The nature of leukocyte shape changes in the pulmonary capillaries

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Redenbach, Darlene M., Dean English, and James C. Hogg. The nature of leukocyte shape changes in the pulmonary capillaries. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L733–L740, 1997.—The size discrepancy between leukocytes [white blood cells (WBCs)] and pulmonary capillaries requires WBCs to deform. We investigated the persistence of this deformation on cells leaving the capillary bed and the role played by the cytoskeleton. Isolated rabbit lungs were perfused in situ via the pulmonary artery with effluent fractions collected from the left ventricle. Washout curves from cell counts in each fraction confirmed that WBCs are preferentially retained over erythrocytes. WBC deformation present on exit from the circulation was compared with that present after recovery in paired fractions, fixed immediately or 60 min later. These cells were compared with cells recovered from the capillary in perfused fixative or fixed in peripheral blood. Our results show that leukocyte deformation persisted after the cells exited the pulmonary circulation. This deformation was associated with minimal submembranous F-actin staining, and microtubule distribution and cell polarization were unchanged. We conclude that cytoskeletal changes that occur during WBC deformation in the pulmonary capillaries are minimal and differ from those known to occur in actively migrating cells during chemotaxis.

neutrophil; pulmonary capillary; deformation; cell polarity; cytoskeleton; cell shape; polymorphonuclear leukocytes

The pulmonary capillary bed is made up of an interconnecting network of ~10^11 short segments ranging in width from 1 to 15 µm with a mean diameter of 7.5 ± 2.3 (SD) µm and length of 14.4 ± 5.8 (SD) µm (9, 24). The number of segments in a single pathway, from an arteriole to venule, has been variously estimated from 50 to 100 (14, 15), and 38% (human) to 67% (rabbit) of them are too narrow to accommodate the spherical diameter of a polymorphonuclear leukocyte (PMN; see Ref. 9). Assuming a normal distribution of capillary segment diameters in the pathways from the arteriole to venule, all of the pathways must contain one or two segments that would restrict circulating cells. This assumption is consistent with the recent report of Wiggs and co-workers (25) who found that PMN with a mean diameter of ~7 µm are delayed to the same degree as nondeformable beads 4 µm in diameter during a single pass through the lung. This shows that the PMN have the capability of rapidly reducing their minimum diameter by ~3 µm to negotiate the restriction imposed by the capillary bed. Because the capillary bed is made up of such a large number of parallel pathways, the pressure drop across individual segments is very small. This means that the cells may negotiate the narrow capillary segments by active shape changes that could involve active locomotion of the circulating WBCs through the restrictions. A delay in restoring their spherical shape after deformation is of interest because maintenance of the shape change required to negotiate the first restriction would facilitate passage through subsequent restrictions in a microvascular bed. The present study was undertaken to examine the deformation and recovery of the PMN in the pulmonary capillary bed and to determine the nature of the cytoskeletal changes associated with these deformations.

MATERIALS AND METHODS

Materials

Buffers. Washout buffer was composed of (in mM) 136 NaCl, 2.7 KCl, 8.2 Na2PO4, 1.5 KH2PO4, 0.7 CaCl2, 0.5 MgCl2-6H2O, and 5.5 glucose, pH 7.4. Washout buffer fixative was composed of 2.5% glutaraldehyde or 1% paraformaldehyde (EM grade; Electron Microscopy Sciences, Fort Washington, PA) made in washout buffer. All buffers were used at 37°C.

Immunohistochemical stains and antibodies. Blood cells were stained with Giemsa stain (BDH, Toronto, ON, Canada). Microtubules were identified with SA6 anti-α-tubulin monoclonal antibody (final dilution 1:4,000 of a 10 mg/ml stock), a generous gift from Dr. David Brown, University of Ottawa, ON, Canada (1). Filamentous actin (F-actin) was localized with tetramethylrhodamine B isothiocyanate (TRITC)- or fluorescein isothiocyanate (FITC)-conjugated phalloidin (reconstituted in 1 ml methanol stock, evaporated and diluted 1:200; Molecular Probes, Eugene, OR). Nuclei were identified with either Giemsa stain or Hoechst 33342 fluorescent nuclear probe (diluted 1:200; Sigma, St. Louis, MO). Secondary antibodies were either FITC- or TRITC-conjugated mouse immunoglobulin G (IgG; Sigma).

