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# Protective Effect of High-Density Lipoprotein-Based Therapy in a Model of Embolic Stroke

Bertrand Lapergue, MD; Juan-Antonio Moreno, PhD; Bao Quoc Dang, BSc; Michèle Coutard, PhD; Sandrine Delbosc, PhD; Guy Raphaeli, MD; Nathalie Auge, PhD; Isabelle Klein, MD, PhD; Mikael Mazighi, MD, PhD; Jean-Baptiste Michel, MD, PhD; Pierre Amarenco, MD; Olivier Meilhac, PhD

**Background and Purpose**—High-density lipoprotein (HDL) levels are inversely associated with stroke incidence, suggesting a protective effect. Using a rat model, we tested the hypothesis that HDL exerts direct vasculo/neuroprotective effects when administered during the acute phase of embolic stroke.

**Methods**—After embolic occlusion, Sprague-Dawley rats were randomly treated intravenously with purified HDL versus saline immediately (2, 10 mg/kg) or 3 or 5 hours (10 mg/kg) after stroke. The effects of HDL were assessed blindly 24 hours later by evaluating neurological deficit score and measuring the infarct volume and blood–brain barrier breakdown. Protease activities and neutrophil infiltration were also evaluated.

**Results**—HDL injection immediately after stroke (10 mg/kg) reduced by 68% the mortality at 24 hours ( $P=0.015$ ). HDL administration immediately or at 3 or 5 hours after stroke also reduced cerebral infarct volume by 74%, 68%, and 70.7%, respectively ( $P=0.0003$ ,  $P=0.011$ , and  $P=0.019$ ;  $n=17$  per group). The neurological deficit at 24 hours in the HDL-treated group was decreased versus the saline-treated group ( $P=0.015$ ). Ischemia-induced blood–brain barrier breakdown was significantly reduced in HDL-treated rats versus controls ( $P=0.0045$ ). Neuroprotective effects of HDL were associated with decreased neutrophil recruitment in the infarct area ( $P=0.0027$ ) accompanied by reduced matrix metalloproteinase gelatinase activity. Immunostaining showed that HDL was associated with endothelial and glial cells, and also that intercellular adhesion molecule-1 expression was decreased in vessels within the infarct area.

**Conclusions**—Administration of HDL is neuroprotective when performed up to 5 hours after experimental stroke. This effect may be attributed to the ability of HDL to protect the blood–brain barrier and limit neutrophil recruitment. (*Stroke*. 2010;41:00-00.)

**Key Words:** blood–brain barrier ■ cerebral ischemia ■ lipoproteins ■ leukocytes

During the onset of cerebral ischemia, the role of the blood–brain barrier (BBB) is critical and should be the target for new protective therapies.<sup>1</sup> Epidemiological studies have found an inverse association between high-density lipoprotein (HDL) cholesterol levels and cerebrovascular events.<sup>2</sup> In addition to reversing cholesterol transport, HDL particles exert anti-inflammatory, antiprotease, and antithrombotic effects<sup>3</sup> that may protect endothelial cells from acute injury. Administration of reconstituted HDL has been shown to normalize endothelial dysfunction in patients with hypercholesterolemia or with low HDL levels.<sup>4,5</sup> Polymorphonuclear neutrophils (PMNs) play a key role in acute ischemic cerebral injury and in ischemia-induced BBB disruption,<sup>6,7</sup> where their associated matrix metalloproteinase 9 (MMP-9) participates in BBB breakdown.<sup>7,8</sup> Interestingly, it was demonstrated in vivo that

HDL inhibits cytokine-induced expression of endothelial adhesion molecules and hence reduces PMN adhesion and transmigration.<sup>9</sup> We hypothesized that HDL injection after the onset of stroke (0 to 5 hours) may decrease PMN recruitment in the ischemic area and also may have beneficial effects on cerebral damage after stroke.

## Materials and Methods

Stroke Therapy Academic Industry Roundtable (STAIR) recommendations have been followed to avoid bias due to experimental design.<sup>10</sup>

## Embolic Stroke Model

Animal care and experimental protocols were approved by the Animal Ethics Committee of Inserm/Paris Diderot University–University Paris 7, authorization 75-214. Male Sprague-Dawley rats (Janvier, Le Genest-St-Isle, France) weighing 300 to 350 g,

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were anesthetized with isoflurane mixed with air (4% for induction; 1% during surgery) under spontaneous respiration. Focal cerebral ischemia was induced by embolization of a preformed clot in the middle cerebral artery, as described.<sup>11</sup> Body temperature was maintained at 37°C±0.5 with a heating pad for the duration of surgery. Glycemia, arterial blood pressure, and blood gases were also monitored during surgery.

