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RESEARCH PAPER

Neutrophils contribute to intracerebral haemorrhages after treatment with recombinant tissue plasminogen activator following cerebral ischaemia

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Background and purpose: Polymorphonuclear neutrophils (PMNs) contribute to the vascular damage caused by transient cerebral ischaemia. Here we have evaluated the role of PMNs in intracerebral haemorrhage (ICH) induced in a model of thrombolysis with recombinant tissue plasminogen activator (t-PA) during the acute phase of cerebral ischaemia.
Experimental approach: The middle cerebral artery (MCA) of male spontaneously hypertensive rats was occluded for 1 h followed by reperfusion and, 5 h later, infusion of thrombolytic products (generated *in vitro* by t-PA on autologous clots). Effects of pretreatment (before the MCA occlusion) with vinblastine (4 days before; 0.5 $mg \cdot kg^{-1}$), monoclonal anti-neutrophil antibody (mAbRP3; 12 h, 0.3 $mg \cdot kg^{-1}$) or saline on ICH, neutrophil infiltration, MCA vascular reactivity and brain infarct volume were assessed, 24 h after the beginning of reperfusion.
Key results: Depletion of circulating neutrophils significantly reduced t-PA-induced ICH (vinblastine, 4.6 ± 1.0; mAbRP3, 5.2 ± 1.0 vs. saline, 10.8 ± 2.7 haemorrhages; $P < 0.05$). This depletion was associated with a decrease in cerebral infiltration by neutrophils and a decrease of endothelium-dependent, vascular dysfunction in isolated MCA, induced by the ischaemia/reperfusion and t-PA treatment. Brain infarct volume was significantly decreased after vinblastine treatment (159 ± 13 mm^3 vs. 243 ± 16 mm^3 with saline; $P < 0.01$) but not after depletion with mAbRP3 (221 ± 22 mm^3).
Conclusions and implications: Our results showed that pharmacological depletion of PMNs prevented t-PA-induced ICH, in parallel with a decrease in cerebral infiltration by PMNs and a decreased endothelial dysfunction in cerebral blood vessels.
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Keywords: stroke; haemorrhages; t-PA; neutrophils; vascular endothelium

Abbreviations: ICH, intracerebral haemorrhage; I/R, ischaemia/reperfusion; MCAO, middle cerebral artery occlusion; t-PA, tissue plasminogen activator; TLP, thrombolysis products

Introduction

Although clot lysis with recombinant tissue plasminogen activator (t-PA) is effective in acute ischaemic stroke, it is also associated with a risk of haemorrhage, especially deleterious for the patients (Hacke *et al.*, 1995; The NINDS and Stroke t-PA Study Group, 1995). Many hypotheses have been proposed to explain the intracerebral haemorrhages (ICH) during thrombolysis (Tejima *et al.*, 2001), and using adjunctive pharmacological strategies could be one of the interesting ways to prevent these serious complications.

We previously demonstrated the role of t-PA-induced clot lysis in ICH in a mechanical thrombolysis-related brain bleeding model based on the post-ischaemic perfusion of a solution resulting from the *in vitro* interaction between t-PA and blood clots, leading to thrombolysis products (TLP) (Gautier *et al.*, 2003). In this model, perfusion of TLP increased the severity of ICH in comparison with the perfusion of t-PA alone, and was associated with a larger infarct volume and an increased vascular dysfunction in comparison with damage induced by middle cerebral artery occlusion (MCAO) alone. Moreover, the permeability of the blood-brain barrier (BBB) and metalloproteinase 9 (MMP-9) activity were also affected (Kahles *et al.*, 2005). These results added to those demonstrating impairment of vascular reactivity during t-PA treatment, in parallel to aggravation of

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brain injury (Cipolla *et al*., 2000; Wang and Lo, 2003; Nassar *et al*., 2004; Yang *et al*., 2007).

