Leukocyte-Induced Acute Endothelial Dysfunction in Middle Cerebral Artery in Rabbits
Response to Aggregating Platelets

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Background and Purpose
Recent evidence suggests a possible role for leukocytes in angiospastic reactions of large cerebral arteries. This study examined the effect of activation of endogenous circulating leukocytes on endothelium-dependent relaxation in the middle cerebral artery in rabbits.

Methods
Leukocytes were activated by rapid injection of either 40 μg/kg phorbol 12-myristate 13-acetate, or 0.2 mg/kg N-formyl-methionyl-leucylphenylalanine into the left carotid artery. Control rabbits received an equal volume of vehicle. Concentration-dependent isometric tension responses of the left and right middle cerebral artery to the dilators acetylcholine, ADP, sodium nitroprusside, or calcium ionophore (A23187), as well as to aggregating platelets, were compared in vitro in control animals and in animals killed 10 minutes after the injection of leukocyte activators in normal and leukocyte-depleted rabbits.

Results
In the control animals there was no significant difference in the reactivity of the left and right middle cerebral arteries. The injection of the leukocyte activators led to enhanced contractile responses to aggregating platelets and a significant reduction in the endothelium-dependent relaxation in response to acetylcholine, ADP, and A23187 in the left middle cerebral artery (the injected side), whereas the effect of an endothelium-independent dilator sodium nitroprusside remained unchanged. In leukocyte-depleted rabbits the injection of either of the leukocyte activators used did not induce significant changes in the reactivity of the left middle cerebral artery.

Conclusions
Intravascular leukocyte activation appears to induce an acute disturbance of the endothelium-dependent relaxation. Under these conditions, platelet activation might result in marked angiospastic reactions of large cerebral arteries. (Stroke. 1994;25:2246-2252.)

Key Words • endothelium • leukocytes • platelet aggregation • vasospasm

There is a body of evidence that suggests that polymorphonuclear leukocytes (PMNs) and monocytes are implicated in the development of myocardial and cerebral ischemia. It has been repeatedly demonstrated that leukocytes accumulate in regions with depressed circulation, contributing to the tissue damage. Recently several investigations of direct action of activated leukocytes on coronary and cerebral arteries have been performed that shifted the focus and influenced studies of leukocyte participation in vasomotor reactions of large arteries in cardiovascular disease. Activated PMNs have been shown to produce vasospastic reactions in arteries from different vascular beds in vitro and in vivo. These data suggest that leukocyte-derived vasoactive substances may contribute to cerebral ischemia, especially in the presence of an atherosclerotic lesion of large cerebral arteries. It has been repeatedly demonstrated that leukocytes accumulate in regions with depressed circulation, contributing to the tissue damage. Recently several investigations of direct action of activated leukocytes on coronary and cerebral arteries have been performed that shifted the focus and influenced studies of leukocyte participation in vasomotor reactions of large arteries in cardiovascular disease. Activated PMNs have been shown to produce vasospastic reactions in arteries from different vascular beds in vitro and in vivo. These data suggest that leukocyte-derived vasoactive substances may contribute to cerebral ischemia, especially in the presence of an atherosclerotic lesion of large cerebral arteries. However, it is unlikely that leukocyte-mediated vasomotor reactions are the sole mechanism of their ability to alter the cerebral circulation. Both PMNs and monocytes can interact with the endothelium and the extravascular matrix to induce functional and morphological changes in the endothelial cells. Because of the major importance of the endothelium in regulation of vessel tone and reactivity, it seems possible that leukocyte effects on the vessel wall might be mediated through alterations of the functional state of the endothelium. This suggestion is corroborated by a few recent observations in which even short-term contact of PMNs with vessel segments in vitro has been demonstrated to cause strong depression of endothelium-dependent vascular relaxation. However, the effects of in vivo intravascular leukocyte activation on the endothelium have not been investigated.

This study was designed to test whether acute leukocyte activation in vivo alters the endothelium-dependent relaxation of large cerebral arteries in a direction that would favor vasoconstriction.