### Sample Size Calculation

The study was designed with 80% power to detect a relative 50% difference in cerebral infarct volume between groups (HDL versus placebo). Statistical testing was performed at the 2-tailed  $\alpha$  level of 0.05 using a *t* test. Based on preliminary data indicating that median infarct volume at 24 hours after stroke was 42.1 (interquartile considering 25th to 75th percentile 17.6 to 65.0), we used 17 rats per group.

### Experimental Protocol

Purified HDL (2 or 10 mg of apoA1/kg body weight), low-density lipoproteins (LDL, 10 mg apoB/kg body weight) or saline were administered intravenously to rats immediately after stroke onset (n=17 per group). Four supplemental groups received either saline or HDL (10 mg apoA1/kg body weight) 3 or 5 hours after stroke onset. One single intravenous injection was performed according to previously reported pharmacokinetics of HDL in rats.<sup>12</sup> Computer-based randomization was used to allocate drug regimens to each group. Experiments were blinded and the operator was unaware of group allocation during surgery and outcome assessment. We evaluated the mortality rate and the neurological deficit at 24 hours after stroke onset using a modified Neurological Severity Score, which is a composite of motor, sensory, and balance tests (Supplemental Figure II; available at <http://stroke.ahajournals.org>).<sup>13</sup>

### Exclusion Criteria

Animals were excluded for analysis if the total lesion volume was <5% (n=3 in saline group, n=2 in LDL group, n=5 in HDL groups) or if subarachnoid hemorrhage was present (n=1 in HDL groups). No deaths due to anesthesia or surgery occurred within 3 hours of embolic stroke induction.

### Measurement of Infarct Volume and Brain Edema

Rats were euthanized 24 hours after induction of focal ischemia. Seven coronal sections of the brain (2 mm in thickness) were stained with 2% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) for 20 minutes at room temperature. The infarct volume in animals that died before the 24-hour time point was also evaluated and included in the results. Volume calculation with edema correction were performed blindly using the following formula:  $100 \times (\text{contralateral hemisphere volume} - \text{noninfarct ipsilateral hemisphere volume}) / \text{contralateral hemisphere volume}$ . Brain edema was determined by calculating the volume difference between the 2 hemispheres and dividing by the volume of the left hemisphere. All the ancillary experiments (Evans blue and immunostaining) were performed on additional animals, except for zymography (which is compatible with prior 2,3,5-triphenyltetrazolium chloride staining).

### HDL and LDL Preparation

HDL (density=1.063 to 1.210 g/mL) and LDL (density=1.019 to 1.063 g/mL) were isolated from a pool of plasma from healthy volunteers by ultracentrifugation as described previously.<sup>14</sup> Apoprotein B and apoprotein A1 concentrations were determined by immunonephelometry and did not show any crosscontamination between HDL and LDL. Five different batches of HDL were used for the study. The purity of LDL and HDL fractions (absence of

albumin contamination) was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Coomassie blue staining.

### HDL Labeling With Carbocyanines and Tracking In Vivo

HDL was incubated overnight at 37°C under gentle shaking with 8.5  $\mu\text{g/mL}$  DiIC18 carbocyanines (Molecular Probes Inc) and then separated by ultracentrifugation. Labeled HDL (10 mg apoA1/kg) was administered intravenously immediately after stroke onset (n=6) and fluorescein isothiocyanate–dextran (2000 kDa; Sigma-Aldrich) was injected just before euthanasia (n=3). After decapitation, brain sections were embedded in optimal cutting temperature medium and immediately frozen. Coronal sections (8  $\mu\text{m}$ ; at +0.70 mm posterior to bregma)<sup>15</sup> were prepared with the use of a cryostat. Cell nuclei were stained with 4', 6'-diamidino-2-phenylindole (0.5  $\mu\text{g/mL}$  for 10 minutes) and the sections were observed under an epifluorescence microscope.

### Alanine Transaminase and Aspartate Aminotransferase Quantification for Assessment of Hepatic Function

Blood was sampled at baseline, 1, 3, and 24 hours after stroke onset in each group (n=4 per group). The plasma activities of alanine and aspartate aminotransferase were measured by commercially available kits using an Olympus AU400 spectrophotometer.

