggregation, thrombin activation, and fibrin clot formation.[2]

Thanks to endovascular thrombectomy (EVT) becoming the standard treatment of acute ischemic stroke,[7] thrombi can be retrieved during EVT,[8] enabling histopathological evaluation of the thrombi.[5,9] A thrombus consists of various blood cells and plasma factors with different compositions, which may vary depending on patient characteristics.[8,10] In particular, a previous study showed that thrombus composition may change over time from the initiation of thrombus formation.[11] The difference of composition may reflect thrombosis mechanism and be associated with the outcome of EVT as well as the clinical

outcome of patients. In previous studies, both of those outcomes were worse in the more aged and organized type of thrombus.[8,12]

Monocyte is a subpopulation of leukocytes that plays a key role in inflammation, which includes regulation of innate and acquired immunity by phagocytosis, synthesis of cytokines and chemokines, antigen presentation. and lymphocyte activation.[13] Although monocytes have been considered as bystanders in thrombus formation, a few studies have suggested that monocyte recruitment in the initial thrombus formation process is expected to contribute to thrombus formation and organization.[14,15] Monocytes have also been suggested to form the structures like NETs by themselves.[16-18] However, it remains unclear regarding the roles of monocytes in the thrombus formation, time-dependent recruit of monocytes into the thrombus, relationship of monocytes with extracellular traps (ETs), and association with patient characteristics or clinical outcomes with monocytes in the thrombus.

In this study, we analyzed the time course of monocyte recruitment and infiltration of arterial thrombosis using a $FeCl_3$-induced carotid thrombosis model in mice. We also investigated the relationship between monocyte and ETs using correlation between their counts and expression pattern in thrombus. In addition, we investigated the clinical characteristics and outcomes of patients with higher monocyte composition in thrombus.

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 gram. Mice were placed in a cage with soft bedding and allowed free access to food and water in a temperature-controlled animal facility where standard 12-hours light and 12-hours darkness alternated. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine (Approval No. 2019-0023) and performed according to the Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

$FeCl_3$-induced common carotid artery thrombosis

$FeCl_3$ was used to induce arterial thrombosis in the common carotid artery (CCA).[19,20] Briefly, anesthesia was induced with 5% isoflurane in a mixture of 70% N_2O and 30% O_2 and maintained with 2% isoflurane. During the surgical procedures, body temperature was continuously monitored and maintained at 37.0±0.5℃ using a homeothermic blanket control unit and heating pad with a rectal probe (Harvard Apparatus, Holliston, MA, USA). The mice were placed in a supine position under a surgical microscope, and a midline incision was made at the cervical area to expose and isolate the left CCA. We measured blood flow 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 measuring the baseline blood flow for 5 minutes, arterial thrombosis was induced using $FeCl_3$. A piece of filter paper (F2877; Sigma-Aldrich Inc., St. Louis, MO, USA) soaked with 2 μl of 50% $FeCl_3$ was placed on the midportion of the CCA for 5 minutes. The filter paper was then removed, and the CCA was washed with normal saline before the blood flow was measured again. The probe was carefully positioned on the distal undamaged CCA, and blood flow was monitored for 10 minutes. Arterial occlusion with thrombus formation was defined as

successful when the blood flow decreased to zero. The blood flow data were analyzed using iWorx LabScribe software (version 4.01).[20] Blood flow of CCA was monitored for 10 minutes to confirm the cessation of flow.

Production of aged thrombus in mice

After successful thrombotic occlusion (cessation of blood flow for 10 minutes) by $FeCl_3$, an aged thrombus was produced by tight ligation of the distal end of the CCA for predefined periods of thrombus maturation (0.5, 1, 2, 3, 6, 24, 48 and 72 hours). After the thrombus maturation, blood flow was recorded for 10 minutes to check whether the cessation of blood flow was maintained. The CCA was, thereafter, washed and excised.

Immunohistochemistry of mouse thrombus

The excised CCA containing thrombus was fixed in 4% paraformaldehyde and embedded in paraffin. The CCA paraffin-embedded block was sectioned longitudinally into 4-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). Phosphate-buffered saline (PBS) was used for cooling and washing. Then, the sections were immersed in 10 mM glycine in PBS for 10 minutes at room temperature. The sections were blocked with blotto containing 1% horse serum and 5% nonfat milk in Tris-buffered saline for 20 minutes. The primary antibodies for mouse thrombus were anti CD68 (ab125212, 1:200, Abcam, Cambridge, UK) for monocytes and citrullinated histone H3 (ab5103, 1:800; Abcam, Cambridge, UK) for ETs. The primary antibodies were incubated for overnight at 4°C. The sections were incubated for 30 minutes at 37°C with 1:200-diluted biotin-conjugated secondary antibodies (goat anti-rabbit IgG, BA-1000, or goat anti-rat IgG, BA-9401; Vector Laboratories, Newark, 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, Newark, CA,

USA), NovaRed (NovaRed Kit; Vector Laboratories, Newark, CA, USA) was used to develop the peroxidase signal. The sections were counterstained with hematoxylin, dehydrated, and mounted using Permount mounting solution medium (Fisher Scientific, Fair Lawn, NJ, USA).

Analysis of thrombus size and composition in mice

We used the Stereo Investigator imaging system (MBF Bioscience, Williston, VT, USA) equipped with a light microscope (Axio Imager D2; Carl Zeiss, Oberkochen, Germany) to obtain the images of stained thrombi. By encircling the contour of the entire thrombus manually at low magnification (50x), we measured the largest area of the thrombus using the built-in function of Stereo Investigator imaging system. Thereafter, the image was captured at a high magnification (400x) using the Virtual Slice module.[21] CD68-positive and histone H3-positive cells were manually counted using a light microscope equipped with a motorized stage (MAC 6000 System, MBF Bioscience, Williston, VT, USA). All measurements were performed in a blinded manner.

