Paradoxical attenuation of leukocyte rolling in response to ischemia-reperfusion and extracorporeal blood circulation in inflamed tissue

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Submitted 7 July 2004; accepted in final form 24 January 2005

Schäfer, Stephan C., Desiree N. Sehrt, Markus Kamler, Heinz Jakob, and Hans-Anton Lehr. Paradoxical attenuation of leukocyte rolling in response to ischemia-reperfusion and extracorporeal blood circulation in inflamed tissue. Am J Physiol Heart Circ Physiol 289: H330–H335, 2005; doi:10.1152/ajpheart.00674.2004.—In contrast to acute preparations such as the exteriorized mesentery or the cremaster muscle, chronically instrumented chamber models allow one to study the microcirculation under “physiological” conditions, i.e., in the absence of trauma-induced leukocyte rolling along the venular endothelium. To underscore the importance of studying the naive microcirculation, we implanted titanium dorsal skinfold chambers in hamsters and used intravital fluorescence microscopy to study venular leukocyte rolling in response to ischemia-reperfusion injury or extracorporeal blood circulation. The experiments were performed in chambers that fulfilled all well-established criteria for a physiological microcirculation as well as in chambers that showed various extents of leukocyte rolling due to trauma, hemorrhage, or inflammation. In ideal chambers with a physiological microcirculation (<30 rolling leukocytes/mm vessel circumference in 30 s), ischemia-reperfusion injury and extracorporeal blood circulation significantly stimulated leukocyte rolling along the venular endothelium and, subsequently, firm leukocyte adhesion. In contrast, both stimuli failed to elicit leukocyte rolling in borderline chambers (30–100 leukocytes/mm), and in blantly inflamed chambers with yet higher numbers of rolling leukocytes at baseline (>100 leukocytes/mm), we observed a paradoxical reduction of leukocyte rolling after ischemia-reperfusion injury or extracorporeal blood circulation. A similar effect was observed when we superfused leukotriene B4 (LTB4) onto the chamber tissue. The initial increase in leukocyte rolling in response to an LTB4 challenge was reversed by a second superfusion 90 min later. These observations underscore 1) the benefit of studying leukocyte-endothelial cell interaction in chronically instrumented chamber models and 2) the necessity to strictly adhere to well-established criteria of a physiological microcirculation.

The multistep concept of leukocyte-endothelial cell interaction proposes early leukocyte tethering and rolling along the endothelial wall followed by firm leukocyte adhesion and transendothelial emigration; each consecutive step involves distinct adhesion molecules and distinct chemoattractant and/or adhesion-promoting mediators (5, 21, 23, 29). It is widely established that leukocyte rolling along the endothelium constitutes a prerequisite for subsequent leukocyte adhesion and emigration (22). However, Kanwar and co-workers (21) identified an enormous redundancy in this system: even an inhibition of leukocyte rolling by >90% with fucoidin or selectin antagonists exerted no significant effect on subsequent firm leukocyte adhesion.

In intact animal organisms, the study of leukocyte-endothelium interaction can be accomplished by intravital microscopy on acutely prepared tissues (e.g., hamster cheek pouch, cremaster muscle, and exteriorized mesentery) or on chronically instrumented tissues [e.g., the dorsal skinfold chamber model in hamsters (10, 28) and mice (27)]. Although in most acutely prepared tissues, there is always “spontaneous” leukocyte rolling under baseline conditions (7), baseline leukocyte rolling is virtually absent in carefully executed chronically instrumented tissues and is even considered a marker of tissue inflammation (for review see Ref. 28).

