Protective effects of leukopenia and tissue plasminogen activator in microvascular ischemia-reperfusion injury

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Bertuglia, Silvia, and Antonio Colantuoni. Protective effects of leukopenia and tissue plasminogen activator in microvascular ischemia-reperfusion injury. Am. J. Physiol. Heart Circ. Physiol. 278: H755–H761, 2000.—Ischemia shifts the anticoagulant/procoagulant balance of the endothelium in favor of activation of coagulation. We studied whether cheek pouch microcirculation of leukopenic hamsters was protected by tissue plasminogen activator (tPA) (50 µg·100 g body wt) against ischemia-reperfusion injury. Adherent leukocytes, total perfused capillary length (PCL), permeability increase, and arteriolar and venular red blood cell (RBC) velocity were investigated by fluorescence microscopy. Measurements were made at control, 30 or 60 min of ischemia, and at 30 or 60 min of reperfusion. Hamsters were made leukopenic by treatment with cyclophosphamide (20 mg/100 g body wt ip, 4 days before the experiment), which decreased circulating leukocyte count by 85–90%. Leukopenic hamsters undergoing 30 min of ischemia followed by 30 min of reperfusion showed no significant decrease in PCL or increased permeability. Leukopenic hamsters undergoing 60 min of ischemia followed by 60 min of reperfusion presented a significant decrease in microvascular perfusion where PCL was 28 ± 7% of baseline, low-flow conditions, and increased permeability. In leukopenic hamsters treated with tPA there was complete protection of capillary perfusion with no significant changes in permeability or arteriolar and venular RBC velocity. In conclusion, thrombus formation may be an additional and independent factor that with leukocyte-mediated mechanisms determines ischemia-reperfusion injury.

Ther is considerable evidence that leukocytes are responsible for microvascular injury during ischemia and reperfusion (1, 2, 14). Leukocyte capillary plugging and venular adhesion would lead to activation of the inflammatory cascade and to release of superoxide anions (O2-) and proteases with impairment of microvascular flow (7, 13, 25). Reports on microvascular protection provided by leukopenia against ischemia-reperfusion are, however, conflicting. Leukopenia had no effect on tissue injury in the mesentery, and tissue injury was eliminated by leukopenia in the cremaster (8). No significant changes were detected in ischemia-reperfusion lung injury in neutrophil-depleted rats (16). A similar reduction in microvascular flow after 6 h of reperfusion was observed in control dogs and in leukopenic dogs where the latter had no fibrillation events (4).

Endothelial cells are the major sites of synthesis of tissue plasminogen activator (tPA), which regulates the fibrinolytic system (19, 22, 29). Oxygen deprivation during ischemia shifts the natural anticoagulant/procoagulant balance of the endovascular wall favoring activation of coagulation. Recent data show that tPA inhibits superoxide anion production in human neutrophils in vitro and improves mortality in patients with acute myocardial infarction (21, 24, 27). Our previous work (5, 8, 17, 28) shows that 30 min of ischemia and 30 min of reperfusion is associated with decreased capillary perfusion, leukocyte activation, and generation of free radicals in hamster cheek pouch microcirculation. Leukocytes appear to have a central role in thrombosis, suggesting that leukocyte-dependent processes in ischemia-reperfusion injury may have significant effects on fibrinolytic processes.

Our aim was to analyze the role of the fibrinolytic system and leukocytes in ischemia-reperfusion injury, thus we measured microvascular perfusion after 60 min of ischemia and 60 min of reperfusion in nonleukopenic and leukopenic hamsters. A group of leukopenic hamsters was treated with tPA. We measured the number of adherent leukocytes, the total perfused capillary length (PCL), the permeability increase, and arteriolar and venular red blood cell (RBC) velocity. Studies were made in the cheek pouch microvascular model using fluorescence microscopy.

MATERIALS AND METHODS

Male Syrian hamsters (80–100 g; Charles River, Como, Como, Italy) were randomly assigned to six different treatment groups. Control hamsters (CH60, n = 7) and leukopenic hamsters (LH60, n = 7) were treated with vehicle infusion and subjected to a period of 60 min of ischemia and 60 min of reperfusion. Control (CHtPA, n = 7) and leukopenic groups (LHtPA, n = 7) were treated with tPA (human rtPA, Sigma Chemical, St. Louis, MO) (50 µg·100 g body wt) iv infusion, injected 5 min before 60 min of ischemia and 60 min of reperfusion). Another group of leukopenic hamsters (LH30, n = 7) was subjected to 30 min of ischemia and 30 min of reperfusion and compared with control hamsters (CH30, n = 7) submitted to the same procedure.

Leukopenia was induced by treatment with cyclophosphamide (Endoxan-Asta, Asta Medica) (20 mg/100 g body wt ip, 4 days before the experiments). White blood cell and platelet counts were made before the treatment and after preparation.