### Evans Blue Extravasation

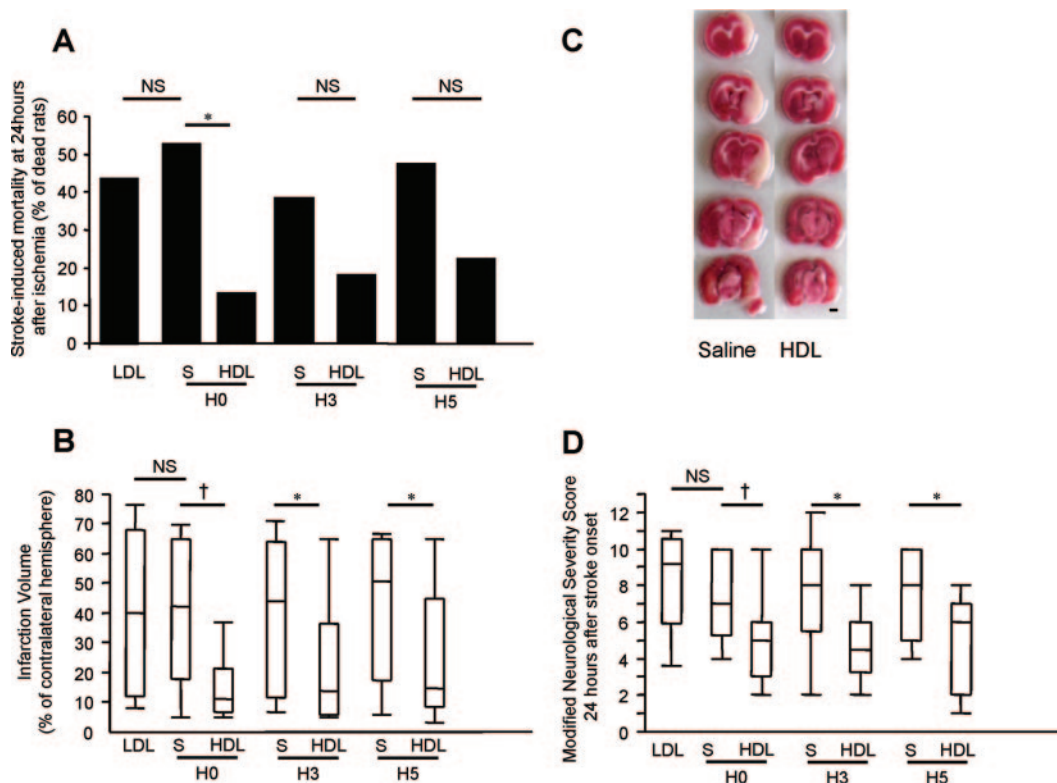
BBB permeability was quantitatively evaluated using fluorescence detection of extravasated Evans blue dye. Rats were treated with HDL (10 mg/kg) or saline immediately after stroke induction (n=6 per group). Two percent Evans blue in saline was then infused (4 mL/kg intravenously) 24 hours after clot injection. After 3 hours, rats were deeply anesthetized with pentobarbital and transcardially perfused with saline to wash out the intravascular dye. Brains were removed, cut into 2-mm coronal sections, embedded in optimal cutting temperature, and frozen. Ten-micron sections (at +0.70 mm posterior to bregma) were prepared. Evans blue extravasation was observed by fluorescence microscopy and was quantified semiautomatically with 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 primary antibodies. A mouse monoclonal antirat endothelial cell antigen antibody (2.5  $\mu\text{g/mL}$ ; Serotec) was used to detect vessels, a polyclonal rabbit antimyeloperoxidase (16.5  $\mu\text{g/mL}$ ; Dako) to detect PMNs, an antiglial fibrillary acidic protein (5.8  $\mu\text{g/mL}$ ; Dakocytomation) to detect astrocytes, and an anti-NF200 (17  $\mu\text{g/mL}$ ; Sigma-Aldrich) to visualize neurons. A monoclonal mouse antirat intercellular adhesion molecule-1 (10  $\mu\text{g/mL}$ ; Biologend) was also used. We included nonimmune IgG in each set of experiments as the primary antibody to test the specificity of the signal and used Alexa-Fluor 488 or 555 as secondary antibodies. Immunostaining was analyzed with a fluorescent microscope interfaced with a digital capture system. The number of immunostained cells was determined semiautomatically with morphometry software (Histolab 6.1.5; Microvision Instruments). All immunohistological evaluations were carried out by an observer who was blinded to the treatment.

### Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis Gelatin Zymography

Immediately after decapitation and brain removal, ipsilateral ischemic brain tissue and the corresponding contralateral



**Figure 1.** A, Treatment with HDL immediately (H0) after stroke onset reduced mortality compared with saline alone. B, Treatment with HDL immediately (H0) or 3 (H3), or 5 (H5) hours after stroke onset reduced ischemic lesion volume compared with saline alone. Treatment with LDL was without effect on mortality or lesion volume (A–B). C, Representative 2,3,5-triphenyltetrazolium chloride-stained coronal sections from saline- and HDL-treated groups. Note larger infarct areas (white) in saline-treated controls. Scale bar=2 mm. D, Treatment with HDL significantly decreased neurological deficit 24 hours after stroke onset compared with the saline-treated group.  $n=17$  per group,  $*P<0.05$ ;  $\dagger P<0.01$ ; NS indicates nonsignificant; S, saline.

nonischemic area were carefully dissected out and separately incubated in RPMI 1640 (10  $\mu\text{L}/\mu\text{g}$  wet tissue) containing antibiotics and antimycotics (Gibco) for 24 hours at 37°C to collect proteases released by the brain tissue ( $n=7$ ). After centrifugation (3000 g, 10 minutes, 20°C), equal volumes of conditioned medium were electrophoresed in the presence of 0.2% sodium dodecyl sulfate in 10% polyacrylamide gels containing 2.5 mg/mL gelatin under nonreducing conditions. Gelatinolytic activity was quantified by densitometry using National Institutes of Health Image 1.42q software.

### In Situ Gelatin Zymography

Ten-micron coronal sections (at +0.70 mm posterior to bregma) were preincubated with either phosphate-buffered saline alone or with 10 mmol/L 1,10-phenanthroline (a broad range MMP inhibitor) for 2 hours at room temperature. They were then incubated with the fluorogenic substrate DQ-Gelatin (40  $\mu\text{g}/\text{L}$ ; Molecular Probes) in zymography buffer: 50 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 5 mmol/L  $\text{CaCl}_2$ , 200  $\mu\text{mol}/\text{L}$  sodium azide for 2 hours at 37°C, followed by 10% neutral formalin fixation ( $n=4$  per group). Proteolytic activity was detected as green fluorescence using an epifluorescence microscope. A control section was incubated in zymography buffer without DQ-Gelatin to detect possible tissue autofluorescence.

### Statistical Analysis

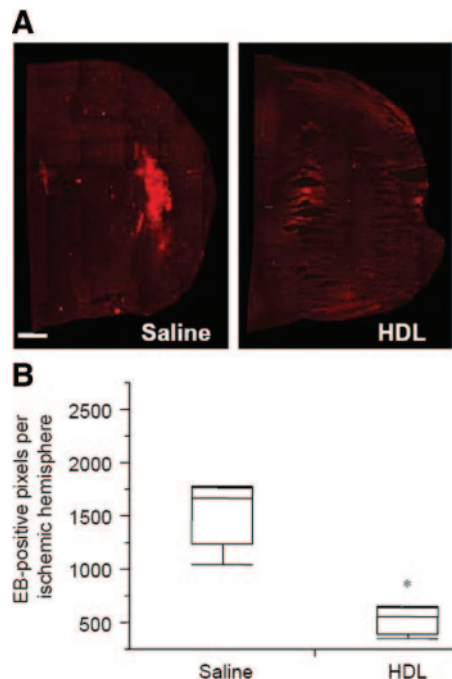
Data are presented as medians (quartiles) for continuous variables and percentages for qualitative variables. We analyzed data by either a Mann-Whitney  $U$  test or, in cases in which  $>1$  group is compared, a Kruskal-Wallis test followed, and if  $P<0.05$ , by a Mann-Whitney  $U$  test. Comparison of mortality 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 7.0.1. The number of animals analyzed is provided in the figure legends.

## Results

### HDL had Beneficial Effects on Death, Infarct Volume, and Neurological Deficit

Compared with saline injection, intravenous administration of HDL immediately after the onset of stroke significantly decreased stroke-related deaths at 24 hours (Figure 1A). A 68.4% reduction in death rate was observed for a dose of 10 mg/kg ( $P=0.015$ ). Relative to saline-treated controls, administration of HDL also significantly reduced infarct size immediately after stroke ( $P=0.0003$ ) and at 3 and 5 hours ( $P=0.011$  and  $P=0.019$ , respectively). This protective effect was dose-dependent (Supplemental Figure I, available online at <http://stroke.ahajournals.org>). Accordingly, the neurological deficit (Figure 1D) at 24 hours after stroke onset was decreased in the HDL-treated group ( $P=0.015$ ). Purified LDL did not reduce infarct size or stroke-related death relative to the saline-injected group ( $P=0.75$  and  $P=0.66$ , respectively). Because HDL has been reported to induce hepatic damage, we assessed circulating hepatic enzyme levels; plasma levels of aspartate aminotransferase and alanine aminotransferase taken during the first 24 hours after stroke did not differ after HDL infusion compared with baseline.