Surgical Procedure

New Zealand White rabbits (2.6 ± 0.5 kg) were anesthetized with 40–60 ml of 0.25 g α-chloralose in 50 ml normal saline plus 20 ml of 32% urethan to deep anesthesia, which was maintained with 50% urethan, intravenous fluid replacement, and 1,000 units heparin. The rabbit was placed on a Harvard respirator via tracheostomy at an initial respiratory rate of 25–30 cycles/min with a tidal volume of 7 ml/kg and then was adjusted to establish physiological blood gas parameters that were monitored from a cannulated carotid artery. The heart and lungs were exposed via midsternal thoracotomy. A single tie was placed around the pulmonary trunk and ascending aorta to isolate the pulmonary circulation after which the pulmonary trunk and left ventricle were cannulated.

Washout Procedure

The lungs were perfused with buffer at a rate of 20 ml/min using an infusion pump (Cole-Palmer). In 11 experiments,
effluent fractions were collected from the left ventricle at 1-s intervals for 25 s, followed by 5-s intervals to a total of 225 s. The pulmonary circulation was then perfused with buffered fixative consisting of either 1.6% paraformaldehyde for immunocytochemical studies or 2.5% glutaraldehyde for morphological studies, with equal results. Preliminary experiments were performed to verify that the pulmonary vascular and airway pressures remained within the physiological range during the experimental procedure. In a series of seven experiments, the effluent was collected at 3-s intervals via a two-channeled cannula directly into paired preweighed vials to compare cells fixed immediately with those allowed to recover their shape. One of these vials contained concentrated fixative at a volume estimated to give a final concentration of 1.6% paraformaldehyde, and the other had fixative added 60 min after collection to achieve the same concentration of fixative. Immediately after fixation, tubes from both groups were weighed, centrifuged, and resuspended in 1 ml of fresh 1.6% paraformaldehyde.

Cell Counts

In experiments performed without fixation of the effluent, the number of white blood cells (WBCs) and red blood cells (RBCs) in each buffer fraction were counted on a Coulter counter (model S880). To determine WBC and RBC counts in experiments in which cells were collected in paired fractions, total cell counts were calculated using Coulter counter values obtained from the samples before the addition of fixative at 60 min. The cells in the effluent from lungs perfused with fixative were counted using a hemocytometer.

Morphology

After the rabbit lungs were fixed by perfusion, they were immersed in glutaraldehyde of the same concentration as that in the perfusate for a minimum of 24 h. These lungs were then processed using a standard procedure that has been previously described in this laboratory (9).

Cell shape analysis. Cell shape analysis was performed on the first 20 intact WBCs encountered on slides made from samples obtained at the peak, shoulder, and plateau of the washout curve and from those washed from the lung by fixative at the end of the experiment. Cells were stained in suspension with Giemsa and were examined using a custom-designed morphometry program (BioView Colour Image Processing System; Infrascan, Vancouver, BC, Canada) to locate the longest diameter and the centroid of the cell. The length determination program was then used to measure the longest diameter and the shortest diameter, defined as that passing through the centroid at right angles to the longest diameter. The "shape ratio" was calculated as the longest diameter divided by the shortest diameter.