Materials and Methods
Experiments were performed on 49 Fauve de Bourgogne rabbits weighing 2.3 to 2.8 kg that were anesthetized with ketamine (10 mg/kg IV) and chloral hydrate (200 mg/kg IV). The trachea was cannulated, and the animals were ventilated with room air. A catheter was placed in a femoral artery for continuous recording of blood pressure. The left lingual artery was exposed and cannulated with a small polyethylene catheter, and the external carotid and thyroid arteries were ligated at their origin for selective injection of leukocyte activators.
into the cerebral circulation. Body temperature was maintained at 37°C to 38°C with a heating pad.

Two main activators of PMNs and monocytes were used. The first one, 12β-methyl-15β-acetoxy-20α-cholesterol aceta (PMA), was dissolved in dimethyl sulfoxide with further dilution in isotonic phosphate buffer immediately before use. It was injected into the left common carotid artery over 15 seconds in a dose of 40 μg/kg, 0.2 mL/kg. This procedure of PMA injection has been repeatedly shown to produce simultaneous platelet and leukocyte intravascular activation and aggregation as microemboli in rabbits.7-11 In the second test, papaverine (3 x 10^-4 mol/L) was added to the bath to obtain the maximum relaxation. The dilator effects were expressed as percentage of the papaverine-induced maximum relaxation. The dilator effects of acetylcholine, sodium nitroprusside, ADP, and calcium ionophore, A23187, were evaluated on the rings routinely precontracted with a submaximal concentration of histamine (10^-5 mol/L). The results are expressed as mean±SEM, and n refers to the number of rats. Differences between means were evaluated using ANOVA. All calculations were performed by means of SPSS PC+ statistical software. A probability value less than .05 was considered statistically significant.

Autologous platelets were separated as described in detail by Shimokawa et al. Briefly, the blood was stabilized with acid-citrate-glucose anticoagulant, and an equal volume of this anticoagulant was added to the platelet-rich plasma obtained by centrifugation; the mixture was centrifuged for 20 minutes at 1600 rpm. The platelet pellet was resuspended in a small volume of the anticoagulant, and a platelet count was obtained manually using Neubauer's chamber and special diluents (Labo-Moderne).

Five groups of animals were studied. The first group was the vehicle-injected animals (n=14). The reactivity of the left and right MCA was compared in this control group after the standard volume of vehicle was administered. In the second group (n=14) and third (n=9) groups, effects of PMA and of fMLP, respectively, on the MCA reactivity were studied at 10 minutes after the injection. In the fourth (n=7) and fifth (n=5) groups, effects of PMA and of fMLP, respectively, on the MCA reactivity were evaluated in the same way after previous leukocyte depletion. For leukocyte depletion 72 hours before the experiment, rabbits were treated with 1.5 mg/kg IV mecloretamine. None of these rabbits showed subsequent decline in condition or body weight. The number of leukocytes and platelets before the mecloretamine treatment and immediately before the injection of activators was calculated manually using Neubauer's chamber and special diluents (Labo-Moderne).

All pharmacological agents used were obtained from Sigma Chemical Co. Drugs were dissolved in distilled water except A23187, which was prepared in dimethyl sulfoxide and diluted in buffer immediately before use. Drugs were added at 100 times their final bath concentration. Dimethyl sulfoxide concentration did not exceed 0.01% and had no effect on vascular responses.

The results are expressed as mean±SEM, and n refers to the number of rats. Differences between means were evaluated using ANOVA. All calculations were performed by means of SPSS PC+ statistical software. A probability value less than .05 was considered statistically significant.

Results

There were no significant differences among any of the groups of rabbits studied regarding the amplitude of the MCA contraction evoked by either KCl (100 mmol/L) or histamine (10^-5 mol/L), the latter being used for vessel precontraction in our experiments.