The link between brain injury, vascular impairment and t-PA-related ICH is not clearly defined. Reperfusion-generated free radicals are mediators of the extent of the infarct and of the impairment of vascular function (Petrault *et al*., 2004), and use of antioxidant drugs concomitant to thrombolysis prevented ICH and decreased infarct size (Lapchak and Araujo, 2005). Neutrophils mediate inflammation and contribute to vascular and parenchymal damage (Del Zoppo and Mabuchi, 2003). Pharmacological inhibition of neutrophil emigration from the vasculature or neutrophil depletion after transient MCAO reduced the post-ischaemic alterations of vascular endothelium and in parallel reduced the infarct size (Emerich *et al*., 2002; Petrault *et al*., 2005). Activation of MMP-9 is responsible for matrix degradation and increased permeability of the BBB, associated with ICH (Kelly *et al*., 2006), and inhibition of MMP-9 prevented t-PA-related ICH (Pfefferkorn and Rosenberg, 2003). Interestingly, MMP-9 is up-regulated by t-PA (Ning *et al*., 2006), and the increased MMP-9 levels observed in infarcted areas were related to neutrophil infiltration into brain (Rosell *et al*., 2008).

In the present study, we evaluated in our thrombolysis-related brain haemorrhage model, the contribution of polymorphonuclear neutrophils (PMNs) on t-PA-induced haemorrhages, and in parallel, on vascular endothelial function and infarct volume, both affected during ischaemia and thrombolysis with t-PA. We used two methods to deplete neutrophils, a non-specific agent (vinblastine) and the more specific anti-neutrophil monoclonal antibody (mAbRP3).

Methods

Animals

All experiments were performed in strict accordance with the guidelines of the National Institutes of Health and French Department of Agriculture. In our model, male spontaneously hypertensive rats (SHR) (Elevage Janvier, France), 10 weeks old and weighing 270 to 320 g, were used. The choice of SHR was based on the role of hypertension as a risk factor for haemorrhage during thrombolysis (Gautier *et al*., 2003).

Experimental design

Three different groups of SHR were randomly selected: vehicle-treated group ($n = 9$), vinblastine-treated group ($n = 9$) and mAbRP3-treated group ($n = 9$). All rats were submitted to ischaemia/reperfusion (I/R) and perfused with a t-PA/clot solution, simulating thrombolysis. The rats included in the study were those that completed the whole protocol and had a histologically proven ischaemia.

Neutrophil depletion was induced by i.v. injection of vinblastine (0.5 mg·kg^{-1}, 0.15 mL; EG Labo) 4 days before I/R or by i.p. injection of mAbRP3 (0.3 mg·kg^{-1}, 1 mL; BD Pharmingen) 12 h before ischaemia and again during ischaemia (2 mL) (Sekiya *et al*., 1990). The vehicle group received saline (0.9%).

Twenty four hours after reperfusion, animals were killed and tissues processed to assess vasoreactivity in the MCA and for immunohistochemical and histomorphometric analysis. Blood samples were collected before surgery for MCAO and 24 h later, to count leucocytes and PMNs (Machine XE 2100, Sysmex).

Cerebral ischaemia and t-PA treatment

Under anaesthesia (chloral hydrate 300 mg·kg^{-1}), focal cerebral ischaemia was induced for 60 min by an occlusion of the right MCA with an intraluminal filament inserted in the external carotid artery to the internal carotid artery. Subsequently, the right jugular vein was cannulated with a polyethylene catheter (24 gauge). Physiological parameters (temperature, mean arterial pressure, arterial blood pH, PaO_2 and $PaCO_2$) were controlled throughout the experiments. After the occlusion, animals recovered from anaesthesia and were allowed to eat and drink freely. There was no evaluation of possible neurological deficits. To mimic thrombolysis *in vivo*, a solution of TLP was prepared *in vitro*, as follows. Autologous blood (0.2 mL) was taken from the jugular vein and left for 5 h to form a thrombus. To obtain TLP for perfusion, the thrombus was then fragmented, and t-PA [1.5 mL of t-PA 20 mg·mL^{-1}; this is equivalent to 10 mg·kg^{-1} (6 mL·kg^{-1}) of t-PA for each rat] was applied to the pieces of thrombus for 30 min. The resulting solution (TLP solution, containing mainly plasmin; total volume 2 mL) was collected and perfused slowly (over 1 h) into the rat, 6 h after ischaemia (Gautier *et al*., 2003).

