Studies on the Interaction Between GP-180-Deficient Neutrophils and Vascular Endothelium

By Michael R. Buchanan, Carol A. Crowley, Richard E. Rosin, Michael A. Gimbrone, Jr., and Bernard M. Babior

A patient whose neutrophils lack the glycoprotein gp-180 shows an increased susceptibility to bacterial infections. Neutrophils from this patient migrate abnormally both in vivo and in vitro. To examine the basis for this abnormality in migration, a study was carried out on the interaction of gp-180-deficient neutrophils with artificial surfaces and with human endothelial cell cultures. Compared with normal neutrophils, gp-180-deficient neutrophils showed decreased adhesion to cold-insoluble globulin-coated plastic surfaces, and their ability to spread on this substratum was greatly impaired. In contrast, gp-180-deficient neutrophils interacted in a normal fashion with endothelial mono-

In the blood stream, neutrophils exist in two rapidly equilibrating pools: the circulating pool, consisting of neutrophils suspended freely in the blood, and the marginated pool, consisting of cells loosely associated with the vascular endothelium.1-3 Under normal circumstances, only 50%-60% of the neutrophils in the blood stream are in the marginated pool, but the fraction of cells in this pool can be increased to nearly 100% by maneuvers such as the intravenous administration of endotoxin1 or exposure of blood to dialysis membrane,6 procedures that may affect neutrophil behavior through their ability to release chemotactic factors, such as complement component C5a.5,6 Tissue neutrophils are probably derived from the pool of marginated cells, which appear to migrate out of blood vessels by passing between the endothelial cells,7 possibly in response to a chemotactic gradient.

Recently, endothelial cell cultures have been used as an in vitro model system to investigate the mechanisms of interaction of neutrophils with the vascular wall.7-15 These investigations have demonstrated that, in the presence of divalent cations, neutrophils will attach to matrix constituents.7,11,12 Such preferential attachment of neutrophils is not seen with a variety of other cultured cells, including vascular smooth muscle cells, fibroblasts, epithelial cells, and malignant cells of various origins.7,10,11,13 Attachment to endothelium is augmented by erythrocytes8 and chemotactic factors,8,11,12 but does not seem to require plasma.9 Neutrophils that are attached to endothelial surfaces tend to retain a spherical configuration.8,11 If incubated on a confluent endothelial monolayer for a sufficient length of time, some of the attached neutrophils will pass between abutting cells and spread between the endothelium and the subjacent surface.7,9 These and other data14,15 suggest that the attachment of neutrophils to cultured endothelial cells is a satisfactory model of margination and that the migration of neutrophils from the upper to the subjacent surface of endothelial monolayers could represent a model of the initial stages of neutrophil egress from vessels into tissues.

We recently described a seriously infected patient whose neutrophils were unable to migrate into a Rebuff window and failed to spread on plastic.16,17 Gel electrophoretic analysis of these neutrophils showed them to lack a 180,000-dalton glycoprotein (gp-180) that was present in normal cells.6 The functional deficiencies characteristic of these neutrophils raised the possibility that an abnormality in their interaction with the vascular endothelium formed the basis for the patient's clinical problems. To test this possibility, we studied the interaction of the patient's neutrophils with artificial surfaces and cultured human endothelial cells in vitro and examined the patient's marginated neutrophil pool in vivo.

*This protein was originally reported to have an apparent molecular weight of 110,000 daltons.14 Subsequent measures showed that this figure was in error. The correct value was determined to be 180,000 daltons.
GP-180-DEFICIENT PMN AND ENDOTHELIUM

MATERIALS AND METHODS

Preparation of Neutrophil Suspensions

Neutrophils were isolated from anticoagulated venous blood by dextran sedimentation and centrifugation through Ficoll-Hypaque as previously described. The cells were suspended in ice-cold calcium-free Hank's balanced salt solution (HBSS, M.A. Bioproducts, Walkersville, Md.) and stored on ice. Immediately before use, they were centrifuged at 400 g for 5 min and resuspended in a calcium-containing Tyrodes-albumin solution. Both normal adult (control) and patient neutrophil suspensions were processed in parallel, and cell viability, as determined by Trypan blue exclusion just prior to the adhesion assay, was 95% and 87%, respectively.

Preparation of Plastic and Glass Surfaces

Thermonox plastic coverslips (No. 1½, 15-mm diameter, Lux Scientific Co., Newbury Park, Calif.) and glass coverslips (no. 1943-00019, 12mm dia., Belco Glass, Inc., Vineland, N.J.) were pretreated for 2 hr at 37°C with (1) calcium-free HBSS containing no additions; (2) 100 µg/ml cold-insoluble globulin (CIG; generously supplied by Dr. T. Maciag, Department of Pathology, Beth Israel Hospital, Boston, Mass.); or (3) 200 µg/ml poly-d-lysine (Sigma Chemical Co.). The coverslips were then rinsed 3 times with calcium-free HBSS and used immediately in the neutrophil adhesion assay.

