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High-density Lipoprotein–based Therapy Reduces the Hemorrhagic Complications Associated With Tissue Plasminogen Activator Treatment in Experimental Stroke

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Background and Purpose—We have previously reported that intravenous injection of high-density lipoproteins (HDLs) was neuroprotective in an embolic stroke model. We hypothesized that HDL vasculoprotective actions on the blood–brain barrier (BBB) may decrease hemorrhagic transformation-associated with tissue plasminogen activator (tPA) administration in acute stroke.

Methods—We used tPA alone or in combination with HDLs in vivo in 2 models of focal middle cerebral artery occlusion (MCAO) (embolic and 4-hour monofilament MCAO) and in vitro in a model of BBB. Sprague–Dawley rats were submitted to MCAO, n=12 per group. The rats were then randomly injected with tPA (10 mg/kg) or saline with or without human plasma purified-HDL (10 mg/kg). The therapeutic effects of HDL and BBB integrity were assessed blindly 24 hours later. The integrity of the BBB was also tested using an in vitro model of human cerebral endothelial cells under oxygen–glucose deprivation.

Results—tPA-treated groups had significantly higher mortality and rate of hemorrhagic transformation at 24 hours in both MCAO models. Cotreatment with HDL significantly reduced stroke-induced mortality versus tPA alone (by 42% in filament MCAO, P=0.009; by 73% in embolic MCAO, P=0.05) and tPA-induced intracerebral parenchymal hematoma (by 92% in filament MCAO, by 100% in embolic MCAO; P<0.0001). This was consistent with an improved BBB integrity. In vitro, HDLs decreased oxygen–glucose deprivation–induced BBB permeability (P<0.05) and vascular endothelial cadherin disorganization.

Conclusions—HDL injection decreased tPA-induced hemorrhagic transformation in rat models of MCAO. Both in vivo and in vitro results support the vasculoprotective action of HDLs on BBB under ischemic conditions. (Stroke. 2013;44:699-707.)

Key Words: blood–brain barrier ■ hemorrhagic transformation ■ high-density lipoproteins ■ ischemic stroke ■ tissue plasminogen activator

Recombinant tissue plasminogen activator (tPA) is the only effective fibrinolytic treatment at the acute stage of ischemic stroke (IS). However, its use remains restricted to carefully selected patients within 4.5 hours after stroke onset and it is associated with an increased risk of brain edema and secondary hemorrhagic transformation (HT).1–4 Therefore, there is a need for development of treatments that would improve the safety and efficacy of tPA. The disruption of the blood–brain barrier (BBB) has been shown to be involved in edema and HT after tPA treatment in IS.5,6 Ischemic injury and tPA display cumulative deleterious effects on the neurovascular unit (BBB, neurons, and astrocytes) at the acute phase of stroke.5,7 High-density lipoproteins (HDLs) have been proposed as a new neuro- and vasculoprotective treatment in IS.8,9 Beyond their well-documented action of reverse cholesterol transport, HDLs have pleiotropic effects (ie, anti-inflammatory, antioxidant, and more generally endothelial protective effects).10,11 We recently reported that intravenous administration of HDLs isolated from human plasma, up to 5 hours after the onset of ischemia, was effective in reducing the infarct volume by maintaining BBB integrity in a rat model of embolic stroke.5

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Here, we sought to assess the potential of HDL therapy in combination with tPA in 2 different models of focal cerebral ischemia in rats. We speculated that beneficial effects of HDLs on BBB integrity could allow reduction of edema and HT induced by tPA injection in IS.