Assessment of ischaemia and evaluation of haemorrhages

Rats were anaesthetized with pentobarbitone (1 mL of 55 mg·mL^{-1}) and the chest opened. Saline (NaCl 0.9%; 60 mL) was perfused intracardially. Then the brain was rapidly removed and frozen at –80°C. Coronal slices (20 μm) were cut at 12 levels according to the stereotaxic section map of Paxinos and Watson. To locate the ischaemia, all sections were stained with cresyl fast violet, a marker of non-necrotizing cells, to permit differentiation between ischaemic and normal tissue. Infarct volume (in mm^3) was measured by using a numerical integration of the respective ischaemic areas for all sections per animal. The volume was corrected for oedema by using the following equation: corrected infarct volume = infarct volume × (right hemisphere volume/left hemisphere volume). Three defined sections (+0.48, –0.92 and –3.30 mm relative to Bregma) were histologically examined for ICH, without knowledge of the treatment group. The incidence of ICH was scaled according to a modified, previously described, method (Niessen *et al*., 2003): 0 = no haemorrhages, 1 = multiple macroscopically visible haemorrhages (seen as petechiae). Severity was estimated as the number of petechial haemorrhages per infarct area. In some animals, *in vivo* magnetic resonance imaging was performed just before death in a 7 tesla narrow bore small animal imaging system (Biospec 70/20 USR, Bruker Biospin, Wissembourg, Germany). We acquired two dimensional T2-weighted images, using turboRARE pulse sequence: TR2500 ms, TE65 ms, FOV: 4 × 4 cm, matrix: 256 × 256, RARE factor 8.

Myeloperoxidase immunohistochemistry

Neutrophil infiltration was assessed by quantifying myeloperoxidase (MPO), an enzyme expressed in neutrophils

(Matsuo *et al.*, 1994). After fixation, sections were blocked and incubated with a 1:500 dilution of rabbit polyclonal anti-MPO primary antibody (DAKO) overnight at 4°C versus a negative control. Sections were then incubated with biotinylated goat anti-rabbit secondary antibody for 3 h at room temperature, followed by an ABC process (ABC kit, Vector) and finally treated with diaminobenzidine. Neutrophil infiltration was quantified at one coronal level (+0.48 mm relative to Bregma) counting positive cells on six adjacent fields of 1 mm^2 in the ischaemic zone. As controls, we used brain sections of sham rats, submitted to surgery for MCAO, without advancing the intraluminal filament into the internal carotid artery and t-PA treatment.

Analysis of the vasoreactivity of isolated MCA

Endothelium-dependent and independent relaxations were assessed in a Halpern arteriograph (Living Systems Instrumentation, Burlington, Vermont, USA) (Petrault *et al.*, 2005). A proximal segment of the right MCA was mounted on two glass cannulas perfused with oxygenated Krebs solution maintained at 37°C and pH 7.4. Experiments were performed under no-flow conditions. The lumen diameter was measured by image analysis and recorded.

All mounted and pressurized arteries were stabilized at a transmural pressure of 100 mmHg for 1 h before experiments. Concentration–response curves for the relaxation to acetylcholine (Ach) were constructed by cumulative addition of Ach (0.001 to 10 $\mu mol \cdot L^{-1}$). NO-mediated relaxations were determined with a single concentration of sodium nitroprusside (SNP 10 $\mu mol \cdot L^{-1}$) on MCA pre-constricted with 5-HT. The vasorelaxant responses observed in MCAs from the three groups of SHR (vehicle, vinblastine and mAbRP3) were compared with responses measured in MCAs from a control group of normotensive Wistar-Kyoto male rats, which served as control group for physiological conditions (Dupuis *et al.*, 2005). Relaxation responses were expressed as percentage of increase of the diameter of the pre-constricted artery.

Statistical analysis

All values were expressed as mean ± standard error mean (SEM). Continuous variables (infarct volumes, number of haemorrhages and MCA relaxation) were compared with a one-way ANOVA followed by a post hoc protected least significant difference (PLSD) Fisher test, if variance analysis was significant. A X^2 analysis was performed to compare results expressed as frequency. A value of $P < 0.05$ was considered to indicate statistically significant differences between means.

Results

Physiological parameters (temperature, blood pressure and gases) remained within the normal range during the 1 h ischaemia and the beginning of reperfusion in all groups. Mortality was low and comparable in the different groups: two vehicle-treated rats and two mAbRP3-treated rats died before the end of the study protocol. At 24 h, group sizes were: vehicle, $n = 7$; vinblastine, $n = 9$ and mAbRP3, $n = 7$.

