

Extracellular Chromatin Is an Important Mediator of Ischemic Stroke in Mice

Simon F. De Meyer, Georgette L. Suidan,* Tobias A. Fuchs,* Marc Monestier, Denisa D. Wagner

Objective—Recently, a growing number of studies have revealed a prothrombotic and cytotoxic role for extracellular chromatin. Cerebral ischemia/reperfusion injury is characterized by a significant amount of cell death and neutrophil activation, both of which may result in the release of chromatin. The goal of this study was to assess the effect of extracellular chromatin in ischemic stroke using a mouse model of transient middle cerebral artery occlusion.

Methods and Results—Similar to reports in stroke patients, we observed increased levels of circulating nucleosomes and DNA after ischemic stroke in mice. In addition, we observed that general hypoxia also augmented extracellular chromatin. We hypothesized that targeting extracellular chromatin components would be protective in ischemic stroke. Indeed, treatment with recombinant human DNase 1 significantly improved stroke outcome. Neutralization of histones using an antihistone antibody was also protective as evidenced by smaller infarct volumes, whereas increasing levels of extracellular histones via histone infusion exacerbated stroke outcome by increasing infarct size and worsening functional outcome.

Conclusion—Our results indicate that extracellular chromatin is generated and is detrimental during cerebral ischemia/reperfusion in mice. Targeting DNA and histones may be a new therapeutic strategy to limit injury resulting from ischemic stroke. (*Arterioscler Thromb Vasc Biol.* 2012;32:1884-1891.)

Key Words: stroke ■ chromatin ■ histones ■ DNase 1

Stroke is a leading cause of death and permanent disability worldwide.¹ It is primarily caused by the obstruction of cerebral arteries.² Currently, early thrombolysis with tissue plasminogen activator is the only available therapeutic option for acute thromboembolic stroke. However, tissue plasminogen activator–mediated thrombolysis is only recommended in the limited time window of up to 3 hours after the onset of stroke symptoms as later applications are associated with the risk of severe intracerebral hemorrhage.³ Various trials testing new thrombolytics, platelet aggregation inhibitors, or anticoagulants failed to improve treatment of ischemic stroke patients.^{4–6} Thus, to develop safer and more effective stroke therapeutics, a better understanding of the pathogenic mechanisms of thrombotic stroke development and the resulting cerebral injury is warranted.

Tissue damage after cerebral ischemia is caused by the interaction of complex pathophysiological processes, including platelet and leukocyte recruitment upon reperfusion, promoting both thrombosis and inflammation.⁷ In the ischemic area, cell death and neutrophil activation may lead to the release of nuclear chromatin consisting of DNA and histones. Our group recently showed that these extracellular DNA

traps represent a new link between inflammation/infection and thrombosis.⁸ These DNA traps provide a stimulus and scaffold for thrombus formation.⁸ Extracellular nucleosomes were recently shown to promote coagulation and intravascular thrombus formation.⁹ Furthermore, histones are potent mediators of platelet activation and aggregation^{8,10} and are shown to be cytotoxic.¹¹ Nucleosome levels are known to be elevated in many conditions where cells are stressed, such as trauma, cancer, and autoimmune disease, all of which have thrombotic complications associated with the disease progression.¹² Recently, markers of extracellular DNA traps were detected in the thrombus and plasma of mice and baboons subjected to deep vein thrombosis, an example of inflammation-enhanced thrombosis.^{8,13} Interestingly, significantly elevated concentrations of DNA and nucleosomes have also been found in stroke patients.^{14–17} DNase 1 is present in plasma where it can facilitate chromatin breakdown after cell death.^{18,19} Serum levels of DNase 1 were reported to be elevated in the clinical setting of myocardial ischemia,²⁰ and a polymorphism resulting in a less active DNase 1 is associated with myocardial infarction,²¹ indicating that this endonuclease could play a protective role in cardiovascular disease.

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From the Immune Disease Institute, Boston, MA (S.F.D.M., G.L.S., T.A.F., D.D.W.); Program in Cellular and Molecular Medicine, Children's Hospital Boston, Boston, MA (S.F.D.M., G.L.S., T.A.F., D.D.W.); Department of Pediatrics, Harvard Medical School, Boston, MA (S.F.D.M., G.L.S., T.A.F., D.D.W.); Laboratory for Thrombosis Research, KULeuven Kulak, Kortrijk, Belgium (S.F.D.M.); and Temple Autoimmunity Center, Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, PA (M.M.).

*These authors contributed equally to this work.

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Correspondence to Denisa D. Wagner, PhD, Immune Disease Institute, Harvard Medical School, 3 Blackfan Circle, 3rd Floor, Boston, MA 02115. E-mail wagner@idi.harvard.edu

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In this study, we examined the generation of extracellular chromatin by hypoxic conditions and in ischemic stroke using a mouse model of transient middle cerebral artery occlusion (tMCAO). We show that markers of extracellular DNA traps are elevated in both models, and we provide evidence that extracellular chromatin is a potential therapeutic target in ischemic stroke.

Materials and Methods

Animals

Wild-type C57BL/6 mice were from Jackson Laboratory (Bar Harbor, ME). All animals were 8- to 10-week-old males except for the hypoxia experiments in which female animals were also used. Animals had free access to standard chow and water and were kept on a light/dark cycle of 12 hours. All experimental procedures were approved by the Animal Care and Use Committee of the Immune Disease Institute.

Materials

Recombinant human DNase 1 (Dornase alpha, Pulmozyme) was purchased from Genentech Inc (San Francisco, CA). Calf thymus histones were purchased from Worthington Biochemical Corp (Lakewood, NJ). Antibodies against histone H2A/H4 were isolated from cell culture supernatants of hybridoma clone BWA3 by affinity chromatography on protein G columns as described.²² Purified antibodies were dialyzed against saline and were characterized by ELISA as described (Figure 1 in the online-only Data Supplement).