Thrombus analysis in patients with stroke

To determine the characteristics of stroke patients with monocyte-rich thrombus, we used registry data and thrombi obtained during EVT in patients with arterial occlusion of the anterior circulation at Severance Hospital in Korea. This study was approved by the Institutional Review Board of Severance hospital (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, 102 consecutive patients whose thrombi were obtained and analyzed between September 2014 and May 2020 were included. The thrombi were immediately fixed in 4% paraformaldehyde, embedded in paraffin blocks, and stored until use. Immunohistochemistry was performed as described for mouse thrombus. The primary antibodies used for human thrombus were anti-glycophorin A (ab129024, 1:400; Abcam, Cambridge, UK) for erythrocytes, anti-CD42b (ab134087, 1:100; Abcam, Cambridge, UK)

for platelets, anti-fibrinogen (ab34269, 1:200; Abcam, Cambridge, UK) for fibrin/fibrinogen, neutrophil elastase (ab68672, 1:200; Abcam, Cambridge, UK) for neutrophils, anti-Histone H3 (ab5103, 1:100; Abcam, Cambridge, UK) for ETs, and anti-CD68 (ab125212, 1:100; Abcam, Cambridge, UK) for monocytes. 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, Newark, CA, USA) was used as a secondary antibody for all primary antibodies. Peroxidase signal was developed using a 3,3'-diaminobenzidine solution (D5637; Sigma-Aldrich Inc., St. Louis, MO, USA). After staining, the paraffin-embedded sections were scanned using a digital scanner (Aperio AT2; Leica Biosystems, Wetzlar, Germany). Automated composition analysis was performed to analyze the images using Fixed Region-of-interest based Image Analysis.[22] The fractions of glycophorin A, CD42b, fibrinogen, neutrophil elastase, Histone H3, and CD68-positive areas were calculated as the percentage pixel density of the total thrombus area.

Immunofluorescence staining of human thrombus

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 the primary antibodies against CD68 (MA5-13324; 1:50; Thermofisher, IL, USA) for monocytes and citrullinated histone H3 (ab5103; 1:50; Abcam, Cambridge, UK) for ETs. All slices were incubated for 1 hour at room temperature with Alexa Fluor 488-labeled donkey anti-mouse IgG (A21202; 1:200, Thermofisher, IL, USA) and Alexa Fluor 647-labeled donkey anti-rabbit IgG (ab150075; 1:200, Abcam, Cambridge, UK) and mounted with Vectashield H-100 mounting medium containing DAPI (Vector Laboratories, Newark, CA, USA). Images were acquired using an LSM780 laser scanning confocal microscope (Axio Imager.D2; Carl Zeiss, Oberkochen, Germany).

Clinical data and outcomes in patients with stroke

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. The etiology of stroke was determined using the Trial of ORG 10172 in Acute Stroke Treatment classification.[23] Recanalization was assessed using the modified thrombolysis in cerebral infarction (TICI) score on conventional angiography immediately after EVT.[24] Successful recanalization was defined as TICI 2b or 3. Functional outcomes were determined at 3 months using the modified Rankin scale (mRS). Functional independence was defined as an mRS score<3, while functional dependence or death were defined as an mRS score≥3. Hemorrhagic transformation was determined on magnetic resonance imaging or computed tomography which were taken around 24 hours after EVT or at any time of clinical worsening. Hemorrhagic infarction (HI) was defined as hemorrhagic transformation with petechiae but no space-occupying effect, while parenchymal hematomas (PH) was defined as a typical homogenous lesion with sharp hematoma border with or without mass effect.[25]

Statistical analysis

To determine the temporal changes of monocyte recruitment and ET formation in arterial thrombus of mouse, the Jonckheere-Terpstra test was performed. In post-hoc analysis, CD68-positive and histone H3-positive cell counts at baseline (10 minutes 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 measure the correlation between the number of CD68-positive cells and the number of histone H3-positive cells in mouse thrombus.

To analyze the clinical characteristics of patients with monocyte-rich thrombus, patients were categorized into the higher monocyte and lower monocyte groups based on the median value of the monocyte fraction in thrombus. 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 counts or fraction of monocytes and those of neutrophils, ETs, erythrocytes, platelets, and fibrin in thrombus. Multivariable logistic regression analysis was performed to determine the factors associated with functional independence at 3 months (mRS<3). Stepwise method was used for the selection of variables from those with $P<0.05$ in univariable analysis to build multivariable logistic regression model. We used firth corrected multivariable logistic regression due to the small cell of successful recanalization. Statistical analyses were performed using R version 4.3.1 (http://www.R-project.org; R Core Team, Vienna, Austria). Statistical significance was set at $P< 0.05$.

III. RESULTS

Animals and aged thrombus

Ninety mice (45 males and 45 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 (baseline [10 minutes after thrombotic occlusion], 0.5, 1, 2, 3, 6, 24, 48 and 72 hours).

Temporal changes in monocyte infiltration and ET formation in mouse thrombus

CD68-positive cell (monocyte) counts initially increased in time-dependent manner starting from 3 hours after thrombus formation and then decreased from 48 hours (Figures 1A and 2A) ($p<0.001$). Likewise, Histone H3-positive cell (ETs) counts increased in time-dependent manner from 0.5 hours after thrombus formation, and then decreased from 48 hours ($p<0.001$) (Figures 1B and 2B).

