Granulocyte Adhesion, Deformability, and Superoxide Formation in Acute Stroke

Armin J. Grau, MD; Elaine Berger; K.-L. Paul Sung, PhD; and Geert W. Schmid-Schönbein, PhD

Background and Purpose: Impaired rheological properties of as well as cytotoxic substances produced by granulocytes may contribute to tissue damage in acute ischemic stroke. To assess changes in the properties of circulating granulocytes, we measured their adhesion, deformability, and superoxide generation in the first 3 days after ischemic stroke.

Methods: Granulocytes from 18 male patients and 20 age- and risk-matched controls were investigated. Adhesion to murine laminin-, human fibronectin-, and bovine serum albumin-coated surfaces was measured with and without the stimulus phorbol myristate acetate and the antiadhesion antibody IB4. Superoxide anion formation was assessed by the reduction of ferricytochrome C. In a subgroup of 10 patients and 11 controls, granulocyte deformability was determined using the micropipette aspiration technique.

Results: The patients had significantly greater granulocyte adhesion to laminin \( (p<0.005) \) and fibronectin \( (p<0.05) \) but not bovine serum albumin. Cell stimulation enhanced the differences between the groups, whereas the antiadhesion antibody inhibited adhesion in both patients and controls. There were no significant differences in granulocyte deformability. Superoxide production by granulocytes was significantly lower in the patients without the stimulus \( (p<0.05) \) and with 10 nM phorbol myristate acetate \( (p<0.005) \).

Conclusions: These findings suggest that circulating granulocytes in ischemic stroke exhibit increased adhesive properties, a feature that represents one of the risk factors for granulocyte entrapment, impairment of microvascular flow, and tissue injury. (Stroke 1992;23:33-39)

In recent years the role of leukocytes in the pathogenesis of ischemic vascular disease has become a focus of research.\(^1\) Evidence from animal experiments suggests an important contribution of leukocytes in the pathophysiology of ischemia in the heart,\(^1\) intestine,\(^3\) skeletal muscle,\(^4\) and other organs as well as in hemorrhagic shock.\(^5\) Animals rendered granulocytopenic or leukopenic in different models of ischemic stroke showed a significantly better outcome than controls.\(^6-8\) In humans, elevated leukocyte counts have been reported to be a risk factor for cerebral infarction.\(^9\) The leukocyte count after ischemic stroke correlates with the severity of neurological impairment and infarct size.\(^10\)

Leukocytes, specifically granulocytes, may mediate acute tissue damage in the brain by impairing microvascular flow and by cytotoxic reactions with the tissue. Reperfusion after ischemia is considered to be a risk, and not only a benefit, since it is accompanied by enhanced retention of leukocytes in the microcirculation.\(^11\) Several clinical studies found decreased leukocyte filterability in acute and chronic cerebrovascular disease.\(^12-14\) These alterations could be caused by increased rigidity, increased adhesiveness, or a combination of these factors. The current study was designed to evaluate separately the adhesive and viscoelastic properties of circulating granulocytes after acute ischemic stroke. Adhesion of granulocytes to bovine serum albumin, laminin, and fibronectin was measured. Neutrophilic granulocytes seem to possess specific binding sites for laminin\(^15\) and fibronectin.\(^16\) Laminin is the major glycoprotein of mammalian basement membranes,\(^17\) and it is used to attach granulocytes to collagen.\(^18\) Fibronectin is an important cell surface and extracellular matrix glycoprotein and can support granulocyte adhesion to endothelial cells.\(^19\) The viscoelastic properties of individual granulocytes were determined using the micropipette aspiration technique.\(^20,21\) In addition, the spontaneous and stim-
ulated production by granulocytes of the superoxide anion (O$_2^-$) as one of the potentially tissue-damaging free radicals was measured.