Effect of vinblastine or mAbRP3 on neutrophils

Animals pretreated with vinblastine or mAbRP3 had a significant reduction in circulating PMNs before the surgery for MCAO (falls of 98% and 54% respectively) and 24 h later (99% and 35% respectively; data not shown). After cerebral I/R and perfusion of TLP, neutrophils were found to have infiltrated the infarct zone (281 ± 117 PMN in vehicle-treated group vs. 2 ± 1 in vehicle sham-operated rats; $P < 0.05$). Treatment with vinblastine or mAbRP3 significantly reduced brain neutrophil infiltration, during I/R and TLP perfusion ($P < 0.05$) (Figure 1). There was no neutrophil infiltration in the contralateral hemispheres.

Effect of neutrophil depletion on ICH

The ICHs induced by t-PA were confined to infarcted areas (Figure 2A). In vehicle-treated rats, I/R and TLP perfusion induced visible petechial haemorrhages (Figure 2B). Neutrophil depletion was associated with a reduction of the incidence of haemorrhage (–33% and –29% respectively; Table 1). When observed, the numbers of petechial haemorrhages were significantly decreased after neutrophil depletion (Table 1, $P < 0.05$). No haematomas were seen.

Effect on neutrophil depletion on cerebrovascular endothelium function

Vascular reactivity, assessed by 5-HT-induced contraction and endothelium-independent, relaxant responses to SNP, was similar in all groups (Table 2). As observed for vehicle-treated rats, I/R accompanied by TLP perfusion induced a significant loss ($P < 0.05$) in endothelium-dependent relaxing responses

Table 1 Histological examination of incidence and severity of intracerebral haemorrhages after ischaemia/reperfusion and tissue plasminogen activator treatment in rats treated with vehicle (saline 0.9%), vinblastine (0.5 $mg \cdot kg^{-1}$) or mAbRP3 (0.3 $mg \cdot kg^{-1}$)

	Histological score		*Severity*
	0 = no haemorrhage	*1 = macroscopically visible haemorrhage*	*Mean number of petechial haemorrhages ± SEM*
Vehicle ($n = 7$)	1	6	10.8 ± 2.7
Vinblastine ($n = 9$)	3	6	4.6 ± 1.0*
mAbRP3 ($n = 7$)	2	5	5.2 ± 1.0*

*$P < 0.05$ vs. vehicle.

