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Review

The Molecular Mechanisms of Post-Adhesive Transmigration of T Cells

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Abstract. Leukocyte extravasation is an essential phenomenon in inflammatory responses of the body. However, less is known about the mechanisms of transendothelial migration of leukocytes subsequent to their adhesion to the endothelium. It could be considered that at least three different cellular responses participate in the transmigration of adherent leukocytes: 1) polarization of adherent cells in cell shape, 2) interactions between adherent cells and molecules bound to the endothelial surface to stimulate migration through the junction between adjacent endothelial cells, and 3) co-ordination with endothelial cells to open the junction. Molecules involved in these events are discussed in this review.

Key words: transmigration; endothelial cells; T cells; molecular mechanism.

Introduction

Continuous recirculation of lymphocytes between blood and tissue allows immune surveillance of the body for an insult of foreign pathogens. In humans, 4.5×10^{11} lymphocytes extravasate from the blood into various organs each day, and back through lymphatics to the blood[39]. This lymphocyte recirculation markedly increases the opportunity that a lymphocyte should encounter foreign antigen presented on an antigen-presenting cell (APC), by which a prompt and efficient response to eliminate the antigen from the body can be achieved. Inflammatory stimuli in peripheral tissues also promote emigration and accumulation of lymphocytes as well as granulocytes from circulating blood. The mechanisms of lymphocyte recirculation and accumulation at inflammatory sites have been intensively studied as a basic response in the immune system. For the last 15 years, the biological functions of various adhesion molecules, including integrins and the regulation of their expression have been elucidated. In addition, endothelial cells have come to be routinely cultured to study interaction with leukocytes. These advances have led to the defining of adhesion molecules involved in leukocyte adhesion to endothelial cells, which is essential for leukocytes to emigrate from vessels.

At present, it is generally accepted that the emigration process consists of three sequential steps with some overlap[38]. The initial step is leukocyte attachment to and rolling along the luminal surface of the endothelium, which is mediated by selectin-carbohydrate interaction. Thereafter, the leukocyte integrins are activated during this contact with the endothelial surface, by

Abbreviations used: LFA-1 – lymphocyte function-associated molecule-1, ICAM-1 – intercellular adhesion molecule-1, IFN – interferon, RANTES – regulated upon activation, normal T cell expressed and secreted.

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which tight adhesion and spreading of the leukocytes to the endothelial surface occur. Subsequently, the final step for the emigration of leukocytes is accomplished by transmigration of the adherent cells across the vessel wall. This step comprises leukocyte transmigration (diapedesis) and penetration of the subendothelial basement membrane. The former involves locomotion of the adherent cells to nearby endothelial cell-to-cell junctions and crawling through them. Compared with the adhesion processes, however, transmigration in this last step has not been fully studied, since it is difficult to dissect the event of adhesion and transmigration. I discuss the molecular mechanisms of lymphocyte transmigration in this review including our recent study[26], although available data are still limited even at present.

Endothelial Adhesion of T Cells and Their Subsequent Transmigration

All T cells adhering to endothelial cells via integrins do not automatically migrate through the endothelium. For example, *in vivo* studies showed that only 1/3 of T cells bound to the endothelium of high endothelial venules (HEV) in murine Peyer's patches can migrate, and other T cells detach after transient binding to HEV[3]. It is also known that, in lymphocyte recirculation in sheep, memory T cells preferentially emigrate to non-lymphoid tissues such as skin, while naive T cells do so through HEV[23]. These findings indicate that there is a specific mechanism to promote extravasation of particular subsets of T cells that adhere to the endothelium. To study the mechanism of T cell transmigration, we developed an *in vitro* model in which monolayers of human umbilical vein endothelial cells (HUVEC) are cultured on collagen gels[25]. This culture system allows quantitative evaluation, as well as direct observation under reverse microscopy, of T cells that adhered but not migrated to HUVEC and those that migrated through HUVEC monolayers into the collagen gels below. Furthermore, migrated T cells can be examined for their cell surface markers by being released from the gels. In the culture system, about 20–30% and up to 6% of added T cells, respectively, adhere to and migrate through unstimulated HUVEC monolayers.

Interestingly, the expression of CD45RO is different between adherent and migrated T cells: although adherent cells consist of approximately equal numbers of $CD45RO^+$ and $CD45RO^-$ cells, migrated cells are largely $CD45RO^+$ memory cells expressing CD4 and $CD44^{hi}$ [2, 5, 25]. Such phenotypic difference is more impressive for CD26 expression: adherent-only T cells are negative for CD26, whereas migrated T cells mostly exhibit $CD26^{hi}$ [5, 25] (Table 1). The characteristic phenotype of migrated T cells is similar to that of T cells accumulated in chronic inflammatory tissues[20].

Table 1. Phenotype of T cells adherent to and migrating through unstimulated human umbilical vein endothelial cell monolayers *in vitro*

	Adherent cells	Migrated cells
CD4/CD8	0.8*	3.5
CD26	–	++(high)
CD31	+	–
CD45RO	+	++
CD45RA	+	–

Adherent and migrated T cells were obtained from co-cultures with endothelial cell monolayers on collagen gels. The data were cited from references[2, 5, 25].

* The ratio of cell number.

– negative; + partially positive; ++ almost positive.