Materials and Methods
Animal Procedures and Experimental Design
Animal care and experimental protocols were approved by the Animal Ethics Committee of the INSERM-University Paris 7, authorization 2010/13/698-0002. Male Sprague–Dawley rats (Janvier, France), weighing 300 to 350 g, were anesthetized by isoflurane mixed with air (4% for induction; 1% during surgery), under spontaneous respiration. Two different models of focal cerebral ischemia were used: embolic middle cerebral artery occlusion (eMCAO) and transient filament MCA occlusion (fMCAO). Focal cerebral ischemia was induced by intraluminal occlusion of the middle cerebral artery for 4 hours. eMCAO was induced by injection of a preformed clot at the origin of MCA as already described. Continuous laser Doppler flowmetry (VMS, Moor Instrument) was used to monitor regional cerebral perfusion to ensure the adequacy of MCA occlusion (perfusion decreased to <20% of preischemic baselines). Animals were assigned to 1 of 4 groups (n=12 per group, except for the tPA-treated group, n=24): control (saline treatment only), tPA alone (10 mg/kg), HDLs alone (10 mg/kg intravenous, jugular vein) (Figure IV in online-only Data Supplement), and tPA plus HDLs. Continuous tPA or saline infusion (for 30 minutes; Harvard Apparatus Infusion Pump) started 4 hours after stroke onset, immediately before recanalization (after withdrawal of the filament in fMCAO), or 4 hours after clot placement in the MCA. One single intravenous injection was performed according to previously reported pharmacokinetics of HDLs in rats. Plasma density was adjusted to 1.25 with KBr and overlaid with KBr saline solution (d=1.22). The second ultracentrifugation was performed at 100 000 g at 20°C for 20 h at 10°C. The density of the bottom fraction containing HDLs was adjusted to 1.25 with KBr and overlaid with KBr saline solution (d=1.22). The second ultracentrifugation was performed at 100 000 g at 10°C. After this step, HDL fractions, representing the top layer of the tube, were recovered as a single band and were then extensively rinsed with saline and concentrated using a centrifugal concentrating device (cut off 10 kDa; Vivascience, Stonehouse, UK). All fractions were desalted either by dialysis against saline or by concentrifugation and 3 washes with saline.

Exclusion Criteria
Animals were excluded for analysis if the following occurred: subarachnoid hemorrhage (n=1 in eMCAO and 2 in fMCAO) or inadequacy of MCA occlusion (n=3 in eMCAO and 1 in fMCAO). One death owing to anesthesia or surgery occurred within 4 hours of stroke induction (1 in fMCAO).

Measurement of Intracranial Hemorrhage and Infarct Volume
Rats were euthanized 24 hours after induction of focal ischemia with a lethal dose of isoﬂurane and perfused through the heart with 10 mL of heparin in 0.9% saline. The brain was rapidly removed. Seven coronal sections of the brain (2 mm in thickness) were immediately made and numeric images were captured for quantification of hemorrhage. Intracerebral hemorrhage was classified by an examiner, unaware of the rat’s regimen, into three groups: no hemorrhage (0); hemorrhagic infarction (H), defined as individual or several petechiae in the core or at the borders of the ischemic area; and parenchymal hematoma (P), when a large area of blood was observed within the core of the infarct.41,42 Moreover, areas of HT from these 7 unstained brain sections were measured (hemorrhage area, mm²) using Photoshop software by semiautomatic selection of extravasated erythrocytes as previously described. The slices were then immediately stained with 2% 3,3′-dihexyloxacarbocyanine chloride (Sigma-Aldrich) for 20 minutes at room temperature. Volume calculation with edema correction was performed blindly using the following formula: 100x(contralateral hemisphere volume−infarct ipsilateral hemisphere volume)/contralateral hemisphere volume. After 2,3,5-triphenyltetrazolium chloride staining, the slice at ±0.70 mm posterior to bregma was fixed in paraformaldehyde 3.7%, cryoprotected, embedded in optimal cutting temperature medium and immediately frozen for immunohistological analysis.

IgG Extravasation
BBB permeability was evaluated 24 hours after stroke by using fluorescence detection of IgG extravasation in the fMCAO model as previously described. Using a cryostat, 10-µm sections were prepared from the coronal slice (at ±0.70 mm posterior to bregma) previously frozen in optimal cutting temperature medium. Sections were incubated overnight at 4°C with donkey antirat IgG antibodies (5 µg/mL; Molecular Probes) labeled with Alexa Fluor 488. IgG extravasation in the ischemic area was quantified semiautomatically using morphometry software (Histolab 6.1.5, Microvision Instruments).