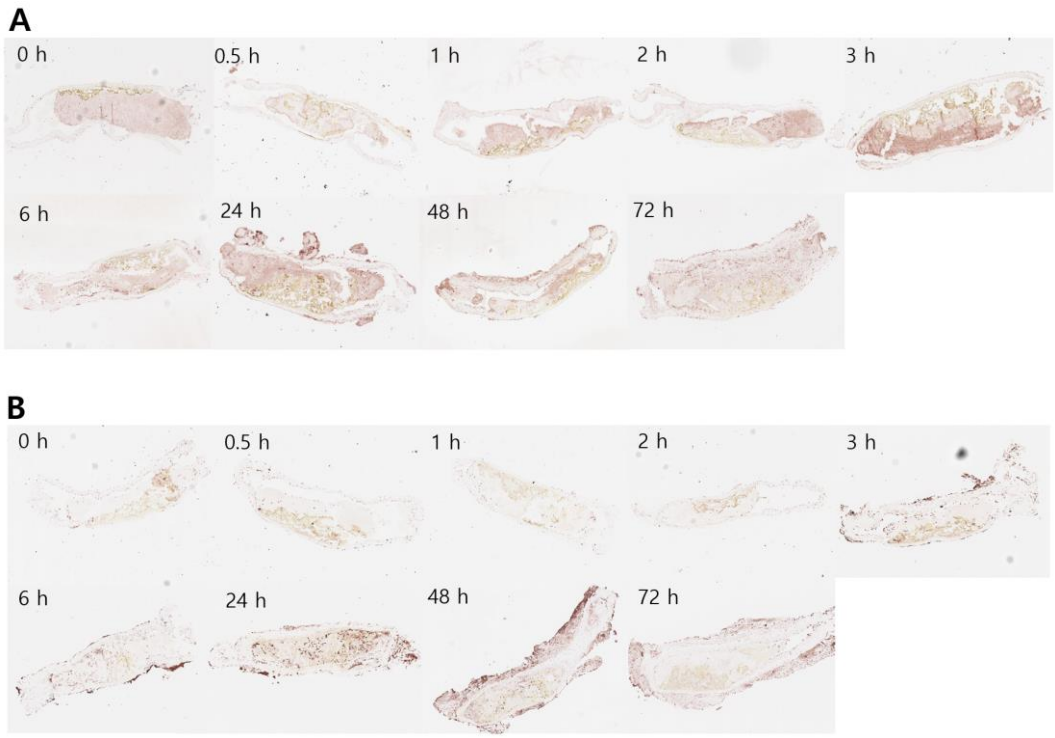

Figure 1. Representative immunohistochemistry of CD68-positive cells (A) and histone H3-positive cells (B) in arterial thrombus of mice.

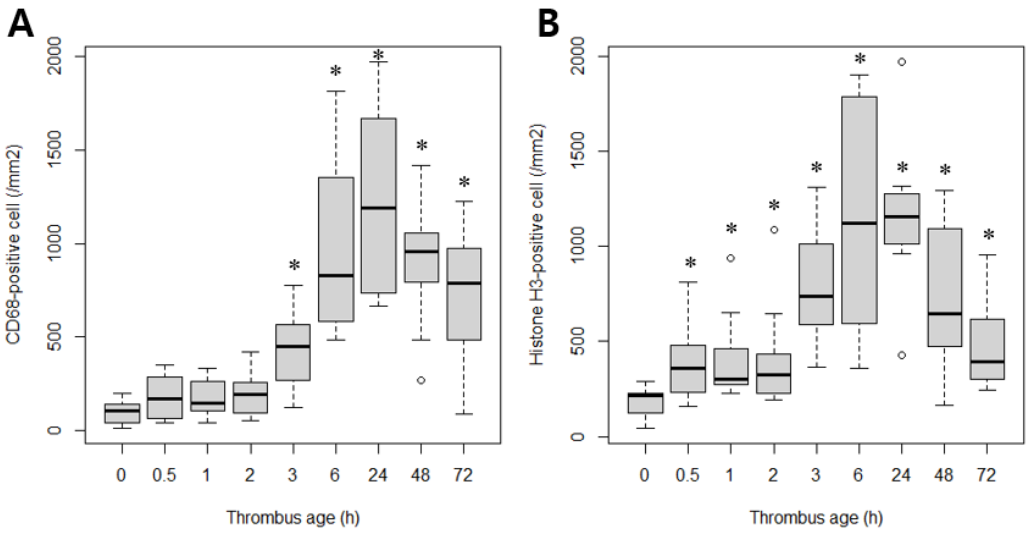

Figure 2. Temporal course of CD68-positive cells (A) and histone H3-positive cells (B) after inducing carotid thrombosis in mice using $FeCl_3$. Counts of both cells increase in a time-dependent manner from 3 hours (all $p<0.01$) for CD68 and 0.5 hours for Histone H3, and then start to decrease from 48 hours (both $p<0.01$) (Jonckheere-Terpstra test). * $p<0.05$ in comparison with 0 hour by Mann–Whitney U test

Association of monocytes with ETs in mouse and human thrombus

Representative images of immunohistochemistry staining for arterial thrombus of human are presented in figure 3. The number of monocytes were strongly correlated with that of ET, both in mouse model ($r=0.705$, $p<0.001$) (Figure 4A) and in thrombus retrieved from stroke patient ($r=0.366$, $p<0.001$) (Figure 5B). Furthermore, confocal immunofluorescence images in human thrombus showed that CD68-positive cells colocalized with histone H3-positive cells (Figure 4B).

