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REVIEW

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# Forging the endothelium during inflammation: pushing at a half-open door?

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**Abstract** During an inflammatory response, changes in the adhesive properties of the endothelium occur that enable normally non-adherent blood-borne leukocytes to adhere and subsequently to traverse the endothelium through small gaps at inter-cellular junctions. This review concentrates on the role played by inter-endothelial adhesion molecules during transmigration and the way in which their expression may be regulated during inflammation. We show that the final "open" signals that lead to the formation of clefts between adjacent endothelial cells may be derived from inflamed tissue underlying the endothelium and from activated leukocytes.

**Keywords** Inter-endothelial adhesion molecules · Transmigration · Inflammation · Endothelium · Inter-cellular junctions

## Introduction

Most of us living in the Western world take it for granted that we will overcome an infection, with or without the help of medical intervention. We do not doubt the fact that cells of our immune system will leave their site of production, migrate in the blood, and arrive ready and rearing to go at exactly the spot where the infection began. Nevertheless, the biology behind this so-called inflammatory response is little short of a miracle. Our vascular system is not an open-ended network of pipes and channels that simply empty their contents into every organ and tissue. Rather, circulating blood is contained within a closed system, bound by endothelial cells whose main purpose is to limit permeability and prevent the loss of blood constituents and volume. The vascular endothelium thus controls the passage of macromolecules and leukocytes. In the resting state, the endothelium presents a non-thrombogenic surface that is not permissive to the passage of cells out of the bloodstream and into the underlying tissues. During an inflammatory response, however, changes in the adhesive properties of the endothelium occur that enable normally non-adherent blood-borne leukocytes to adhere and subsequently traverse the endothelium through small gaps at inter-cellular junctions. Precisely how these gaps form is a complex issue. The focus of this review will be the role played by inter-endothelial adhesion molecules during transmigration and the way in which their expression may be regulated during inflammation. We will try to show that the final "open" signals that lead to the formation of clefts between adjacent endothelial cells may be derived from inflamed tissue underlying the endothelium and from activated leukocytes.

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## Migration of cells of the immune system

The immune system protects the body from invading microorganisms and parasites and surveys the tissues for expression of aberrant self-antigens. The principal players in this defense system are the leukocytes, and they are well equipped to cope with virtually any pathogen. Most pathogens have extremely high proliferation rates, and prevention of systemic infections and damage needs a rapid response from both innate and adaptive immune systems. In other words, granulocytes, monocytes, and lymphocytes have to migrate to the site of infection and quickly. The mechanisms of innate immunity are always present and can be rapidly mobilized as a first-line defense against the infection. Innate immunity was thought to represent a non-specific immune response, but it is now clear that it has considerable specificity and is capable of discriminating between pathogens and self. Recognition of pathogens is mediated by germline-encoded pattern-recognition receptors, including Toll-like receptors in mammals (Akira et al. 2001). The cells of the innate immune system may either deal successfully with the infection or at least hold it in check while the more

powerful forces of the adaptive immune response are brought into play. The cells of the innate immune system are produced rapidly and in large numbers when required and generally have a short lifespan, being eliminated once they have exerted their function. Their migration is unidirectional, out of the bloodstream and across inflamed endothelium. By contrast, the response of the adaptive immune system, i.e., of the lymphocytes, is relatively slow, since the number of cells with receptors specific for any given pathogen is extremely low. Nature has responded to this disadvantage in a remarkable way, and lymphocytes have complex migration patterns. Mature non-activated lymphocytes continuously recirculate between the blood and lymphoid tissues (Ager 1994; Pabst and Binns 1989; Salmi and Jalkanen 1997; Wiedle et al. 2001). Lymphocyte extravasation occurs in the post-capillary venules at specialized vascular sites called high endothelial venules (HEVs; Anderson and Anderson 1976; Anderson et al. 1976; Baekkevold et al. 2001; Girard and Springer 1995). In humans, HEVs are found in all secondary lymphoid organs, with the exception of the spleen. Endothelial cells of HEVs have a specialized morphology, together with the specific expression of several genes that increase their adhesive properties for lymphocytes (Girard et al. 1999). The recirculation of naive lymphocytes has been the subject of many recent reviews (Girard and Springer 1995; Salmi and Jalkanen 1997; Wiedle et al. 2001). Back to that trick of nature: if a lymphocyte encounters its specific antigen in the secondary lymphoid organ, then its migration pattern changes. This change is brought about by modifications in the surface expression of adhesion molecules and chemokine receptors that together re-direct the activated lymphocyte to sites of inflammation, thereby maximizing the chances that a primed lymphocyte will arrive at the site of infection (for a review, see Muller 2002). Coupled with this is the fact that lymphocytes that have been activated by antigen are "harbored" as memory cells that can respond more quickly to subsequent encounters with the same antigen. The outcome is the generation of a pool of antigen-specific lymphocytes that can quickly arrive at the site of an infection and rapidly expand their numbers.

