Neutrophil Diapedesis: Paracellular or Transcellular?

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To reach an inflammatory site in the interstitium, circulating neutrophils (PMN) must first traverse the endothelial barrier. Whether PMN emigrate between endothelial cells (paracellular pathway) or through the endothelial cells proper (transcellular pathway) is controversial. Herein, we present anatomic, functional, and teleological arguments that support both points of view. An attempt is also made to reconcile this apparent controversy.

One of the classic features of inflammation is infiltration of the affected tissue by polymorphonuclear leukocytes (PMN). For this to occur, circulating PMN must undergo adhesive interactions with the endothelium that allow them to become activated, flatten, and emigrate across the endothelial lining of microvessels. The molecular determinants of these adhesive interactions have been studied extensively, and there is a general consensus regarding the sequence of events that lead to PMN being placed in a position to emigrate into tissue (3, 12). Initially, a weak adhesive interaction occurs between circulating PMN and the endothelium that results in saltatory movement of PMN along the endothelium, a phenomenon referred to as rolling. Rolling allows the PMN to remain in close apposition to the endothelium, and, if there are locally generated inflammatory mediators present, the PMN become activated. Once activated, the PMN form stronger adhesive interactions with the endothelium that lead to their arrest. Subsequently, the PMN emigrate across the endothelium. Unlike the case for the initial adhesive interactions (rolling and adhesion), about which there is a general consensus on the mechanisms involved, little is known regarding the mechanisms involved in PMN transendothelial migration. Even the basic premise of whether PMN emigrate between endothelial cells (paracellular pathway) or through the endothelial cells proper (transcellular pathway) is controversial.

Evidence in favor of paracellular diapedesis

Prevailing consensus holds that circulating PMN emigrate from the intravascular compartment into the interstitium by passing between adjacent endothelial cells, i.e., using a paracellular pathway (11). Both in vivo and in vitro studies using electron and light microscopy have captured migrating PMN extending pseudopodia between endothelial cells to pass through the endothelial barrier in response to a chemotactic gradient. For PMN to be able to use the paracellular pathway for transendothelial migration, the adhesive coupling between adjacent endothelial cells must be disrupted and endothelial cells must separate from one another sufficiently to allow passage of the PMN. Many inflammatory mediators used to simulate inflammation in vitro can, per se, induce the formation of gaps in cultured endothelial cell monolayers, i.e., endothelial cell retraction. However, since large gaps between endothelial cells are rarely observed in in vivo models of inflammation, this phenomenon may represent an exaggerated effect of inflammatory mediators in in vitro systems. Cultured endothelial monolayers have underdeveloped interendothelial adhesion junctions compared with their counterparts in vivo, and thus inflammatory mediators may more readily produce endothelial cell retraction in vitro than in vivo (12). The in vivo correlate to this phenomenon is increased macromolecular leakage across interendothelial junctions observed in response to inflammatory mediators. Together, the in vitro studies lend credence to the idea that endothelial cells can separate from each other in response to inflammatory mediators, albeit the extent of separation may be exaggerated.

Evidence that PMN can induce endothelial cell retraction is primarily derived from in vitro studies that have addressed the mechanisms involved in PMN-mediated endothelial cell injury (12). This injury was nonlytic in nature and manifested as endothelial cell detachment from monolayers grown on nonporous surfaces. This endothelial cell detachment occurred 3–6 h after layering of activated PMN on the surface of endothelial cell monolayers and has been attributed to PMN-derived elastase. Since detached endothelial cells are rarely observed in in vivo models of inflammation, PMN-mediated endothelial cell detachment also appears to be an exaggeration of an in vivo event due to the experimental conditions imposed in vitro. In these systems (endothelial monolayers grown on nonporous surfaces), the PMN are not allowed to transmigrate and move away from the endothelial cell monolayer. Thus they remain in close proximity to the endothelial cells and persist in proteolytic aggravation of the monolayer, eventually inducing endothelial cell retraction and subsequent detachment. If the activated PMN are allowed to migrate across endothelial cell monolayers, gross endothelial cell retraction and detachment is rarely observed.