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of the cheek pouch. Measurements were made before ischemia and at 30 or 60 min after the start of reperfusion.

The cheek pouch was surgically prepared as previously reported (2). Anesthesia was induced by pentobarbital sodium injection (Nembutal, 5 mg/100 g body wt ip). The animals were tracheotomized, and the right carotid artery and femoral vein were cannulated to measure blood pressure and to administer additional anesthesia and fluorescent tracers. The cheek pouch was gently everted and fixed to a special stage of the microscope, and a thin black blade was inserted through a small incision between the upper and lower layers of the pouch. The membrane was suffused with a 36 ± 0.5°C Ringer solution (4 ml/min) with 5% CO2-95% N2 adjusted to pH 7.35.

Atraumatic microvascular clips were placed on the proximal part of the cheek pouch to achieve complete ischemia for 30 or 60 min. The clamp was then removed, and microcirculation was observed after 30 or 60 min.

Observations were made with a fluorescent microscope (Leitz Orthoplan) and a filter block (Ploempak, Leitz) fitted with a long-working distance objective [×4, numerical aperture (NA) 0.14; ×20, NA 0.25] and a ×10 eyepiece. Epi-illumination was provided by a xenon 150-W lamp using the appropriate filters for FITC bound to dextran (molecular weight 150,000, 50 mg/100 g body wt iv as 5% wt/vol solution) and appropriate filters for acridine red (Chroma, Stuttgart, Germany) (1 mg/100 g body wt in 0.3 ml) and a supplemental injection of acridine red (Leitz KG1). The animals received an intravenous injection of acridine red (Chroma, Stuttgart, Germany) (1 mg/100 g body wt in 0.3 ml) and a supplemental injection (final volume 0.3 ml · 100 g body wt iv) with 5% CO2-95% N2 adjusted to pH 7.35.

Table 1. Effects of leukopenia and tPA on ischemia-reperfusion

<table>
<thead>
<tr>
<th></th>
<th>CH30</th>
<th>CH60</th>
<th>CH1PA</th>
<th>LH30</th>
<th>LH60</th>
<th>LH1PA</th>
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<tbody>
<tr>
<td>n</td>
<td>30</td>
<td>30</td>
<td>10</td>
<td>14</td>
<td>14</td>
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<tr>
<td>Leukocytes/ 100 µm venules</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>2.0 ± 0.7</td>
<td>1.9 ± 0.7</td>
<td>1.5 ± 0.7</td>
<td>2.0 ± 0.4</td>
<td>1.5 ± 1.0</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>Reperfusion</td>
<td>19.0 ± 1.5‡</td>
<td>22.1 ± 2.4‡</td>
<td>7.0 ± 0.9*</td>
<td>1.4 ± 0.4</td>
<td>3.5 ± 1.5</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>PCL</td>
<td>9,990 ± 550</td>
<td>9,480 ± 450</td>
<td>10,080 ± 400</td>
<td>9,080 ± 560</td>
<td>8,900 ± 300</td>
<td>9,900 ± 420</td>
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<tr>
<td>Permeability</td>
<td></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>0.05 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.02 ± 0.01‡</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>Ischemia</td>
<td>0.25 ± 0.06*</td>
<td>0.35 ± 0.06*</td>
<td>0.20 ± 0.04*</td>
<td>0.09 ± 0.02†</td>
<td>0.15 ± 0.03‡</td>
<td>0.06 ± 0.03‡</td>
</tr>
<tr>
<td>Reperfusion</td>
<td>0.60 ± 0.09*</td>
<td>0.89 ± 0.07*</td>
<td>0.75 ± 0.04‡</td>
<td>0.10 ± 0.04‡</td>
<td>0.20 ± 0.06‡</td>
<td>0.08 ± 0.04‡</td>
</tr>
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Values are means ± SD; n, no. of venules observed. Effects of leukopenia and tPA after 30 min or 60 min of ischemia-reperfusion in leukopenic groups (LH30, LH60, LH1PA) compared with controls (CH30, CH60, CH1PA). tPA, tissue plasminogen activator. Adherent leukocytes are expressed as number/100 µm of venular length; perfused capillary length (PCL) after reperfusion as percentage of baseline; and permeability is given in terms of gray levels normalized relative to baseline. *P < 0.05 compared with baseline; †P < 0.01 compared with baseline; ‡P < 0.01 compared with control group.
**CH60 group.** Adhesion of leukocytes was higher after 60 min of reperfusion compared with that observed in CH30 animals (Table 1), whereas PCL decreased significantly (42 ± 4% of baseline, 9,480 ± 450 µm), see Table 1. Figure 1 reports the time course of the percentage decrease in PCL compared with the baseline in the experimental groups. The increase in permeability was pronounced in venules (Table 1), whereas RBC velocity in arterioles and venules decreased significantly at 60 min of reperfusion to 0.65 ± 0.15 and 0.37 ± 0.10 from 1.25 ± 0.20 and 0.80 ± 0.19 mm/s (baseline); \( P < 0.01 \) compared with baseline (Fig. 2).