Determination of Nucleosome Levels in Plasma

To measure nucleosome and DNA levels in plasma, blood was collected from the retro-orbital sinus (49:1 vol/vol of blood:0.5 mol/L EDTA). Plasma was prepared by centrifuging anticoagulated whole blood for 5 minutes at 2300g. Plasma supernatant was carefully removed and centrifuged again for 5 minutes at 2300g to remove any remaining blood cells. Plasma was stored at -80°C until analysis. Nucleosome levels were measured using the Cell Death Detection ELISA or Cell Death Detection ELISA^{PLUS} (Roche, Indianapolis, IN). This assay allows for the relative quantification of histone-complexed DNA fragments (mono- and oligonucleosomes).

Quantification of Plasma DNA

Plasma was diluted 1:10 in PBS, and 50 μL of diluted plasma was mixed with 50 μL SytoxGreen in PBS (2 $\mu\text{mol/L}$; Invitrogen, Grand Island, NY) to label DNA. Fluorescence was recorded in a fluorometer (Fluoroskan; Thermo Fisher Scientific, Waltham, MA). Autofluorescence was determined in samples mixed with PBS without SytoxGreen and subtracted.

Hypoxia Treatment

Mice were exposed to normobaric hypoxia at 6% oxygen for 24 hours or were housed at normal air room pressure. For hypoxia treatment, animals were placed in a controlled atmosphere animal chamber (A-15274-P; Biospherix, Lacona, NY). Hypoxia was achieved by substituting nitrogen for oxygen using a Pro:ox model 110 compact oxygen controller (Biospherix). Mice were given food and water ad libitum.

Induction of Cerebral Ischemia

Focal cerebral ischemia was induced by 60- or 120-minute tMCAO. Mice were anesthetized with 2% isoflurane/oxygen mixture. After a midline skin incision in the neck, the proximal common carotid artery and the external carotid artery were ligated, and a standardized silicon rubber-coated 6.0 nylon monofilament (6021; Doccoc Corp, Redlands, CA) was inserted and advanced via the right internal

carotid artery to occlude the origin of the right middle cerebral artery. Operation time per animal did not exceed 15 minutes. The intraluminal suture was left in situ during the complete occlusion time. Then animals were reanesthetized, and the occluding monofilament was withdrawn to allow reperfusion. In animals undergoing a sham treatment, the exact same procedure was followed except that the monofilament was only inserted very briefly (<2 seconds) into the common carotid artery without advancement into the right internal carotid artery. Some animals were exclusively used for laser Doppler flowmetry (Periflux 5000; Perimed, Kings Park, NY) to monitor regional cerebral blood flow in the middle cerebral artery territory (6 mm lateral and 2 mm posterior from bregma). Regional cerebral blood flow consistently decreased to <10% of baseline values upon occlusion of the middle cerebral artery by the monofilament. Upon filament withdrawal, regional cerebral blood flow was restored to $\approx 75\%$ of baseline flow (Figure II in the online-only Data Supplement). Sham surgeries did not cause a drop in regional cerebral blood flow (not shown). Arterial blood oxygenation was measured using a small rodent oxymeter thigh sensor (Mouse^{OX}; STARR Life Sciences, Oakmont, PA) and did not change during the course of the surgery or during the 24-hour reperfusion period (not shown).

DNase 1 or Histone Treatment

Treatment of wild-type animals with recombinant human DNase 1 was performed by an intraperitoneal administration of 50 μg of recombinant human DNase 1 (rhDNase 1) 15 minutes before surgery. Five minutes before reperfusion, 10 μg of rhDNase 1 was given via retro-orbital intravenous administration. To maintain rhDNase 1 levels, a second intraperitoneal dose of 50 μg was given 12 hours after the first dose. A delayed rhDNase 1 treatment regimen was also used, in which mice were injected 1 hour after the start of reperfusion with 50 μg rhDNase 1 IP plus 10 μg rhDNase 1 IV, followed by 50 μg IP 12 hours later. The half-life of rhDNase 1 in circulation is ≈ 22 hours after infusion into DNase knockout mice.²³

Treatment with antihistone H4 antibody (BWA3) consisted of a single intravenous retro-orbital bolus injection of antibody (BWA3 or IgG1 isotype control antibody) at a concentration of 10 mg/kg 5 minutes before reperfusion.

For treatment with histones, mice received 10 mg/kg of calf thymus histones by retro-orbital intravenous administration, immediately after the start of reperfusion.

Assessment of Infarct Volume

Mice were euthanized 24 hours after initiation of tMCAO. Brains were quickly removed and cut into 2-mm-thick coronal sections using a mouse brain slice matrix. The slices were stained with 2% 2,3,5-triphenyl-tetrazolium chloride (Sigma-Aldrich, St. Louis, MO) in PBS to visualize healthy tissue and unstained infarctions. Sections were photographed with a digital Nikon D70 camera, and infarct areas (white) were measured blindly using Image J software (National Institutes of Health, Bethesda, MD; <http://rsb.info.nih.gov/ij.html>). Edema formation was measured by comparing the volume of the ischemic hemisphere with the volume of the control hemisphere. None of the experiments resulted in significant edema formation that could bias infarct volume.

Bederson Score

Neurological function was assessed, blinded for the mouse genotype, 24 hours after initiation of tMCAO, using the modified Bederson score.²⁴ This test determines global neurological function according to the following scoring system: 0, no deficit; 1, forelimb flexion; 2, decreased resistance to lateral push; 3, unidirectional circling; 4, longitudinal spinning; 5, no movement.

Grip Test

The grip test was performed as described.²⁵ A mouse was placed on a wooden bar (3 mm diameter, 40 cm long) attached to 2 vertical

supports 40 cm above a flat surface. When placing the mouse on the bar midway between the supports, the experiment was rated according to the following system: 0, falls off; 1, hangs onto bar by 2 forepaws; 2, same as for 1, but attempts to climb onto bar; 3, hangs onto bar by 2 forepaws plus 1 or both hindpaws; 4, hangs onto bar by all 4 paws plus tail wrapped around bar; 5, escape (mouse able to reach one of the supports).