PMN-derived elastase can induce endothelial cell retraction and detachment within monolayers. This is analogous to the use of proteases to expand endothelial cells, i.e., the protease-treated cells retract and detach from the substratum, but after treatment with protease inhibitors, they can be reseeded and continue to grow normally. Interestingly, both endothelial...
cell retraction and PMN transendothelial migration can be prevented by protease (e.g., elastase) inhibitors. These latter observations indicate that PMN use endogenous elastase to induce endothelial cell retraction of sufficient magnitude to allow them to pass between adjacent endothelial cells without harming them. Recent studies suggest that elastase-mediated PMN transendothelial migration is a highly regulated process that does not require PMN degranulation (3). The activated PMN do not secrete elastase into the extracellular milieu; rather, they mobilize elastase to the membrane and localize it to the migrating front, e.g., the pseudopodia penetrating between adjacent endothelial cells. That PMN degranulation is not necessary for their transendothelial migration is further supported by the observation that PMN cytoplasts (anuclear PMN devoid of granules) migrate across endothelial monolayers in response to a chemotactic gradient just as efficiently as normal PMN. Again, the transendothelial migration of cytoplasts can be prevented by elastase inhibitors.

Endothelial cells are held together by adhesion junctions composed of transmembrane proteins (11). Thus for neutrophils to pass between endothelial cells the adhesive interactions of these junctions must be disrupted. There are two

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**FIGURE 1.** Diagrammatic representation of possible mechanisms mediating paracellular diapedesis in vitro (A) and transcellular diapedesis in vivo (B). A: PMN diapedesis through endothelial cell-cell junctions involves disassembly of endothelial cadherin/catenin complexes (blue bars). This may occur either proteolytically through surface-bound elastase (red circles) or by activating adhesion-mediated intracellular signaling. B: adherent PMN inserts filopodia-like processes into forming endothelial caveolae or vesiculo-vacuolar organelles (1), thereby gaining access to the abluminal side (2). Endothelial processes engulf the luminal portion of the PMN (3) and reseal the vessel before the PMN migrates into the tissue (4). Arrows, endothelial intercellular junctions.
adhesion junctions relevant to PMN transendothelial migration: adherens junctions and tight junctions. Adherens junctions consist of vascular-endothelial (VE)-cadherin/catenin complexes. VE-cadherin has an extracellular domain that homotypically interacts with VE-cadherin on adjacent endothelial cells. The cytoplasmic domain of VE-cadherin associates with intracellular β- or γ-catenins. These VE-cadherin/catenin complexes are connected to the actin cytoskeleton via α-catenin (Fig. 1A). Tight junctions consist of multiple transmembrane proteins, including occludin and claudins 1 and 2, and associated intracellular proteins, such as ZO-1, -2, and -3, which link the tight junction proteins to the cytoskeleton. Of these two adhesion junctions, the effect of PMN transendothelial migration on adherens junctions has received the most attention.

Recent studies using confocal microscopy indicate that PMN adhesive interactions (adhesion and transendothelial migration) can result in local disruption of the adherens junction proteins (10). Two populations of PMN were captured adhering to endothelial cell monolayers: 1) those underneath which there was no disruption of the continuity of the adherens junction proteins and 2) those underneath which there was disruption of the adherens junction proteins. The disruption of adherens junction continuity by adherent PMN was a local phenomenon, since adherens junctions further away from the PMN were not affected. A systematic analysis revealed that adherens junction proteins beneath adherent PMN were sequentially affected, i.e., β-catenin was lost before VE-cadherin. PMN captured in the process of transendothelial migration were invariably associated with the loss of all adherens junction proteins at the site of migration. Again, there was no effect on adherens junction integrity at sites further removed from the migrating PMN. Furthermore, evidence for the importance of adherens junction disruption in PMN transendothelial migration includes the following: 1) in vivo, antibodies against VE-cadherin increased PMN emigration, and 2) in vitro, antibodies against VE-cadherin increased PMN transendothelial migration and induced reorganization of endothelial actin cytoskeleton, resulting in the formation of gaps between endothelial cells.

With respect to tight junction complexes, recent studies have made similar observations. PMN transendothelial migration disrupted ZO-1 and -2 continuity only at the site of PMN penetration into the interendothelial junctions (11). Widespread discontinuities in tight junctions were not noted.