## The multi-step adhesion cascade

In both inflammatory and homeostatic leukocyte migration, immune cells have to breach the vascular barrier, a process often referred to as transmigration or extravasation or diapedesis. As mentioned above, constitutive recirculation occurs essentially within lymph nodes across HEVs; this will not be dealt with further here, except to make the important point that HEVs represent a specialized endothelium. Their unique morphology has almost certainly evolved to facilitate high lymphocyte traffic, a hypothesis supported by the presence, in chronically inflamed non-lymphoid tissues, of HEV-like vessels that are believed to support lymphocyte recruitment (Dawson et al. 1992; Duijvestijn et al. 1987). The nature of the junctional composition of HEVs is not completely understood, but it is tempting to speculate that there may be similarities with inflamed endothelium. Outside of the lymphoid organs, the endothelium changes in response to an infection in the underlying tissue: a normally non-permissive surface for the adhesion of circulating leukocytes becomes permissive, supporting the recruitment of high numbers of inflammatory cells from the passing bloodstream. A multi-step adhesion cascade has been proposed consisting of four steps (Butcher and Picker 1996; Carlos and Harlan 1994; Springer 1994). Transmigration represents the final step in this cascade. In the first step, leukocytes roll on endothelial cells. This is followed by a triggering step in which there is a rapid activation of leukocyte integrins via G-protein-coupled receptors, and then a third step at which point the leukocyte adheres tightly onto the endothelial surface. Finally, diapedesis occurs and the leukocyte crawls through the junction between apposing endothelial cells. An understanding of the first three steps has proceeded rapidly over the past ten years.

### Leukocyte rolling

The first step involves initial tethering of the leukocyte followed by reversible rolling. Rolling is generally mediated by interaction between selectins and glycosylated ligands and leads to a slowing down of the cells in the bloodstream. There are three selectins: L (leukocyte)-selectin, P (platelet)-selectin and E (endothelial)-selectin. L-selectin is expressed on virtually all non-activated leukocytes, and the ligands for this selectin are constitutively expressed on HEVs. Hence, this selectin is responsible for the trafficking of naive lymphocytes through lymph nodes (Berg et al. 1989). Upon activation of lymphocytes by antigen, L-selectin is down-regulated, and this prevents the re-entry of antigen-experienced cells into lymph nodes (thus facilitating their re-direction to sites of inflammation; Hafezi-Moghadam et al. 2001; Rigby and Dailey 2000). The down-regulation of L-selectin involves transcriptional regulation and protein shedding by surface matrix metalloproteases (Preece et al. 1996). E-selectin and P-selectin are expressed on vascular endothelium activated by inflammatory stimuli (Lasky 1995; Ley and Tedder 1995). E-selectin is up-regulated by de novo protein synthesis and mediates the binding of granulocytes and some lymphocytes to activated endothelium. P-selectin is sorted to specialized granules (Weibel-Palade bodies) and translocates to the cell surface within minutes of stimulation by thrombin, histamine, and complement components (Hattori et al. 1989; McEver et al. 1989), which are produced during an inflammatory response. During chronic inflammation, P-selectin may be expressed stably at the cell surface. The rolling phase is not mediated exclusively by the selectins. Notably, the integrin $\alpha 4\beta 1$ (VLA-4) can support rolling and tethering on vascular cell adhesion molecule-1 (VCAM-1), an integrin ligand expressed on activated

endothelium (Alon et al. 1995; Berlin et al. 1995; Henderson et al. 2001). In addition, CD44, a transmembrane glycoprotein expressed in a wide variety of cell types, can function as an adhesion receptor to mediate leukocyte rolling on hyaluronan (Clark et al. 1996; DeGrendele et al. 1996), a component of the extracellular matrix and a physiologic ligand of CD44 (Aruffo et al. 1990). Hyaluronan expression is upregulated on endothelial cells following pro-inflammatory stimuli (Mohamadzadeh et al. 1998; Nandi et al. 2000), and CD44 is upregulated in activated lymphocytes (DeGrendele et al. 1997; Lesley et al. 1994). A recent report has demonstrated that the cell surface density of CD44 is a critical factor for CD44-dependent cell rolling on hyaluronan (Gal et al. 2003).

### Activation of integrins and tight adhesion

Rolling and tethering are reversible events and must be replaced by a strong adhesion if the leukocyte is to cross the endothelial barrier. In the second step of the adhesion cascade, the leukocyte is stimulated by chemokines, some of which are constitutively produced and appear to fulfil housekeeping functions, but many of which are only produced upon cell activation. For example, endothelial cells exposed to bacterial products or primary inflammatory cytokines, such as lipopolysaccharide (LPS), tumour necrosis factor-$\alpha$ (TNF-$\alpha$), or interleukin-1 (IL-1), produce monocyte chemotactic proteins (MCPs) (CCL7, CCL8, CCL13), RANTES (CCL5), fractalkine (CX3CL1), and macrophage inflammatory proteins (CCL3, CCL4) (Baggiolini and Dahinden 1994; Ben-Baruch et al. 1995; Mantovani et al. 1997, 1998). At least some chemokines have been demonstrated to associate, through a heparin-binding domain, with proteoglycans on the luminal surface of endothelial cells (Tanaka et al. 1993). The effects of chemokines on leukocytes are mediated by G-protein-coupled receptors, and leukocyte responses to these molecules depend on the presence of the relevant receptor.

Upon stimulation of a leukocyte through chemokine receptors, the activity of its integrins is increased, and this brings about the third step of the cascade, a tight adhesion of the leukocyte onto integrin ligands on the endothelium. Integrins are heterodimers formed by a combination of one alpha and one beta chain (Shimizu et al. 1999). The integrins $\alpha 4\beta 1$ (VLA-4) and $\alpha L\beta 2$ (LFA-1) have major functions in leukocyte-endothelial cell interactions (Hu et al. 1993; R.S. Larson and Springer 1990; Lobb and Hemler 1994; Stewart et al. 1995). The regulation of integrin activity can be acheived in several ways: by a change in integrin affinity (Ginsberg et al. 1992), by association of the integrin with the cytoskeleton (Kucik et al. 1996), or by clustering (van Kooyk et al. 1994). Integrin ligands are members of the Ig superfamily; intercellular cell adhesion molecule-1 (ICAM-1) and ICAM-2 bind to LFA-1, VCAM binds to VLA-4. ICAM-1 and VCAM are up-regulated on endothelium stimulated by IL-1, TNF-$\alpha$, and LPS (Dustin et al. 1986; Wellicome et al. 1990).