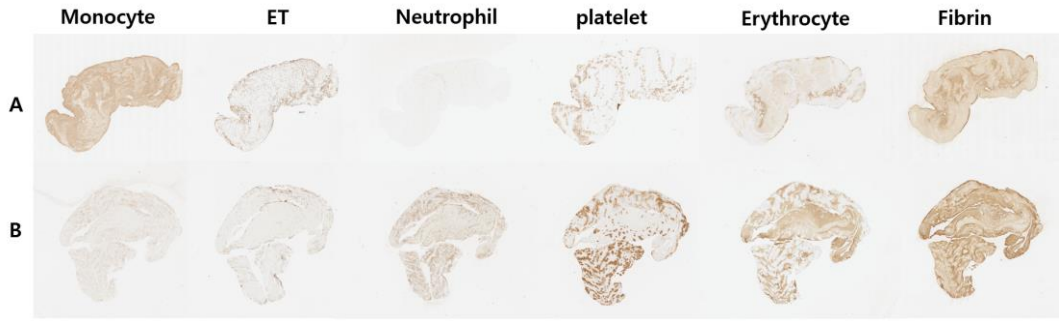

Figure 3. Representative immunohistochemistry of thrombi with higher monocyte (A) and lower monocyte (B) composition that were obtained during endovascular thrombectomy in patients with stroke. The primary antibodies for immunohistochemistry were anti-elastase Ab for neutrophils, anti-glycophorin A Ab for erythrocyte, anti-CD42b Ab for platelet, anti-fibrinogen/fibrin for fibrin, anti-Histone H3 Ab for ET and anti-CD68 Ab for monocyte.

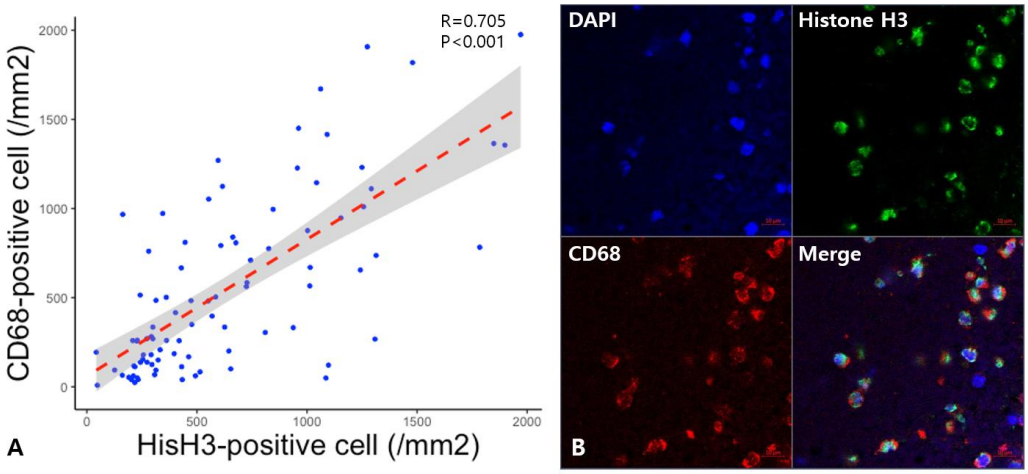

Figure 4. Correlation between the number of CD68-positive cells and that of histone H3-positive cells in arterial thrombus of mouse. There was strong correlation between counts of monocytes and those of histone H3-positive cells (Pearson's correlation test) (A). Representative confocal immunofluorescence images of CD68-postive cells and histone H3-postive cells in thrombus of stroke patient. CD68-postive cells and histone H3-postive cells are colocalized (B).

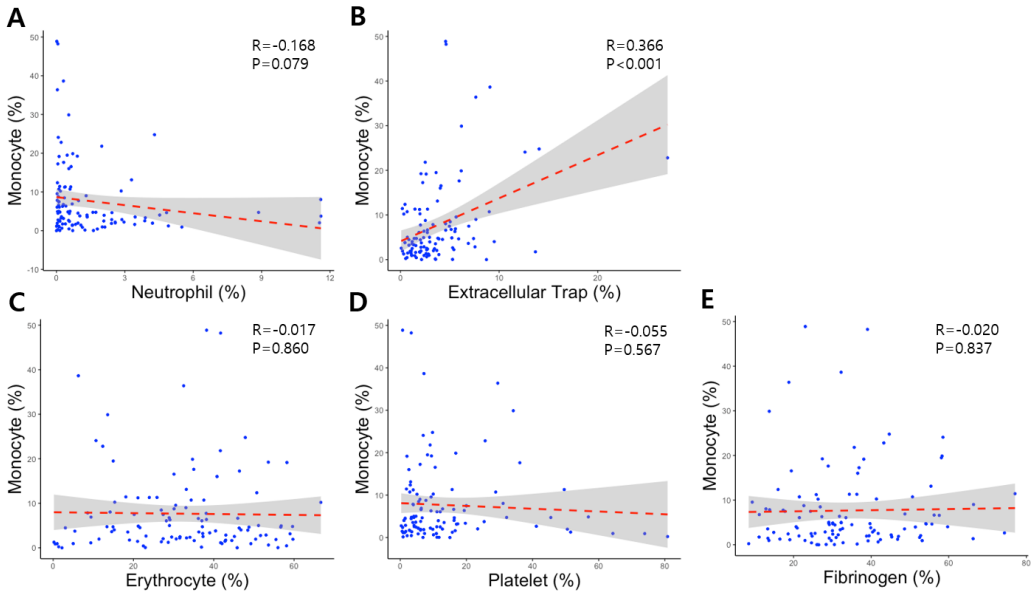

Figure 5. Correlation of the fraction of monocytes and that of neutrophil. (A), extracellular traps (B), erythrocyte (C), platelet (D), and fibrinogen (E) in thrombi obtained from patients with stroke. The fraction of monocytes was positively correlated with that of extracellular traps (B) (Pearson's correlation test).

Characteristics of stroke patients with higher monocyte fraction in thrombus

A total of 102 patients were included in the analysis. The median fraction of monocyte in thrombi was 4.47% (IQR [1.93-9.41]). Baseline characteristics were not different between the higher monocyte group and the lower monocyte group except for platelet counts, which were higher in the higher monocyte group (median 228×10^9/L, IQR [177-267] vs. median 186×10^9/L, IQR [164-225]) (p=0.036) (Table 1).