Potential mechanisms involved in paracellular diapedesis

The mechanism by which PMN-endothelial cell adhesive interactions disrupt the adherens junction is unclear. One possibility is that adherent PMN use endogenous proteases to degrade adherens junction proteins (Fig. 1A). For example, elastase inhibitors were able to diminish the extent of PMN-induced loss of the adherens junction proteins VE-cadherin and β-catenin (2). Other studies (11) indicate that activated PMN can degrade VE-cadherin and that an elastase inhibitor can prevent this degradation. Furthermore, purified neutrophilic elastase produces degradation products of VE-cadherin similar to those noted after degradation of this adherens junction component by activated PMN. Together, it seems likely that PMN-derived elastase plays a role in the disruption of the adherens junction.

PMN-endothelial cell adhesive interactions could also signal the endothelial cells to actively participate in PMN transendothelial migration by separating from each other, i.e., interendothelial gap formation (Fig. 1A). This contention is supported by the following lines of evidence. Adherent PMN can induce increases in endothelial cell Ca²⁺ levels, and chelating this Ca²⁺ results in an inhibition of PMN transendothelial migration. Furthermore, inhibition of endothelial myosin light-chain kinase (a key determinant of interendothelial cell gap formation) reduced PMN transendothelial migration (11). Finally, as pointed out above, adherent PMN affect the intracellular β-catenin before the plasma membrane-spanning VE-cadherin. Together, these observations indicate that endothelial cells can be induced to actively participate in PMN transendothelial migration by retracting from one another.

Evidence in favor of transcellular diapedesis

Many early and more recent reports that examined leukocyte diapedesis in vivo using an ultrastructural approach indicate that the majority of PMNs exit the vasculature transcellularly, i.e., through pores or passages that traverse the endothelial cytoplasm. Examination of serial sections using transmission electron microscopy suggest that PMNs can migrate through regions that are not associated with identifiable cell-cell junctions (4, 5, 8). It has been argued that even serial sectioning does not unequivocally prove that a cell-cell junction was not close by but rather that it may have been missed. In addition, it may be difficult to identify electron-dense adherens junctions, particularly in those regions in which the endothelium is <0.5 μm in height. Finally, recent in vitro data indicate that cell-cell adherens junctions molecularly disassemble during PMN diapedesis (10), and therefore one would not expect to see them morphologically.

Scanning electron microscopy images provide more compelling evidence that PMNs penetrate endothelial cells through openings that are not associated with endothelial cell-cell contacts (4, 9). PMNs that are in the process of diapedesis appear to have a dumbbell shape, with a constriction in the region at the level of the endothelium (4, 5, 7, 8, 9, 13, 15). Depending on how far diapedesis has progressed, a bubble-like extension is seen either protruding into the vessel lumen or extending into the interstitium (5, 9, 11, 15). This indicates that the leukocytes are squeezing through a circular pore of a small, limited diameter rather than inducing the retraction of individual endothelial cells from one another. The endothelial cell and the leukocyte remain in close contact throughout diapedesis. The endothelium often appears to spread along the luminal side of the PMN surface to completely engulf it.
thereby closing the luminal gap before the PMN is released into the tissue matrix (4, 5, 8). This process has frequently been observed during emigration in vivo but has not been demonstrated to occur during transendothelial migration in vitro.

**Potential mechanisms involved in transcellular diapedesis**

Although it is difficult to apply stringent mechanistic approaches in in vivo studies, there is sufficient circumstantial evidence to support several possible mechanisms by which PMN emigrate transcellularly. It is conceivable that the transcellular pores through which PMN migrate are a result of proteolytic damage to the endothelium. However, it has been pointed out that, at least ultrastructurally, the endothelium remains undamaged and continues to form caveolae and endothelial vesicles even in membrane regions that are in close contact with the PMN (13). It is more likely that PMN, which appear to migrate for short distances along the apical endothelial surface, seek out thin regions of the endothelium, such as fenestrae. Fenestrae can be opened (50 nm in diameter) or subtended by a diaphragm the thickness of two plasma membranes (devoid of cytoplasm). Thus the PMN could readily pass through opened fenestrae or proteolytically pass through diaphragmed fenestrae without damaging the endothelium proper. Fenestrae may represent an important pathway for PMN emigration in those organs that contain fenestrated microvessels (e.g., gastrointestinal mucosa, secretory glands).