Preparation of Human Endothelial Cell-Coated Coverslips

Human endothelial cells, harvested from umbilical cord veins, were replicate-plated and grown to confluence on Thermonox plastic coverslips in M199 medium (M.A. Bioproducts) supplemented with 20% fetal calf serum (M.A. Bioproducts), as previously described.

Neutrophil Adhesion Assay

The adhesion of neutrophils to the various surfaces was measured by a modification of the monolayer adhesion assay described by Curwen et al. Briefly, coverslip cultures, or plastic or glass coverslips treated as indicated above, were rinsed in calcium-free HBSS and transferred to 16-mm plastic wells (Cluster-24 plates; Costar, Cambridge, Mass.) containing 75 µl of neutrophil suspension (5000 cell/mm). Each coverslip was incubated for 30 min at 37°C under static conditions, then removed and rinsed 3 times in calcium-free HBSS to remove all loosely attached neutrophils. Three rinses were required to remove all loosely attached cells; further washings did not alter the number of cells associated with the surface. Some coverslips were processed for quantitation of adherent neutrophils by light microscopy, while others were processed for scanning electron microscopy.

Quantitation of Neutrophil Adherence

Artificial surfaces. Each coverslip was transferred to a plastic well containing fresh HBSS. Random areas of each coverslip were photographed using a Nikon inverted phase-contrast microscope at 200x primary magnification. Each photograph was coded and a grid counting method used to determine the number of neutrophils adhering per unit area and the proportion of these that had spread on the surface.

Endothelial cell monolayers. After incubation with neutrophils, coverslip cultures were fixed for 30 min at 22°C in 2% glutaraldehyde (0.1 M sodium cacodylate, 0.2 M calcium chloride, pH 7.35) and then stained with Wright's stain. Ten random areas of each coverslip were photographed using a Zeiss photomicroscope at 110x primary magnification. Black and white negative slides of each area were projected onto a screen and the number of neutrophils adherent to, or spread beneath, the endothelial monolayer were determined by three observers in a manner similar to that described by Pearson et al. and Beeley et al. 7

Preparation for light and electron microscopy. Each coverslip was fixed in 2% glutaraldehyde and postfixed in 1% osmium tetroxide, both for 30 min at 22°C, then dehydrated in a series of graded ethanol. After critical-point drying from CO2, in a Samdri critical point dryer (model PVT-3) and sputter coating with platinum-gold (50A), the coverslips were examined in an AMR-1000 A scanning electron microscope.

Some culture monolayers also were embedded in Epon and transverse sections prepared for light and transmission electron microscopy, as previously described.

RESULTS

The abnormality initially demonstrated in gp-180-deficient neutrophils was their failure to spread on a plastic surface. In the current study, experiments with several types of artificial surfaces confirmed this abnormality. Results obtained using plastic surfaces are shown in Table 1. Under standard assay conditions, comparable numbers of gp-180-deficient and normal neutrophils attached to uncoated plastic coverslips. However, adhesion of defective neutrophils to plastic coated with cold-insoluble globulin (the circulating counterpart of fibronectin) was significantly reduced compared with control neutrophils. In addition, the spreading of gp-180-deficient cells was severely defective on both surfaces (Table 1, Fig. 1). In contrast, attachment, and especially spreading, of neutrophils was greatly augmented when the plastic was coated with polylysine, a substance known to promote interactions between cells and underlying substrates by means of powerful electrostatic forces that attract the negatively charged cell surface to the highly cationic polymer. It is of interest that the spreading defect of gp-180-deficient cells, though very pronounced on

<table>
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<th>Table 1. Interaction Between Neutrophils and Artificial Surfaces</th>
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<tr>
<td>Total Number Adherent*</td>
</tr>
<tr>
<td>[No. of Cells/mm² Surface (% spread)]</td>
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<td></td>
</tr>
<tr>
<td>Control</td>
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<td>Patient</td>
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*Data are expressed as mean ± SEM, n = 5; see Materials and Methods for experimental details.
†The number in parentheses represents the percentage of adherent neutrophils that were spread upon the surface, as is illustrated in Fig. 1.
‡Significant difference between patient and control neutrophils (p < 0.005)
other surfaces, could not be demonstrated on the polylsine-coated plastic. Comparable results were obtained with similarly treated glass surfaces (data not shown).