The present study was motivated by a laboratory accident: in a series of ischemia-reperfusion experiments, the strict criteria for a “good” chamber were not applied, and mildly or even overtly inflamed chambers were included in the experiments. Only when the data for leukocyte-endothelium interaction before and after reperfusion injury were reviewed did we discover that although in this series of experiments the stimulation of postischemic leukocyte adhesion was comparable to that in previous studies, there was virtually no postischemic increase in leukocyte rolling, which was in striking contrast to previous experience on the same model (25, 26). On analysis of the data obtained in individual postcapillary venules, we found that leukocyte rolling increased significantly in those vessels that had only few rollers at baseline (i.e., good chambers) but was virtually unchanged in vessels with moderately enhanced baseline rolling and even dropped paradoxically in vessels with robust baseline rolling (i.e., inflamed chambers). The present series of experiments was performed to validate this accidental observation and to extend it to another pathophysiological stimulus of leukocyte rolling under the condition of extracorporeal blood circulation (18, 19).

Furthermore, we saw parallels of this accidental observation with well-established differences in exogenously stimulated leukocyte rolling between acutely prepared and chronically instrumented microvascular beds. For instance, superfusion of an acutely prepared hamster cheek pouch microcirculation with leukotriene B4 (LTB4) significantly reduces baseline leukocyte rolling within only a few minutes, in parallel with an increase in leukocyte adhesion (7). Yet, in the same species, LTB4 superfusion onto a chronically implanted observation chamber conversely increases leukocyte rolling and leukocyte adhesion simultaneously (25). Despite this contrasting effect of LTB4 on leukocyte rolling, the adhesion-promoting effect of LTB4 on
leukocytes is comparable in both situations (7, 25). Consistent
with this line of reasoning, Ley and co-workers (30) observed
a time-dependent alteration in leukocyte rolling flux after acute
exeriorization of the cremaster muscle in wild-type mice as
well as in other animal species. Leukocyte rolling flux reached
its maximum 40–60 min after the beginning of the surgical
procedure, in agreement with earlier findings in the hamster
cheek pouch and the mouse and rat mesentery (30).
In this report, we provide experimental evidence that the
attenuation of leukocyte rolling by LTB4 superfusion in acutely
prepared tissues represents an unphysiological response that
can be mimicked by a second superfusion of LTB4. This
concept is further supported by the demonstration that leuko-
cyte rolling in response to ischemia-reperfusion injury (25, 26)
or extracorporeal blood circulation (18, 19) is significantly
blunted and even paradoxically reversed in inflamed chambers,
which show increased leukocyte rolling at baseline.

MATERIALS AND METHODS

Animal model. The dorsal skinfold chamber preparation in awake
Syrian Golden hamsters was used for intravital microscopy. The
experimental preparation used in this study is very similar to that
described previously in detail (10, 25, 28), with only minor modifi-
cations. Briefly, inbred 6- to 8-wk-old (55–70 g body wt) Syrian
Golden hamsters were fed standard rodent diet and allowed water ad
libitum; they were anesthetized by injection of pentobarbital sodium
(60 mg/kg body wt ip; Narcoren, Merial, Hallbergmoos, Germany).
The entire back of the animal was shaved, and two titanium frames
were implanted so as to sandwich the extended double layer of the
skin. One layer of the skin was completely removed in an 18-mm-
diameter circular area, and the remaining layer, consisting of epider-
mis, subcutaneous tissue, and a thin striated muscle skin, was covered
with a coverslip incorporated in one of the frames. The dorsal skinfold
chambers were well tolerated by the animals, i.e., they showed no
signs of discomfort and no adverse effects on feeding and sleeping
habits. An indwelling catheter was implanted into the right jugular
vein in the same session. The experiments were conducted in accor-
dance with the national and institutional guides for the care and use of
animals.