Corner Test

For the corner test,²⁶ a mouse was placed on a flat surface between 2 vertical boards arranged at a 30° angle with a small opening along the joint between the 2 boards to encourage entry into the corner. The mouse was placed between the 2 angled boards facing the corner and half way to the corner. When entering deep into the corner, the mouse rears forward and upward, then turns back to face the open end. The nonischemic mouse turns either left or right, but the ischemic mouse preferentially turns toward the nonimpaired, ipsilateral (right) side. The turns in one versus the other direction were recorded from 10 trials for each test. Turning movements that were not part of a rearing movement were not scored. Results are expressed as the number of right turns of 10 valid trials. Mice that were not able to walk (Bederson score 4 or 5) were excluded.

Statistical Analysis

Data are expressed as mean±SEM. All statistical analysis was performed using Prism 4 (version 4.0b; GraphPad Software, Inc, La Jolla, CA). To compare groups, the unpaired 2-tailed Student *t* test was used. *P* values <0.05 were considered statistically significant.

Results

Hypoxia Elevates Circulating Levels of Nucleosomes

It is well established that conditions associated with hypoxia, such as exposure to high altitude or long-haul air travel, are associated with a hypercoagulable state and form a risk factor for venous thrombosis.^{27,28} Because extracellular DNA traps promote thrombosis and coagulation,^{8,9} we investigated the effect of hypoxia on circulating markers of extracellular chromatin. Mice were exposed to normobaric hypoxia at 6% oxygen for 24 hours. Control mice were housed at normal room air oxygen concentration. Immediately after exposure, blood was collected to determine nucleosome levels in plasma (Figure 1). We performed 4 independent experiments, and in each experiment mice that experienced hypoxia showed significantly higher plasma levels of nucleosomes compared with the normoxic mice. Overall, plasma nucleosome levels in mice that received hypoxia treatment (1.35 ± 0.11 , $n=25$) were more than 3-fold increased over the values of normoxic mice (0.44 ± 0.05 , $n=19$, $P<0.0005$). No difference was observed between male and female animals. These results indicate that hypoxia is associated with the liberation of extracellular chromatin components, which could contribute to an increased risk for thrombosis.

Extracellular Chromatin Markers Are Increased After Stroke

Based on our observation that general hypoxia leads to increased levels of extracellular chromatin markers, we decided to investigate the effect of cerebral ischemia/reperfusion injury on circulating levels of extracellular chromatin components. Mice were subjected to 2 hours of tMCAO, followed by 22 hours of reperfusion. Before the

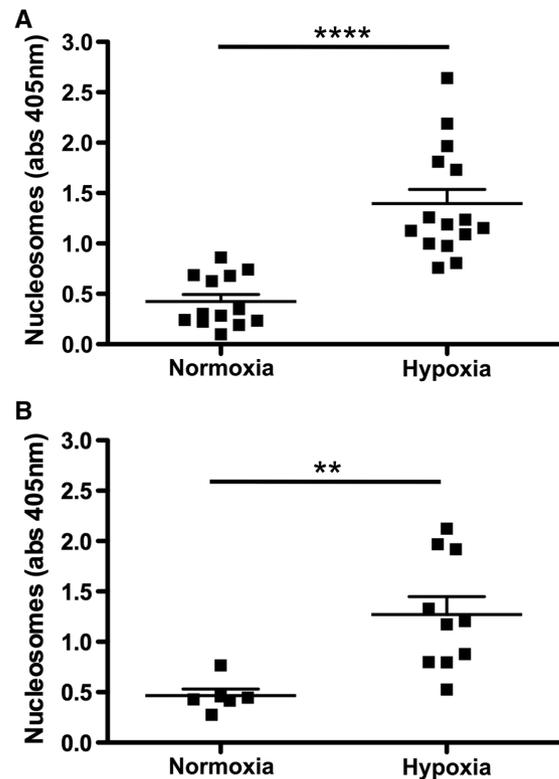


Figure 1. Nucleosome levels are increased after hypoxia. Female (A) and male (B) wild-type animals were kept under normoxic (room air) or hypoxic (hypoxia chamber, 6% O₂) conditions for 24 hours, after which plasma samples were collected. Nucleosome levels were determined in the plasma samples and expressed as nucleosome-specific absorbance levels (Abs 405). Hypoxia significantly increased the levels of circulating nucleosomes (** $P<0.005$; **** $P<0.0001$).

surgical procedure and after reperfusion, blood samples were collected and plasma was prepared. Sham-operated animals underwent the same surgery but without occlusion of the right middle cerebral artery. Levels of circulating DNA and nucleosomes were determined in the plasma samples (Figure 2). Although not statistically significant, the sham procedure showed a trend toward an increase in both parameters over baseline levels, which may be related to tissue damage of the invasive surgery. Nevertheless, in animals that experienced stroke, levels of circulating DNA and nucleosomes were increased several-fold over baseline levels (Figure 2; $P<0.05$). These data indicate that cerebral ischemia/reperfusion injury leads to the generation of extracellular chromatin, resulting in a significant elevation in the amount of these cell death markers in circulation.

Infusion of Recombinant DNase 1 Improves Ischemic Stroke Outcome in Mice

Our group has previously shown that DNase 1 can cleave chromatin/platelet strings, thereby limiting the extent of extracellular DNA trap-mediated thrombosis *in vitro*.⁸ We hypothesized that this enzyme may have a beneficial impact on stroke progression by limiting excessive platelet recruitment and activation by extracellular chromatin generated