### Diapedesis

A picture of this final step in the adhesion cascade is just beginning to take shape. Leukocytes transmigrate by passing between adjacent endothelial cells, the paracellular route. It should be noted, however, that this is not the exclusive pathway for these cells. Early ultrastructural studies suggested that lymphocytes could pass through the body of an endothelial cell (Marchesi and Gowans 1964), and recently, Feng and colleagues (1998) have demonstrated that, under certain situations, in vivo neutrophils emigrate from inflamed venules via a transcytotic pathway, i.e., through the body of an endothelial cell. For these studies, N-formyl-methionyl-leucyl-phenalanine (FMLP) was injected as an inflammatory agent. Hence, for at least one stimulus and one cell type, a transcytotic pathway of extravasation exists. Whether other inflammatory cells take this route in response to different stimuli remains, for the present, controversial. Furthermore, it will be important to identify the molecules involved.

If the leukocyte adopts the paracellular route, and the evidence to date suggests that much of the time it does, then the challenge facing it is daunting, since endothelial cells are connected to each other by an impressive array of endothelial junctional structures that the leukocyte must breach (for a review, see Johnson-Leger et al. 2000). These junctions are present in order to maintain the integrity of the endothelium and to regulate vascular permeability. Four different types of endothelial junctions have been described: tight junctions, gap junctions, adherens junctions, and syndesmosomes, or complexus adherentes, which are present to varying degrees along the vascular tree (for reviews, see Dejana et al. 1995, 1996, 1997; Dejana and Del Maschio 1995; Vestweber 2000). These junctions resemble the homologous structures in epithelium, but their spatial organization is less ordered, possibly reflecting the need for constantly changing requirements of permeability and cellular transmigration. The various junctions have been extensively reviewed, and so their descriptions here will be restricted to features that are important for understanding the remainder of this review.

## Inter-endothelial junctions

### Tight junctions

These are the most apical junctions, i.e., closest to the luminal surface, and they form a very close contact between adjacent cells. They appear as a series of discrete sites of apparent membrane fusion, involving the outer leaflet of the plasma membranes of adjacent cells. Three types of transmembrane proteins, occludin, claudins, and junctional adhesion molecule (JAM), co-localize with

tight junctions (Furuse et al. 1993, 1998; Martin-Padura et al. 1998). Inside the cells, several cytoskeletal signaling molecules are concentrated in the tight junctional area (Gumbiner 1996).

### Adherens junctions

These junctions are cellular membrane contacts formed by transmembrane glycoproteins called cadherins. Cadherins physically attach the cell membrane to an intracellular undercoat network of cytoplasmic proteins and actin microfilaments via their cytoplasmic tails (Geiger and Ayalon 1992; Tsukita et al. 1992), a process mediated by intracellular catenins (Kemler 1993). In addition to the neuronal cadherins, the endothelium expresses a specific cadherin, VE-cadherin. VE-cadherin is localized at intercellular junctions in all endothelia (Ayalon et al. 1994; Lampugnani et al. 1992), and the extracellular domain is responsible for $Ca^{2+}$-dependent homophilic adhesion. Like most other cadherins, the cytoplasmic tail is linked to the cytoskeleton via the catenins and p120 in the endothelium.

Historically, the adherens junction has been considered a key player in endothelial permeability, and the VE-cadherin:catenin complex has received and continues to receive wide acclaim as the main regulator of permeability in microvascular endothelium. This may be justified if we consider permeability for macromolecules but it is only part of the story concerning transmigration. Indeed, the correlation between leukocyte transmigration and changes in vascular permeability is far from clear. In the following sections, we describe the junctional molecules that play a role in transmigration, and the reader will probably come to the conclusion, as we have, that the assignment of a molecule to a particular junctional structure does not determine its role in this process. Indeed, with recent advances in our understanding of this event, a picture is beginning to form of transmigration as a dynamic process in which tight junctional, adherens, and gap junctional proteins collaborate to bring about the final step of the adhesion cascade across an inflamed endothelium.

### Gap junctions

Gap junctions are clusters of transmembrane hydrophilic channels that allow direct exchange of ions and small molecules (Beyer 1993). They consist of a pair of interacting hemichannels (connexons), contributed by each of the co-operating cells. Each channel comprises six polypeptide subunits, or connexins, arranged around a central pore. Three connexins are constitutively expressed on vascular endothelium, connexin (Cx) 37, 40, and 43 (Bruzzone et al. 1993; Larson et al. 1997; Polacek et al. 1997; Reed et al. 1993; van Rijen et al. 1997). Interestingly, the connexins on endothelial cells are mainly located at cell-cell contacts (van Rijen et al. 1998).

## Inflammation and the endothelium

When a tissue is damaged, it alerts the body's defense system in what has been termed the inflammatory response. What is perceived as damage or injury may be one or more of many factors: vessel occlusion leading to hypoxia, physical damage, bacterial infection, or viral infection. During infection, for example, extracellular bacteria may stimulate tissue-resident macrophages to produce a battery of cytokines and other inflammatory mediators. Neutrophils are thereby recruited to the site of infection and contribute to the inflammation, establishing a self-perpetuating inflammatory response. The inflammatory response is itself composed of a series of different events that involve soluble factors, changes in endothelial cell adhesiveness and permeability, and recruitment of inflammatory cells. A text-book definition of inflammation is the local accumulation of fluid accompanied by swelling, reddening, and pain, all effects of which stem from the dilation of local blood capillaries, a reduction in blood flow rate, and increased permeability of the endothelium. The changes that occur at the surface of the endothelium "tag" the area as inflamed and act as "exit" signals for circulating inflammatory cells, which can now adhere to and transmigrate specifically at these sites. If we consider these changes, we see that they reflect, intriguingly, the requirements of each step of the adhesion cascade. For example, early on during an inflammatory response, histamine and thrombin are released by mast cells present in damaged or infected tissue. Both these agents trigger the rapid translocation of P-selectin to the endothelial cell surface. E-selectin is up-regulated by de novo protein synthesis following stimulation by inflammatory cytokines (Doukas and Pober 1990; Leeuwenberg et al. 1990). Hence, the rolling and tethering step is facilitated. Chemokines produced specifically at the site of infection mediate the activation of leukocyte integrins, whereas the ligands of these integrins, ICAM-1 and VCAM-1 are up-regulated on the surface of inflamed endothelium.