Subjects and Methods

Eighteen male inpatients from different hospitals in San Diego, Calif., were evaluated during the first 3 days after the onset of ischemic stroke. All patients had a neurological deficit that lasted for >24 hours. A cerebral hemorrhage was excluded by cranial computed tomography on the day of admission. Exclusion criteria were infections or other inflammatory diseases, malignancy, end-stage renal failure, recent surgery or recent myocardial infarction, and medication with corticosteroids. The patients had a mean±SD age of 64±8 (range 47–75) years, 14 had a history of hypertension, seven had diabetes mellitus, and nine were smokers at the time of the stroke. Twelve patients had received at least one medication with 325 mg acetylsalicylic acid before our tests; the others had not received specific treatment for the stroke. The control group consisted of 20 men matched for age (mean±SD 64±10 years, range 45–79 years) and some of the concomitant risk factors; 14 individuals were diagnosed with hypertension, seven suffered from diabetes, and seven were smokers. None had a history of strokes. Nine controls were regularly taking acetylsalicylic acid (325 mg/day, n=8; 162.5 mg/day, n=1) for preventive purposes, and four were asked to take one tablet containing 325 mg acetylsalicylic acid on 2 days prior to venipuncture. The research protocol was approved by the Human Subjects Committee of all participating hospitals.

Following consent, blood was drawn from an arm vein into Vacutainers (Becton Dickinson, Rutherford, N.J.) with sodium heparin as an anticoagulant. Total leukocyte counts were determined manually using a Neubauer chamber, and differential counts were performed in Dulbecco’s phosphate buffered saline (DPBS) (Cellgro, Herndon, Va.) at pH 7.0 and a concentration of 5x10$^6$ cells/ml. Moderate contamination with erythrocytes was accepted to avoid hypotonic lysis, which could be shown to be a major source of granulocyte activation. The following assays were started usually during the fourth, and in rare cases during the fifth hour after phlebotomy.

To assess cell adhesion properties we used protein-coated plastic plates in combination with the myeloperoxidase assay to determine the number of adherent cells after attachment and a standardized rinse. Groups of 12 wells in a 48-well dish were coated each with a 1% solution of bovine serum albumin (Sigma), a 20 µg/ml solution of murine laminin (Sigma), or a 20 µg/ml solution of human fibronectin (gift from Dr. Kurt Gehlsen, La Jolla Institute for Experimental Medicine, La Jolla, Calif.) in DPBS. After 1 hour of incubation at 37°C, the plates were washed twice using an Eppendorf multipipette, and then Medium 199 (Gibco BRL Inc., Gaithersburg, Md.) was added. Half of the wells received the monoclonal antibody IB4 (gift from Dr. Karl Arfors, La Jolla Institute for Experimental Medicine) directed against the CD11/CD18 adhesion complex on the surface of the granulocytes (10 µg/ml final concentration). Then 25 µl of the cell suspension was added to each well. To estimate the degree to which the granulocytes can be stimulated in vitro, the tumor promoter phorbol myristate acetate (PMA)(Sigma; 1 nM final concentration) was added to half of the wells. Thus, each group of 12 wells consisted of four triplets with or without the antibody and with or without the stimulus PMA. The final volume in each well was 0.25 ml. The plates were washed after 30 minutes of incubation at 37°C in a standardized way using 1 ml of buffer applied with an Eppendorf multipipette.

To evaluate the number of adherent cells, the amount of myeloperoxidase in each well was measured and calibrated in terms of the actual cell count using a standard curve determined from each cell preparation. Myeloperoxidase is contained at high concentrations in the primary granules of granulocytes and plays a significant role in their cytotoxic activity. For this purpose the granulocytes were lysed with 0.5% hexadecyltrimethylammonium bromide (HTAB) (Sigma) in 0.05 mol KPO$_4$ at pH 5.4; 15 µl of a standard sample or 0.5% HTAB as the blank was pipetted into a 96-well plate and 55 µl of KPO$_4$ buffer, 20 µl of 0.3 mM H$_2$O$_2$, and 10 µl of 1.6 mM tetrathymethylbenzidine (Sigma) were added. The change of color was measured photometrically at 650 nm. The coefficient of variation of the unstimulated triplets was about 0.20. The standard curves were calculated using a linear least-squares procedure; the correlation coefficient was in all cases >0.97.