**Figure 2.** A, HDL administration immediately after stroke induction decreased ischemia-induced BBB breakdown as observed by fluorescence microscopy (Evans blue appears in red). Scale bar=2 mm. B, Quantification of Evans blue-positive pixels per ischemic hemisphere.  $n=6$  per group,  $*P=0.0066$ .

### HDL Decreased BBB Breakdown and Brain Edema

We measured Evans blue extravasation in the infarct area at 24 hours after stroke onset. Morphometric quantification

revealed that HDL (10 mg/kg) reduced BBB permeability by 64% compared with control ( $P=0.0066$ ; Figure 2). Brain edema was significantly decreased in the HDL-treated group (10 mg/kg) relative to the saline-treated group (18.1% versus 5.7%, respectively;  $P=0.01$ ).

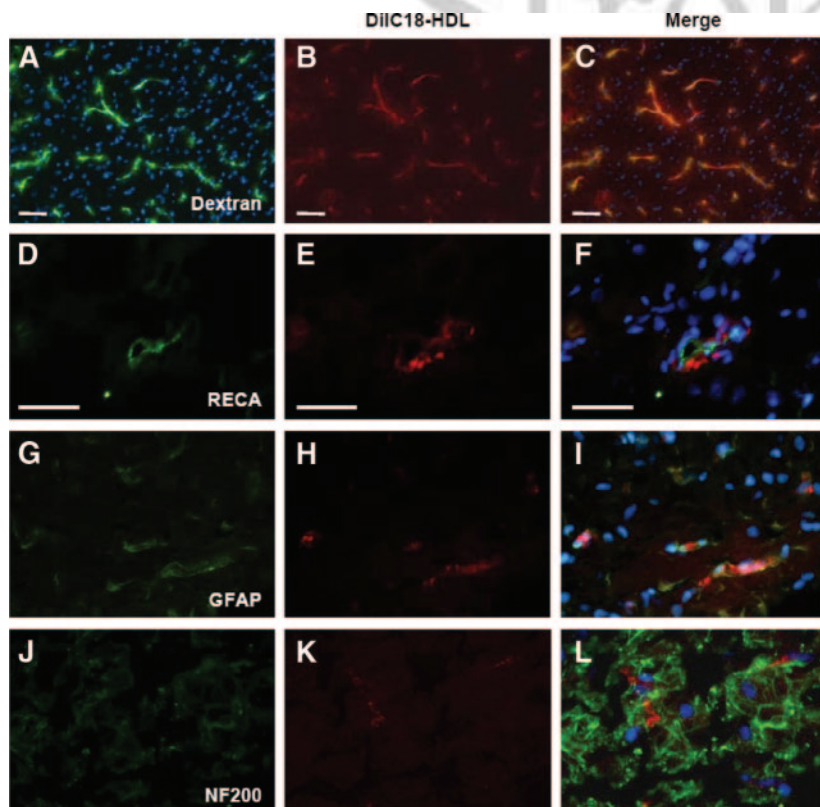
### Labeled HDL Penetrated the Infarct Area

To test whether HDL administration may reach the infarct area and thus directly impact the endothelium, we injected fluorescently labeled HDL immediately after stroke onset. Twenty-four hours later, the rats were injected intravenously with the vascular marker fluorescein isothiocyanate-dextran which was allowed to circulate for 10 seconds before euthanasia. As shown in Figure 3, HDL could penetrate the infarct area and be taken up by endothelial cells and by astrocytes, but no colocalization with neurons was observed.

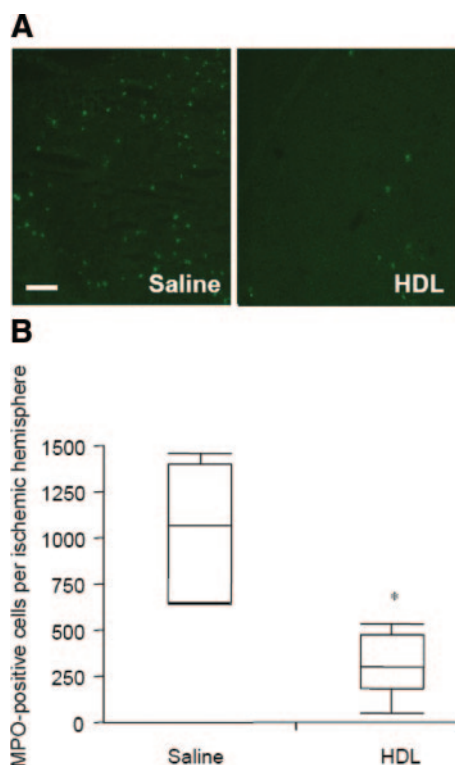
### HDL Reduced PMN Recruitment and Associated MMP Gelatinase Activity in the Infarct Area

Myeloperoxidase immunostaining followed by morphometric quantification (number of myeloperoxidase-positive cells) revealed that injection of 10 mg/kg HDL decreased PMN recruitment by 70% relative to saline-treated controls ( $P=0.027$ ; Figure 4).