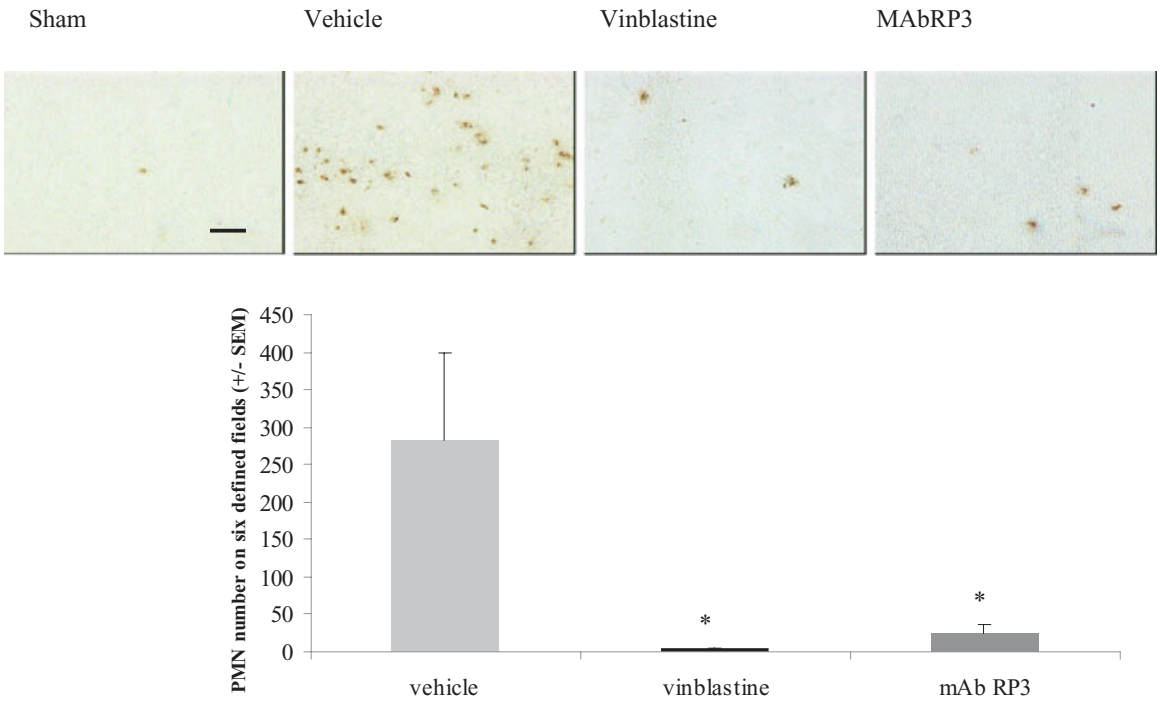

Figure 1 Effect of i.v. administration of vehicle (NaCl 0.9%), vinblastine (0.5 mg·kg^{-1}) or mAbRP3 (0.3 mg·kg^{-1}) on neutrophil infiltration in rats submitted to ischaemia/reperfusion and TLP treatment. Infiltration was quantified by counting cells positive to anti-MPO antibody on six adjacent fields of 1 mm^2 in ischaemic zones. Values are mean ± SEM. *$P < 0.05$ vs. vehicle. Scale bar: 100 µm. MPO, myeloperoxidase; PMN, polymorphonuclear neutrophil; TLP, thrombolysis products.

Table 2 Vasoreactive effects of acetylcholine (Ach), 5-HT and sodium nitroprusside (SNP) on middle cerebral artery

	Control	*24 h after ischaemia/reperfusion and tissue plasminogen activator* treatment		
		Vehicle	*Vinblastine*	*mAbRP3*
Ach 10 µmol·L^{-1} (% relaxation)	20 ± 1.1	9.2 ± 0.5*	23.4 ± 5.1#	20.5 ± 3.8#
EC_{50} (µmol·L^{-1})	0.036 ± 0.008	0.127 ± 0.027*	0.019 ± 0.010#	0.005 ± 0.002#
SNP 10 µmol·L^{-1} (% relaxation)	58.36 ± 5.18	46.1 ± 12.6	66.0 ± 11.6	66.7 ± 19.7
5-HT 1 µmol·L^{-1} (% constriction)	29.41 ± 3.80	34.6 ± 3.0	39.1 ± 3.1	33.4 ± 4.2

The EC_{50} to Ach was calculated from dose–response curves. Values are mean ± SEM.
*$P < 0.05$ vs. control.
#$P < 0.05$ vs. vehicle.

to increasing doses of Ach, as compared with control. This endothelial dysfunction was significantly prevented by neutrophil depletion ($P < 0.05$, Table 2). Sensitivity to Ach (as EC_{50}) was decreased during I/R and TLP perfusion, but this decrease was prevented when PMNs were depleted ($P < 0.05$; Table 2).

Effect of neutrophil depletion on cerebral infarct size

Polymorphonuclear neutrophil depletion induced by vinblastine induced a significant decrease in both total and cortical infarct volume (159 ± 13 mm^3 and 105 ± 14 mm^3 respectively in vinblastine-treated group vs. 243 ± 16 mm^3 and 183 ± 13 mm^3 in vehicle-treated rats; $P < 0.01$) (Figure 3). PMN depletion induced by mAbRP3 was not associated with a significant decrease in total and cortical infarct volumes (221 ± 22 mm^3 and 167 ± 20 mm^3 respectively in mAbRP3-treated rats). There was no difference in striatal infarct size in all three experimental groups.