Immunohistochemistry
Frozen sections were fixed with 3.7% paraformaldehyde and blocked with 10% goat serum. Sections were incubated overnight at 4°C with a rabbit polyclonal to collagen IV (2 µg/mL; Abcam) for detection of basal lamina of intracerebral vessels. Fluorescein isothiocyanate-conjugated Bandeiraea Simplicifolia Lectin I (isolectin B4) (5 µg/mL; Vector Laboratories) was used to visualize endothelial cells (ECs), and a polyclonal rabbit antimonyperoxidase (16.5 µg/mL; Dako) to detect polymorphonuclear neutrophils (PMNs). The number of immunostained cells was determined semiautomatically using morphometry software (Histolab 6.1.5, Microvision Instruments). Nonimmune rabbit IgG was included in each set of experiments as primary antibody to test the specificity of the signal. We used Alexa-Fluor 555 as secondary antibodies. Immunostaining was analyzed with a fluorescent microscope interfaced with a digital capture system. All immunohistological evaluations were performed by an observer who was blinded to the treatment. For semiquantification of the collagen type IV expression, 3 fields of each territory (right MCA and contralateral area) were acquired using a x10 objective. A threshold of fluorescence intensity, which encompasses positive vessels on the contralateral image, was applied to each corresponding ipsilateral image. Data are presented as a percentage of the positive immunoreactivity area of the contralateral area (pixel). Immunohistochemistry analysis was performed in the fMCAO model.

Isolation of Lipoproteins and In Vivo Tracking of HDLs
Lipoproteins were isolated from a pool of heparinized plasma of healthy volunteers by ultracentrifugation. In brief, plasma density was adjusted to d=1.22 with KBr and overlaid with KBr saline solution (d=1.063). Ultracentrifugation was performed at 100 000g for 20 h at 10°C. The density of the bottom fraction containing HDLs was adjusted to 1.25 with KBr and overlaid with KBr saline solution (d=1.22). The second ultracentrifugation was performed at 100 000g overnight at 10°C. After this step, HDL fractions, representing the top layer of the tube, were recovered as a single band and were then extensively rinsed with saline and concentrated using a centrifugal concentrating device (cut off 10 kDa; Vivascience, Stonehouse, UK). Al fractions were desalted either by dialysis against saline or by concentrifugation and 3 washes with saline.

For tracking in vivo, HDLs were labeled with carbocyanines. HDLs were incubated overnight at 37°C under gentle shaking with 8.5 µg/mL DiIC18 carbocyanines (Molecular Probes Inc, USA) and then separated by ultracentrifugation. Labeled HDLs (10 mg apoA1/kg) were administrated intravenously in the fMCAO model (4 hours of MCAO) immediately after stroke onset (n=3). Rats were euthanized.
at 1, 3, and 24 hours after stroke onset. After decapitation, brain slices were embedded in optimal cutting temperature medium and immediately frozen. Coronal sections (8 μm) (at +0.70 mm posterior to bregma)26 were prepared with the use of a cryostat. Isocitron B4 (Vector Laboratories) was used to identify ECs as already described.26 Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (0.5 μg/mL for 10 minutes) and the sections were observed under an epifluorescence microscope.

Effect of HDLs on tPA Activity.

The potential effect of HDLs on tPA activity was measured in vitro using recombinant tPA or ex vivo on plasma after injection of tPA±HDLs. The amidolytic activity of tPA (400 μg/mL) was assessed by using SPECTROZYME chromogenic substrate (methylsulfonyl-n-cyclohexyltyrosyl-glycyl-arginine para-nitroanilide acetate; American Diagnostica) P-444 substrate in the presence or absence of HDLs (0.4 g/mL). The kinetics of amidolysis by tPA with 1 mM SPECTROZYME chromogenic substrate at 37°C were monitored by measuring absorbance at 405 nm for 2 hours with a multispan spectrophotometer (BMG Labtech). Initial rates of tPA activity were calculated from plots of 405 nm versus time (mDO/min). For ex vivo experiments, tPA (10 mg/kg) was administered via the femoral vein (10% bolus and 90% during 20 minutes) in rats with or without HDLs (10 mg/kg). Blood was sampled from the jugular vein in citrate-containing tubes, 3 minutes after administration of tPA, HDLs, or tPA+HDLs. Plasma samples were diluted 20-fold with phosphate buffered saline containing 0.1% human serum albumin (ABCyS) and 0.01% Tween 20 in 96-well microtiter plates. tPA amidolytic activity was determined as described above. Measurements were performed in triplicate (n=3 per group of rats) and expressed as mean±SD.