Intravital fluorescence microscopy. Between the implantation of
the observation chamber and the microscopic investigation, a recovery
period of 72–96 h was allowed to eliminate the effects of anesthesia
and surgical trauma on the microvasculature. Epi-illumination (100-W
fluorescent lamp attached to an Axiohot intravital microscope, Zeiss, Jena,
Germany) and a ×20 water immersion objective (total magnification
×560; Zeiss) were used to select 10 regions of interest per chamber,
each containing ≥1 characteristic draining venule with a diameter of
20–50 μm. Rhodamine 6G (Sigma-Aldrich Chemie, TauKitchen,
Germany) and a ×20 water immersion objective (total magnification
×560; Zeiss) were used to select 10 regions of interest per chamber,
each containing ≥1 characteristic draining venule with a diameter of
20–50 μm. Rhodamine 6G (Sigma-Aldrich Chemie, TauKitchen,
Germany) was administered intravenously immediately before the
microscopic studies to visualize leukocyte rolling and firm adhesion to
the vessel wall. To minimize the phototoxic effect, we used J)
rhodamine 6G, which is far less phototoxic than acridine orange (37),
as a marker of leukocytes and 2) an intermediary amplifier as well as
a fluorescent light dimmer. In this way, it is possible to dim light
exposure to <20% of the output power of a 100-W fluorescent lamp
(Fluo Arc HBO 100). In pilot studies, we tested the potential photo-
toxic effect of this setup with an observation period up to 4 h and a
reapplication of rhodamine 6G after 2 and 4 h. Under these conditions,
we observed no significant effect of light exposure on the number of
rolling or sticking leukocytes. Leukocytes were classified according
to their interaction with endothelial cells as rolling and adherent leuko-
cytes as previously described in detail (25). The microscopic images
were recorded with a high-resolution black-and-white camera (Kappa,
Gleichen, Germany) on S-VHS video tape. A computer-controlled
stepping motor (Märzhäuser, Wetzlar, Germany) was used to investi-
gate the identical sites of interest at baseline and 2 and 4 h after 2 h
of pressure-induced ischemia (25) or 20 min of extracorporeal circu-
lation (18, 19), respectively, or at defined times after leukotriene
superfusion (25).

Extracorporeal blood circulation. The experimental protocol was
conducted as described elsewhere (18, 19). The experiments were
performed in the laboratory of Markus Kamler (University of Essen
Medical School). Briefly, extracorporeal blood circulation was intro-
duced via a micro-roller pump (Alitea, Stockholm, Sweden) and
Silastic tubing (1 mm ID, 60 cm long; Migge, Heidelberg, Germany)
shunted between the carotid artery and the jugular vein in 20 animals.
The sterilized extracorporeal circuit was primed with 1 ml of Ringer
solution, and the flow rate was adjusted to 1 ml/min. Extracorporeal
blood circulation was then continued for a total of 20 min (19). The
animals tolerated these procedures very well and showed no signs of
discomfort or changes in blood pressure or heart rate. The observation
chamber temperature was 28°C, which declined only slightly during
extracorporeal blood circulation. The percentage of the cardiac output
represented by the extracorporeal blood circulation is ~3%/min. If the
total blood volume of the hamster and the flow rate of the pump are
considered, 21 ml of blood are transferred through the tube system
within 30 min.

Ishemia-reperfusion. The experimental protocol was performed as
described previously (25, 28). The experiments were performed in the
laboratory of Hans-Anton Lehr (University of Mainz Medical School). In 20 animals, a 2-h period of ischemia was induced by
application of gentle pressure on the muscle against a coverslip with
a silicone pad and an adjustable screw that was just sufficient to empty
the blood vessels, as described previously (25, 26). With the help of
a computer-assisted stepping motor-driven microscope stage, the
same vessel segments that had been recorded at baseline were investi-
gated again at 2 h of reperfusion.

Topical leukotriene application. The experimental protocol was
performed as described previously (25). LTB4 (Amersham Buchler,
Braunschweig, Germany) or its vehicle (1% ethanol in 0.9% saline)
was superfused directly onto the striated muscle within the observa-
tion window at 20 nmol/l for 3 min. The superfusate was then washed
away with physiological saline solution, and stimulated leukocyte
rolling was quantified by intravital microscopy at 15, 30, 60, and 90
min, as well as 15 min after renewed leukotriene superfusion at 20
nmol/l.