What about the final step of the adhesion cascade? Is there a similar regulation of junctional proteins by inflammatory signals? Diapedesis occurs at bi-cellular junctions between two apposing endothelial cells at an array of junctional complexes (for a review, see Muller 2001). The presence of these junctions might be expected to hinder the passage of transmigrating cells, and indeed, several studies have demonstrated that transmigrating leukocytes may take the path of least resistance. Burns and colleagues (1997, 2000) have demonstrated that neutrophil transendothelial migration across monolayers of human umbilical vein endothelial cells (HUVECs) occurs preferentially at tri-cellular corners where the borders of three endothelial cells intersect, and where both tight and adherens junctions are discontinuous. Furthermore, prior culturing of the HUVECs with astrocyte-conditioned medium (to induce a higher frequency of tight junctions) does not affect neutrophil migration across HUVEC monolayers (Burns et al. 1997). Since

tri-cellular corners represent less than 10% of the total endothelial border area, this implies that neutrophil transmigration may be highly selective for tri-cellular junctions. In the same study, monocytes showed less of a preference for such junctions, and so tri-cellular migration may be specific to neutrophils. For leukocytes that "choose" the bi-cellular pathway, evidence is mounting that they actually employ the junctional molecules to aid their passage through the junctions. In this respect, we should perhaps consider endothelial junctions not so much as being fences, but more as representing bridges where the molecules present enable the progression of the leukocyte through the inter-endothelial cleft. In the following section, we have detailed the molecules that are present at these junctions and that have demonstrated roles in transmigration and we discuss the way in which their expression and localization may be influenced during an inflammatory response.

## Junctional molecules involved in leukocyte transmigration: regulation by inflammatory conditions

### PECAM-1

PECAM-1, a member of the immunoglobulin superfamily, is expressed diffusely on the surface of platelets and most leukocytes (DeLisser et al. 1994; Watt et al. 1995). In addition, it is concentrated at the intercellular junctions of endothelial cells (although not associated with a particular junctional structure), where it can support cell-cell adhesion (Ayalon et al. 1994). PECAM has been implicated as a critical mediator of monocyte and neutrophil transendothelial migration in vivo, and also in vitro across HUVEC monolayers (Bogen et al. 1994; Liao et al. 1997; Muller et al. 1993). Whereas the absence of PECAM in knockout mice does not affect the number of monocytes undergoing transmigration, PECAM-deficient mice show an accumulation of neutrophils at the basement membrane of postcapillary venules in the mesentery, suggesting an important role for this molecule in the migration of neutrophils across the trans-basement membrane (Duncan et al. 1999). Further support for this has come from a recent study by Dangerfield and colleagues (2002) who have demonstrated, by using intravital microscopy, that homophilic PECAM-1 interactions induce the up-regulation of integrin $\alpha 6\beta 1$expression on transmigrated neutrophils, and that this interaction is required for neutrophil migration through the perivascular basement membrane. $\alpha 6\beta 1$ is the principal leukocyte laminin receptor, and laminin is a key component of all venular basement membranes (Timpl 1989). The way in which inflammatory signals impinge upon PECAM function is not yet clear. The combined treatment of HUVECs with the inflammatory cytokines TNF-$\alpha$ and interferon-$\gamma$ (IFN-$\gamma$) has been demonstrated to result in a re-distribution of PECAM out of inter-cellular junctions; this is correlated with changes in PECAM-cytoskeleton association (Romer et al. 1995). One explanation for the removal of PECAM from endothelial cell junctions during inflammation is that its removal may reduce adhesive interactions between adjacent endothelial cells, thus enhancing diapedesis. Alternatively, or additionally, this re-distribution may "free" junctional PECAM, thus providing a pool of PECAM that can interact with transmigrating leukocytes. Indeed, the recent findings of Mamdouh et al. (2003) demonstrate that PECAM is actually targeted to areas of transmigrating monocytes. Here, the authors demonstrate the presence of a membrane network that lies just beneath the surface of the plasma membrane at HUVEC cell borders and that is connected to the junctional surface. PECAM-1 is found in this compartment and constitutively recycles evenly along endothelial cell borders. During transendothelial migration across HUVEC monolayers, however, recycling PECAM is targeted to areas of the junction at which the migration of monocytes is taking place. The authors suggest that this may provide a source of endothelial PECAM for the leukocyte to engage via homophilic interaction with its own PECAM. The continuous addition of PECAM further and further into the junction may create a haptotactic gradient through the junction. Indeed, a role for PECAM as a molecular zipper was first suggested 6 years ago (Bianchi et al. 1997), and so the elucidation of a mechanism by which this may occur is an exciting advance in our understanding of transendothelial migration. Targeting of PECAM to transmigrating cells would both provide a scaffold to support the leukocyte through the endothelial cleft, and a signal to prepare the cell for subsequent migration through the basement membrane (Dangerfield et al. 2002).