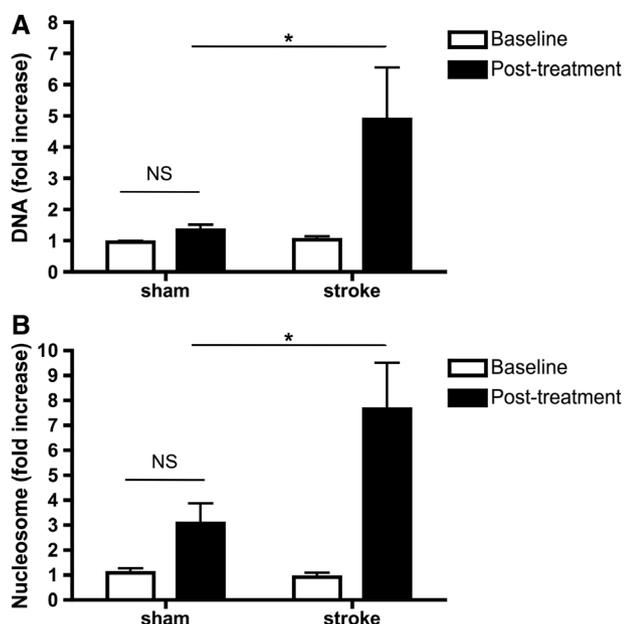


Figure 2. DNA and nucleosome levels are increased after cerebral ischemia/reperfusion. Mice were subjected to 2 hours of transient middle cerebral artery occlusion (tMCAO), followed by 22 hours of reperfusion. Before the surgical procedure (baseline) and after 22 hours of reperfusion (post-treatment), blood samples were collected and plasma was prepared. Sham-operated animals underwent the same surgical procedures but without occlusion of the right middle cerebral artery. DNA levels (**A**, $n=9$ for each group) and nucleosome levels (**B**, $n=6$ for each group) were measured and normalized over baseline levels of sham-treated mice. Both DNA and nucleosome levels slightly increased in some animals after sham treatment, but this difference was not significant (NS). DNA and nucleosome levels were significantly higher in all animals that experienced stroke ($*P<0.05$).

upon cerebral ischemia/reperfusion injury. To test this, we treated mice with rhDNase 1 (50 μg IP before surgery, followed by 10 μg IV before the start of reperfusion and 50 μg IP 12 hours after the first intraperitoneal dose). Compared with vehicle-treated animals, mice that received rhDNase 1 developed $\approx 40\%$ smaller infarcts (81.45 \pm 9.08 mm^3 versus 50.67 \pm 8.74 mm^3 , $P<0.03$; Figure 3A). Treatment of mice with rhDNase 1 also led to a dramatic improvement in their functional outcome as shown by 3 separate tests assessing neurological and motoric behavior. Compared with vehicle-treated mice, the Bederson test score (1.79 \pm 0.30 versus 3.00 \pm 0.22 for vehicle versus rhDNase 1-treated animals, respectively, $P<0.03$), the grip test score (1.80 \pm 0.31 versus 3.07 \pm 0.35 for vehicle versus rhDNase 1-treated animals, respectively, $P<0.02$), and the corner test result (8.41 \pm 0.87 versus 5.46 \pm 0.93 for vehicle versus rhDNase 1-treated animals, respectively, $P<0.04$) were all significantly better in mice treated with rhDNase 1 (Figure 3B). To investigate whether the protective effect of rhDNase 1 is still maintained when rhDNase 1 treatment is delayed, we began to administer rhDNase 1 treatment 1 hour after the start of reperfusion (50 μg IP plus 10 μg IV followed by 50 μg IP 12 hours later). Interestingly, we observed that animals that received rhDNase 1 at this late time point developed smaller infarcts (62.68 \pm 4.40 mm^3) compared with the control group, treated with vehicle (81.19 \pm 4.88 mm^3 , $P<0.009$; Figure 3C).

When comparing early rhDNase 1 treatment (Figure 3A) with delayed rhDNase 1 treatment (Figure 3C), the decrease in infarct volumes was lower in the latter, but still highly statistically significant ($P<0.009$). Functional outcome scores were, however, not significantly improved in the delayed treatment group.

Targeting Histones Is Protective in Ischemic Stroke

Nucleosomes are segments of DNA that are wound around a protein core of histones. It has been shown that extracellular histones are highly cytotoxic toward endothelium, induce intravascular thrombosis, and even cause death when administered at very high doses (75 mg/kg).¹¹ Furthermore, sublethal concentrations of histones aggregate platelets and cause thrombocytopenia in a dose-dependent manner in mice.¹⁰ To assess whether extracellular histones are of pathological significance in the progression of ischemic stroke, we infused 10 mg/kg antihistone H2A/H4 antibody (BWA3)²² 5 minutes before reperfusion (Figure 4). This antibody was previously shown to rescue mice from extracellular histone-mediated death in a mouse model of sepsis.¹¹ Administration of histone-neutralizing antibody resulted in a protective effect compared with animals treated with IgG1 isotype control antibody. The infarct volumes of BWA3-treated mice were $\approx 30\%$ smaller than in IgG1-treated mice (46.00 \pm 5.82 mm^3 versus 63.43 \pm 4.29 mm^3 , $P<0.05$; Figure 4A). Neurological/motoric outcome as measured by the Bederson and grip test scores were generally better in BWA3-treated mice, although this difference was not significant (Figure 4B).

Elevated Concentrations of Extracellular Histones Exacerbate Ischemic Stroke

To further confirm the detrimental role of histones in cerebral ischemia/reperfusion injury, we infused mice that underwent tMCAO with a low dose (10 mg/kg) of calf thymus histones immediately after the start of reperfusion. Control animals received sterile saline. This low dose did not affect platelet counts of the treated mice (Figure III in the online-only Data Supplement) nor blood pH (not shown). We observed significantly larger infarcts in mice that were treated with histones compared with control mice (86.10 \pm 5.57 mm^3 versus 66.65 \pm 6.16 mm^3 , respectively, $P<0.05$; Figure 5A). This increase was functionally relevant as the Bederson score was significantly worse in histone-treated mice ($P<0.05$; Figure 5B). Taken together, our results indicate that histones and extracellular chromatin are major contributors to reperfusion injury after stroke.

Discussion

In this study, we demonstrate that markers of extracellular chromatin are increased in hypoxic conditions and after ischemic stroke. Treatment of mice submitted to experimental stroke with rhDNase 1 or histone-neutralizing antibodies has a protective effect indicating an important role for extracellular chromatin in the development of ischemic stroke and thus suggesting that DNA and histones may be relevant therapeutic targets for this condition.

