Direct Arterial Damage and Neurovascular Unit Disruption by Mechanical Thrombectomy in a Rat Stroke Model

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Abstract

Mechanical thrombectomy (MT) is a standard treatment for acute ischemic stroke that could cause hemorrhagic complications. We aimed to evaluate the pathology of MT-induced arterial damage and neurovascular unit (NVU) disruption in relation to tissue-type plasminogen activator (tPA) injection for acute ischemic stroke. We induced transient middle cerebral artery occlusion in male SHR/Izm rats for 2 hours. This was followed by reperfusion with/without tPA (3 mg/kg) and "rough suture" insertion that mimicked MT once or thrice (MT1 or MT3). Compared with the control group, the tPA+MT3 group presented with an increase in the cerebral infarct and hemorrhage with severer IgG leakage. Moreover, structural damage reaching the tunica media was detected in the MT3 and tPA+MT3 groups. The tPA+MT3 group presented with increased matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF) expression with some MMP9-positive cells expressing a neutrophil marker myeloperoxidase. Furthermore, basal lamina detachment from astrocyte foot processes was observed in the tPA+MT1 and tPA+MT3 groups. These findings suggest that MT causes direct arterial damage, as well as VEGF and MMP9 upregulation, which results in NVU disruption and hemorrhagic complications in acute ischemic stroke, especially when combined with tPA.

Significance Statement

Mechanical thrombectomy (MT) is among the standard treatments for acute ischemic stroke; however, it can cause hemorrhagic complications. In this study, we established a novel MT-treated rat model of ischemic stroke and found that MT caused direct arterial damage. Moreover, it increased the cerebral infarct and hemorrhage volumes when combined with tPA. Further, MT upregulated VEGF and MMP9 expression, which caused neurovascular unit disruptions that led to hemorrhage. In addition, MMP9 localized in myeloperoxidase -positive cells suspected to be neutrophils near the injured vessel area.

1. Introduction

Early reperfusion therapies using tissue-type plasminogen activator (tPA) and mechanical thrombectomy (MT) has become a standard treatment for acute ischemic stroke, especially for large vessel occlusions in the anterior circulation (Powers et al., 2018). However, intracerebral hemorrhage remains a complication of early reperfusion therapy. Furthermore, the frequency of stent retriever passing during an MT procedure is positively correlated with the re-canalization rate. Further, increased intracranial hemorrhage results in worse clinical outcomes (Baek et al., 2018). Consequently, several clinical trials are being performed currently to compare the effectiveness of direct MT without tPA and post-tPA secondary MT.

Neurovascular unit (NVU) disruption is among the main mechanisms implicated in reperfusion injury, which is a hemorrhagic complication of reperfusion therapy (Khatri et al., 2012). Previous studies have shown that tPA toxicity may

compromise endothelial surface integrity. Moreover, MT may cause direct vessel injury in the intimal and medial arterial wall layers. Both mechanisms are possibly associated with NVU damage (Reil et al., 2000). In addition, a clinical study on MT reported NVU damage observed using contrast-enhanced magnetic resonance imaging, as well as a risk of intracerebral hemorrhage and poor early neurological recovery in acute ischemic stroke (Renú et al., 2017). However, the mechanisms underlying MT-induced arterial damage and NVU disruption in ischemic stroke remain unclear. Consequently, we aimed to evaluate the pathology of arterial damage and NVU damage caused by tPA and MT in a novel rat model of acute ischemic stroke mimicking MT after transient middle cerebral artery occlusion (tMCAO).

2. Methods and Materials

2.1 Vascular Injury by Rough Suture

In this study, we mimicked human arterial wall damage caused by MT with stent retriever in rats. In clinical settings, stent retrievers can cause arterial wall damage and hemorrhagic complications in elderly patients with arteriosclerotic vascular disease, hypertension, or hyperlipidemia. Stent retriever devices have been shown to induce specific structural changes of the arterial wall, including endothelial denudation and internal elastic lamina fracture (Gory et al., 2013). In this study, we established a rat model with similar histopathological changes. Since a stent retriever cannot be inserted into the rat internal carotid artery due to its huge diameter, we established a novel rat model.