Relaxation of precontracted MCA segments in response to an endothelium-independent dilator, sodium nitroprusside, was similar in the left and right MCAs of control animals and after PMA or fMLP injection (Fig 1). In control animals, ACh (receptor-mediated endothelium-independent dilator) and A23187 (non-receptor-mediated endothelium-dependent dilator) caused comparable relaxation of the left and right MCA (Figs 2 and 3). However, after either PMA or fMLP injection, the concentration-response curves of response to these agonists in the left MCA (injected side) were shifted to the right, and the magnitude of their effects was significantly reduced, especially in PMA-injected animals (Figs 2 and 3). In the right MCA, ACh and A23187 produced concentration-dependent dilation similar to that in control animals.

In our conditions, ADP induced only moderate concentration-dependent relaxation even in control rabbits (Fig 4). This relaxation was of comparable magnitude in the left and right MCAs of control animals and in the right MCA of PMA- or fMLP-injected animals. In the left MCA of PMA- and fMLP-injected rabbits, the
FIG 1. Concentration-response curves show dilator effects of sodium nitroprusside on the middle cerebral artery (MCA) of control rabbits (A, n=8) and after 4β-phorbol 12β-myristate 13α-acetate (B, n=8) and N-formyl-methionyl-leucylphenylalanine (C, n=5) injection into the left carotid artery. Open circles indicate right MCA; closed circles, left MCA. Values are mean±SEM and are expressed as percent of the maximal relaxation induced by 3×10^{-4} mol/L papaverine.

effect of ADP was drastically reduced, in some cases to almost total disappearance (Fig 4).

After the treatment with meclorethamine, the number of circulating leukocytes was significantly reduced to 12% to 18% of baseline value (Table). The differential count of the leukocytes remained unchanged, and the platelet count was also unaffected by meclorethamine (Table). Investigation of MCA reactivity to endothelium-dependent agonists showed that, after leukocyte depletion, the difference in the concentration-response curves between the left and right MCAs almost totally disappeared in both PMA- and fMLP-injected rabbits (Fig 5). In non-precontracted segments of the MCA from control animals, aggregating platelets caused comparable contractions of the left and right MCA (Fig 6). After both PMA and fMLP injection, platelets induced similar contractions of non-precontracted segments of the right MCA, whereas significantly greater contractions were evoked in segments of the left (injected) MCA (Fig 6). In segments of the right MCA (n=3) from PMA- or fMLP-injected animals, aggregating platelets induced further contraction of 20.7±7.5% in PMA-injected animals and 23.1±8.8% in fMLP-injected animals (P<.05 with respect to the constriction of the right MCA).

Discussion

Intravascular leukocyte activation is likely to produce regional circulatory disorders. Numerous investigations have shown that intravascular injection of PMA produces strong leukocyte-mediated microcirculatory disturbances, depression of blood flow and tissue metabolism, and edema formation in the lung,17,18 the heart,5,27-29 and the brain.9,30 Similarly, fMLP injection also produces vasoconstriction and microcirculatory disorders in these organs.3,6,20 Supporting these findings, the present study demonstrates for the first time that intravascular leukocyte activation by either PMA or fMLP injection into the carotid artery markedly suppresses the endothelium-dependent relaxation in the MCA.
It can be speculated that, after the injection of PMA or fMLP into the left common carotid artery, the passage of activated leukocytes through the dependent MCA caused leukocyte-endothelium interactions to occur, resulting in acute failure of vascular relaxation. Such alterations in vasoreactivity, induced in vivo in the present experiments, seem similar to those induced in vitro in other investigations.13-15 Very similar changes in MCA reactivity were produced by the two leukocyte activators, supporting the hypothesis that the effects observed were mediated via intravascular activation of leukocytes. Both PMA and fMLP activate PMNs and monocytes, producing intravascular aggregation, adhesion to the endothelium, and release of oxidants, proteases, and lipid metabolites.2 fMLP is a more selective leukocyte agonist, whereas PMA can also activate platelets in vivo. However, both activators also have binding sites on the endothelium and smooth muscle cells, the role of which is poorly understood.2 Thus, in principle, our data also could be the result of direct effects of the activators on the vessel wall.