Immunohistochemistry

The cells perfused from the lung with buffer or buffered fixative were processed for localization of tubulin (SA6 anti-α-tubulin, 1 mg/ml stock diluted 1:400), F-actin (FITC- or TRITC-conjugated phalloidin, 1:50–1:200), and nuclei (Hoechst 33342; Sigma; excitation 365: nm; barrier: 395 nm; emission: 515–556 nm), TRITC (excitation: 546 nm; barrier: 580 nm; emission: 590 nm), and bis-benzamide (Hoechst 33342; Sigma; excitation 365: nm; barrier: 395 nm; emission: 420 nm).

Confocal Microscopy

The cytoskeleton of cells fixed in the capillary were observed on a Biorad MRC 600 confocal laser scanner (Bio-Rad, Burlingame, CA) attached to an upright Nikon Optiphot 2 microscope equipped with a ×60 oil immersion objective (Nikon, Mississauga, ON, Canada). With the use of 0.1-µm steps, three-dimensional fluorescent images were compiled from 120 consecutive optical sections to localize the microtubule organizing center (MTOC) and the cellular distribution of F-actin in fixed cells.

Data Analysis

For the shape ratio, data means were compared using a one-tailed Student’s t-test (significance, P = 0.05). For comparison between groups on the washout curve, two-way analysis of variance was determined (significance, P = 0.05), and specific comparisons were made using the sequential rejective Bonferroni comparison. All statistical analyses were performed using Systat software with errors expressed as ± SD.

RESULTS

Figure 1 shows data from a single experiment in which cell counts are expressed as a percentage of the total cells washed from the lung. The WBC-to-RBC ratio at the peak of the curve (leading edge of perfusate from the left ventricle) at 1.77 ± 0.77 was not significantly different from that of peripheral arterial blood (common carotid artery) at 1.68 ± 0.23. Figure 2 shows WBC and RBC curves constructed from combined data from 11 experiments in which the values are expressed as a percentage of their peak values. Comparison of the WBC and RBC curves fitted with 95% confidence intervals showed that washout of WBCs was delayed compared with RBCs. Increasing the flow after the WBC curve reached a plateau resulted in a small increase in the number of cells in the effluent with an immediate return to the plateau level (n = 2, data not shown).

The switch from buffer to buffered fixative perfusion did not change the number of leukocytes recovered in the effluent. Similar results were obtained with either fixative. Microscopic examination of lung tissue that
had been fixed by perfusion after the washout procedure showed that the majority of blood cells remaining in the perfused lung were elongated leukocytes located in capillary segments (Fig. 3). Examination of the WBCs perfused from the lung by buffered fixative, after the initial buffer washout, showed that those cells possess the same deformed shape as WBCs remaining in the capillary (Fig. 3, inset). Figure 4 shows cumulative washout data of seven experiments in which the cells were perfused from the lung with buffer and were collected in paired samples that were either fixed immediately or were deferred for 60 min. Comparisons of shape analysis (mean shape ratio) of cells that were fixed immediately with cells that were fixed 60 min later are shown in Fig. 5. Cells collected at the peak of the washout curve showed a similar spherical shape with either immediate or deferred (late) fixation. However, those cells collected at the shoulder and plateau of the washout curve (see Fig. 4) and fixed immediately were elongated compared with those that were allowed to recover for 60 min before fixation (late fix). Grouping cells by size reveals that cell elongation increased in frequency and degree over the washout period (Fig. 6) but did not reach values of cells perfused out of the lung by fixative.

Although cells recovered by perfusing them out of the lung with fixative were elongated, they showed no evidence of lamellipodia or uropod formation, which is typical of a leading or trailing edge in migrating cells. Furthermore, there was no consistent change in the position of the nucleus, which was typical of a polarized pattern seen in migrating WBCs (Fig. 7). In the deformed WBC, microtubules radiated from a perinuclear center and extended throughout the cell (Figs. 7 and 8). The MTOC, which is considered to occur at the point of divergence of the microtubule arrays, was localized in cells stained with anti-tubulin antibodies (Fig. 8). The MTOC was most frequently located in a lateral position adjacent to the nucleus and did not show any preference for a polarized position at either end of the nucleus of these elongated cells. Unlike the intense F-actin staining seen in activated neutrophils, the F-actin staining in these cells was pale, appearing to accumulate in the submembranous area occupied by the ectoplasm, an area generally devoid of membranous organelles. The actin distribution at the cell surface was explored further using anti-tubulin and F-actin probes (Fig. 9). The three-dimensional images revealed the radiation of microtubules from randomly positioned focal centers, with no evidence of preference for a leading or trailing edge. These confocal images also verified that cortical F-actin was distributed evenly and was not consistent with the intense F-actin staining reported in activated or migrating cells.