Oxygen radical formation was measured by the reduction of horse heart ferrocyanochrome C (Sigma) using 96-well dishes. Briefly, 125 µl DPBS, 25 µl granulocyte suspension (5x10$^6$ cells/ml), and 100 µl cytochrome C (2.5 mg/ml) were added to each well. To stimulate the cells, 25 µl of the buffer was substituted by different concentrations of PMA or the chemotactic substance formyl-Met-Leu-Phe (FMLP)(Sigma). All concentrations were tested in triplicate, and in a fourth well 25 µl of superoxide dismutase (Sigma, 1 mg/ml) was added instead of buffer to determine the superoxide-independent cytochrome reduction. The absorption at 550 nm was read in a microplate reader immediately after addition of the stimulus and after incubation for 30 minutes at 37°C.
In a subgroup of 10 patients we studied the viscoelastic properties of neutrophilic granulocytes using a micropipette system. The mean±SD age of the 10 men was 60±7 (range 47–72) years. Three patients were diagnosed with diabetes mellitus and seven with hypertension, six were smokers, and seven had received treatment with acetylsalicylic acid prior to the phlebotomy. The control group consisted of 11 male volunteers with a mean±SD age of 56±12 (range 38–79) years. Three controls had diabetes, seven had hypertension, five were smokers, and seven had taken acetylsalicylic acid before the test.

Blood was obtained as described above and transferred into sterile 15 ml polystyrene tubes (Dow Corning, Corning, N.Y.). The erythrocytes were allowed to sediment at room temperature for 20–45 minutes. Leukocyte-rich plasma was aspirated and diluted 1:20 with sterile filtered and endotoxin-tested DPBS (Sigma) adjusted to pH 7.35, 285 mosm. A small round chamber was mounted on the stage of an inverted microscope and filled with 950 µl DPBS containing 5% autologous plasma; 30–50 µl of the cell suspension was added. The cells were viewed with a ×100 objective and ×20 eyepiece and projected via a video camera on a television monitor at a final magnification of about ×5,000. All length measurements were calibrated with a 50×2 µm stage micrometer (Graticules Ltd, Towbridge, England). The cell deformation together with a video time display was recorded on a videotape recorder. Micropipettes were stretched in a pipette puller (model P-80/PC, Sutter Instruments Co., San Rafael, Calif.) to a small tip size with an average internal radius of about 1.8 (range 1.6–2.2) µm and filled with DPBS.

The tip of the pipette was positioned close to the surface of a granulocyte with the help of a hydraulic micromanipulator (Narishige Scientific Instrument Laboratory, Tokyo, Japan). A constant negative pressure of 490 dyne/cm² (approximately 0.5 cm H₂O) was applied for 5 or 10 seconds to aspirate a portion of the cell into the micropipette. Under such high optical resolution, granulocytes can be readily differentiated from other cells by their size and the granules in their cytoplasm. Cells were randomly selected for aspiration, and neither cells with a platelet attached to the surface nor granulocytes that were in contact with other leukocytes were excluded. Cells with a pseudopod were aspirated between 90° and 180° from the pseudopod. The use of endotoxin-free buffer and a constant room temperature led to a low degree of cell activation. More than 30 granulocytes were aspirated, and on average the first 20 well-recorded cell deformation histories were used for analysis. The experiments could be completed in most cases within 2 hours but no later than 3 hours after phlebotomy. The time course of cell deformation was determined during single-frame replay on a television screen. The length of the aspirated tongue was measured at eight times: during the first second and then once every 4 seconds. Granulocytes exhibit an initial rapid deformation followed by a slow creep (Figure 1). The displacements were analyzed by use of a viscoelastic model for the granulocyte, with a cortical shell at tension T₀ and a viscoelastic cytoplasm with a viscosity coefficient µ and an elasticity coefficient K. These parameters describe the deformability of a cell within the error of the measurement for small deformations (tongue size less than radius.
TABLE 1. Granulocyte Adhesion in Stroke Patients and Controls