Discussion

In the present work, we demonstrated that ICH associated with the use of t-PA in cerebral ischaemia can be partially prevented by depletion of PMNs. In our experimental model of thrombolysis-related brain haemorrhage, vinblastine and mAbRP3 decreased the incidence and severity of ICH. In parallel, these treatments reduced neutrophil infiltration in infarcted areas as well as preventing the endothelial dysfunction induced by the combination of I/R and TLP perfusion. The prevention of t-PA-induced ICH was not associated with

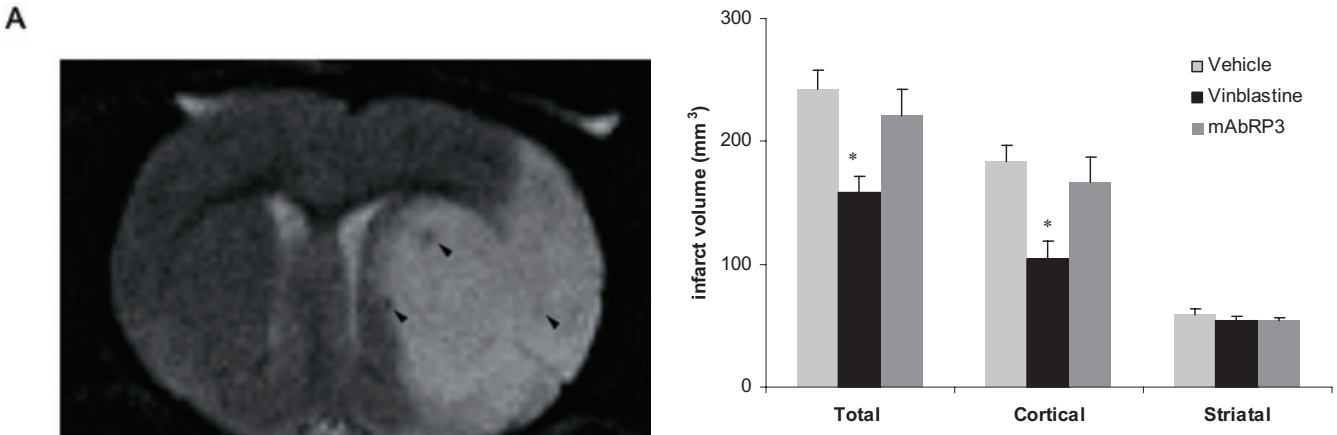

Figure 3 Effect of i.v. administration of vehicle (NaCl 0.9%), vinblastine (Vb: 0.5 mg·kg^{-1}) or mAbRP3 (0.3 mg·kg^{-1}) on total, cortical and striatal infarct volume (corrected for oedema). All rats were submitted to ischaemia/reperfusion and thrombolysis products treatment. (*$P < 0.05$ vs. vehicle). Volumes are expressed in mm^3 (mean ± SEM).

Figure 2 Effect of i.v. administration of vehicle (NaCl 0.9%), vinblastine (0.5 mg·kg^{-1}) or mAbRP3 (0.3 mg·kg^{-1}) on haemorrhagic risk in rats submitted to ischaemia/reperfusion and thrombolysis products treatment. Intracerebral haemorrhages were confined to infarct areas as seen *in vivo* on T2-weighted MRI images (A). Petechial haemorrhages were macroscopically visible on histological sections (B).

a decrease in infarct size, as treatment with vinblastine or mAbRP3 did not have the same protective effect.

In our experimental model of thrombolysis-related brain haemorrhage, we provided evidence of the crucial role of neutrophils in t-PA-induced ICH. Previous studies have already highlighted the role of neutrophils in the physiopathology of cerebral ischaemia and neuronal death, through vascular and brain damage (Emerich *et al.*, 2002; Petrault *et al.*, 2005; McColl *et al.*, 2007; Wang *et al.*, 2007). In haemorrhagic consequences of cerebral ischaemia, the role of neutrophils is complex because circulating neutrophils do not seem to be significant contributors (Harris *et al.*, 2005) whereas the numbers of cerebral infiltrating neutrophils are significantly associated with these complications, via MMP-9 (Wang and Lo, 2003; Rosell *et al.*, 2008). In spontaneous ICH, the role of neutrophils is controversial as inflammatory mediators released from activated neutrophils participate in the functional deficit after ICH, whereas administration of G-CSF, stimulator of the neutrophil granulocyte lineage, has beneficial effects on this ICH-associated deficit, through an anti-inflammatory effect (Park *et al.*, 2005). Our work showed for the first time that neutrophils are directly implicated in t-PA-induced ICH in cerebral ischaemia.