In situ zymography showed an increase in the overall gelatinase activity in the ipsilateral infarct area compared with the homologous contralateral control area. After HDL administration, this increase in gelatinase activity appeared less important than in the saline-treated group (Figure 5A). At 24 hours after stroke onset, sodium dodecyl sulfate-



**Figure 3.** HDL penetrated the infarct area and was taken up by endothelial cells and astrocytes but not by neurons. A, Fluorescein isothiocyanate-dextran (70 kDa) injected 10 seconds before euthanasia (blue: nuclear staining by 4', 6'-diamidino-2-phenylindole). B, Same field showing carbocyanine-labeled HDL (DiI18-HDL, red) 24 hours after injection. C, Merged images. D, Rat endothelial cell antigen (RECA) staining. E, H, K, DiI18-HDL (red) 24 hours after injection. G, Immunostaining for glial fibrillary acidic protein (GFAP). J, Immunostaining for neurofilaments (NF200). F, I, L, Merged pictures. Results are representative of 3 independent experiments. Scale bar=50  $\mu\text{m}$ .



**Figure 4.** HDL injected immediately after stroke decreased neutrophil recruitment in the stroke area as shown by myeloperoxidase (MPO) immunostaining (green). A, Immunostaining for MPO on brain sections 24 hours after stroke. Scale bar=200  $\mu$ m. B, Quantification of MPO immunostaining signal (n=6 per group, \* $P=0.027$ ).

polyacrylamide gel electrophoresis gelatin zymography showed an increased activity of MMP-9 in the ischemic area versus the contralateral noninfarcted area. MMP-9 activity was reduced in the infarct area in the HDL-treated group compared with the control group (Figure 5B–D). Similar results were obtained for the neutrophil-associated homodimeric form of MMP-9 (Figure 5B–D).

#### HDL Decreased Intercellular Adhesion Molecule-1-Positive Vessels in the Infarct Area

We postulated that decreased PMN recruitment in the HDL-treated group may be due to a reduced expression of intercellular adhesion molecule-1 (ICAM-1). Immunostaining for ICAM-1 revealed a decrease in ICAM-1-positive vessels in the HDL-treated group compared with controls (Figure 6).

#### Discussion

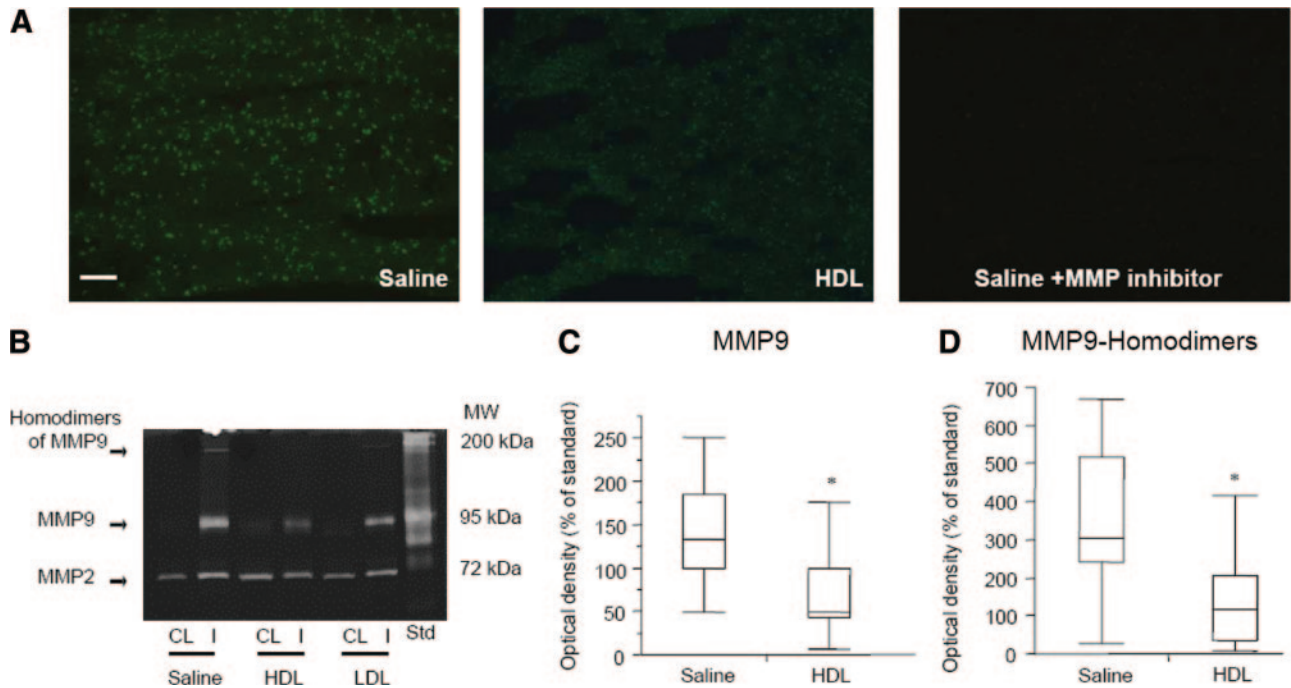
There is mounting epidemiological evidence suggesting that HDL may be protective against cerebrovascular ischemia.<sup>2</sup> The present study shows a beneficial effect of purified human HDL given intravenously within 5 hours after the onset of cerebral ischemia in a rat model of embolic stroke. This embolic model of focal cerebral ischemia is the closest model to human pathophysiology because it is admitted that 80% of strokes in humans involve an embolic mechanism.<sup>14</sup>