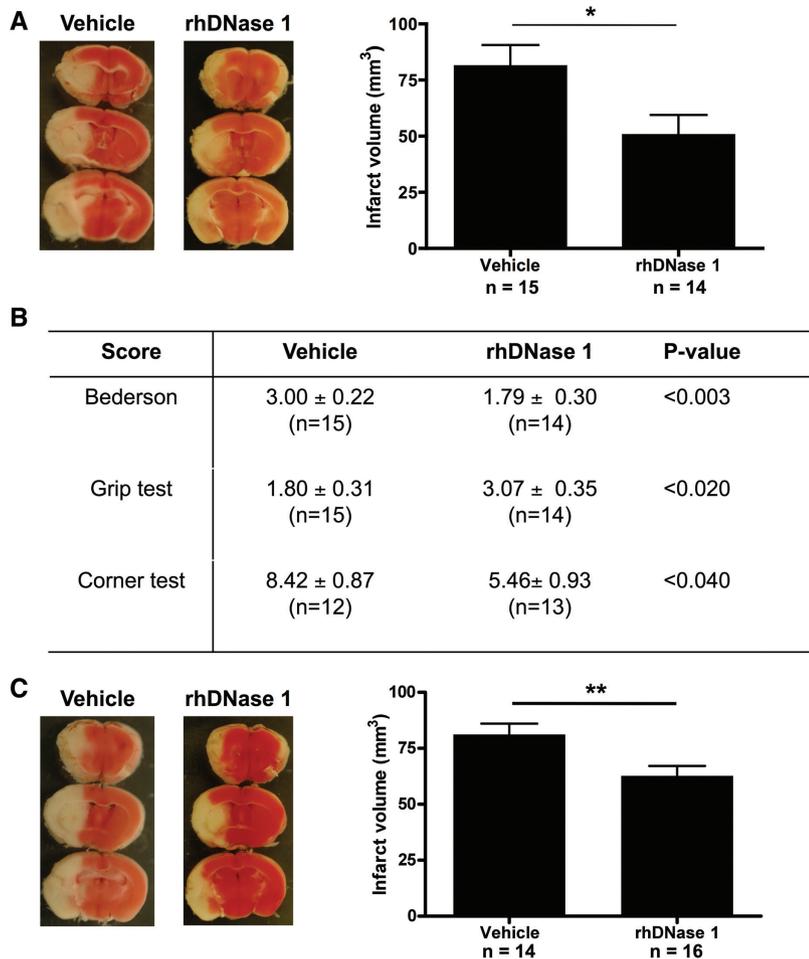


Figure 3. Recombinant human DNase 1 (rhDNase 1) improves outcome after ischemic stroke. Wild-type mice treated with rhDNase 1 or vehicle were subjected to 1 hour of transient middle cerebral artery occlusion (tMCAO) and 23 hours of reperfusion, after which mice behavior was tested and brains analyzed. **A**, Early treatment with rhDNase 1 (started before reperfusion). Representative 2,3,5-triphenyl tetrazolium chloride stains of 3 consecutive coronal brain sections (left), on which ischemic infarctions appear white. Brain infarct volumes as measured by planimetry (right). Mice receiving early treatment with rhDNase 1 developed significantly smaller infarctions than control (vehicle) mice. **B**, Neurological Bederson score, grip test score, and corner test results of the experiment shown in **A**. On all 3 parameters, mice treated early with rhDNase 1 showed a significantly better neurological outcome compared with control mice ($*P<0.05$). **C**, Delayed treatment with rhDNase 1 (started 1 hour after reperfusion). Representative 2,3,5-triphenyl tetrazolium chloride stains of 3 consecutive coronal brain sections (left). Brain infarct volumes as measured by planimetry (right). Mice receiving the delayed treatment with rhDNase 1 also developed significantly smaller infarctions than control (vehicle) mice.

Extracellular Chromatin Markers Are Increased After Hypoxia and Ischemic Stroke

We observed that general hypoxia leads to an increase in nucleosome levels in plasma. The hypoxia level used in our study is similar to that found at extreme altitudes such as experienced by mountaineers. High altitude is known to be associated with thrombotic complications.²⁸ Recently, it was reported that spending time even at moderate altitude was a potential risk factor for thrombosis.²⁹ The origin of circulating nucleosomes released by hypoxia is unknown, but because chromatin is both procoagulant and prothrombotic^{8,9} it is plausible that it contributes to the higher incidence of thrombosis in hypoxic conditions. This observation led us to postulate that ischemia may similarly result in the generation of extracellular chromatin that in the case of stroke may worsen brain injury. We therefore examined whether tMCAO would have an effect on circulating levels of extracellular chromatin. We observed that experimental ischemic stroke indeed results in a significant increase in both DNA and nucleosome levels in circulation. These experimental results in mice are in agreement with clinical observations that DNA/nucleosome levels are elevated in stroke patients. With ischemic cell damage being a dynamic process with considerable interindividual variation, the striking association between circulating DNA/nucleosome levels and stroke severity has always been surprising.³⁰ Indeed, these levels strongly correlate with stroke severity and are associated with

morbidity, mortality, and degree of disability.^{14–17} In one study, assessment of nucleosomes, S100 protein, neuron-specific enolase, C-reactive protein, and leukocytes 3 days after stroke revealed that only nucleosomes provided independent prognostic information concerning the 1-year recovery period.¹⁴ Liberation of chromatin and its degradation products from damaged cells seems a plausible mechanism for increased DNA/nucleosome levels during and after stroke. It is difficult to identify the cellular origin of the circulating DNA/nucleosomes. Undoubtedly, dying neurons release chromatin, which could cross the disrupted blood-brain barrier. Given the well-established role of neutrophils in the pathophysiology of ischemic stroke,⁷ it is also tempting to speculate that neutrophils could lead to significant chromatin release through the formation of neutrophil extracellular traps. Neutrophil extracellular traps promote thrombosis and bind red blood cells, which could further hinder efficient reperfusion of ischemic areas.⁸ We recently demonstrated that extracellular chromatin, likely originating from neutrophils, contributes to the pathogenesis of deep vein thrombosis (DVT) in mice.¹³ Interestingly, besides neutrophils, mast cells, monocytes, and eosinophils also release extracellular DNA traps in response to inflammatory stimuli and reactive oxygen species.^{31–33}

rhDNase 1 Is Protective in Ischemic Stroke

After tMCAO, both platelet-dependent thrombus formation and coagulation play a crucial role in stroke progression, implying