Our data also suggested that neutrophils were involved in t-PA-induced haemorrhages by their interactions with cerebral vasculature rather than by their effects on cerebral tissue. Vinblastine and mAbRP3 prevent post-ischaemic and TLP-induced endothelial dysfunction as demonstrated by the effect on the relaxing responses of the MCA, which is an indirect index of arteriolar damage, as the proximal part of the artery is structurally different from the distal part. The specific depletion of neutrophils induced by mAbRP3 diminished the haemorrhagic risk of thrombolysis and prevented the (I/R and TLP)-induced alterations of endothelial response to Ach (a marker of endothelial function), to the same extent as vinblastine, while there was a difference between the two treatments in terms of their effect on infarct size. This difference could be explained by the pleiotropic effect of vinblastine, inducing a non-specific depletion of leukocytes and also interfering with other cell types, such as platelets. This difference in reduction of infarct size between vinblastine and mAbRP3 was disclosed in the SHR, which are more susceptible to ischaemia than normotensive rats (Coyle, 1986). We have previously demonstrated that the same dose of mAbRP3 was protective in normotensive rats (Petrault *et al.*, 2005). This difference is interesting for the purpose of our work, as it demonstrates the lack of correlation between decreased infarct size and reduced t-PA-induced ICH, although severity and duration of ischaemia are often described as a risk factor for haemorrhagic sequelae after thrombolysis (Larrue *et al.*, 1997; Thomalla *et al.*, 2007). These data highlight a stronger link between vascular lesions and thrombolysis-related haemorrhages, through leukocyte–endothelium interactions.

The vascular impact of the *in situ* activation of PMN has been described in various experimental models of I/R (Akopov

et al., 1994; Ishikawa *et al*., 2004). It is probably linked to rolling and adhesion of PMN to the endothelium, following increased expression of adhesion proteins (selectins and cell adhesion molecules) (Okada *et al*., 1994). However, neutrophil adhesion alone cannot explain all the vascular lesions, and a major role is probably also played by migration of PMN towards the infarct zone across the endothelium and, more generally, through the vessel wall (Justicia *et al*., 2006). Given that neutrophils are responsible for vascular damage (loss of tight junctions and proteolysis of the extracellular matrix among others), this phenomenon can favour the extravasation of red blood cells and thus ICH (Dijkhuizen *et al*., 2002). Direct proof comes from the fact that PMN were observed to accumulate inside microvessels in the infarct area in parallel to t-PA-induced ICH (Kano *et al*., 2000). Moreover, in human stroke, infiltrated PMN in infarcted areas are associated with breakdown of the BBB, degradation of basal lamina (via MMP-9 activation) and extravasation of blood (Rosell *et al*., 2008).

The mechanisms by which neutrophils, vessels and t-PA interact remain to be determined in our model. Plasmin, resulting from clot lysis with t-PA, has already been implicated in the physiopathology of haemorrhagic complications, as plasmin is capable of activating leukocytes and thus increases BBB breakdown and vascular permeability (Montrucchio *et al*., 1996; Xue and Del Bigio, 2001). In turn, PMN are responsible for the activation and release of metalloproteases during cerebral ischaemia (Asahi *et al*., 2000; Justicia *et al*., 2003). It is now well proven that these proteases aggravate the infarction and contribute to the haemorrhagic risk of thrombolysis (Aoki *et al*., 2002; Sumii and Lo, 2002; Castellanos *et al*., 2003; Gidday *et al*., 2005). Moreover, PMNs seem to be the main source of MMP-9 in haemorrhagic areas and particularly around brain microvessels (Rosell *et al*., 2008). Finally, t-PA directly stimulated release of MMP-9 by degranulation of neutrophils (Cuadrado *et al*., 2008).

Our results underline the importance of the contribution of neutrophils, probably through vascular damage, in the physiopathology of the t-PA-related ICH, independent of the infarct lesions. The prevention of leukocyte–endothelium interactions could constitute a development pathway for adjuvant treatments for thrombolysis, which would broaden the therapeutic window of t-PA. Some pharmacological agents (such as adhesion protein inhibitors or anti-PMN antibodies) may well be useful in this respect.

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Conflict of interest

None.

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