We prepared two suture types. The first was the smooth suture (silicone-coated 4-0

nylon thread with a diameter of 380 μm and a smooth surface; Figure 1A), which was used for the previously described classical tMCAO method (Abe et al., 1988). The second was a newly designed rough suture (silicone-coated 4-0 nylon thread with a diameter of 380 μm and a surface fully coated with rough white fused alumina [20 μm in diameter, Naniwa Abrasive Manufacturing, Osaka, Japan] checked with a light microscope; Figure 1B). The rough suture was inserted from the external carotid artery and passed through the internal carotid artery bifurcation to mildly damage the arterial intima. In a pilot study, the filament size and coating materials were adjusted while checking for resulting pathological changes until the appropriate condition was determined. Moreover, the top of the rough suture was round and soft to prevent vessel perforation causing subarachnoid hemorrhage.

2.2 Experimental Groups and Treatment

Male stroke-resistant spontaneously hypertensive (SHR/Izm) rats (12 weeks old, bodyweight 280-310 g; total n = 43; SLC, Shizuoka, Japan) were acclimatized to standard rat cages for two weeks under conventional laboratory conditions with a 12/12-hour light-dark cycle and controlled humidity and room temperature of 23-24 °C. The animals were fed rat pellets (MF, Oriental Yeast, Tokyo, Japan) and water was provided *ad libitum*. Each cage contained one rat and was cleaned weekly with paper pulp bedding (Oriental Yeast, Tokyo, Japan). Male rats were used since estrogen may enhance post-ischemic stroke neurogenesis and behavioral recovery in female animals (Park et al., 2019, Suzuki et al., 2009). All animal experiments complied with the protocols approved by the Animal

Committee of the Graduate School of Medicine and Dentistry, Okayama University (OKU#2018288). The rats were divided in the following six experimental groups: the control group (n = 6), MT1 group (n = 6), MT3 group (n = 7), tPA group (n = 7), tPA+MT1 group (n = 6), and tPA+MT3 group (n = 11) (Figure 1C).

2.3 Focal Cerebral Ischemia, tPA Administration, and Insertion of the Rough Suture

All the rats were anesthetized using a nitrous oxide/oxygen/isoflurane mixture (69/30/1%) administered through an inhalation mask. The right carotid bifurcation was exposed and the external carotid artery was legated distal to the bifurcation. Next, a smooth suture was inserted through the stump of the external cerebral artery and gently advanced for middle cerebral artery occlusion. After occlusion for 2 hours, we either administered a vehicle (physiologic saline, intravenously, 0.5 mL) or tPA (Grtpa, Mitsubishi Tanabe Pharma Corporation, Osaka, Japan, intravenously, 3 mg/kg). The nylon embolus was withdrawn for blood flow restoration in the MCA territory. For MT, the rough suture was similarly inserted and pulled out once or thrice. Finally, the incision was closed. The rectal temperatures were maintained at 37.0 °C by placing the animals on a heating bed (Model BWT-100; Bio-Research Center, Nagoya, Japan). We measured the cortical cerebral blood flow in the right MCA territory during ischemia through laser-Doppler flowmetry using an ALF21 (Advance, Tokyo, Japan). We detected increased infarct volume with a two-sided 5% significance level and a power of 80% with a necessary sample size of 6 rats per group. As a result, we used 43 rats; among them, 5 rats that died from intraoperative complications were excluded.

2.4 Tissue Preparation

At 24 hours after reperfusion, the rats (total n = 38: 6, control; 6, MT1; 6, MT3; 6, tPA; 6, tPA+MT1; and 8, MT3+tPA) were anesthetized using intraperitoneal pentobarbital injections (40 mg/kg) and transcardially perfused with ice-cold phosphate-buffered saline (PBS) and 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4). The whole brain was removed and immersed in the same fixative for 12 hours at 4 °C. After washing with PBS, the tissues were sequentially transferred into 10%, 20%, and 30% (wt/vol) sucrose solutions, embedded in powdered dry ice, and stored at -80 °C. We prepared 15-μm thick sections using a cryostat at -18 °C, mounted on silane-coated glass slides, and stored at -80 °C.

2.5 Histology and Single Immunohistochemistry Analysis

To identify ischemic lesion areas, the brain sections were stained using cresyl-violet as Nissl staining and examined with a light microscope (Olympus BX-51; Olympus Optical, Tokyo, Japan). The sections were cut at 2, 0, -2, -4, and -6 mm from the bregma. We measured the infarct area in these 5 sections by counting pixels using NIH ImageJ software (National Institutes of Health, Bethesda, MD) and calculated the infarct volume by multiplying the infarct area by a 2-mm thickness (Kawai et al., 2011).