### CD99

Similar to PECAM-1, CD99 is concentrated at the borders between endothelial cells and is a critical mediator of monocyte transmigration in vitro through HUVEC monolayers (Schenkel et al. 2002). Again, similar to PECAM, the blockade of CD99 on either the monocyte or the endothelial cell prevents diapedesis, thus indicating the homophilic interaction between leukocyte and endothelial cell as playing a major part in monocyte transmigration. CD99 appears to control a step in diapedesis distinct from and distal to that controlled by PECAM-1 (Aurrand-Lions et al. 2002; Schenkel et al. 2002). There is no evidence, to date, that suggests that the localization or expression of CD99 on endothelial cells may be regulated by inflammatory signals.

### JAMs

As a brief introduction to the JAMs, the authors would like to alert the reader to some confusion in the naming of these molecules, which were cloned simultaneously by independent laboratories. A table has been provided (Table 1) listing the alternative names that have appeared

**Table 1** Past and present designations of the JAM family of proteins

| Old names | Reference | Ligands | New designation |
|---|---|---|---|
| JAM-1 | Martin-Padura et al. 1998 | JAM-A<br>$\alpha L\beta 2$ (LFA-1) | JAM-A |
| JAM2 (man)<br>JAM-3 (mouse)<br>VE-JAM (man) | Cunningham et al. 2000<br>Aurrand-Lions et al. 2000<br>Palmeri et al. 2000 | $\alpha 4\beta 1$<br>JAM-C | JAM-B |
| JAM3 (man)<br>JAM-2 (mouse) | Arrate et al. 2001<br>Aurrand-Lions et al 2001 | JAM-C<br>$\alpha M\beta 2$ (Mac-1)<br>$\alpha X\beta 2$ | JAM-C |

for these molecules and including the officially accorded nomenclature that will be henceforth adopted by the scientific community (Muller 2003).

*JAM-A*

JAM-A was the first member of this "sub-family" of adhesion molecules to be cloned (Martin-Padura et al. 1998). It belongs to the Ig superfamily, possessing two extracellular Ig domains, and interacts homotypically in confluent monolayers of Chinese hamster ovary (CHO) cells. Confocal and immunoelectron microscopy has shown that JAM-A co-distributes with tight junctional components. Furthermore, several PDZ-containing proteins have been demonstrated to associate with the PDZ-binding motif in the cytoplasmic domain of JAM-A, including cingulin, occludin, ZO-1, afadin, CASK, and PAR-3/ASIP (Bazzoni et al. 2000; Ebnet et al. 2000, 2001). A monoclonal antibody directed against JAM-A (BV11) inhibited spontaneous and chemokine-induced monocyte migration across an endothelial cell monolayer in vitro and monocyte infiltration in a model of skin inflammation in vivo (Martin-Padura et al. 1998). Furthermore, combined treatment with the inflammatory cytokines TNF-$\alpha$ and IFN-$\gamma$ induced the disappearance of JAM-A from intercellular junctions and its re-distribution on the cell surface, whereas the total amount of surface JAM-A was unchanged (Ozaki et al. 1999). This suggests a mechanism whereby the function of JAM-A may be regulated during inflammation. Interestingly, such combined cytokine treatment resulted in reduced transendothelial migration under static conditions, although there was no effect under flow conditions. The real significance of these findings has only recently come to light: Ostermann and colleagues (2002) have identified LFA-1 as a ligand for JAM-A and have shown that JAM-A-LFA-1 interaction is involved in the tight adhesion of leukocytes (memory T cells) to HUVEC under static and flow conditions, when the endothelial monolayer is stimulated with TNF-$\alpha$ and IFN-$\gamma$, i.e., when JAM-A is expressed at the apical surface. On the other hand, when JAM-A is in the junctions of unstimulated endothelial cells, it apparently plays a role rather in the transmigration of these cells. JAM-A has also been shown to contribute to LFA-1-mediated transmigration of neutrophils across unstimulated HUVEC under flow conditions. Interestingly, the membrane proximal Ig domain of JAM-A supports its interaction with LFA-1, whereas the membrane-distal domain is responsible for its homophilic dimerization at inter-endothelial junctions (Ostermann et al. 2002). We should perhaps take a lesson from these studies, which add a new level of complexity to the role played by junctional molecules during leukocyte recruitment. Indeed, these molecules may demonstrate a plasticity of function, being involved for example in adhesion or diapedesis, depending on their sub-cellular localization, and this localization may itself be regulated by physiological parameters.

*JAM-B and JAM-C*

Two other JAM family members have since been cloned (Arrate et al. 2001; Aurrand-Lions et al. 2000, 2001; Cunningham et al. 2000; Palmeri et al. 2000). Like the prototype, JAM-A, both have integrin ligands: $\alpha 4\beta 1$ for JAM-B, and $\alpha M\beta 2$ and $\alpha X\beta 2$ for JAM-C. Furthermore, JAM-B and JAM-C appear to be binding partners. The interaction between these two family members is intriguing, since JAM-B is expressed on HEVs of lymph nodes and tonsils (Palmeri et al. 2000) and also on the endothelium of arterioles in and around inflammatory and tumour foci (Liang et al. 2002). JAM-C, in addition to its expression on endothelium, is expressed on activated T cells, platelets, B cells, monocytes, dendritic cells, and NK cells in man (Johnson-Leger et al. 2002; Liang et al. 2002; Santoso et al. 2002). The role of these two JAMs in transmigration is not yet clear. JAM-B/JAM-C interactions may play a part in leukocyte trafficking during inflammation. Nevertheless, the findings that JAM-C is also expressed by the endothelium (Aurrand-Lions et al. 2001), and that both JAM-B and JAM-C have integrin ligands on circulating cells, suggest that several interactions may occur depending on the physiological setting. Our laboratory has shown that murine lymphocytes, which do not themselves express JAM-C, transmigrate endothelial cells in a JAM-C-dependent manner (Johnson-Leger et al. 2002). Furthermore, an antibody directed against JAM-C inhibits the migration of human peripheral blood mononuclear cells across monolayers of unstimulated HUVECs in vitro. We do not yet know the mechanism(s) involved in this antibody-mediated block, but clearly the possibilities are manifold.