In general, control experiments in published work have shown that intra-arterial injection of these activators in the absence of leukocytes does not induce any action on the vascular bed,4,9,27-29 and no in vitro effects have been observed on endothelium-dependent reactions in concentrations that activate leukocytes.31 However, in some experiments using relatively high concentrations of the activators in vitro, fMLP produced vessel relaxation and nitric oxide release, and PMA has depressed endothelium-dependent reactions.32-33 Such phenomena are most unlikely to be significant in our experiments. Nevertheless, we performed a special investigation of PMA and fMLP effects on the MCA reactivity after intracarotid injection in leukocyte-depleted animals. The data obtained clearly show that, after the decrease in the leukocyte count by 80% to 90% of baseline value, neither PMA nor fMLP induced significant changes in MCA responses to endothelium-dependent dilators. Thus, the effects of these agents on the endothelium-dependent responses observed in this study may be considered to be mediated via intravascular activation of leukocytes. It is not, however, unlikely that some other factors are involved in the leukocyte-induced endothelial dysfunction, especially when leukocyte activation was provoked by the less selective agonist PMA. Our data show that the PMA-induced reduction in the vascular responses to all endothelium-dependent dilators studied is more marked than that induced by fMLP injection. Although the exact mechanisms are not clear, this difference might be associated with simultaneous activation of leukocytes and platelets after intracarotid injection of PMA. Presumably, activated platelets can elicit additional PMN activation and adhesion to the vessel wall, since such an interaction of these cells has been documented by many in vitro investigations.14-16
Hematologic Values After Leukocyte Depletion with 1.5 mg/kg IV Meclorethamine

<table>
<thead>
<tr>
<th>Hematologic Values</th>
<th>Group 4 (n=7)</th>
<th>Group 5 (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leukocyte count, x 10^3</td>
<td>9.0±0.9</td>
<td>9.1±1.2</td>
</tr>
<tr>
<td>Differential leukocyte count, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMNs</td>
<td>43.4±6.9</td>
<td>40.7±5.8</td>
</tr>
<tr>
<td>Monocytes</td>
<td>5.2±1.6</td>
<td>7.0±1.5</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>41.8±7.9</td>
<td>48.4±6.8</td>
</tr>
<tr>
<td>Platelet count, x 10^3</td>
<td>335±24</td>
<td>312±19</td>
</tr>
</tbody>
</table>

PMNs indicates polymorphonuclear leukocytes.

Furthermore, it has been shown that PMA can modify the state of the endothelial cells, resulting in enhanced adhesion of PMNs with more marked generation of superoxide anions.35

The mechanisms of the leukocyte-mediated alterations of the MCA reactivity are not clear. They appear to be associated with a selective depression of endothelium-dependent relaxation without alterations in the smooth muscle cell reactivity because only vasodilatory responses to the endothelium-dependent dilators ACh, ADP, and A23187 were blunted; sodium nitroprusside, an endothelium-independent dilator, produced the same relaxation of the MCA in control rabbits and rabbits injected with PMA or fMLP. Under our conditions, intravascular leukocyte activation evoked a similar reduction of responses to receptor-mediated (ACh, ADP) and non-receptor-mediated (A23187) dilators, indicating that the endothelial dysfunction observed was not associated with disturbances of receptor activation in the endothelial cells. Apparently, the dysfunction might be induced by a direct action on the production and/or release of vasodilators, particularly prostacyclin and endothelium-derived relaxing factor (EDRF), in the endothelium. Such effects could be associated with the release of free radicals from activated leukocytes, which have been demonstrated to impair the production and accelerate the deactivation of EDRFs.1-12 Interestingly, the same variant of the endothelial dysfunction has been observed after ischemia-reperfusion.24,36 The endothelial dysfunction caused in vivo is stable, and it may be persistently observed after removal of vessel segments and their preparation for testing the effects of agonists.24-36 The similarity in character of such dysfunction caused by ischemia-reperfusion (see Reference 36...
for review) and by intravascular activation of leukocytes (the present work) corroborates the hypothesis that leukocytes may be involved in the pathogenesis of the ischemia-reperfusion-induced endothelial dysfunction in cerebral and coronary arteries.\(^{36,37}\)