**DISCUSSION**

These results confirm a previous report (9) showing that PMN are deformed in the capillary bed and extend those observations by showing that the deformation is maintained as PMN transit from the capillary bed to the left ventricle. They also show that this cell deformation is not accompanied by morphological evidence of actively migrating cells.

Previous studies have shown that PMN have a median transit time of 60 s in the pulmonary capillary bed (14). Using direct observation by video microscopy through lung windows, Gebb et al. (12) and Lien et al. (18, 19) have shown that the WBCs frequently stop during a single transit through the vascular bed. This differs markedly from the RBCs that have a median transit time of ~1 s (14) even though the maximum diameters of RBCs and WBCs are similar (9). This difference in transit time is due to the RBC discoid shape, which allows it to fold rapidly and to negotiate capillary restrictions ~300 times faster than leukocytes (4, 5, 11), resulting in WBC margination in the lung (7, 8, 10).

Recent studies by Wiggs and co-workers (25) have shown that PMN have the ability to undergo a rapid
change in diameter from ~7 to 4 µm during a single pass through the pulmonary circulation. Our purpose was to determine the nature of this deformation, its persistence after the cells leave the capillary bed, and the cytoskeletal changes that accompany this change in shape.

Intracellular events associated with leukocyte migration have been shown to be specific to the environment in which they migrate (21). Nonpolarized leukocytes suspended in solution undergo a random extension of actin-rich filopodia without becoming polarized (20, for review, see Ref. 22). In contrast, cells exposed to chemoattractant gradients reorganize their nuclei, Golgi apparatus, and microtubules. If they are able to form contacts with a rigid substrate, they extend actin-rich cell processes and migrate in the direction of the attractant (17, 22, 23). Sullivan and Mandell (23) have shown that cells in contact with a three-dimensional substrate behave quite differently. PMN were placed on a firm substrate with a “gel” overlay to provide three-dimensional contact and then were exposed to a chemoattractant gradient. The cells moved slowly toward the chemoattractant without accumulation of an F-actin-rich zone in their leading edge. Cells not covered by the gel polarized, extended F-actin-rich processes, and migrated. These data suggest that organelle polarization is influenced by the presence of chemoattractants, whereas the F-actin accumulation in cell processes depends on the nature of surface contacts available.

Our data show that filopodia or lamellipodia formation, the hallmark of active migration on a substrate, is lacking on WBCs recovered from the pulmonary capil-
laries. However, the movement of WBCs surrounded by an endothelial tube probably differs from cells migrating on rigid surfaces, and this could explain the lack of F-actin-rich filopodia or lamellipodia. Tensegrity, a structural system first described by Buckminster Fuller (20a) and based on tensional integrity provided by discontinuous compression elements, has been proposed to explain the mechanical response of cells to alterations in shape that are derived both externally and internally (for review see Ref. 17). The tensegrity model of cell shape predicts the formation of actin-dense stress fibers when cells exert a linear pull on a rigid substrate but does not require F-actin redistribution for changes in global shape when the inward pull is not focused on a limited number of rigid cell-substrate contacts (16, 17). Our findings are consistent with the tensegrity model of cell shape, which has the single requirement that the cell surface be under continuous tension. This force is provided by the F-actin-based contractile system, which provides centripetal force on the cell membrane. Microtubules, on the other hand, provide an opposing centrifugal force that may be regulated locally. The tensegrity model predicts actin-rich lamellipodia formation only in cell deformation associated with adhesion to a rigid substrate but not when adhesion does not occur as in leukocyte transit under physiological conditions.