Table 1. Baseline characteristics of patients according to monocyte composition in thrombus

	Higher Monocyte group (N=50)	Lower Monocyte group (N=52)	P-value
Demographic			
Age, years	75.5 [69.0-83.8]	73.0 [64.0-81.5]	0.318
Sex, male	22 (44.0)	24 (46.2)	0.827
Comorbidities			
Hypertension	36 (72.0)	34 (65.4)	0.472
Diabetes mellitus	18 (36.0)	14 (26.9)	0.323
Dyslipidemia	16 (32.0)	17 (32.7)	0.940
Coronary artery disease	8 (16.0)	4 (7.7)	0.193
Atrial fibrillation	32 (64.0)	31 (59.6)	0.649
Peripheral artery disease	0 (0.0)	1 (1.9)	>0.999
Previous stroke	15 (30.0)	10 (19.2)	0.206
Active cancer	3 (6.0)	8 (15.4)	0.127
Current smoker	6 (12.0)	7 (13.5)	0.825
Laboratory findings			
Hemoglobin, g/dL	13.0 (10.95-15.05)	13.1 (10.98-15.22)	0.771
Hematocrit, %	38.8 [36.3	39.5 [36.7-44.0]	0.625
White blood cell, 10^3/μl	8480 [6768-9715]	7090 [6095-9265]	0.160
Platelet, 10^9/L	228 [177-267]	186 [164-225]	0.036
ESR, mm/h	13.0 [5.3-30.3]	13.5 [4.0-30.0]	0.799
C-reactive protein, mg/L	4.1 [2.2-14.7]	4.6 [0.8-16.0]	0.424
Total cholesterol, mg/dL	140 [120-175]	155 [134-177]	0.179
HDL-cholesterol, mg/dL	41.5 (30-53.0)	43.0 (31.3-54.7)	0.520
LDL-cholesterol, mg/dL	86.5 [63.0-113.0]	87.0 [71.3-107.0]	0.659
Triglyceride, mg/dL	88.0 [69.0-137.0]	98.5 [75.8-131.0]	0.549
D-dimer, ng/mL	750 [426-1477]	1045 [477-2935]	0.166

Fibrinogen, mg/dL	300 [279-347]	306 [253-343]	0.620
BUN, mg/dL	17.6 [13.0-21.8]	16.9 [13.5-21.6]	0.843
Creatinine, mg/dL	0.82 [0.70-1.08]	0.86 [0.73-0.93]	0.920
Albumin, mg/dL	4.0 [3.8-4.3]	4.1 [3.8-4.4]	0.438
Glucose, mg/dL	110 [95-135]	115 [102-140]	0.368
PT, second	11.7 [11.3-12.3]	11.6 [11.0-13.3]	0.899
aPTT, second	31.0 [28.4-33.8]	29.1 [27.2-31.6]	0.060
Stroke classification			0.960
Cardioembolism	33 (66.0)	33 (63.5)	
Large artery atherosclerosis	6 (12.0)	7 (13.5)	
Undetermined	11 (22.0)	12 (23.1)	
Intravenous tPA	18 (36.0)	16 (30.8)	0.575
Initial NIHSS	12.0 [6.3-19.0]	13.5 [9.0-19.0]	0.471

Data are presented as a mean ± standard deviation, median [IQR], or number (%).
ESR, erythrocyte sedimentation rate; HDL-cholesterol, high-density lipoprotein-cholesterol; LDL-cholesterol, low-density lipoprotein-cholesterol; BUN, blood urea nitrogen; PT, prothrombin time; aPTT, activated partial thromboplastin time; tPA, tissue plasminogen activator; NIHSS, National Institutes of Health Stroke Scale

In comparison of factors related to EVT, occlusion site, rate of successful recanalization, and onset-to-recanalization time did not differ between the groups (Table 2). The fraction of neutrophil was significantly lower and the fraction of ET was significantly higher in the higher monocyte group than in the lower monocyte group (Table 2). In terms of clinical outcome, functional independence was more frequent (54.0% vs. 32.7%, p=0.030) and parenchymal hematoma was less frequent (8.0% vs. 28.8%, p=0.007) in the higher monocyte group than the lower monocyte group (Table 2).

Table 2. Comparison of variables related to endovascular thrombectomy according to monocyte composition in thrombus

	Higher monocyte group (N=50)	Lower monocyte group (N=52)	P-value
Occlusion site			0.125
Proximal ICA	9 (18.0)	17 (32.7)	
Distal ICA	12 (240)	5 (9.6)	
M1	16 (32.0)	19 (36.5)	
M2	13 (26.0)	11 (21.2)	
Successful recanalization	46 (92.0)	49 (94.2)	0.713
Thrombus composition, %			
Platelet	8.0 [4.0-16.3]	7.2 [3.2-15.2]	0.545
Fibrinogen	32.3 [23.8-44.3]	30.6 [24.1-41.8]	0.501
Erythrocyte	32.6 (17.3-47.9)	31.7 (14.1-49.3)	0.772
Neutrophil	0.4 [0.1-0.9]	1.1 [0.2-2.6]	0.023
Extracellular trap	3.8 [2.4-5.6]	2.1 [1.2-3.4]	0.001
Procedure time, minutes	28.5 [22.3-46.5]	31.0 [24.8-43.0]	0.242
Onset-to-recanalization time, minutes	381 [188-540]	264 [203-597]	0.789
Functional independence	27 (54.0)	17 (32.7)	0.030
Death at 3 months	10 (20.0)	14 (26.9)	0.410
Hemorrhagic infarction	16 (32.0)	15 (28.8)	0.729
Parenchymal hematoma	4 (8.0)	15 (28.8)	0.007
Symptomatic hemorrhage	4 (8.0)	4 (7.7)	1.000

Data are presented as a mean ± standard deviation, median [IQR], or number (%).
ICA, internal carotid artery; M1, horizontal segment of middle cerebral artery before bifurcation; M2, insular segment of middle cerebral artery