### Connexins

The last few reviews dealing with endothelial junctions and leukocyte transmigration might have stated that gap junctions appear to play no role during transmigration. Given the experimental evidence or, more precisely, its lack, this was a fair assessment of the situation. However, recent data suggest that gap junctional coupling between leukocytes and the endothelium may play a role in modulating transendothelial migration (Zahler et al. 2003). Cx43 is expressed on stimulated leukocytes (Jara et al. 1995), and human T, B, and NK lymphocytes derived from peripheral blood and secondary lymphoid organs express Cx40 and Cx43 (Oviedo-Orta et al. 2000). Communication via gap junctions during transmigration was first described by Oviedi-Orta and colleagues (2002). In their study, the authors demonstrated, by using dye-transfer experiments, that lymphocytes and endothelial cells generate functional gap junction channels during transmigration in vitro. More recently, Zahler et al. (2003) have demonstrated that gap junctional coupling exists between adherent neutrophils and HUVECs in vitro and that this coupling is reduced when the HUVECs are stimulated with TNF-$\alpha$. More importantly, leukocyte transmigration is enhanced when gap-junctional coupling is inhibited, suggesting a regulatory role for this coupling during transmigration.

## Signalling at endothelial cell junctions

Whereas the precise details and sequence of events taking place during transendothelial migration remain to be determined, there is an overwhelming body of data in support of paracellular migration. A paucity of data describing transcytosis suggests that this pathway may represent a minor one, perhaps being restricted to particular cell subsets or stimuli. The scientific community awaits clarification concerning this issue, which is an interesting topic for future research. Our current view of leukocyte emigration is, therefore, migration through a cleft between apposing endothelial cells. Hence, the two key aspects of this process are leukocyte migration and endothelial junction widening. Cellular migration is the result of choreographed changes in cell morphology and adhesion properties and is regulated by the actin cytoskeleton. In a recent review that deals with molecular events taking place within transmigrating leukocytes, Worthylake and Burridge (2001) highlight the importance of integrin activity and actin organization, which gives rise to dramatic changes in cell shape and adhesive properties in response to inflammatory signals. We should like to spend the remainder of this current review by considering how the widening of endothelial junctions may occur during inflammation. To this end, we have come full circle and will now consider the review's title. By a "half-open door" we do not wish the reader to imagine gaping holes in the endothelium, to which the leukocyte merely has to crawl and then slip through. Such a state of affairs would have fairly catastrophic consequences for the organism, since prolonged endothelial hyper-permeability is a serious and life-threatening clinical complication. Nevertheless, the evidence suggests that, during inflammation, the endothelium of the post-capillary venules becomes locally hyper-permeable, and that this is accompanied by the formation of minute gaps between adjacent endothelial cells. There is leakage of fluid from the blood, which provides the underlying tissue with plasma proteins such as complement factors, immunoglobulins, and coagulation factors (for a review, see van Hinsbergh and van Nieuw Amerongen 2002). In other words, the increase in permeability is an attempt to "heal" the injured tissue. Vasoactive agents such as histamine induce the formation of these tiny gaps between endothelial cells (Baluk et al. 1997; Hirata et al. 1995). In vitro, histamine-induced increases in permeability depend on an increase in cytoplasmic calcium (van Hinsbergh and van Nieuw Amerongen 2002). The increase in cytoplasmic calcium ions causes a calcium-dependent activation of the myosin light chain kinase (MLCK), an enzyme that phosphorylates myosin light chains. Phosphorylation of the myosin light chain can induce actin polymerization and endothelial cell retraction (Goeckeler and Wysolmerski 1995; Moy et al. 1993, 1996; Sheldon et al. 1993), and this may be sufficient to create the small gaps. Alternatively, agents such as histamine may act directly on junctional proteins, thereby regulating their presence in the junction. Hence, under inflammatory conditions, gaps may form even in the absence of leukocytes. In reality, these gaps are extremely small (approximately 1.5 $\mu$m), probably too small to allow the passage of leukocytes. However, it is conceivable that leukocytes use these tiny "footholds" to begin engaging the junction. Perhaps, via interaction with junctional molecules, they actively participate in widening pre-existing gaps, to just the right width to allow them to pass! There is, indeed, experimental evidence to suggest that leukocytes contribute to endothelial gap formation: stimulated neutrophils induce myosin light chain phosphorylation via activation of MLCK and isometric tension in endothelial cell monolayers, whereas unstimulated neutrophils have no effect on either parameter (Hixenbaugh et al. 1997). What about other signals generated at inter-endothelial junctions during inflammation? This is, as yet, an area that awaits clarification. Nevertheless, if we consider the proteins present in these junctions, it is apparent that most, if not all, have recognized signaling functions.