Image analysis. Unbranched venules (25–50 μm diameter, >250
μm long) were selected for observation. Fluorescently labeled leuko-
cytes moving in the periphery of the axial stream across an imaginary
line perpendicular to the axis of the vessel were considered rolling
leukocytes. These cells were counted for 30 s and assessed as a
fraction of the microvessel circumference, which was calculated from
the microvessel diameter, which was assessed by a computer-assisted
image analysis (4) and with the assumption of cylindrical vessel
geometry. Leukocyte rolling velocity was assessed by quantifying the
time (in seconds) needed by the leukocytes for a displacement of 200
μm. Leukocyte adhesion was quantified as cells that remained on the
same spot on the endothelial lining during 30 s, expressed as the
number of cells per endothelial surface (as calculated from the
microvessel diameter and a defined length of 200 μm) (25, 26).

Statistical analysis. Values are means ± SD. Wilcoxon’s test was
performed for intergroup comparisons, and Spearman’s rank correla-
tion was performed to compare rolling and adherent leukocytes at
baseline vs. the difference between stimulated minus baseline rolling
leukocytes and adherent leukocytes in ischemia-reperfusion and
extracorporeal blood circulation-treated animals. Leukocyte rolling
velocity under baseline conditions was compared with that under stim-
ulated conditions, and Wilcoxon’s test was performed for intergroup
comparisons. Differences of rolling leukocyte velocity under baseline
and stimulated conditions were assessed and plotted against the
change in the number of leukocytes rolling under baseline and stimulated conditions (see Fig. 4).

RESULTS

The effect of extracorporeal circulation on leukocyte rolling is shown in Fig. 1. In analogy to previous observations (18, 19), extracorporeal blood circulation rapidly induced leukocyte rolling along the endothelium. However, this effect was seen only in optimal chamber preparations, in which <30 leukocytes were rolling under baseline conditions. When even mildly inflamed chambers were included in the experiments, the rolling-promoting effect of extracorporeal blood circulation was inconsistent and no longer statistically significantly different from baseline conditions (Fig. 1). When we included obviously inflamed chambers, in which ≥100 leukocytes rolled at baseline, in the experiments, extracorporeal blood circulation resulted in a paradoxical reversal of stimulated leukocyte rolling, with fewer leukocytes rolling after than before extracorporeal blood circulation. However, because of the enormous standard deviations of rolling responses in inflamed chambers, this effect reached no statistical significance. In agreement with previous observations (18, 19), extracorporeal blood circulation did not affect vessel diameters (not shown).

The effect of ischemia-reperfusion injury on leukocyte rolling is shown in Fig. 2. In analogy with previous experiments (25, 26), ischemia-reperfusion resulted in a significant increase in leukocyte rolling along the endothelium. Consistent with the observations after extracorporeal blood circulation (Fig. 1), this effect was seen only in optimal chambers (<30 leukocytes rolling at baseline) and was subsequently lost in mildly inflamed chambers (30–80 rolling leukocytes) or even paradoxically reversed in obviously inflamed chambers (>90 rolling leukocytes at baseline). Baseline leukocyte rolling as a marker of chamber inflammation had virtually no significant impact on stimulated leukocyte adhesion to the endothelium, with a slight, albeit nonsignificant, trend toward reduced postischemic leukocyte adhesion in more inflamed chambers (Fig. 2, inset). In agreement with previous experiments (25, 26), ischemia-reperfusion injury had no effect on microvessel diameters (not shown).