<table>
<thead>
<tr>
<th>Combination</th>
<th>Bovine serum albumin</th>
<th>Laminin</th>
<th>Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients</td>
<td>Controls</td>
<td>Patients</td>
</tr>
<tr>
<td>IB4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMA 0.1 nM</td>
<td>n</td>
<td>Mean±SD</td>
<td>n</td>
</tr>
<tr>
<td>−</td>
<td>18</td>
<td>17.0±7.5</td>
<td>20</td>
</tr>
<tr>
<td>+</td>
<td>18</td>
<td>55.4±20.7*</td>
<td>20</td>
</tr>
<tr>
<td>− +</td>
<td>17</td>
<td>1.5±1.1†</td>
<td>18</td>
</tr>
<tr>
<td>+ +</td>
<td>16</td>
<td>3.0±3.1</td>
<td>18</td>
</tr>
</tbody>
</table>

Values are % adherent cells. PMA, phorbol myristate acetate. *p<0.005 and p<0.05, respectively, different from controls by Mann-Whitney U test.

of the pipette). Increases in the values of these parameters reflect stiffening of the cell. In addition, we assessed larger cell deformations by means of the ratio of the tongue length after 1 and 5 seconds to the square of the radius of the pipette; this index of deformation is largely independent of the size of the pipette.

The significance of differences between the patient and control groups was evaluated using the nonparametric Mann-Whitney U test. In the micropipette experiments the medians of the values in each individual were compared.

Results

There were no significant differences between the groups in leukocyte counts (7,800±1,800 cells/nl in patients versus 6,800±2,450 cells/nl in controls), granulocyte counts (4,750±1,450 cells/nl in patients versus 3,700±1,800 cells/nl in controls), or mononuclear cell counts (3,000±1,100 lymphocytes and monocytes/nl in patients versus 3,100±1,000 cells/nl in controls).

Unstimulated granulocytes failed to show a significant difference between the groups in albumin-coated wells, but adhesion to laminin and fibronectin was significantly greater for patient granulocytes (Table 1). Stimulation with 1 nM PMA led to greater absolute differences between the groups on all protein surfaces. The addition of IB4, with or without PMA, reduced the adhesion of granulocytes in both groups. Adhesion tended to remain higher in the patients although the differences between groups were not significant in most cases because of high interindividual variability.

Superoxide anion generation by the circulating granulocytes was lower in the patients for both stimulated and unstimulated cells (Table 2). The differences between patients and controls were significant without stimulus and with 10 nM PMA. At 1 nM PMA a wide variation in stimulation among samples was found. The difference between patients and controls was maximal at 10 nM PMA, whereas at the highest concentration (100 nM PMA) the difference between the groups was smaller. No significant differences were found after receptor-mediated superoxide stimulation with FMLP.

There were no significant differences with respect to cell size between stroke patients and control subjects (Table 3). Figure 1 demonstrates the deformation history of a granulocyte in the micropipette experiment at four times. Such cell deformation could be observed in all individuals. The average deformability parameters, based on the cortical shell model (TQ, μ, and K), had a tendency toward lower values in the patients, indicating more deformable cells. But neither the average deformability parameters nor the relative displacement after 1 and 5 seconds differed significantly between groups (Table 3). The interindividual and intraindividual variations as assessed with the coefficient of variation tended to be smaller in the patients.

Discussion

The focal influx of leukocytes into ischemic brain lesions is a well-known histopathologic finding both experimentally and clinically. Accumulation of granulocytes in low-flow regions was detected in the first hours postischemia, suggesting an early contribution of these cells in hemostatic and inflammatory processes after ischemia. In addition to their role in inflammation, granulocytes influence flow in the microcirculation. The high cytoplasmic stiffness of granulocytes leads to a transit time through capillaries longer than for erythrocytes. In the case of a reduced local pressure or altered rheological properties of the granulocytes, retention of these cells in the microcirculation may be prolonged and might even become permanent.