For brain hemorrhage analysis, we performed iron staining using an enhanced Perl reaction. Briefly, the sections were incubated with Perl solution (5% potassium ferrocyanide and 5% HCl; 1:1) for 45 minutes, washed in distilled water, and incubated

with 0.5% diamine benzidine tetrahydrochloride and nickel for 60 minutes (Wu et al., 2003). The hemorrhage area was measured using the same method for infarct area measurement. Further, hematoxylin-eosin (H&E) staining was performed to evaluate vessel wall structural damage that enhanced filament rugosity. To estimate the rat IgG leakage levels, the sections were incubated with biotin-labeled rabbit anti-rat IgG antibody (1:500; Vector Laboratories, PK-4004). Immunoreactivities were developed in horseradish peroxidase streptavidin-biotin complex solution (Vectastain ABC kit; Vector Laboratories, PK-6104) and incubated with diaminobenzidine tetrahydrochloride. Further immunohistochemistry was performed using rabbit anti-matrix metalloproteinase-9 (MMP-9) antibody (1:200; Abcam, ab38898, AB_776512) and rabbit anti-vascular endothelial growth factor (VEGF) antibody (1:200; Abcam, ab46154, AB_2212642). For the negative control, we stained some brain sections using a similar procedure without the primary antibody. A light microscope (Olympus BX‐51, Tokyo, Japan) was used to examine the sections. The investigators who performed the surgical procedures and immunohistochemistry analyses were blinded to the tPA and MT assignments.

2.6 Double Immunofluorescence Analysis

To detect MMP9-positive cell types, double immunofluorescence analyses were performed for MMP9 and myeloperoxidase (MPO), a neutrophil marker. To determine NVU damage caused by mechanical injury, double immunofluorescence analyses for glial fibrillary acidic protein (GFAP) and collagen IV were performed using the following primary antibodies: goat anti-GFAP antibody (1:500; Millipore, MAB3402, AB_94844);

rabbit anti-collagen IV antibody (1:500; Novotec, NOT20441-1, AB_2827429); and mouse anti-MPO antibody (1:200; Abcam, ab90810, AB_2146325). Immunoreactivities were visualized using an appropriate fluorescent secondary antibody, mouse anti-rabbit IgG antibody conjugated with Alexa 555 (Thermo Fisher Scientific), and goat anti-mouse IgG antibody conjugated with Alexa 488 (Thermo Fisher Scientific). The treated sections were scanned and examined at 100x magnification for the confocal microscopy (LSM-510; Zeiss, Jena, Germany).

2.7 Semiquantitative Analysis and Vascular Dissociation Index

To semiquantitatively evaluate rat IgG and staining intensity, we used stained sections of the caudate-putamen (1.2, 0.7, and 0.2 mm rostral to the bregma, Paxinos, and Watson; 1982) from each rat, as well as three areas in the ipsilateral infarcted cortex, contralateral cortex, and the area near the injured artery. Each section was randomly chosen and captured at 200x magnification for light microscopy. The staining intensity was measured using image processing NIH ImageJ software. We evaluated basement membrane detachment from the astrocyte end-feet through discriminating staining of collagen IV/GFAP double-labeled sections. The treated sections were evaluated by randomly selecting three striatum levels and four areas in the ipsilateral peri-infarcted cortex and near the injured artery area. We confirmed the border between the ischemic core and peri-infarcted lesion using cresyl-violet staining of adjacent sections as previously described (Omori et al., 2002). We measured the area between the astrocyte end-feet and the basement membrane of each blood vessel, as well as the length of each

blood vessel. Subsequently, we calculated the area-to-length ratio as the vascular dissociation index (Yamashita et al., 2009).

2.8 Statistical Analysis

All statistical analyses were performed using GraphPad Prism (version 8.3, GraphPad Software Inc., San Diego, CA, SCR 002798). All data were expressed as the mean ± standard deviation or median (interquartile range). Since the data were non-normally distributed, analyses were performed using the Kruskal–Wallis variance analysis test followed by a Dunn's multiple comparison test. Statistical significance was set at $P < 0.05$.