In Vitro BBB Model

Cell Culture

The human brain EC line hCMEC/D3 was kindly provided by Dr P.O. Couraud.25 Cells were cultured in complete endothelial basal medium-2 (EBM +2.5% of fetal calf serum and supplements containing hydrcortisone and growth factors).

Cell Treatments

Before each experiment, cells were washed 3 times with phosphate buffered saline and then incubated with 400 μg/mL of HDL solution. Oxygen–glucose deprivation (OGD) conditions were obtained by using Dulbecco’s Modified Eagle’s Medium without glucose (Gibco) versus 1 g/L glucose Dulbecco’s Modified Eagle’s Medium for non-OGD conditions. Oxygen deprivation was obtained by using a hypoxia chamber (Billups-Rothenberg) where atmospheric air was replaced with a gas mixture (0% O2, 5% CO2 and 95% N2 air product). Dulbecco’s Modified Eagle’s Medium used for OGD conditions was equilibrated with the same mixture.

In Vitro Permeability Measurements

For permeability experiments, hCMEC/D3 cells were seeded at 5x10³ cells/cm² on collagen inserts (pore controllable ﬁber ﬁbers, 0.4 μm pore size, Millicell 24-well plates, Millipore) in complete endothelial basal medium-2. Cells were grown for 10 days postconfluence before use. Permeability was assessed using fluorescein isothiocyanate-labeled dextran (70 kDa molecular weight). Fluorescein isothiocyanate-dextran (0.385 mg/mL) was added to the inserts, which were transferred every 15 minutes for 45 minutes to collecting wells containing 600 μL of fresh medium. Two hundred microliters from the collecting wells were transferred into a 96-well plate, and the dextran fluorescence was measured on the microplate reader at 485 nm (excitation) and 538 nm (emission). Fluorescence was converted to dextran concentration using a standard curve. The volume cleared was calculated from the ratio of dextran concentration in each sample to the applied concentration (0.385 mg/mL). The endothelial permeability coefﬁcient (Pe) (cm/min) was calculated by determining the volumes of clearance of dextran with or without cells. The cleared volume was calculated from the ratio of initial dextran concentration (0.385 mg/mL) to that in the collecting wells and plotted against time. Permeability surface (PS) was calculated from 1/PS=1/PS–1/PSf, where PSf is the slope of clearance in the presence of cells and PS is the slope for a blank insert (cell free).27 Pe=PS/S, where S is the surface area of the insert (0.7 cm²).

For cell culture, functionality assay, and mRNA expression analysis, please see online-only Data Supplement.

Immunocytoﬂuorescence

HCMED/D3 cells were seeded at 5x10³ cells/cm² onto collagen-coated labteks in complete endothelial basal medium-2. Cells were grown for 7 days postconfluence before use. After treatment, cells were ﬁxed in 3.7% paraformaldehyde for 30 minutes and stored in phosphate buffered saline at 4°C. Rabbit polyclonal antihuman vascular endothelial (VE) cadherin (1:200; Bender Med Systems) was used as primary antibody, followed by a secondary antibody conjugated to Alexa 555 (Invitrogen). Negative controls using nonimmune rabbit IgGs at the same concentration as anti-VE cadherin were included in each set of experiments to check for nonspecific staining.

Statistical Analysis

Data are presented as medians (quartiles) for continuous variables, or mean (±SD) for immunostaining experiments (Figure 3B; Figure 4B in online-only Data Supplement) and percentages for qualitative variables. We analyzed data by using a Kruskal–Wallis test followed by pairwise Mann–Whitney U tests. Comparison of mortality and intracerebral hemorrhage between groups was performed using the Fisher exact test. A 2-tailed value of P<0.05 was considered signiﬁcant. Data were analyzed using JMP 7.01. The number of animals analyzed (n) is provided in the ﬁgure legends.

Sample Size Calculation

The study was designed with 80% power to compare the proportion of parenchymal hematoma in tPA+HDL-treated group versus tPA alone. For proportions of 0.7 versus 0.2 (P=0.05), which are similar values from preliminary experiments, a sample size of 11 would be required.