Table 2. Superoxide Anion Generation by Granulocytes in Stroke Patients and Controls

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Patients (n=17)</th>
<th>Controls (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.8±0.9*</td>
<td>1.7±1.6</td>
</tr>
<tr>
<td>Phorbol myristate acetate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 nM</td>
<td>2.0±3.3</td>
<td>3.7±5.0</td>
</tr>
<tr>
<td>10 nM</td>
<td>13.2±7.5†</td>
<td>20.9±6.4</td>
</tr>
<tr>
<td>100 nM</td>
<td>21.4±6.2</td>
<td>25.4±5.5</td>
</tr>
<tr>
<td>Formyl-Met-Leu-Phe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 μM</td>
<td>2.3±1.6</td>
<td>3.4±1.9</td>
</tr>
<tr>
<td>1.0 μM</td>
<td>2.9±1.8</td>
<td>4.1±2.8</td>
</tr>
<tr>
<td>10 μM</td>
<td>2.4±1.4</td>
<td>3.3±2.4</td>
</tr>
</tbody>
</table>

Values are mean±SD nM superoxide anion/325,000 granulocytes/30 min. *p<0.005 and p<0.005, respectively, different from controls by Mann-Whitney U test.
The lack of a significant increase in cell deformability controls in regard to factors such as diabetes mellitus, but this observation has so far not been reproduced after stroke. There may also be alternative explanations for the unaltered deformability. There could be in the current study may be due to the absence of high levels of activator in the plasma, which could contribute to the impaired filterability in acute and recent stroke but could not be detected with the current sample size. Furthermore, stiff and adhesive cells, which could be formed in the presence of high levels of activator in the plasma, are unlikely to be present in circulating peripheral venous blood. Such cells tend to leave the bloodstream during their passage through the microcirculation. Stiff and adhesive granulocytes may even be trapped in the lesion per se. Therefore, we conclude that the decreased filterability of circulating granulocytes after stroke may be mainly due to greater adhesiveness.

Granulocytes that have been stimulated may become refractory to subsequent activation. Thus, prior in vivo stimulation could be an underlying reason for our observation of a decrease in superoxide anion production by circulating granulocytes in the patients. The enhanced adhesion of granulocytes after stroke may play a role here as well since it has been documented that adherent granulocytes show a delayed onset but a sustained burst of respiratory activity compared with suspended cells. Our data give no evidence for a significant contribution of circulating granulocytes to the recently reported phenomenon of elevated plasma levels of free radicals in acute stroke. Our measurements were limited to a single instant after the onset of ischemia, and the time course of the events is currently unexplored. Furthermore, the free radical production of cells in the ischemic lesion and the surrounding penumbra may differ from that of cells in the circulation.

Almost certainly, the increased adhesiveness can be regarded as a reaction to the ischemic lesion in the brain. But there is evidence suggesting that a disturbed leukocyte rheology persists even months after stroke and that an elevated leukocyte count is a predictor of stroke and transient ischemic attack. This suggests that leukocytes may also play an initiating role in the etiology of cerebrovascular disease. Further clinical studies are needed in this respect to establish the role of granulocyte adhesion and free radical formation in ischemic stroke and to clarify the contribution of mononuclear cells.

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<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients (n=10)</th>
<th>Controls (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of measurements (min)</td>
<td>55±19</td>
<td>43±9</td>
</tr>
<tr>
<td>Pipette radius (Rp) (μm)</td>
<td>1.87±0.14</td>
<td>1.89±0.14</td>
</tr>
<tr>
<td>Cell radius (μm)</td>
<td>4.41±0.05</td>
<td>4.45±0.11</td>
</tr>
<tr>
<td>Cell cortex tension (dyne/cm)</td>
<td>0.041±0.003</td>
<td>0.044±0.007</td>
</tr>
<tr>
<td>Viscosity coefficient (dyne·sec/cm²)</td>
<td>39.8±4.0</td>
<td>45.1±10.7</td>
</tr>
<tr>
<td>Elasticity coefficient (dyne/cm²)</td>
<td>597±148</td>
<td>992±462</td>
</tr>
<tr>
<td>Deformation after 1 sec/Rp² (1/μm)</td>
<td>0.420±0.035</td>
<td>0.409±0.052</td>
</tr>
<tr>
<td>Deformation after 5 sec/Rp² (1/μm)</td>
<td>0.591±0.060</td>
<td>0.621±0.105</td>
</tr>
</tbody>
</table>

Values are mean±SD.
References


39. Zimmerman JI, Shellhammer JH, Parrillo JE: Quantitative analysis of polymorphonuclear leukocyte superoxide anion...


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**KEY WORDS** • cerebral ischemia • granulocytes • superoxide
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