3. Results

3.1 Infarct Volume, Intracranial Hemorrhage, and IgG Leakage

The postoperative survival rates at 24 hours in the control, MT1, MT3, tPA, tPA+MT1, and tPA+MT3 groups were 100% (n = 6), 100% (n = 6), 85.7% (n = 6), 85.7% (n = 6), 100% (n = 6), and 72.7% (n = 6), respectively (Figure 2A). Compared with the control, MT1, and tPA+MT1 groups, the tPA+MT3 group had significantly increased infarct volumes (*p = 0.046, Dunn's multiple comparison test; Figure 2B, C). Intracranial hemorrhage, including hemorrhagic infarction and very minor hemorrhage, was observed in 31 rats (4, control; 3, MT1; 5, MT3; 5, tPA; 6, tPA+MT1; and 8, tPA + 3MT). Compared with the control group, the tPA+MT3 group had greater hemorrhage volume (*p = 0.0102, Dunn's multiple comparison test; Figure 2D). The H&E staining revealed cracks reaching

the tunica media of the intracranial cerebral artery in the MT3 and tPA+MT3 groups (Figure 3A, arrowheads). IgG staining revealed IgG leakage mainly near the injured vessel area (Figure 3B, arrowheads). Compared with the control group, semi-quantitative analysis revealed significantly greater IgG leakage in the tPA+MT1 and tPA+MT1 groups (****p < 0.0001; Dunn's multiple comparison test), tPA+MT3 group (**p = 0.0011; Dunn's multiple comparison test), and MT3 group (*p = 0.0403; Dunn's multiple comparison test) (Figure 3C & D).

3.2 Immunoreactivity for MMP9 and VEGF and Double Immunostaining

There was increased MMP9 and VEGF expression in the ischemic area and near the injured vessel area in the MT1, MT3, tPA, tPA+MT1, and tPA+MT3 groups. There was significantly increase MMP9 pixel intensity in the tPA+MT1 (*p = 0.0181, Dunn's multiple comparison test) and tPA+MT3 groups (***p = 0.0006, Dunn's multiple comparison test) compared with the control group (Figure 4A, B). Double immunostaining for MMP9 and MPO revealed MMP9 localization in MPO-positive cells with segmented nuclei (Figure 4C). In addition, compared with the control group, the tPA+MT3 group had a significantly increased VEGF pixel intensity in the infarcted area and near the injured vessel area (*p = 0.0408, Dunn's multiple comparison test; Figure 4D, E).

There were increased vascular dissociation indexes between the basal lamina (collagen IV) and astrocyte foot processes (GFAP) in the tPA+MT3 group (*p = 0.0048, Dunn's multiple comparison test, Figure 5A, B).

4. Discussion

Compared with the control group, there were significantly increased infarct and hemorrhage volumes in the tPA+MT3 group (Figure 1B-D). Further, the MT3 and tPA+MT3 groups had structural damage reaching the tunica media (Figure 3A). The tPA+MT1 and tPA+MT3 groups had significant IgG leakage within the peri-injured vessel territory (Figure 3B-D). The tPA+MT3 group showed increased MMP9 and VEGF expression in the ischemic lesion and peri-injured vessel territory (Figure 4A, B, D, and E) with some of the MMP9-positive cells expressing MPO (Figure 4C). Finally, the tPA+MT1 and tPA+MT3 groups showed significant detachment of the basal lamina from astrocyte foot processes (Figure 5A, B).

In rats, endogenous IgG is distributed in NVU breakdown areas (Richmon et al., 1998), which is related to hemorrhagic complications in ischemic stroke (Hamman et al., 1999). In our study, the observed IgG leakage (Figure 2) was indicative of NVU damage in the vessels of the infarct area, as well as mechanically injured vessels. MMP9, which is a zinc-dependent endopeptidase that degrades basement membranes, is mainly localized in infiltrating neutrophils, endothelial cells, and activated microglia (Rosell et al., 2008). MMP-9 gene deletion was shown to significantly reduce post-tMCAO NVU disruption, which was associated with decreased degradation of tight junction proteins (Asahi et al., 2001). Therefore, MMP9 activation could cause NVU disruption and subsequent hemorrhagic transformation (Wang et al., 2004). Furthermore, tPA can directly induce MMP-9 release from neutrophils (Cuadrado et al., 2008) and cause NVU

damage (Yamashita et al., 2009). These processes have been suggested to contribute to post-tPA hemorrhagic transformation. Moreover, the VEGF signaling cascade is located upstream of MMP9 (Kanazawa et al., 2011) and plays an important role in NVU damage. Neutrophils are recruited to the intimal surface of mechanically injured arteries within 1-24 hours of injury (Roque et al., 2000) and migrate to the peri-infarct lesion in the acute ischemic stroke phase (Perez-de-Puig et al., 2015) where they activate both MMP9 (Rosell et al., 2008) and VEGF (Chodobski et al., 2003).