In organs containing continuous microvessels (e.g., skin, muscle), the PMN may use caveolae or pinocytotic vesicles and insert filopodia into them to penetrate the endothelial barrier. These structures, between 50 and 100 nm in diameter, are believed to mediate protein transport across the endothelium and provide an extrajunctional route for vascular protein leakage during inflammation. Recently, caveolae have been shown to form vesiculo-vacuolar organelles, which can form small continuous membrane-bound passageways across the cytoplasm of an endothelial cell (6). The formation of such caveolae and vesicles has been demonstrated to continue in endothelial surface membrane regions that are in contact with adhering PMNs (13). In addition, PMNs that closely adhere to the endothelial cell surface often extend finger-like filopodia into indentations in the apical plasma membrane, thereby reshaping the endothelial surface (4, 7, 8). The protruding force of an adhering filopodium into forming vesiculo-vacuolar organelles may lead to the emergence of this filopodium at the abluminal endothelial side (Fig. 1B), where it can readily interact with the extracellular matrix and spread out along it (Fig. 1B).

A concentration of F-actin and microfilaments in leading pseudopodia of PMN has been demonstrated during diapedesis in vivo (15) and in vitro (3) and may generate the force required to pull advancing cellular processes along through the transcellular pores. The pores may widen to a size of 3-5 μm, through which the leukocyte can readily traverse. This widening of the initial vacuolar pore may be accomplished in part by forces generated by tension in the cortical microfilament system present in the spherical part of the leukocyte that still protrudes into the lumen of the vessel. In fact, a concentration of F-actin in the caudal region of transmigrating PMNs has been observed during late stages of diapedesis (15). In addition, cytoskeletal rearrangement in the endothelial cytoplasm may aid the widening of the transendothelial pore, as has been previously suggested (14). The process of pore formation would be accompanied by a spreading of apical endothelial membrane regions along the PMN cell surface, leading to the eventual engulfment of the luminal PMN portions by the endothelium, as has been demonstrated in electron micrographs (Fig 1B). This active participation of the endothelium would aid in the speedy rescaling of the endothelial lining, thereby limiting protein leakage.

**Summary**

From this review of the evidence, it is apparent that PMN can use both paracellular and transcellular pathways to traverse the endothelial barrier during inflammation. It is worth pointing out that PMN transendothelial migration in vitro favors the paracellular pathway, whereas PMN transendothelial migration in vivo favors the transcellular pathway. If one teleologically assumes that the transmigrating PMN will choose the path of least resistance, then there may be an explanation for the apparent discrepancy between results obtained using in vitro and in vivo approaches.

In endothelial cell monolayers grown in culture, the most attenuated regions are near cell-cell junctions, and it is therefore not surprising that this is the preferred site where diapedesis occurs. Furthermore, in cultured endothelial cells the intercellular junctions are unstable structures that are being constantly disassembled and reassembled. Such a region would be readily accessible by the pseudopodia of transmigrating leukocytes. There is also evidence that, even in vitro, the paracellular pathway used by PMN during diapedesis is specifically selected, i.e., diapedesis occurs preferentially at tricellular corners rather than between two endothelial cells (1). Adherens junctions and tight junctions are discontinuous at these tricellular corners, thus presenting the path of least resistance for the transmigrating PMN. In contrast, interendothelial junctures are much better developed in vivo and are more resistant to protein movement across them. Thus the PMN may preferentially use pores in the thin region of the endothelial cells, such as fenestrae or caveolae, as the path of least resistance.

To attribute the preferential pathway (paracellular vs. transcellular) used by PMN during diapedesis to whether in vitro or in vivo approaches were used to study this phenomenon is, notably, an oversimplification. There are many examples of departures from this paradigm. For example, there is evidence indicating that PMN can use the paracellular pathway in vivo and the transcellular pathway in vitro. Furthermore, if the transcellular pathway is the preferential one for PMN diapedesis in vivo, why does interfering with junctional proteins dramatically alter PMN diapedesis in vivo? It could well be that this argument will persist for years to come, much like the argument regarding whether transendothelial protein movement occurs preferentially via the paracellular or the transcellular pathway. One can envision that the arguments regarding the route of transendothelial migration of PMN may, in part, be attributed to the nature of the inflammatory response or the
nature of the vascular bed in which it occurs. Hopefully, further studies will provide additional information that will allow for the resolution of this controversy.

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References