An example of the cheek pouch microvasculature after 60 min of ischemia and 60 min of reperfusion is shown in Fig. 3. It is possible to observe vessels with intraluminal clotting, and the increase in permeability is evident.

**CH30 group.** Leukopenia and tPA in Ischemia Reperfusion

All cyclophosphamide-pretreated groups were severely leukopenic at the beginning of the experiments compared with the control group and remained so throughout. Leukocytes were 789 ± 30/mm³ compared with baseline.
with the baseline value of 5,890 ± 350/mm³. There were no adherent leukocytes in venules in baseline conditions, and there was no significant increase in the number of adherent leukocytes after ischemia-reperfusion in this group (Table 1).

Platelets did not change significantly after treatment with cyclophosphamide when compared with baseline (250,000 ± 60,000/mm³ vs. 290,000 ± 50,000/mm³, respectively).

LH 30 hamsters had a slight change in PCL that was 90 ± 6% of baseline (9,080 ± 560 µm) (Fig. 1). Leukopenia significantly attenuated the increased microvascular permeability induced by 30 min of ischemia and reperfusion (Table 1). Arteriolar and venular RBC velocity (n = 27) were 1.35 ± 0.20 and 0.90 ± 0.10 versus 1.30 ± 0.15 and 0.92 ± 0.15 mm/s during baseline (Fig. 2).

LH 60 hamsters had a significant decrease in PCL that was 28 ± 7% of baseline (8,990 ± 300 µm). Permeability increased compared with baseline; however, leakage was reduced compared with control (Table 1). Arteriolar and venular RBC velocities (n = 27) were 0.45 ± 0.10 and 0.23 ± 0.09 versus 1.40 ± 0.25 and 0.95 ± 0.20 mm/s during control, respectively, and at 60 min of reperfusion (Fig. 2). In Fig. 4, we show the cheek pouch microcirculation in baseline conditions and after 60 min of ischemia followed by 60 min of reperfusion in a leukopenic hamster. In Fig. 5, we present an example of intravascular clotting at the end of 60 min of reperfusion in a leukopenic hamster.

In the LH tPA group, there was no significant decrease in microvascular perfusion because PCL was 97 ± 1% of baseline (9,900 ± 420 µm) (Fig. 1). Permeability did not increase significantly when compared with baseline (Table 1). At 60 min of reperfusion, arteriolar and venular RBC velocity (n = 27) were 1.30 ± 0.15 and 0.89 ± 0.10 versus 1.45 ± 0.25 and 1.00 ± 0.22 mm/s at baseline, respectively (Fig. 2).

In Fig. 6 we show the cheek pouch microcirculation in control conditions and after 60 min of ischemia followed by 60 min of reperfusion in a leukopenic hamster treated with tPA.

Mean arterial pressure (MAP) and heart rate were 90 ± 7 mmHg and 280 ± 10 beats/min under baseline conditions and did not change significantly after reperfusion. Leukopenia and tPA did not affect MAP and heart rate significantly.

**DISCUSSION**

Our study shows that early injury in the cheek pouch microcirculation during ischemia-reperfusion is leukocyte dependent, whereas the late injury is caused by the inhibition of the fibrinolytic system. In leukopenic hamsters treated with tPA microvascular flow was maintained with no change in the PCL, microvascular permeability, and RBC velocity when compared with the baseline. Activation of the fibrinolytic system and leukocyte-mediated effects during ischemia may be key components contributing to cause microvascular dysfunction and the no-reflow phenomenon.

Leukocyte depletion preserves capillary perfusion and reduces macromolecular leakage of the cheek pouch microcirculation after 30 min of complete ischemia followed by 60 min of reperfusion. Leukopenia does not improve capillary perfusion after 60 min of ischemia followed by 60 min of reperfusion because of a significant reduction in the PCL, increased microvascular permeability, and low arteriolar and venular RBC velocity. The protection provided solely by the fibrinolytic system is apparent in terms of the lower leukocyte adhesion seen in tPA-treated animals under control conditions; however, this does not significantly preserve arteriolar and venular velocities and functional capillary density during ischemia and reperfusion.

Our data show that microvascular damage after 30 min of ischemia and 30 min of reperfusion (2, 3) were completely counterbalanced by leukopenia. We did not...
detect a relevant increase in permeability in leukopenic hamsters after 30 min of ischemia and reperfusion. The slight increase in permeability is probably caused by the absence of inflammatory mediators released by leukocytes (20). The relative protection exerted by leukopenia may depend on the low levels of circulating cytokines, oxygen free radicals, and tPA inhibitor.