### VE-cadherin

The endothelium specifically expresses VE-cadherin, which is localized at intercellular junctions in all endothelia and is linked to the cytoskeleton via $\beta$-catenin and $\gamma$-catenin. VE-cadherin is a target of agents that increase vascular permeability, such as vascular endothelial growth factor (VEGF), histamine, and thrombin (Esser et al. 1998; Rabiet et al. 1994, 1996). The addition of an

antibody against VE-cadherin to HUVEC monolayers leads to a marked reorganization of the actin cytoskeleton and increases monolayer permeability. In addition, neutrophil migration across HUVEC monolayers is enhanced upon antibody treatment. These effects are accompanied by a change in the distribution of VE-cadherin (Hordijk et al. 1999). Furthermore, the in vivo administration of an antibody directed against VE-cadherin resulted in increased vascular permeability in the heart and lungs (Corada et al. 1999). Esser and colleagues (1998) propose that tyrosine phosphorylation of VE-cadherin regulates vascular permeability. In an in vitro model, VEGF stimulated the migration of endothelial cells and induced an increase in the paracellular permeability of HUVECs. These effects correlated with an increase in phosphotyrosine labeling at cell-cell contacts, and VE-cadherin, $\beta$-catenin, $\gamma$-catenin, and p120 were phosphorylated on tyrosine. At the same time, there was a change in the pattern of VE-cadherin staining from a continuous linear labeling to a zigzag pattern, a change previously shown to be correlated with the loss of junctional integrity. Allport and colleagues (2000) have reported that VE-cadherin and associated catenin molecules transiently disappear during in vitro transmigration of monocytes. Real-time imaging has since demonstrated, in vitro, that monocytes and neutrophils migrate to endothelial cell-cell contacts bearing continuous VE-cadherin staining. A gap is formed in the VE-cadherin at the point at which the leukocyte interacts with the endothelium, and the cell migrates through this gap (Shaw et al. 2001). Indeed, the leukocytes appear literally to "push aside" the VE-cadherin. What is perhaps particularly interesting about this study is that the leukocytes also migrated through small pre-existing gaps in VE-cadherin staining, widening them first in order to pass through them. In relation to this, it should be noted that the HUVEC monolayers used for the transmigration assays were stimulated with TNF-$\alpha$. It would be interesting to know whether such "pre-existing" gaps were also present in unstimulated monolayers, or whether this represents an inflammation-induced event. VE-cadherin plays an essential role in the regulation of endothelial cell contacts and is therefore likely to be a target of signaling during transmigration. Furthermore, its removal from areas where monocytes and neutrophils are actively transmigrating is a strong argument for its regulation being partially mediated by the leukocyte itself. Whether this "pushing aside" involves the phosphorylation of VE-cadherin remains to be determined.

Vascular endothelial protein tyrosine phosphatase (VE-PTP) and VE-cadherin have been recently demonstrated to interact via their membrane proximal extracellular domains (Nawroth et al. 2002). Furthermore, induction of VE-PTP in CHO cells decreased the tyrosine phosphorylation of VE-cadherin (probably via recruitment and/or activation of another phosphatase) and, at the same time, increased cell-contact integrity. Since VE-PTP is a receptor-type phosphatase expressed at the surface of endothelial cells, one might imagine a mechanism whereby activated leukocytes bind to VE-PTP and modulate its activity. Alternatively, or additionally, VE-PTP may be regulated directly by inflammatory cytokines acting locally on endothelial cells.

### PECAM-1

PECAM-1 has two distinct immunoreceptor tyrosine-based inhibitory motifs in its cytoplasmic domain (for a review, see Newman and Newman 2003). Tyrosine phosphorylated PECAM can recruit SH2 domain-containing signaling proteins, notably SHP-2. PECAM-1 can be phosphorylated on tyrosine in virtually all endothelial cells following mechanical stimulation (Osawa et al. 1997) or cell adhesion (Bird et al. 1999). Src family kinases appear to be involved in this phosphorylation, although a non-Src family kinase may also play a role. PECAM is also reported to interact with phosphorylated $\beta$-catenin, and this interaction may link PECAM to the actin cytoskeleton (Matsumura et al. 1997). $\gamma$-Catenin does not need to be phosphorylated to bind to PECAM. Interestingly, the addition of anti-PECAM antibodies to human NK cells results in actin rearrangements. Upon stimulation with inflammatory cytokines, PECAM is redistributed away from endothelial junctions (Romer et al. 1995). Its subsequent engagement by PECAM expressed on transmigrating cells may provide signals that induce actin rearrangements in endothelial cells.

### JAM-A

Upon activation of platelets by agonists such as thrombin and collagen, JAM-A is phosphorylated by protein kinase C. Furthermore, stimulation with thrombin leads to phosphorylation of JAM-A on serine 284 (Ozaki et al. 2000), and this is correlated with a change in the distribution of JAM-A, which subsequently forms clusters at several sites of cell-cell contact. Whether a similar mechanism operates in endothelial cells remains to be determined. Certainly JAM-A is relocalized following combined treatment of HUVECs with TNF and IFN-$\gamma$ (Ozaki et al. 1999), although the signaling events involved in this process await elucidation.

In summary, signals generated at endothelial cell junctions may be derived from both inflammatory signals and activated leukocytes, the combined effects of which may serve to induce endothelial cell retraction and hence gap widening.

## Transmigration and changes in vascular permeability

As discussed above, changes in vascular permeability occur during inflammation. If pre-existing minute gaps are widened by transmigrating leukocytes, then we might expect to see dramatic increases in permeability during this event. However, there is no correlation between transmigration and permeability changes. Some in vitro

studies have demonstrated that vascular permeability does increase during the migration of leukocytes toward endothelial cell-cell contacts (Del Maschio et al. 1996; Tinsley et al. 1999). Conversely, others have shown that the migration of neutrophils through HUVEC monolayers in response to chemoattractants does not induce any change in electrical resistance or permeability for albumin (Huang et al. 1988). Such discrepancies may arise from the use of different leukocyte:endothelial cell ratios (Huang et al. 1993), and from the comparison of essentially different mechanisms such as adhesion and transmigration. Indeed, when neutrophil adhesion was not accompanied by transmigration, this led to an increase in permeability, perhaps because there was no transmigrating cell to "plug" the gap induced by its adhesion (Huang et al. 1993).