Characteristics of stroke patients associated with functional outcomes

In univariable analysis, factors associated with functional independence at 3 months included blood albumin, successful recanalization, and higher monocyte composition. Meanwhile, age, blood level of erythrocyte sedimentation rate, C-reactive protein, D-dimer, fasting glucose, initial NIHSS, occlusion site, and parenchymal hematoma were associated with functional dependence or death at 3 months (Tables 3 and 4). In multivariable analysis, factors that were significantly associated with functional dependence or death at 3 months were age (odds ratio [OR] 0.926, 95% confidence interval [CI] 0.879-0.976, p=0.004), fasting glucose level (OR 0.981, 95% CI 0.966-0.996, p=0.011), initial NIHSS (OR 0.836, 95% CI 0.753-0.927, p=0.001), proximal ICA occlusion (OR 0.106, 95% CI 0.015-0.756, p=0.025). Independent predictors of functional independence at 3 months were albumin level (OR 9.540, 95% CI 2.060-44.192, p=0.004), successful recanalization (OR 141.659, 95% CI 4.758-4218.049, p=0.004), and higher monocyte composition in thrombus (OR 4.954, 95% CI 1.467-16.724, p=0.010) (Table 5).

Table 3. Demographic data and laboratory results associated with functional outcomes at 3 months

	Functional independence at 3 months (N=44)	Functional dependence or death at 3 months (N=58)	P-value
Demographic			
Age, years	70.0 [64.0-78.3]	79.0 [66.8-85.0]	0.003
Sex, male	22 (50.0)	24 (41.4)	0.386
Comorbidities			
Hypertension	28 (63.6)	42 (72.4)	0.344
Diabetes mellitus	11 (25.0)	21 (36.2)	0.227
Dyslipidemia	16 (36.4)	17 (29.3)	0.451
Coronary artery disease	5 (11.4)	7 (12.1)	0.913
Atrial fibrillation	25 (56.8)	38 (65.5)	0.371
Previous stroke	13 (29.5)	12 (20.7)	0.303
Active cancer	2 (4.5)	9 (15.5)	0.109
Current smoker	7 (5.61)	6 (7.39)	0.404
Laboratory findings			
Hemoglobin, g/dL	13.2±1.66	13.0±2.36	0.614
Hematocrit, %	39.3 [36.4-43.1]	39.0 [36.2-44.2]	0.743
White blood cell, 10^3/μl	7420 [5815-9068]	8225 [6678-10300]	0.151
Platelet, 10^9/L	200 [165-234]	205 [169-273]	0.383
ESR, mm/h	9.5 [4-18.5]	16.5 [6.3-38.5]	0.032
C-reactive protein, mg/L	2.95 [1.10-6.85]	9.25 [2.50-25.20]	0.004
Total cholesterol, mg/dL	149.4±39.0	154.6±39.6	0.515
HDL-cholesterol, mg/dL	43.0±11.8	41.7±11.4	0.583
LDL-cholesterol, mg/dL	81.5 [64.8-106.0]	89.0 [69.8-109.0]	0.482
Triglyceride, mg/dL	83.0 [61.5-139.0]	102.0 [77.0-130.0]	0.121
D-dimer, ng/mL	540 [368-1298]	1056 [587-2784]	0.007
Fibrinogen, mg/dL	288 [245-321]	314 [272-357]	0.093

BUN, mg/dL	16.9 [13.6-20.9]	17.6 [13.4-22.3]	0.423
Creatinine, mg/dL	0.87 [0.70-0.98]	0.84 [0.72-1.02]	0.917
Albumin, mg/dL	4.1 [4.0-4.4]	4.0 [3.7-4.3]	0.027
Glucose, mg/dL	104 [91-118]	122 [105-166]	<0.001
PT, second	11.5 [11.0-12.2]	11.9 [11.2-12.9]	0.060
aPTT, second	30.3 [27.8-34.0]	29.7 [27.2-34.0]	0.246
Stroke classification			0.824
Cardioembolism	27 (61.4)	39 (67.2)	
Large artery atherosclerosis	6 (13.6)	7 (12.1)	
Undetermined	11 (25.0)	12 (20.7)	
Intravenous tPA	17 (38.6)	17 (29.3)	0.322
Initial NIHSS	9.0 [5.0-13.3]	17.5 [12.0-20.0]	<0.001

Data are presented as a mean ± standard deviation, median [IQR], or number (%).
ESR, erythrocyte sedimentation rate; HDL-cholesterol, high-density lipoprotein-cholesterol; LDL-cholesterol, low-density lipoprotein-cholesterol; BUN, blood urea nitrogen; PT, prothrombin time; aPTT, activated partial thromboplastin time; tPA, tissue plasminogen activator; NIHSS, National Institutes of Health Stroke Scale

Table 4. Endovascular thrombectomy-related factors associated with functional outcomes at 3 months

	Functional independence at 3 months (n=44)	Functional dependence or death at 3 months (n=58)	P-value
Occlusion site			0.033
Proximal ICA	6 (13.6)	20 (34.5)	
Distal ICA	9 (20.5)	8 (13.8)	
M1	14 (31.8)	21 (36.2)	
M2	15 (34.1)	9 (15.5)	
Successful recanalization	44 (100.0)	51 (87.9)	0.018
Thrombus composition, %			
Platelet	8.6 [3.9-13.9]	7.1 [3.8-16.5]	0.927
Fibrinogen	35.7±14.9	32.4±14.1	0.262
Erythrocyte	33.0±15.3	31.5±17.4	0.645
Neutrophil	0.5 [0.1-2.0]	0.7 [0.3-2.0]	0.225
Extracellular traps	3.4 [2.5-4.6]	2.1 [1.4-4.9]	0.092
Monocyte	4.9 [2.0-11.1]	3.6 [1.8-7.8]	0.184
Higher monocyte composition	27 (61.4)	23 (39.7)	0.030
Higher ET composition	27 (61.4)	24 (41.4)	0.046
Procedure time, minutes	26.5 [21.8-48.5]	29.5 [24.5-43.0]	0.242
Onset-to-recanalization time, minutes	357 [186-448]	407 [216-689]	0.197
Hemorrhagic infarction	13 (29.5)	18 (31.0)	0.871
Parenchymal hematoma	2 (4.5)	17 (29.3)	0.001