Conversely, leukopenic hamsters after 60 min of ischemia followed by 60 min of reperfusion showed a marked decrease in PCL, indicating a pronounced reduction in microvascular blood flow, concomitant to a moderate increase in permeability associated to the no-flow conditions in capillaries and venules. Therefore, it would appear that leukocyte adhesion is not the major mechanism causing the no-reflow phenomenon during ischemia-reperfusion injury because capillary occlusions occurred in the absence of leukocytes. Our data show that both nonleukopenic and leukopenic hamsters were damaged by the longer period of ischemia, indicating that inadequate activation or inhibition of fibrinolysis contributes to ischemia-reperfusion injury.

In leukopenic hamsters treated with tPA, capillary perfusion was completely preserved, as indicated by the unchanged perfused capillary length and the lack of increased macromolecular leakage.

tPA is produced by endothelial cells that line the vascular system. However, recent data show that capillaries and small microvessels but not the endothelium of larger pulmonary vessels stain positive for tPA. Pulmonary vessels that contain endothelial cell-derived tPA were consistently between 7 and 30 µm in diameter (15). Therefore, it has been suggested that the steady-state level of plasma tPA is maintained through its constitutive production by endothelial cells of the smaller vessels of the vascular system.

Our results indicate that there was interruption of flow in arterioles and venules in leukopenic hamsters, which was counterbalanced by tPA. Therefore, tPA might play a role in maintaining flow in arterioles and venules, through the maintenance of capillary patency, thus contributing to the reduction of the no-reflow phenomenon after reperfusion. Furthermore, the protec-
tion afforded by tPA against the increase in microvascular permeability might be related to the reduction of $O_2$ due to tPA (24).

Leukocytes have a profound influence on the fibrinolytic system because the no-reflow phenomenon during ischemia followed by reperfusion appears to be modulated by tPA in leukopenic hamsters. Recent data show a greater tendency of fibrinolytic inhibition in sepsis in nonleukopenic patients; furthermore, in shock conditions, levels of tPA inhibition were high in both the nonleukopenic and leukopenic groups (9). Thus thrombosis does not appear to be a factor in ischemia-reperfusion damage in hemorrhagic shock. Conversely, our data show that thrombosis contributes to 60-min ischemia-reperfusion injury, whereas this is not the case for leukopenic hamsters treated with tPA.

Inhibition of the fibrinolytic system under ischemia may determine intravascular coagulation, thereby contributing to the capillary no-reflow phenomenon. It is likely that tPA works by lysing intravascular thrombi and improving reperfusion, thereby reducing capillary occlusion. However, platelets are present in leukopenic animals and their activation may trigger the release of secretory products, leading to microvascular obstruction and vascular endothelium damage during ischemia (12, 26). It has been reported that the cause of preoperative ischemia and myocardial infarction in coronary artery bypass graft patients may depend on the increased number of platelet-endothelial interactions in the microvasculature (23).

Our findings suggest a progression of thrombotic disorders in leukopenic hamsters after a prolonged period of ischemia that might be related to platelet aggregation.

In leukopenic hamsters after 60 min of ischemia there were significantly lower arteriolar and venular RBC velocities, whereas these were in the normal range in leukopenic hamsters treated with tPA. Leukocytes are a factor in determining blood viscosity (10), thus leukopenia may lower blood viscosity and improve RBC velocity after 30 min of reperfusion. After 60 min of ischemia-reperfusion the fibrinolytic system might become unbalanced, leading to accelerated intravascular clotting, which increases blood flow resistance, lowering perfusion.

Recent data showed preservation of endothelial-dependent vasorelaxation after 45 min of occlusion and 45 min of reperfusion in the mesenteric artery of adhesion molecule-deficient mice (25). In neutrophil-poor pigs, neutrophils and platelets can influence the vasocostrictive responses of arteries (18). The level of inflammatory mediator thromboxane B2 was lower in leukocyte-depleted patients during cardiopulmonary bypass thus reducing the inflammatory response (6).

Therefore, it was suggested that a decrease in tPA release in response to decreased flow might be also responsible for decreased endothelial cell responsiveness during ischemia and reperfusion (11). Consistent with these data leukopenic hamsters treated with tPA had normal RBC velocity and capillary blood flow. Furthermore, leukopenic animals were protected from the damage to the capillary barrier caused by adherent leukocytes.

In conclusion, leukopenia and tPA are protective in ischemia-reperfusion injury suggesting that the no-reflow phenomenon may be a consequence of the inhibition of the fibrinolytic system during ischemia and reperfusion. Our data are consistent with a progression of thrombotic disorders in leukopenic hamsters as a function of the duration of ischemia. Furthermore, ischemia and reperfusion damage may be limited by the protective effect exerted by tPA during the ischemic period on the endothelium.

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