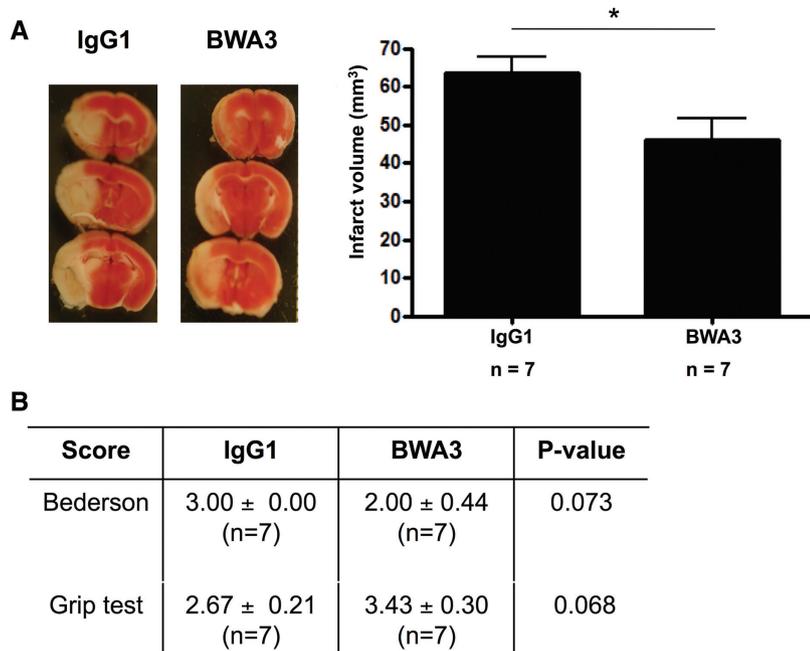


Figure 4. Histone neutralization is protective in ischemic stroke. Wild-type mice treated with a histone-neutralizing antibody (BWA3) or isotype control antibody (IgG1) were subjected to 1 hour of transient middle cerebral artery occlusion (tMCAO) and 23 hours of reperfusion, after which mice behavior was tested and brains analyzed. **A**, Representative 2,3,5-triphenyl tetrazolium chloride stains of 3 consecutive coronal brain sections (left), on which ischemic infarctions appear white. Brain infarct volumes as measured by planimetry (right). Mice treated with BWA3 developed significantly smaller infarctions in comparison with mice treated with the isotype control ($*P < 0.05$). **B**, Functional Bederson and grip test scores. Neutralization of histones (BWA3) showed a tendency for a better neurological outcome compared with IgG1 control treatment. Treatment with vehicle gave the same results as treatment with IgG1 antibody (not shown, $P = 0.12$).

that new components inhibiting thrombosis and coagulation could become beneficial in stroke treatment.³⁴ We previously showed in vitro that both thrombolytic (tissue plasminogen activator) and DNase activity were needed to dissolve clots containing chromatin and that DNase 1 can cleave chromatin/platelet strings, thereby limiting the extent of extracellular DNA trap-mediated thrombosis in vitro.⁸ rhDNase 1 is a readily available drug that is currently used for the treatment of cystic fibrosis where extracellular chromatin is generated.³⁵ In this study, we show that rhDNase 1 has a protective effect on cerebral ischemia/reperfusion injury, even when administered 1 hour after onset of reperfusion. Based on our previous findings, a plausible explanation for our observation of the protective effect of rhDNase 1 could be that rhDNase 1 removes excessive chromatin material that could act as thrombogenic DNA traps blocking the microcirculation. Our findings on ischemic stroke are

in accordance with our observations in a mouse model of DVT, where infusion of rhDNase 1 protected mice from flow restriction-induced DVT, most probably by cleaving extracellular DNA traps. This notion is further supported by recent findings by von Brühl et al,³⁶ who described that neutrophil extracellular DNA traps promote mouse DVT growth via a mechanism that is dependent on platelet glycoprotein Ib and factor XII. Also in this study, rhDNase 1 reduced DVT. Interestingly, glycoprotein Ib and factor XII have been shown to be important mediators of experimental stroke, suggesting that similar mechanisms might also apply in cerebral ischemia/reperfusion injury.^{37–40} Furthermore, polyphosphate polymers potentially initiate fibrin formation via the factor XII-driven intrinsic pathway and hence are potent activators of coagulation, possibly by acting as templates to assemble clotting proteins.^{41–43} Recently, it was shown that this blood coagulation-triggering activity positively