In contrast to the marked difference between normal and gp-180-deficient cells with respect to their behavior on artificial surfaces, the cells were indistinguishable with respect to their behavior on cultured endothelial monolayers. Similar numbers of normal and gp-180-deficient cells were found to adhere to the upper surfaces of endothelial cells (Table 2), retaining their spherical configuration (Fig. 2). Moreover, the proportion of cells that had migrated beneath the monolayer also was similar to control (Table 2). GP-180-deficient neutrophils attached to the upper surfaces of cultured endothelial cells and others lodged beneath the monolayer are shown in Fig. 3.

These results suggest that gp-180-deficient neutrophils interact in a normal fashion with vascular endothelial cells. In vivo evidence that this may be so was sought by measuring the size of the marginated neutrophil pool in the gp-180-deficient patient. As shown in Fig. 4, epinephrine administration on two separate occasions caused substantial increases in the patient’s blood neutrophil counts. The patient thus appears to have a normal marginated neutrophil pool, an in vivo finding consistent with the observations made with cultured endothelial cells.

Table 2. Interaction Between Neutrophils and Human Endothelial Cell Cultures

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<th>Total Number Adherent</th>
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<tr>
<td></td>
<td>(No. of Cells/sq mm Surface)</td>
<td>(%)†</td>
</tr>
<tr>
<td>Control</td>
<td>646 ± 46</td>
<td>67 ± 2</td>
</tr>
<tr>
<td>Patient</td>
<td>627 ± 77</td>
<td>59 ± 6</td>
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*Data are expressed as mean ± SEM, n = 10.
†This value represents the percentage of the total number of neutrophils associated with the coverslip culture that were identified beneath the endothelial monolayer.
DISCUSSION

The patient with gp-180 deficiency was originally admitted with a severe localized bacterial infection and a neutrophil count of 93,000 cu mm. Despite this very high white count, his neutrophils failed to move into a Rebuck skin window. Even when the patient is well, his white count remains high (20,000–30,000 per cu mm) and cuts heal slowly. These findings indicate that gp-180-deficient neutrophils have difficulty moving from blood vessels into the surrounding tissues.

In attempting to explain this abnormality, two possibilities may be considered: (1) gp-180-deficient neutrophils fail to interact normally with vascular endothelium and therefore are unable to leave the blood vessels, or (2) gp-180-deficient neutrophils penetrate the vascular endothelium in a normal fashion, but, once out of the blood vessels, they cannot interact sufficiently well with interstitial elements to migrate normally through extravascular tissues. The present experiments suggest that the latter is the case. GP-180-deficient neutrophils adhere to endothelial monolayers in normal numbers, migrate between abutting endothelial cells to spread beneath the monolayer in a maneuver highly suggestive of in vivo egress, and marginate normally in vivo. In contrast, these cells fail to spread onto surfaces on which normal neutrophils spread readily. The former observations strongly suggest that gp-180-deficient neutrophils interact normally with endothelium, while the latter provides indirect evidence for an abnormal interaction with extravascular constituents. The results with cold-insoluble globulin are of special interest, since the closely related extracellular matrix glycoprotein,
fibronectin, has been implicated in the attachment and spreading of various cells onto collagen, an ubiquitous component of extravascular tissues.21

A point that remains to be investigated is the effect of chemotactic factors on the interaction of gp-180-deficient neutrophils with endothelial monolayers. These agents, which are thought to promote the migration of neutrophils from the vascular system to sites of inflammation, have been shown both to augment the adhesion of neutrophils to endothelial cells in vitro6,11,12 and to induce them to aggregate and marginate in vivo.5 The fact that the gp-180-deficient patient has a normal marginated pool suggests that the effect of chemotactic factors on the interaction between his neutrophils and cultured endothelial cells is likely to be normal. More direct evidence concerning this question will be sought in future studies.

Insofar as the gp-180-deficient neutrophils interact in a normal fashion with vascular endothelium despite a marked impairment in their interaction with other surfaces, the present experiments provide further evidence for the special relationship between neutrophils and endothelial cells that has been postulated by previous workers.7,12,14 The basis for this relationship is not known, but recent data suggest that the endothelial cell plays an active role in this interaction.12,14

**Fig. 4.** Presence of a marginated neutrophil pool in the gp-110-deficient subject. The marginated pool was measured according to the method of Joyce et al.,22 using 0.1 mg epinephrine infused over 10 min as the demarginating agent. The infusion was started at time zero. Measurements in the patient were made on 2 consecutive days: the control was a normal adult volunteer.

**ACKNOWLEDGMENT**

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**REFERENCES**


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