One mechanism by which "plugging" of endothelial cell junctions may occur during transmigration is suggested from the recent studies of Mamdouh and colleagues (2003). PECAM-bearing membrane is targeted to areas of the junction at which monocytes are transmigrating, thereby increasing the contact area between leukocytes and endothelial cells (Mamdouh et al. 2003). This may serve to maintain the integrity of the endothelium during transendothelial migration, thus preventing leakage of plasma proteins (see commentary by Ager 2003). PECAM may turn out to be one of several junctional molecules that are re-targeted to endothelial surfaces contacting transmigrating leukocytes.

## Platelets and inflammation

One aspect of inflammation that is sadly neglected in many reviews dealing with the inflammatory recruitment of leukocytes is the role played by platelets. This is certainly a misguided omission since platelets undoubtedly play a very important role in leukocyte recruitment at sites of inflammation. An intact endothelium normally prevents platelet activation and adhesion, but upon endothelial injury, sub-endothelial collagen and von Willebrand factor become exposed, thereby supporting platelet adhesion and activation (Fuster et al. 1992; van Zanten et al. 1994). This leads to the recruitment of an increasing number of platelets. Subsequently, interactions occur between P-selectin expressed at the surface of adhered activated platelets and PSGL-1 on circulating leukocytes (Sako et al. 1993), and in this way, immobilized platelets can capture leukocytes from flowing blood (Hagberg et al. 1998; Lalor and Nash 1995). The initial association leads to increased levels of the integrin Mac-1 on leukocytes (Neumann et al. 1999), further supporting interactions with platelets. Platelets have been shown to play an important role in the delivery of leukocytes to endothelium (Diacovo et al. 1996a, 1998), and platelets adherent to sub-endothelial matrix components support the rolling, adhesion, and transmigration of leukocytes (Diacovo et al. 1996b; Kuijper et al. 1997). Of particular relevance to this review is the expression of PECAM-1 (Newman et al. 1990; Stockinger et al. 1990), JAM-A (Malergue et al. 1998; Martin-Padura et al. 1998), and JAM-C on platelets (Santoso et al. 2002). Since all these molecules have demonstrated or proposed roles in leukocyte migration across endothelial cells, it is conceivable that leukocytes transmigrate monolayers of activated platelets lining an injured endothelium by using similar molecular interactions. In an exciting recent "twist" to the tale, Mac-1 expressed on neutrophils and monocytes has been identified as a ligand for platelet JAM-C (Santoso et al. 2002). JAM-A has been demonstrated to promote platelet aggregation (Kornecki et al. 1990; Naik et al. 1995), and interestingly, the stimulation of platelets by thrombin induces a change in the distribution of JAM-A, which forms clusters at several sites of platelet-platelet contact.

## Conclusions

Although a great deal of progress has been made in understanding the final step of leukocyte emigration at sites of inflammation, the molecular nature of the signals and cross-talk that occur between a leukocyte and the endothelium remain to be clarified. It is important to remember that inflammation itself is very complex and induces a wide variety of changes in the endothelium, some of which are clearly independent of the leukocyte. It would make biological sense if these early tissue-derived signals primed the endothelium for later signals delivered by adherent activated leukocytes—a true division of labor. In this way, the task of the leukocyte becomes much easier, and yet the barrier function of the endothelium is preserved: the leukocyte may widen pre-existing gaps in the endothelial monolayer and, at the same time,

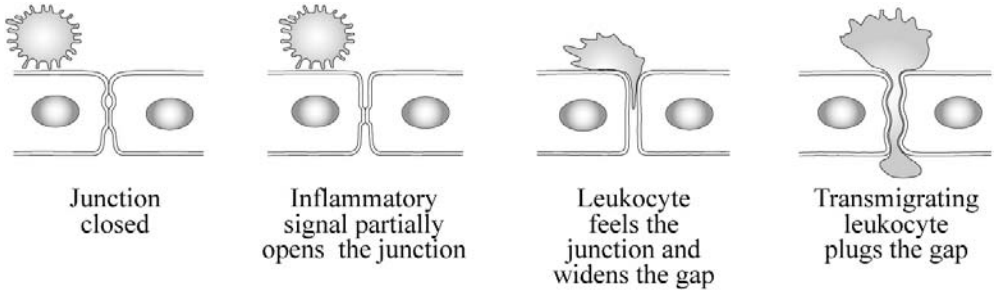


**Fig. 1** Leukocytes widen pre-existing gaps in the endothelial monolayer and, at the same time, plug the gap as they pass through the inter-endothelial cleft

plug the gap as it passes through the inter-endothelial cleft (Fig. 1). The study by Mamdouh and colleagues (2003) provides a novel mechanism by which the integrity of the endothelium might be maintained during transendothelial migration.

Whereas it is always intellectually appealing to conceive a model in which each molecule in a biological system plays a specific part and in a particular sequence under a given set of conditions, we may have to accept that transendothelial migration is, for the moment, too complex to be explained by arrows and boxes. Undoubtedly, all the molecules described to date as playing a role really do have a relevant function, but exactly which molecules and which interactions predominate, and in what sequence, during a cell's journey from the apical surface of the endothelium to its basement membrane is just beginning to be unveiled. Real-time imaging, shear flow experiments, and the use of fluorescently tagged molecules, together with improved transfection protocols, have made important contributions to these advances. To understand precisely the nature of signals involved in transendothelial migration necessitates the perfect in vitro reconstruction of an inflammatory response. With the speed of technological advances in biological research, such a scenario may not be so far away.

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