Results

In Vivo Experiments

Baseline Characteristics

Physiological characteristics (body temperature, glycemia, blood pressure, and blood gases) did not differ across groups throughout the experiments (Table in online-only Data Supplement).

Effect of the Combined Treatment With tPA and HDLs on Mortality, Infarct Volume, and Neurological Deficit

To test whether HDL infusion may be effective in preventing deleterious effects of tPA injected at the reperfusion stage, tPA±HDL solution was administered 4 hours after stroke onset and compared with control groups (saline or HDLs alone), in two models of stroke in rats. tPA increased mortality and infarct volume in both filament and embolic models as detailed in Figure 1. The mortality rate observed in the tPA-treated group was higher in the fMCAO than in the eMCAO (P<0.05) (Table in online-only Data Supplement). Additional treatment with HDLs relative to tPA treatment alone signiﬁcantly decreased infarct volume: −59.8% in eMCAO, P=0.005 and −46.4% in fMCAO (P=0.0001), and mortality: −72.7% in eMCAO, P=0.05 and −41.9% in fMCAO (P=0.009) (Figure 1). HDL treatment alone signiﬁcantly reduced cerebral infarct volume

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Effects of Combined Treatment on BBB Integrity

To assess the effect of the combined treatment on BBB integrity, cerebral edema and IgG extravasation were evaluated as a surrogate marker of BBB disruption during acute stroke.

Large areas of edema surrounding ischemic cerebral vessels were observed only in the tPA-treated group compared with the tPA+HDL group (Figure 3A). Increased IgG extravasation was shown in the ipsilateral ischemic versus the contralateral hemisphere across the different groups. Combined treatment with tPA and HDLs significantly reduced IgG extravasation relative to tPA alone, respectively, 39±3% versus 88±1% of the ischemic area, \( P<0.04 \) (Figure 3B). We then performed immunostaining for collagen IV, a major component of the basal lamina of cerebral microvessels. Twenty-four hours after stroke, the number of collagen IV immunoreactive vessels in the infarcted area was decreased compared with the contralateral hemisphere in the saline group and even more in the tPA-treated group. Combined tPA and HDL therapy was associated with a significant increase in collagen IV immunoreactive vessels relative to tPA alone, suggesting an improved BBB integrity, respectively, 91±3% versus 45±8%, \( P=0.02 \) (Figure 1 in online-only Data Supplement).

HDLs taken up by ECs reduced the expression of adhesion molecules and PMN recruitment

To produce a significant effect on tPA-associated complications, we hypothesized that injected HDLs should reach the ischemic area and be taken up by cerebral ECs. HDL particles were internalized as early as 1 hour after injection (Figure 4) and remained detectable within ECs for 24 hours.

HDLs decreased adhesion molecule expression by cerebral ECs in vitro and limited PMN recruitment in the ischemic area in vivo.

Given the anti-inflammatory effect of HDLs on ECs, we tested their capacity to reduce ICAM-1 and VCAM-1 expression in human cerebral EC culture, in response to tumor necrosis factor-\( \alpha \). These 2 major adhesion molecules play a critical role in PMN diapedesis and infiltration into ischemic tissues.\(^{24} \) We show that HDLs decreased tumor necrosis factor-\( \alpha \)-induced expression of ICAM-1 and VCAM-1 by ECs in a dose-dependent manner (Figure 2 in online-only Data Supplement). We then assessed PMN recruitment in the ischemic area in vivo, in our experimental groups. PMN recruitment was significantly decreased in

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*Figure 1.* Effects of high-density lipoproteins (HDL) and tissue plasminogen activator (tPA) on mortality and infarct volume in focal stroke models (A, transient filament middle cerebral artery occlusion, tMCAO; B, embolic MCAO, eMCAO). A, tPA increased mortality rate compared with the 3 other groups \( (P<0.05) \). HDLs significantly decreased stroke-induced mortality in the tPA+HDL-treated group compared with tPA alone in both models. B, HDL injection significantly decreased infarct volume at 24 hours after stroke versus saline and in combination with tPA versus tPA alone \( (n=12 \text{ per group, except for tPA-treated group, } n=24) \). \( *P<0.05, **P<0.001 \). NS indicates not statistically significant.