In our study, mechanically damage to the arterial intima activated the VEGF and MMP9 pathway with neutrophil migration, which leads to NVU disruption and hemorrhagic transformation (both enhanced by tPA administration). Recent clinical studies have reported no differences in the MT treatment outcomes with or without tPA pre-administration (Tsivgoulis et al., 2016; Broeg-Morvay et al., 2016; Abilleira et al., 2017). Contrastingly, blood-brain barrier disruption with increased infarct volume and poorer clinical outcome has been associated with tPA use and/or more stent retriever passes in patients with acute ischemic stroke (Renú et al., 2017), which is consistent with our findings. This study has several limitations. First, there was a small sample size in each group. Second, we observed a high intracerebral hemorrhage rate than that in human patients treated using thrombectomy with tPA, which could be attributed to our use of a high tPA dose. Therefore, the findings should be interpreted carefully.

In summary, our results suggest that VEGF, MMP9, and neutrophils contribute to NVU disruption. Moreover, they suggest that vascular protection therapy could improve the outcome of patients with severe stroke undergoing tPA and endovascular treatment.

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Conflict of Interest Statement

The authors disclose no potential conflicts of interest.

Author Contributions

All the authors had full access to all data used in this study and take responsibility for the data integrity and the accuracy of the data analysis. Study concept and design: R. S. and K.A. Acquisition of data: R.S. Analysis and interpretation of data: R.S. and T.Y. Drafting of the manuscript: R.S., T.Y. and K.A. Statistical analysis: R.S. Technical, and material support: R.S., T.Y., K.T., N.M., E.N., Y.O., M.T., N.H., and Y.O. Study supervision: T.Y and K.A.

Data accessibility

The data that support the findings of this study are available upon reasonable request.

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Figure Legends

Figure 1

(A) The smooth suture used to occlude the middle cerebral artery. (B) The rough suture with white fused alumina coating used to mildly damage the arterial intima. Scale bar = 500 μm. (C) Six experimental groups including the control, MT1, MT3, tPA, tPA+MT1, and tPA + MT3 groups.

Figure 2

(A) The Survival rates in the 6 experimental groups. (B) Nissl staining of the rat brains. Scale bar = 2 mm. (C) Quantitative analysis showed significantly increased infarct volumes in the tPA+MT3 group compared with the control group. (D) The hemorrhage volume was significantly greater in the tPA+MT3 group compared with the control group (*$p < 0.05$).

Figure 3

(A) H&E staining showing structural damage in the tunica media of the internal carotid artery in the MT3 and tPA+MT3 groups (arrowheads). Scale bar = 10 μm. (B) A coronal section stained with anti-IgG antibody showing IgG leakage (arrowheads) mainly near the injured vessel area. Scale bar = 2 mm. (C) IgG staining of peri-infarcted lesions in the 6 groups. Scale bar = 50 μm. (D) Semiquantitative analysis relatively significantly greater IgG leakage in the tPA+MT3 (****$p < 0.0001$), tPA+MT1 (**$p < 0.01$), and MT3 group (*$p < 0.05$) than in the control group.

Figure 4

(A) Anti-MMP9 staining of the peri-infarct lesion at 24 hours after reperfusion. Scale bar = 50 μm. (B) Compared with the control group, the MMP9 pixel intensity in the tPA+MT1 (*$p < 0.05$) and tPA+MT3 groups was significantly increased (***$p < 0.001$). (C) The double immunostaining for MMP9 and MPO showed MMP9 localization in MPO-positive cells with segmented nuclei.

Scale bar = 10 μm. (D) Anti-VEGF staining in the 6 groups. Scale bar = 50 μm. (E) Compared with the control group, the VEGF pixel intensity was significantly increased in the tPA+MT3 group (*$p < 0.05$).

Figure 5

(A) Double immunostaining of collagen IV and GFAP in the 6 groups. Scale bar = 50 μm. Arrowheads point to the detachment of the GFAP-positive astrocyte end-feet from the collagen IV-positive basement membrane. (B) The tPA+MT3 group had significantly increased vascular dissociation indexes estimating the area between the basal lamina and astrocyte foot processes (*$p < 0.01$).