Data are presented as a mean ± standard deviation, median [IQR], or number (%).
ICA, internal carotid artery; M1, horizontal segment of middle cerebral artery before bifurcation; M2, insular segment of middle cerebral artery; ET, extracellular trap

Table 5. Multivariable logistic regression analysis for factors associated with functional independence at 3 months

	Odds ratio (95% CI)	P-value
Age, year	0.926 (0.879-0.976)	0.004
Albumin, mg/dL	9.540 (2.060-44.192)	0.004
Glucose, mg/dL	0.981 (0.966-0.996)	0.011
Initial NIHSS	0.836 (0.753-0.927)	0.001
Occlusion site		
M1	0.459 (0.083-2.534)	0.372
M2	0.277 (0.043-1.808)	0.180
Proximal ICA	0.106 (0.015-0.756)	0.025
Successful recanalization	141.659 (4.758-4218.049)	0.004
Higher monocyte composition	4.954 (1.467-16.724)	0.010

CI, confidence interval; NIHSS, National Institutes of Health Stroke Scale; ICA, internal carotid artery; M1, horizontal segment of middle cerebral artery before bifurcation; M2, insular segment of middle cerebral artery

IV. DISCUSSION

This study showed that, monocytes were increasingly recruited in a time-dependent manner in arterial thrombosis, starting from 3 hours and then decreasing from 48 hours following the induction of thrombosis in a carotid artery thrombosis model in mice. ETs showed a similar recruit pattern. The fraction of monocytes in thrombus was significantly correlated with that of ETs, both in the mouse model and among stroke patients. Monocytes and ETs were colocalized on confocal immunofluorescence images in thrombi of stroke patients. Patients with a higher monocyte composition in thrombus experienced less frequent occurrence of parenchymal hematoma. Importantly, a higher monocyte composition in thrombus was an independent predictor of functional independence or death at 3 months.

In this study, the number of monocytes in mouse thrombus increased a few hours after the induction of thrombosis. This finding suggests that monocytes may not play a major role in the initial mechanisms of thrombosis but rather in the later process of thrombosis. Traditionally, monocytes in cardiovascular disease or stroke were suggested to play roles similar to those of the other inflammatory cells in the injured tissues,[26] including the production of inflammatory cytokines and facilitating vascular inflammation.[27] However, other studies suggested that monocytes or macrophages may prevent left ventricular thrombus formation,[28] or may contribute resolution of inflammation, tissue repair and remodeling after myocardial infarction.[29-31]

Once, a type of inflammatory cells is activated, it can trigger a chain reaction leading to the recruitment, activation, and degranulation of the other inflammatory cells.[1,32] Macrophage, one of the monocyte family, may attenuate thrombus growth by inhibiting the activation of other inflammatory cells.[33] They may also prevent thrombosis by clearing activated inflammatory cells and their products that are prothrombotic.[34] Furthermore, monocytes and macrophages also can release anti-inflammatory factors, such as IL-10 and TGF-beta, which can inhibit thrombus formation.[28] These findings suggest that monocytes and macrophages may contribute to the attenuation of ongoing thrombus growth.

Of note, the number or fraction of monocytes was correlated with those of ETs in both the mouse model and human arterial thrombus in this study. Additionally, monocytes and ETs were colocalized in confocal immunofluorescence staining. Although the main functions of ETs are protection of host by killing pathogens and avoiding dissemination of them, uncontrolled formation of ET may be involved in the pathogenic mechanism of thrombosis.[35] Previously, ETs were thought to be only produced from neutrophils. However, recent studies have reported that monocytes also produce ETs.[16-18] Although the role of ETs derived from monocytes is not well known, previous studies suggested that monocyte/macrophage-derived ETs may associated with pathology of various diseases, such as renal disease, venous thrombosis, and myocardial infarction.[36,37] Because most of the studies reported the prothrombic effect of ETs, further research is necessary to determine whether monocyte/macrophage-derived ETs has antithrombotic effect as well, especially in the late phase of arterial thrombosis.

In this study, we found that higher monocyte fraction in arterial thrombus of stroke patients was associated with functional independence at 3 months. In addition, patients with higher monocyte fraction less likely had hemorrhagic transformation of parenchymal hematoma. These findings suggest that the presence of monocytes in thrombus is linked to favorable clinical outcomes following EVT.

While neutrophils play a deleterious role during early stage in ischemic injury, macrophages and monocytes are involved in cleaning damaged tissues and initiating repair.[31,33,34] Hemorrhagic transformation and parenchymal hematoma in the ischemic area result from degradation of microvascular basal lamina. Matrix metaloproteinase-9 plays a major role in the basal lamina degradation and resulting hemorrhagic transformation and parenchymal hematoma.[38,39] Neutrophils are one of the sources of matrix metaaloproteins-9. In contrast to neutrophils, monocytes in microthrombi and microvessels may contribute to attenuating microvascular and brain tissue damages during early or subacute stages of cerebral ischemia.

It is uncertain how the higher monocyte fraction is linked to the microvascular and tissue

damage in stroke patients. However, during EVT, distal small arteries may be affected by the embolization of small, fragmented monocyte-rich thrombi. Monocyte-rich microthrombi, which are not visible on conventional angiography, may have a local impact on the microvascular system. Out findings from the thrombosis model in mice indicate that monocytes are not passively trapped but actively recruited to the thrombus. While the mechanism of monocyte recruitment to thrombus is unknown, similar mechanisms may occur in the microvessels of the ischemic brain. Patients with increased monocyte recruitment to the thrombus may also experience increased monocyte recruitment and infiltration into the microvessels and ischemic brain.