The effect of LTB4 superfusion on leukocyte rolling is shown in Fig. 3. In analogy with previous observations (25), leukotriene superfusion resulted in a rapid and statistically significant stimulation of leukocyte rolling along the endothelium, which remained at a rather high level during the subsequent 90 min. On repeated superfusion with LTB4 in the same mode and concentration, we observed a rapid and statistically significant drop in the number of rolling leukocytes (Fig. 3). This ~50% drop in the number of rolling leukocytes is virtually identical in terms of extent and time course to the decline in leukocyte rolling observed on initial LTB4 superfusion onto the cheek pouch preparation observed in the experiments of Dahlen and co-workers (7). In agreement with our previous observations (25) and observations by others (7), leukotriene superfusion did not affect microvessel diameters (data not shown).
The effect of ischemia-reperfusion on leukocyte rolling velocity is shown in Fig. 4. Under the conditions of our experiment, ischemia-reperfusion had no significant effect on leukocyte rolling velocity in noninflamed or in borderline inflamed or blatantly inflamed chambers (Wilcoxon’s test). When calculated for all chambers together, mean leukocyte rolling velocity was 45.3 ± 16.5 μm/s under baseline conditions and did not significantly change after ischemia-reperfusion (46.1 ± 17.9 μm/s). Subanalysis of data for non-inflamed and borderline and blatantly inflamed chambers is shown in Table 1. It becomes apparent that the differences in postischemic leukocyte rolling in the three groups of chambers are neither associated with nor secondary to changes in leukocyte rolling velocity.

**DISCUSSION**

Chamber models have been used for intravitral microscopy, because they allow prolonged observation periods (28, 32) and study of the microcirculation in the absence of anesthesia, which affects microcirculatory responses (9, 31), and in the absence of immediate surgical trauma (32). A classic application of intravitral microscopy in chamber models has been the use of various fluorescent markers to study leukocyte-endothelial cell interaction (26, 28, 32).

The principal finding in the present study is that stimulated leukocyte rolling and, to a much lesser extent, stimulated leukocyte adhesion after ischemia-reperfusion injury and extracorporeal blood circulation are markedly influenced by the extent of leukocyte rolling under baseline conditions as a reflection of prior tissue injury (Figs. 1 and 2). In analogy to previous findings during experimental tissue preconditioning (8, 33, 40), baseline leukocyte rolling was, to a large extent, effectively protected from postischemic leukocyte rolling in the rat mesentery by a mechanism that was thought to involve the preserved synthesis of nitric oxide during ischemia-reperfusion (38).

Davis and co-workers (8) proposed that P-selectin, an adhesion molecule that exists preformed in Weibel-Palade bodies and is rapidly translocated to the cell surface in response to transient oxidative stress (41). Similarly, preconditioning with bradykinin superfusion significantly attenuated the postischemic leukocyte rolling in the rat mesentery by a mechanism that was thought to involve the preserved synthesis of nitric oxide during ischemia-reperfusion (38).

Davis and co-workers (8) proposed that P-selectin, an adhesion molecule that exists preformed in Weibel-Palade bodies and is rapidly translocated to the cell surface in response to appropriate inflammatory stimuli (15) and is centrally involved in postischemic leukocyte infiltration and tissue injury (24), is

Table 1. Effect of ischemia-reperfusion on leukocyte rolling and leukocyte rolling velocity in noninflamed, borderline, and blatantly inflamed chambers