As the intravascular activation of leukocytes causes a severe attenuation of the endothelium-dependent relaxation of the MCA, this phenomenon is likely to favor vasoconstriction of large cerebral arteries. The endothelium-dependent relaxation limits the action of many spasmogenic stimuli and seems to be of particular importance for the prevention of platelet-induced vasoconstrictor reactions. Aggregating platelets release a number of strong vasoconstrictors that can contribute to coronary and cerebral angiospasm.\(^{7,38}\) Under normal conditions, platelet-induced vasoconstriction is prevented by the endothelium-dependent relaxation,\(^{39}\) but endothelial dysfunction due to atherosclerosis\(^ {25}\) or ischemia-reperfusion\(^ {26}\) promotes an exaggerated constrictor response to aggregating platelets, particularly in large cerebral arteries.\(^ {40}\)

Thus, the leukocyte-induced endothelial dysfunction observed in this study could also alter vessel susceptibility to platelet-derived agonists. Our experiments with activated platelets confirm this suggestion, showing significantly increased contractile response in vitro to aggregating platelets in MCAs from rabbits given an intracarotid injection of either PMA or fMLP. In both quiescent and precontracted segments, platelet-induced contraction was almost doubled in the left (injected) MCA compared with that of the right (control) MCA.

The increased MCA responses to aggregating platelets after leukocyte activation are also in agreement with the attenuation of the MCA relaxation by ADP observed in the same conditions. In the basilar artery Shimokawa and coworkers\(^ {41}\) showed that purinergic mechanisms are responsible for the vasorelaxant component of platelet vasoactivity, so that diminished sensitivity of the MCA to ADP appears to be of particular importance for the exaggerated platelet-induced MCA constriction observed in the present study. It is worth mentioning that in our conditions the ADP-induced vasorelaxation of the rabbit MCA was small even in control animals. Thus, platelet activation may be especially dangerous for promoting cerebral angiospasm in conditions when even the ADP-mediated restriction of platelet contractility is eliminated by endothelial dysfunction. Certainly, the depression of ADP-induced relaxation may not be the sole mechanism of the leukocyte-induced increased susceptibility of the MCA to aggregating platelets. During their interactions, activated leukocytes as well as the endothelial cells and platelets can produce a number of highly active factors, such as platelet-activating factor (PAF), which may favor the spastic vessel reactions (see Reference 41 for review). It seems likely that PAF can, at least partly, elicit process(es) of leukocyte-induced endothelial dysfunction, resulting in augmented vessel responses to aggregating platelets.

Thus, the present study indicates that intravascular leukocyte activation alters the reactivity of the MCA, eliminating the endothelium-dependent mechanism of vessel relaxation in response to various agonists. This phenomenon might favor cerebral vasoconstriction, particularly that induced by aggregating platelets. Certainly, the clinical significance of these findings remains debatable.

Acknowledgment

Dr S.E. Akopov received a postdoctoral fellowship from the Ministère de la Recherche et de la Technologie (France).

References

Inflammatory processes in acute stroke are being increasingly investigated in laboratories internationally. As a result of this intensive study, numerous mediators and processes are being implicated as participants in acute ischemic brain damage. Investigators are becoming increasingly aware that events at the blood-endothelium interface in ischemia are multifactorial, and efforts to probe the interactions between putative mediators of ischemic and postischemic brain injury are beginning. In this study, activated polymorphonuclear leukocyte (PMN) interaction with endothelium appears to attenuate endothelium-dependent vasodilation and augment platelet-induced vasoconstriction. Other recent examples of interactions include PMN modulation of tumor necrosis factor-α release by macrophages and the capacity of PMN to stimulate release of growth factors from cultured porcine aortic endothelial cells.

Work of this sort sharpens our concepts of the pathophysiology of acute brain ischemia, but it also brings into sharp relief the complexity inherent in ischemic brain damage.

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Leukocyte-induced acute endothelial dysfunction in middle cerebral artery in rabbits.
Response to aggregating platelets.
S E Akopov, R Sercombe and J Seylaz

Stroke. 1994;25:2246-2252
doi: 10.1161/01.STR.25.11.2246
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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