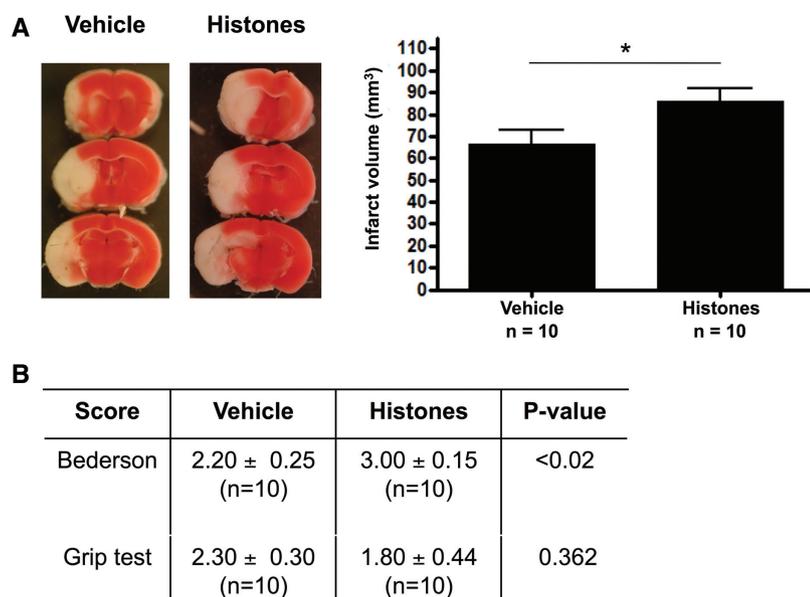


Figure 5. Infusion of histones exacerbates ischemic stroke. Wild-type mice treated with histones or saline (vehicle) were subjected to 1 hour of transient middle cerebral artery occlusion (tMCAO) and 23 hours of reperfusion, after which mice behavior was tested and brains analyzed. **A**, Representative 2,3,5-triphenyl tetrazolium chloride stains of 3 consecutive coronal brain sections (left), on which ischemic infarctions appear white. Brain infarct volumes as measured by planimetry (right). Mice treated with histones developed significantly larger infarctions in comparison with mice treated with vehicle ($*P < 0.05$). **B**, Functional Bederson and grip test scores. Infusion of histones led to worsening of neurological outcome, as seen by increased Bederson score, compared with control treatment.

correlates with polyphosphate polymer size and that short polymers are even able to antagonize the blood clotting capacity of longer polymers by acting as competitors, keeping clotting proteins from assembling effectively together on longer polymers.⁴⁴ Thus, rhDNase 1 could also attenuate blood clotting by cleaving long DNA polymers into smaller, less procoagulant fragments. In addition, we recently showed that von Willebrand factor also interacts with extracellular DNA traps and that von Willebrand factor plays a crucial pathophysiological role in both DVT and stroke.^{8,45–48} Whether von Willebrand factor binding to extracellular DNA further promotes brain injury (that could be reduced by rhDNase 1) remains to be established.

Histones Contribute to Ischemic Stroke Progression

Our results show that the other important chromatin component, histones, also contributes to cerebral ischemia/reperfusion injury and that targeting of histones results in a protective effect. These data are in line with our previous findings that histones also promoted DVT in mice.¹³ Histones promote platelet activation and aggregation,^{8,10} which could promote secondary thrombus formation during the reperfusion phase after ischemic stroke. Also, histones have been shown to be cytotoxic to endothelium, which could affect the blood–brain barrier.^{11,49} Consistent with our findings, Huang et al⁵⁰ reported that histones exacerbate liver ischemia/reperfusion injury and that histone neutralization (using the same antibody as used in our study) protects against injury. Interestingly, these authors demonstrated a cytotoxic effect of histones, mediated by toll-like receptor 9, suggesting that histones serve as new link between tissue damage and activation of innate immunity. Similarly, histones were shown to be an important mediator of death in sepsis¹¹ and after acetaminophen-induced toxicity.⁵¹ In both studies, blocking histones, using the same antibody used in our work, protected mice from death. Recently, it was shown that extracellular histones also enhance plasma thrombin generation by impairing protein C activation,⁵² a mechanism that could contribute to microvascular thrombosis after stroke. Given that activated protein C can cleave histones, thereby reducing their cytotoxicity in sepsis,¹¹ it is interesting to note that activated protein C is also protective in a murine ischemic stroke model.⁵³ Interestingly, activated protein C mutants with reduced anticoagulant activity were still shown to have a protective effect in ischemic stroke.⁵⁴ This further suggests that other mechanisms besides promoting thrombosis could mediate the detrimental effects of histones in cerebral ischemia/reperfusion injury, including cytotoxic, inflammatory, or apoptotic activities or disruption of the blood–brain barrier.

Further studies are now needed to clarify the exact pathogenic mechanisms of histone-induced brain injury and to establish whether histone/chromatin release could also contribute to the cognitive decline observed after invasive procedures such as coronary artery bypass surgeries.⁵⁵

In conclusion, our results indicate that extracellular chromatin and its components are important mediators of ischemic stroke injury. It will be important to further elucidate the exact mechanisms of stroke exacerbation by extracellular chromatin. We propose that antichromatin therapy could lead to new strategies in stroke management. Cleavage of DNA by DNase

1 and neutralization of histones using antihistone antibodies may complement current thrombolytic therapy to limit reperfusion injury. Further studies are needed to investigate the potential synergistic effects of combined therapies targeting different substrates, such as the combination of DNase 1 with tissue plasminogen activator.

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Disclosures

None.

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Extracellular Chromatin Is an Important Mediator of Ischemic Stroke in Mice

Simon F. De Meyer, Georgette L. Suidan, Tobias A. Fuchs, Marc Monestier and Denisa D. Wagner