To our knowledge, only 1 study has reported a neuroprotective effect of HDL, but this effect was preventive because HDL was injected 2 hours before the onset of

stroke.<sup>15</sup> By contrast, in our study, we injected HDL up to 5 hours after the onset of cerebral ischemia and found a therapeutic neuro- and vasculoprotective effect. Given that the half-life of HDL is 7 to 12 hours in rats, we consider 1 single HDL administration to be sufficient in our stroke model.<sup>12</sup> This timeframe for HDL administration could be applicable to humans; however, multiple injections could maximize the effect of HDL, especially in cases of delayed reperfusion. Also, the dose used in this previous study<sup>15</sup> was 120 mg apoA1/kg, a high dose reported to produce hepatic damage in humans.<sup>16</sup> In our study, HDL exhibited a marked effect at 10 mg/kg, which would be compatible with human therapy. This effect was unlikely to be due to a higher recanalization rate. Indeed, mechanical flushing was ruled out because (1) HDL was injected intravenously; and (2) neither LDL nor saline injected in the same way had protective effects. Moreover, we assessed the persistence of the clot at the termination of the internal carotid artery and at the origin of the middle cerebral artery 24 hours after stroke onset and found no significant difference in recanalization rate among HDL-, LDL-, and saline-treated groups.

The therapeutic benefits observed with HDL were likely to be due to the protection of the BBB. As shown by immunostaining in our experimental conditions, HDL is taken up by endothelial cells and glial cells but not by neurons. Our data agree with previous studies, which report a beneficial role of HDL on astrocyte and cerebral endothelial cell function.<sup>17,18</sup> HDL exerts beneficial effects on endothelial cells in response to different types of aggression, such as tumor necrosis factor  $\alpha$ -induced apoptosis *in vitro*.<sup>19</sup> HDL may also prevent ischemia-induced BBB disruption and associated neutrophil extravasation into the cerebral parenchyma through limitation of the overexpression of adhesion molecules by endothelial cells.<sup>20</sup> Indeed, we found that HDL decreased ischemia-related BBB permeability and subsequent neutrophil infiltration. We report that HDL reduced endothelial ICAM-1 expression in the infarct area compared with nontreated rats. This adhesion molecule is involved in neutrophil recruitment and its expression was also reported to be elevated in the infarct area of the human brain using autopsy material.<sup>21</sup> Our results are in accordance with the study of Cockerill et al.<sup>9</sup> In a model of hemorrhagic shock, they found that HDL decreased neutrophil recruitment and the number of ICAM-1-positive vessels within injured tissues.<sup>9</sup> Leukocyte elastase could also participate in neutrophil extravasation by promoting ICAM-1 expression.<sup>22</sup> Under ischemic conditions, neutrophil elastase may also degrade the basal lamina<sup>23</sup> and promote neuronal death.<sup>24</sup> We recently reported a new antielastase property of HDL attributed to its associated  $\alpha$ -1 antitrypsin.<sup>25</sup> This could participate in the beneficial effects of HDL in our rat model of ischemic stroke, but we cannot exclude other protective mechanisms such as the antioxidant, antithrombotic, or anti-inflammatory properties that have been attributed to HDL.