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compared with saline in both stroke models \( (P<0.05) \) but only a trend for decreased mortality was observed \( (P=0.29) \). Combined treatment also improved the neurological outcome relative to tPA treatment alone in both models (MCAO, \( P=0.01 \); eMCAO, \( P=0.01 \); data not shown).

Effects of HDL Treatment on HT and Cerebral Edema Induced by tPA

Because the major complications associated with tPA treatment are HT and edema, we tested the effect of HDLs in our models of stroke on these particular endpoints. Only the groups treated by tPA alone exhibited a high percentage of parenchymal hematoma (P) (MCAO, 62%; eMCAO, 46%), which was strongly associated with mortality \( (P=0.02; \text{ Figure 2}) \). No significant difference was observed for the rate of petechial hemorrhage between groups. Interestingly, the combined treatment of tPA and HDLs markedly decreased the incidence of parenchymal hematoma by \( >90\% \) in both models compared with both tPA alone and saline treatment \( (P<0.001; \text{ Figure 2A}) \). The quantitative analysis of hemorrhage area was also markedly reduced in the tPA+HDL-treated group relative to tPA alone in both MCAO models, 4.33 (interquartile range [IQR], 0–17.56) versus 44.22 (IQR, 29.30–65.49) for tMCAO and 0 (IQR, 0–3.79) versus 41.12 (IQR, 19.23–99.58) for eMCAO, \( P<0.001 \) (Figure 2B).

Effects of Combined Treatment on BBB Integrity

To assess the effect of the combined treatment on BBB integrity, cerebral edema and IgG extravasation were evaluated as a surrogate marker of BBB disruption during acute stroke.

Large areas of edema surrounding ischemic cerebral vessels were observed only in the tPA-treated group compared with the tPA+HDL group (Figure 3A). Increased IgG extravasation was shown in the ipsilateral ischemic versus the contralateral hemisphere across the different groups. Combined treatment
Supplementation with HDLs reduced, at least partially, this hypothesis they may inhibit tPA activity in vitro and ex vivo. Because HDLs display antiprotease properties, we tested the activity in vitro model subjected to OGD with or without HDLs. In our conditions (4 hours of stimulation), OGD induced an increased BBB permeability: $7.8 \times 10^{-3}$ (IQR, $6.2-9.4 \times 10^{-3}$) versus $5.6 \times 10^{-3}$ (IQR, $5.5-6.4 \times 10^{-3}$) in control, $P=0.04$. Interestingly, coincubation with HDLs decreased OGD-induced BBB permeability by 38.5%, $P=0.002$ (Figure 6A). In addition, we looked at VE cadherin, given that it is a pivotal junction protein involved in the maintenance of the restricted permeability of the endothelial barrier. Immunofluorescent staining for VE cadherin, performed on hCMEC/D3 cells 7 days postconfluence, showed that OGD induced a disorganization of intercellular junctions characterized by intracellular patches of VE cadherin. Supplementation with HDLs reduced, at least partially, this phenotype by maintaining the cell–cell junctions (Figure 6B).

**HDLs Prevented tPA-induced BBB Injury In Vitro**

We hypothesized that the beneficial in vivo effects of HDLs at the acute phase of stroke may be, at least in part, owing to protection of the BBB. We thus assessed BBB integrity using an in vitro model subjected to OGD with or without HDLs. In our conditions (4 hours of stimulation), OGD induced an increased BBB permeability: $7.8 \times 10^{-3}$ (IQR, $6.2-9.4 \times 10^{-3}$) versus $5.6 \times 10^{-3}$ (IQR, $5.5-6.4 \times 10^{-3}$) in control, $P=0.04$. Interestingly, coincubation with HDLs decreased OGD-induced BBB permeability by 38.5%, $P=0.002$ (Figure 6A). In addition, we looked at VE cadherin, given that it is a pivotal junction protein involved in the maintenance of the restricted permeability of the endothelial barrier. Immunofluorescent staining for VE cadherin, performed on hCMEC/D3 cells 7 days postconfluence, showed that OGD induced a disorganization of intercellular junctions characterized by intracellular patches of VE cadherin. Supplementation with HDLs reduced, at least partially, this phenotype by maintaining the cell–cell junctions (Figure 6B). Using a selective substrate of tPA, we showed that HDLs did not modify tPA proteolytic activity, in vitro, HDL+tPA: $216 \pm 17$ versus tPA: $244 \pm 35$, nonsignificant (Figure IIIA in online-only Data Supplement). These results were further supported ex vivo in plasma of rats that had received intravenous administration of tPA and HDLs. The proteolytic activity of tPA from rat plasma was not affected by concomitant injection of HDLs, HDL+tPA: $216 \pm 16$ versus tPA: $225 \pm 53$, nonsignificant (Figure IIIB in online-only Data Supplement) suggesting that a combined treatment is possible without interference with the fibrinolytic action of tPA.