Fig. 6. Grouped cell shape data from peak (A), shoulder (B), plateau (C), and FIC (D) samples for 7 experiments. Cells in the IF groups were elongated but did not exceed a shape ratio of 2.0. Maximum shape ratio in LF and FIC cells reached shape ratio values of 5.0.

Fig. 7. Cells perfusion fixed in pulmonary capillaries. Representative leukocytes from FIC fraction. Lymphocytes (A and B) and neutrophils (C and D) are elongated, but the nucleus is typically central. Tubulin (E and G) and F-actin stain (F and H) are evenly distributed.
Signals provided by externally applied deformation of the cell membrane have been proposed to be capable of initiating changes in local actin-membrane interaction through a number of proposed second messenger pathways (for review, see Ref. 6). The formation of microfilament structures is regulated in at least two ways. Filament length is regulated by actin monomer sequestering, filament capping proteins, and actin fila-

Fig. 8. Microtubule organizing centers (MTOCs) in leukocytes from IF fractions. MTOCs, the point of convergence of microtubules identified with anti-tubulin staining, are randomly distributed in cells fixed after being recovered from the pulmonary circulation.

Fig. 9. Confocal images of leukocytes from FIC fractions. WBCs dual stained with tetramethylrhodamine isothiocyanate (TRITC)-phalloidin F-actin (A, C, and E) and fluorescein isothiocyanate (FITC)-5A6 antibody to tubulin (B and D) have been imaged by confocal microscopy and assembled as stereo-paired micrographs. Images (A and B) and (C and D) are from the same cells that were perfused from the lung with fixative. Note the even distribution of F-actin (A and C) compared with the intense F-actin accumulation that occurs in the presence of process formation (E).
ment severing proteins, whereas three-dimensional microfilament structures are modulated by changes in microfilament cross-linking by actin cross-linking proteins. In leukocytes, cytosolic actin binding proteins sequester monomeric actin, effectively raising the cytosolic critical concentration for actin polymerization to 800 µM from 8 µM of purified actin, conferring a powerful buffering capacity on actin monomer to polymer exchange. Deformation of the leukocyte membrane could initiate second messenger-mediated modulation of actin cross-linking events through a separate pathway from actin binding proteins, providing for local buffering of “stiffening” without increased polymer formation (see Ref. 6). This is consistent with our observation of transiently retained shape change without formation of actin filament bundles and with earlier observations by others that PMN retain deformation relative to the degree and duration of deformation (2). Retention of the shape beyond the capillary segment, which would facilitate passage through the subsequent segment, may be mediated by the time course of such actin-associated cross-linking events (3). These observations are consistent with the findings of Weggs and co-workers (25) in which PMN having a mean diameter of 7 µm succeeded in mimicking the passage of rigid beads 4 µm in diameter, implying a limited deformability through rapid reduction in cross-sectional diameter. Furthermore, our results are consistent with the findings of Gebb and co-workers (12) in which leukocytes passing through canine pulmonary capillaries, visualized directly under the visceral plural surface through a window in the chest, were observed to elongate as they crossed capillary segments at the pleural surface, a requirement predicted by earlier modeling of leukocyte transit in pulmonary capillaries by these investigators (13).

In summary, our data show that leukocytes passing through the pulmonary capillary segments undergo deformation, which they retain beyond the capillary segment without evidence of cytoskeletal reorganization or cell polarization. These results suggest that the rapid reduction in PMN diameter required to negotiate restrictions in the capillary bed are different from those observed in cells migrating toward a chemoattractant. We propose that these may be initiated by membrane deformation signals that mediate internal buffering of stiffness to facilitate leukocyte transit through narrow capillaries in the circulation.

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