<table>
<thead>
<tr>
<th>Chamber Type</th>
<th>Baseline</th>
<th>Postischemic</th>
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<tr>
<td>Noninflamed chambers (&lt;30 cells/30 s)</td>
<td>42.6 ± 9.5</td>
<td>44.6 ± 13.6</td>
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<tr>
<td>Rolling velocity, μm/s at baseline</td>
<td>20.8 ± 6.4</td>
<td>122.1 ± 77.7*</td>
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<tr>
<td>Borderline chambers (31–100 cells/30 s)</td>
<td>44.1 ± 13.0</td>
<td>38.3 ± 14.9</td>
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<tr>
<td>Rolling velocity, μm/s at baseline</td>
<td>53.3 ± 24.6</td>
<td>93.6 ± 79.0</td>
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<tr>
<td>Inflamed chambers (&gt;100 cells/30 s)</td>
<td>52.8 ± 29.1</td>
<td>53.5 ± 20.0</td>
</tr>
<tr>
<td>Rolling velocity, μm/s at baseline</td>
<td>122.2 ± 27.0</td>
<td>92.5 ± 53.2</td>
</tr>
<tr>
<td>Rolling leukocytes, n/mm⁻¹ s⁻¹ at baseline</td>
<td>Values are means ± SD of 6–8 animals per group. *P &lt; 0.05 vs. baseline (Wilcoxon’s test).</td>
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reduced as a consequence of ischemic preconditioning. Similarly, Nonaka and colleagues (33) observed in a retinal ischemia-reperfusion model that the maximum number of rolling leukocytes can be significantly reduced by ischemic preconditioning. Finally, Wang and co-workers (40) showed that preconditioning with morphine increases shedding from the neutrophil surface of gp100^\muE, the murine analog to human L-selectin and a key adhesion molecule involved in the initial steps of leukocyte rolling (39). On the basis of our present observation, we speculate that, in analogy to these earlier reports, "inflammatory preconditioning" may play an important role in our studies. The exact mechanisms that are operative in our experiments warrant further investigation.

In an effort to clarify the mechanisms by which elevated leukocyte rolling attenuates stimulated leukocyte rolling, we have performed superfusion studies with LT\textsubscript{B\textsubscript{4}}. LT\textsubscript{B\textsubscript{4}} is a lipoygenase product of arachidonic acid metabolism and is generated primarily by polymorphonuclear (3, 12) and mononuclear phagocytes (36). It has been identified, in chamber models (25) and other models of intravital microscopy (2, 20), as a key mediator of leukocyte-endothelial cell interaction in intact organisms after ischemia-reperfusion injury and as a potent chemotactant responsible for the recruitment of neutrophils to the site of inflammation (34). LT\textsubscript{B\textsubscript{4}} is of particular relevance for our present study because of an apparent discrepancy in the literature concerning the microcirculatory response under different experimental conditions. We report here that 20 nM LT\textsubscript{B\textsubscript{4}} stimulates leukocyte rolling in vivo in the dorsal skinfold chamber model (Fig. 3). This finding is in agreement with data reported by Fox-Robichaud and co-workers (13), who described an almost twofold increase in leukocyte rolling on LT\textsubscript{B\textsubscript{4}} stimulation of cat mesentery. In contrast to these earlier reports, "inflammatory preconditioning" may play an important role in our studies. The exact mechanisms that are operative in our experiments warrant further investigation.

The observation that, despite drastic effects of inflammation on leukocyte rolling after ischemia and after extracorporeal blood circulation, we saw no comparable deleterious effects on postischemic leukocyte adhesion (Fig. 2, inset) underscores the fundamental differences in the mechanisms of leukocyte rolling and leukocyte adhesion (20). Further support for the validity of this observation can also be derived from a recent report in which leukocyte adhesion in response to platelet-activating factor was found unchanged in L-selectin-deficient mice (17). Asako and colleagues (1) stressed that, in acute models of intravital microscopy, the extent of leukocyte rolling depends on the extent of the surgical trauma and that only a very careful surgical preparation results in very low levels of rolling. They concluded that only in carefully instrumented preparations can meaningful observations be made on leukocyte responses to inflammatory mediators [e.g., histamine (1)]. Our present report goes one step beyond this caveat: not only can such leukocyte responses be missed, but they can even be converted to paradoxical, contrary effects. In conclusion, our present study underscores our theory that when chronic chamber models are used for intravital microscopy, the established criteria for the absence of inflammation must be strictly applied to ensure a physiological response to ischemia-reperfusion injury, extracorporeal blood circulation, and potentially other microvascular stimuli of leukocyte-endothelial interaction (28).

REFERENCES