**Discussion**

In the present study, using 2 models of focal stroke in rats, we showed that HDL injection prevented the risk of HT induced by tPA administration in the acute stage of IS. Both in vivo and in vitro results support the vasculoprotective action of HDLs on BBB under ischemic conditions.

1. HDL administration limits HT induced by tPA.

To address whether HDL treatment may decrease HT induced by tPA, we used 2 stroke models with tPA characterized by a high incidence of parenchymal hematoma and associated mortality, as observed in humans with delayed tPA injection. Combination therapy with HDLs decreased the risk of hematoma by at least 90% in both models. These results are
of major importance given that in human patients, hematoma subsequent to tPA treatment are associated with clinical deterioration and poor clinical outcome.

Our results on HT are consistent in 2 different models (eMCAO and fMCAO) which is in accordance with Stroke Therapy Academic Industry Roundtable recommendations.

In the filament model of MCAO, tPA was injected at the time of reperfusion, 4 hours after occlusion, highlighting the (extra) vascular deleterious effects of this potent serine protease without any effect of tPA on clot fibrinolysis. This condition may be similar when patients with acute cerebral artery occlusion are recanalized by thrombectomy. In the embolic stroke model, HT induced by tPA injection was the consequence of both clot dissolution and extravascular action during the reperfusion. It is worth noting that the infarct volume of tPA-treated group did not differ significantly from the saline-treated group. In fact, late administration of tPA (4 hours) has been shown to have no effect on infarct volume of focal IS in rodents. The percentage of hematoma and death differed significantly between the 2 models probably because of the difference in MCA occlusion time. As we have shown in a duplex sonography study, in a similar embolic stroke model, 29% of rats with confirmed MCAO occlusion had undergone recanalization between 1 and 4 hours after stroke onset, whereas MCA occlusion induced by a filament lasted exactly 4 hours.

2. Effects of combined treatment on BBB integrity in vivo and in vitro.

Pleiotropic protective effects of HDLs on ECs have been demonstrated in clinical and preclinical settings. Our in vivo and in vitro data support this beneficial effect on cerebral ECs.
Indeed, in vivo, intravenously injected HDLs were taken up by cerebral ECs within 1 hour after stroke onset (Figure 4), even when the MCA was occluded by the monofilament and could thus exert their cytoprotective effects (antiapoptotic, antiprotease, and anti-inflammatory effects). We speculate that this may be owing to corticocortical collateral vasculature. HDL particles could be detected up to 24 hours after injection, suggesting a potential prolonged effect.

As already reported in various experimental models, HDLs exert potent anti-inflammatory effects and limit PMN recruitment in injured tissues, including in our IS model. Herein, we demonstrate that HDLs limited PMN transmigration in our model of hemorrhagic complications induced by recombinant tPA. In addition, we showed in vitro that HDLs reduced the expression of ICAM-1 and VCAM-1 induced by tumor necrosis factor-α. Tumor necrosis factor-α stimulation is classically used to assess the anti-inflammatory potential of HDLs. This cytokine has also been shown to play a critical role in the acute stage of IS.

Our in vitro study also supports the beneficial effect of HDLs on the BBB. In vitro, using a well-described model of BBB, OGD for 4 hours induced an increase in BBB permeability and VE cadherin disorganization, both prevented by HDLs. We have intentionally chosen a 4-hour OGD period to conform to the timing of our in vivo model and, more importantly, to the therapeutic window in humans. Our data frame well with the previously reported direct action of HDLs or reconstituted HDLs (rHDLs) on ECs. The precise mechanisms of HDL protection on ECs are not completely understood but apolipoprotein A1, lipids surrounding the protein core, sphingosine-1-phosphate, or other proteins associated with HDLs, including antielastase, may provide explanations for their beneficial actions on the BBB. By limiting BBB permeability in OGD conditions, HDLs may prevent tPA extravasation and limit its cytotoxic effects within brain tissue.