In this study, monocytes increased in a time-dependent manner during the first 24 hours in mice. However, in stroke patients who underwent EVT within 24 hours after symptom onset, there was no difference in the time from onset to recanalization between the higher and lower monocyte groups in the thrombus. The time from symptom onset to recanalization may partly reflect the thrombus age. However, the time of symptom onset may not accurately represent the time of initial thrombus formation. This discrepancy may occur due to potential time delays between thrombus formation and embolization to the cerebral artery, especially in patients with cardioembolic stroke including atrial fibrillation. Thrombi might have formed and grown in the cardiac chamber before embolization. Furthermore, the time of symptom onset noted by the patients, or their family can often be inaccurate or unknown.

This study has several limitations. Firstly, thrombosis is induced using the $FeCl_3$ model in mice. $FeCl_3$ causes vascular injury through oxidative stress, leading to red blood cell-mediated platelet recruitment and thrombosis.[20,40-42] Although this model is most widely used for thrombosis research in animals, the mechanism of thrombosis differs from that in stroke patients. Additionally, there are differences in the proportions of leukocyte subsets between human and mice.[43] Therefore, these distinctions should be considered when interpreting findings of this study. Secondly, our explanation regarding the association between monocyte proportions in thrombus and functional outcomes is speculative. In

addition, the association between monocytes and ETs were based solely on the comparison of their counts within thrombus in this study. As such, their interactive roles in thrombosis are uncertain based on this study. Therefore, further studies are necessary to validate our findings and elucidate their underlying mechanisms. Finally, we included patients with anterior circulation stroke for this study. Therefore, interpretation of findings in this study should be restricted to those with anterior circulation stroke.

V. CONCLUSION

This study showed that monocytes and ETs increased in a time-dependent manner during thrombosis. This study also showed that the higher proportion of monocytes in thrombus was associated with favorable functional outcomes and reduced risks of hemorrhagic transformation in stroke patients undergoing EVT.

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ABSTRACT (IN KOREAN)

동맥혈전에서 단핵구 동원과 혈전 조성에 따른 뇌졸중 환자의 특징

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이기정

목적

혈전생성에서 혈소판이나 섬유소 이외에도, 백혈구를 비롯한 여러 염증 관련 세포들이 상호작용한다는 것이 최근 많이 보고되었다. 그러나 염증 반응에서의 단핵구의 역할은 잘 알려져 있지만, 혈전생성에서의 역할은 최근에 연구되기 시작하였다. 본 연구에서는 염화제이철을 이용한 경동맥 폐색 마우스 모델을 통하여 시간에 따라 동맥혈전에 단핵구가 동원되는 양상을 살펴보고, 뇌경색 환자의 혈전제거술로부터 얻은 동맥혈전에서 단핵구의 조성에 따른 환자의 특징을 알아보고자 한다.

방법

암 연구소의 쥐에서 염화철을 이용하여 경동맥 혈전을 유도한 후 각각 30 분, 1 시간, 2 시간, 3 시간, 6 시간, 24 시간, 48 시간, 72 시간 동안 경동맥 원위부를 결찰하여 시간에 따른 혈전을 만들었다. 뇌졸중 환자의 혈전분석에서는 임상 데이터와 동맥내 혈전제거시 얻은 혈전을 이용하였다. 혈전 조성을 확인하기 위해 면역조직화학염색을 하였으며, 세포들의 분포를 확인하기 위해 면역형광염색을 하였다.

결과

90마리 쥐의 혈전에서 CD68 양성 세포 수(단핵구)와 히스톤 H3 양성 세포(세포외 트랩) 수는 각각 3시간, 0.5시간 이후부터 증가하다가, 두 가지 모두 48시간 이후 감소하였다($p<0.001$). 단핵구와 세포외 트랩의 분율은 쥐 모델에서($r=0.705$, $p<0.001$)와, 뇌졸중 환자에서($r=0.366$, $p<0.001$) 모두 양의 상관관계를 보였다. 뇌졸중 환자 혈전의 단핵구와 세포외 트랩은 공초점 면역형광 촬영에서 동일 위치에 있었다. 102명의 뇌졸중 환자들 중 단핵구가 많은 군에서, 혈중 혈소판 농도는 더 높고(중앙값 228×109/L, 사분위간 범위 [177 – 267] vs. 중앙값 186×109/L, 사분위간 범위 [164 – 225], $p=0.036$), 뇌실질출혈의 빈도는 낮았으며(8.0% vs. 28.8%, $p=0.007$), 기능적 독립을 의미하는 좋은 예후의 빈도는 더 높았다 (54.0% vs. 32.7%, $p=0.030$). 로지스틱 회귀분석을 활용한 다변량분석에서 높은 단핵구의 분율은, 기능적 독립을 의미하는 좋은 예후를 예측하는 독립적인 인자였다. (odds ratio 4.954; 95% confidence interval 1.467-16.724, $p=0.010$).

결론

단핵구와 세포외 트랩은 쥐 동맥혈전 분석에서 처음에는 시간 의존적으로 증가하다가, 48시간 이후부터 감소하는 경향을 보였다. 사람과 쥐 혈전 모두에서 단핵구와 세포외 트랩 분율은 연관을 보였다. 혈전 단핵구 분율이 높은 환자는 혈중 혈소판 농도가 높고, 뇌실질출혈이 적었으며, 기능적 독립을 의미하는 좋은 예후의 빈도는 더 많았다. 뇌졸중 환자들의 혈전에서 높은 단핵구 분율은 기능적 독립을 의미하는 좋은 예후를 예측하는 인자였다.

핵심되는 말: 단핵구, 혈전증, 뇌졸중, 세포외 트랩, 혈관내 혈전제거술, 기능적 예후