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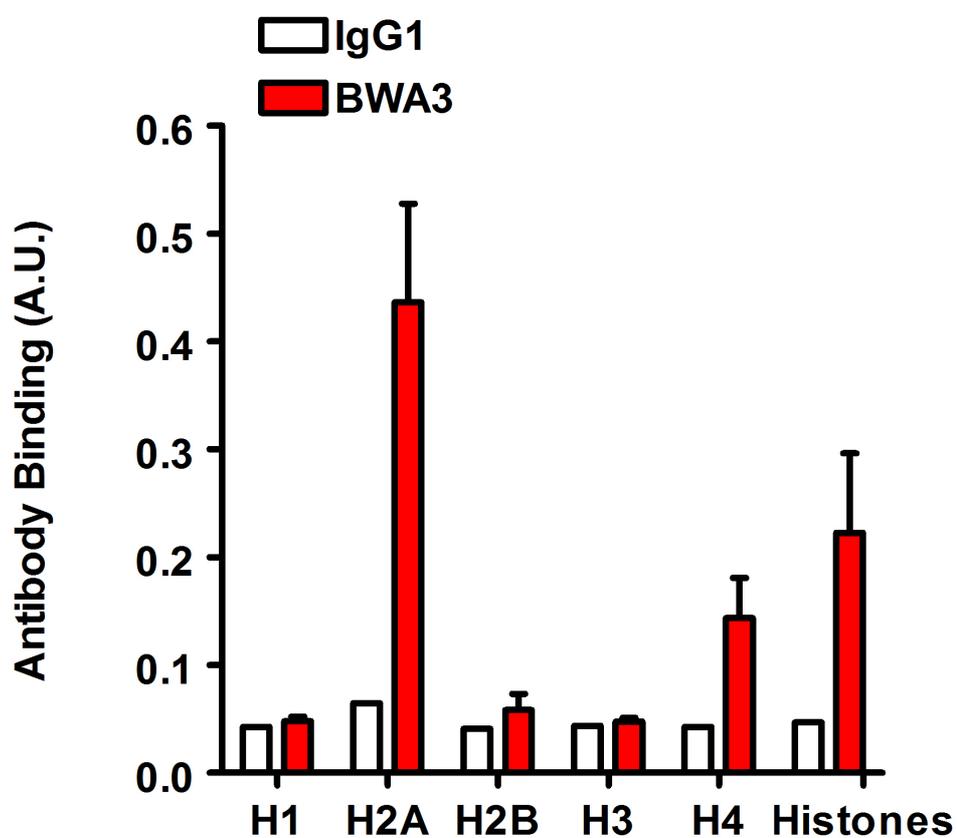
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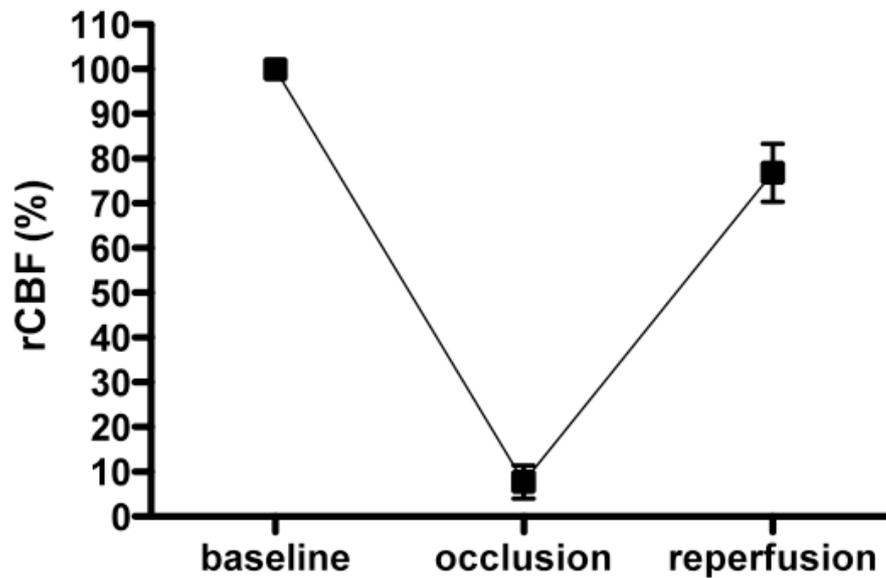
Supplemental Figure I



Supplemental Figure I: BWA3 antibody characterization

Antibodies purified from clone BWA3 and control IgG1 were tested for binding to purified histones H1, H2A, H2B, H3, H4 and a mixture of all histones in an ELISA set-up. BWA3 antibodies bound to H2A and H4 and consequently also to the histone mix. IgG1 did not bind to histones.

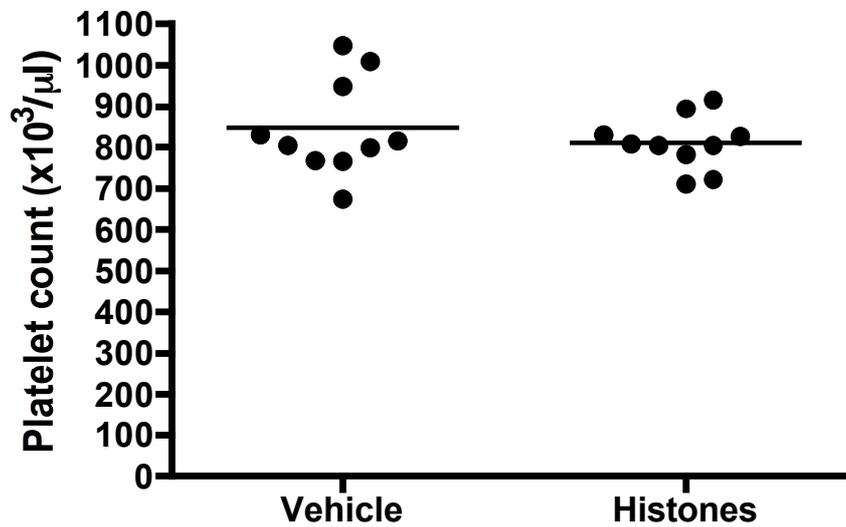
Supplemental Figure II



Supplemental Figure II: Regional cerebral blood flow of the MCA territory

Insertion of the occluding monofilament results in a drop of regional cerebral blood flow (rCBF) to less than 10% of baseline values upon occlusion of the MCA ($7.71 \pm 3.74\%$ of baseline, $n=5$). Upon withdrawal, rCBF is restored to approximately 75% of baseline flow ($76.82 \pm 6.51\%$ of baseline, $n=5$). In sham operated mice, the MCA was never occluded.

Supplemental Figure III



Supplemental Figure III: Platelet counts after histone infusion

Mice that underwent tMCAO were infused with a low dose (10 mg/kg) of calf thymus histones immediately after the start of reperfusion (n=10). Control animals received sterile saline (vehicle, n=10). Blood samples were collected at the time of sacrifice (23 h after reperfusion) and platelet counts were measured using a Hemavet 950FS cell counter (Drew Scientific Inc, Dallas, TX). Data are pooled from two independent experiments.