BBB disruption in response to aggression by tPA and ischemic conditions plays a pivotal role in the initiation of blood component leakage into the cerebral compartment. In humans, the disruption of the BBB assessed by gadolinium extravasation after brain MRI was strongly correlated with the risk of symptomatic HT after tPA administration. HDL-based therapy may have beneficial effects on cerebral ECs and thus reduce complications associated with BBB disruption as we have shown with a decrease in IgG extravasation and collagen IV degradation when HDLs were coinjected with tPA versus tPA alone.

3. In vitro and ex vivo assessment of HDL effects on tPA activity.

Finally, we have shown that HDL did not interact with the proteolytic action of tPA, a serine protease. We assessed a putative interaction between HDLs and tPA given that some proteins carried by HDLs are protease inhibitors. Both tPA and HDL concentrations used in our study are in the range of those used in humans. Our results suggest that the beneficial actions of HDLs in combination with tPA treatment decrease HT without interference with the fibrinolytic effect of tPA.


Our study highlights the potential of HDL-based therapy as an original neuro- and vasculoprotective agent in the acute treatment of stroke. HDL-raising drugs, such as niacin, have been suggested to be neuroprotective in experimental stroke. HDL-based therapy is supported by numerous epidemiological studies that showed that low HDL-cholesterol levels were associated with cerebrovascular events. Recently, among 489 patients with acute IS treated with tPA within 3 hours of stroke onset, high HDL-C levels were significantly associated with a good outcome at 3 months. This suggests that HDL supplementation may be beneficial at the acute stage of stroke. Raising HDL levels encompasses 2 different strategies: HDL infusion and HDL-raising drugs. A single rapid rHDL infusion immediately restored endothelial function in hypercholesterolemic patients or in subjects with low HDL-C. In addition, infusion of rHDLs decreased the lipid burden and the concentration of inflammatory markers in
atherosclerotic plaques. Chronic administration of HDL-raising drugs, such as cholesterylester transfer protein inhibitors or niacin, is currently being evaluated in secondary vascular prevention but the first results seem to be disappointing. Nevertheless, to obtain an effect of HDLs in the acute phase of cerebral ischemia, HDL infusion seems to be more appropriate.

Prophylactic injection of rHDL (120 mg/kg) has been shown to be neuroprotective in 2 different focal stroke models. This dose contrasts with the lower dose (10 mg/kg of plasma HDLs) used in our study. This discrepancy may be explained by the composition of HDLs that is different between HDLs isolated from plasma and rHDLs, composed only of phosphatidyl-choline and ApoA1. The HDL-associated proteome by the composition of HDLs that is different between HDLs used in our study. This discrepancy may be explained to functionality issues. Indeed, studies on HDL function among tors or niacin, is currently being evaluated in secondary vascular prevention but the first results seem to be disappointing.

HDLs consist of numerous subpopulations of particles, which are functionally heterogeneous. Raising HDL levels by pharmacological agents in patients with a metabolic syndrome or other risk factors for stroke may lead to production of dysfunctional HDLs. Indeed, studies on HDL function among type 2 diabetes or coronary heart disease patients have shown that isolated HDLs were dysfunctional or even proinflammatory. Global HDL-raising strategies are still debated owing to functionality issues. We thus hypothesize that rapid infusion of functional HDLs may be beneficial in acute cerebral ischemia.

Limits
We did not use long-term follow-up for our in vivo study and we used HDLs isolated from human plasma. Thus, this is a proof of concept study. Although we have validated our results in 2 different models of IS, they should be replicated by independent research groups. Further studies are needed, especially with rHDLs or HDL mimetics in acute stroke in humans.

Conclusions
Our results suggest that HDL therapy may limit the deleterious effects of tPA in acute IS, such as HT. This could have a strong clinical impact. Reconstituted human HDLs should now be tested in IS patients.

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Disclosures
None.

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