We followed STAIR recommendations to avoid bias associated with experimental studies.<sup>10</sup> One limitation of



**Figure 5.** A, In situ gelatin zymography. HDL immediately after stroke onset decreased MMP gelatinase activity in the ischemic area. MMP inhibition markedly reduced the fluorescent signal, indicating that this signal is specific for MMP activity (n=4 per group). Scale bar=50  $\mu$ m. B–D, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis zymography of brain-conditioned medium and densitometric analysis showed increased of MMP-9 levels after cerebral ischemia. Levels of MMP-9 and MMP-9 homodimers were increased in the ipsilateral infarct area (I) versus contralateral control area (CL) in the saline-treated group at 24 hours after stroke onset. HDL infusion decreased MMP-9 and MMP-9 homodimer levels in the infarct area. LDL had no effect on MMP-9 or MMP-9-homodimer levels. Std indicates standard. \* $P$ <0.05. Representative gelatin zymogram of 7 independent experiments.

our study was that we used purified human HDL without separating the different fractions. Therefore, this HDL preparation contained all the proteins that are associated with HDL,<sup>25</sup> which may themselves contribute to the beneficial effects. Five different pools of HDL were used with similar results, suggesting that small variations in composition, (ie, content of vitamin E, polyunsaturated fatty acids, etc) between the different batches did not significantly impact the protective effect of HDL. Another limitation of our study is that we did not investigate longer outcome for infarction size and neurological deficit after

HDL injection. Nevertheless, our study was designed to evaluate HDL-based therapy in stroke in acute conditions when BBB breakdown and PMN infiltration are both high. In conclusion, we found a beneficial effect of HDL infusion in an in vivo model of embolic stroke up to 5 hours after the onset of ischemia. Further studies are needed to confirm that HDL-based therapy is applicable to acute stroke in humans.

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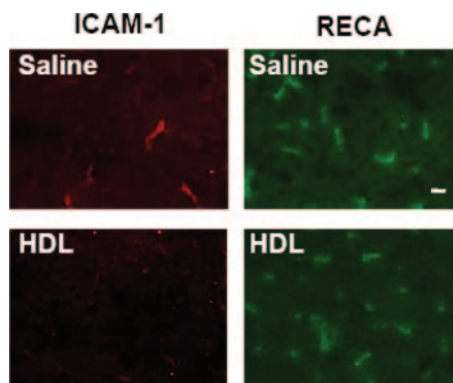
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### Disclosures

None.

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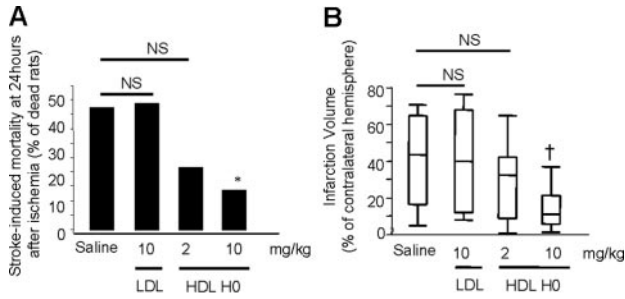


**Figure 6.** HDL decreased ICAM-1-positive vessels in the stroke area. Rat endothelial cell antigen (RECA, green) and ICAM-1 (red) immunodetection in the infarct area (adjacent sections). Results are representative of 4 independent experiments. Scale bar=30  $\mu$ m.

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**Figure I.** Dose effect of HDL administration (2 or 10 mg/kg) immediately after stroke onset. A, Stroke-induced mortality at 24 hours after ischemia. B, Infarction volume at 24 hours after ischemia. n=17 per groups. † $P<0.01$ , \*  $P<0.05$ .

<b>Motor tests</b>	<b>6</b>
<i>Raising rat by the tail</i>	
1. Flexion of forelimb	
1. Flexion of hindlimb	
1. Head moved > 10° to vertical axis within 30s	
<i>Placing rat on the floor</i>	
0. Normal walk	
1. Inability to walk straight	
2. Circling toward the paretic side	
3. Falling over on paretic side	
<b>Sensory tests (vibrissae stimulation)</b>	<b>1</b>
<b>Beam balance tests</b>	<b>6</b>
0. Balances with steady posture	
1. Grasps side	
2. Hugs and one limb falls down	
3. Two limbs fall down or spins (>60s)	
4. Falls off >40s	
5. Falls off >20s	
6. Falls off <20s	
<b>Maximum points</b>	<b>13</b>

**Figure II.** Modified neurological severity score.



# Stroke

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