In addition to the phenotypic analysis, there is evidence that T cell adhesion and transmigration are mediated by different molecules[25]. Although T cell adhesion to HUVEC is markedly diminished by anti-limphocyte function-associated molecule-1(LFA-1) antibodies added with the T cells, transmigration failed to be blocked by the same antibodies added after the T cells had adhered to HUVEC. These findings provide the hypothesis that post-adhesive transmigration is induced by mechanisms other than the LFA-1/intercellular adhesion molecule-1(ICAM-1) pathway mediating T cell--endothelial cell adhesion. It would be difficult to determine whether LFA-1/ICAMs are involved in the process of transmigration without separately assessing adhesion and transmigration, because blocking antibodies against the molecules inhibit T cell-endothelial cell adhesion, which is requisite for transmigration.

Polarization of Adherent T Cells for Transmigration

When lymphocytes are placed on substrates coated with soluble ICAM-1 or anti-LFA-1 monoclonal antibodies (mAb), they become flattened in cell shape and move randomly[7, 14, 16]. Motile cells reveal morphological polarization characterized by extension of lamellipodia and filopodia at the leading edge where actin polymerization actively occurs[27], whereby they move directed by a chemical attractant gradient[4]. Chemoattractants also cause the rearrangement of actin-based

cytoskeleton and change cell shape from a spherical to a polarized form. Importantly, the dynamic redistribution of molecules is associated with the polarization. For example, the receptors for formyl-methionyl-leucil--phenylalanine and urokinase-type plasminogen activator, which are known chemoattractants, are located at the front edge of migrating neutrophils and monocytes, respectively[15]. Similarly, the chemokine receptors CCR2 and CCR5 are concentrated at the leading edge of PHA-activated T cells in response to chemokines[30]. In contrast, several adhesion molecules, such as ICAMs, redistribute towards the tailing edge, called the uropod. The redistribution seems to be a distinctive event in migrating cells, because the cytokine receptors interleukin (IL)-2Rα, IL-2Rβ and tumor necrosis factor (TNF) receptor R1 exhibit no polarization[30]. The concentration of the chemokine receptors to the leading edge would be suitable for cells to migrate to a chemoattractant source.

A similar change in cell shape is observed in the interaction of T cells and HUVEC. When resting T cells were cultured on unstimulated or interferon γ (IFN-γ) stimulated HUVEC monolayers, a small proportion of adherent cells displayed a flattened shape with polarization[26]. In electron microscopic observation, the adherent cells extended filopodia to the intercellular junction between adjacent endothelial cells[26]. Although it is still unclear whether the polarization of T cells bound to endothelial cells is induced by stimulation via adhesion molecules or soluble factors, chemokines may be an inducible factor. Treatment of T cells with pertussis toxin (PT) completely abolishes transmigration with no effects on adhesion to HUVEC[26]. The inhibitory effect of PT may indicate the involvement of chemokines in post-adhesive transmigration, since chemoattractant factors generally act via the PT-sensitive G protein-mediated signaling pathway. PT-treated lymphocytes home poorly to lymph nodes regardless of the preserving a capacity to bind lymph node HEV[17]. A recent report has suggested that transendothelial migration of activated T helper (Th1)--type cells is enhanced by RANTES, a CC chemokine, secreted by IFN-γ-stimulated endothelial cell monolayers, and that anti-RANTES antibodies block transmigration by inhibiting pseudopod formation, but not adhesion, of T cells[19]. These findings support the postulate that polarized adherent T cells claw in the direction of the endothelial junction and pass through it, whereas non-polarized adherent cells remain on the surface of HUVEC. Morphological polarization of T cells following adherence to endothelial cells would be essential for the subsequent transmigration.

Molecules Involved in Post-Adhesive Transmigration of T Cells

Cell polarity with redistribution of chemokine receptors in T cells is exerted by mitogenic activation, chemoattractant stimulus, or the use of adhesion molecules immobilized on the substrate. Transmigration of activated T cells is indeed augmented in the presence of a gradient of chemokines which stimulate cell motility. However, in cocultures of resting T cells and HUVEC monolayers, adherent T cells claw and transmigrate through the monolayers in the absence of exogenous stimulants or a chemokine gradient. What adhesive interactions cause the directional locomotion? As mentioned above, the LFA-1/ICAM-1 pathway is unlikely to be involved in the process of post-adhesive transmigration. Recently, several molecules have been reported as candidates for supporting the transmigration (Table 2).

CD31 (platelet-endothelial cell adhesion molecule-1) is a 130 kDa adhesion molecule belonging to the immunoglobulin superfamily and is expressed on platelets, naive T cells, monocytes, neutrophils and natural killer (NK) cells[28]. Also, CD31 is densely located at the junction of adjacent endothelial cells[29]. In antibody blocking studies, leukocyte CD31 has been implicated to mediate their post-adhesive transmigration between adjacent endothelial cells in a manner of homophilic interaction with the endothelial CD31 and to be involved in the subsequent penetration of the basement membrane in a manner of heterophilic interaction with unknown components of the subendothelial basal lami-

Table 2. Candidates for maediating post-adhesive transmigration of T cells

Molecules	Distribution	References
CD31	monocytes, neutrophils, NK cells, endothelial cells	28, 29
CD47	neutrophils, endothelial cells, epithelial cells	8, 32
4C8	T cells, B cells, NK cells, monocytes, eosinophils	26
NKRP1A (CD161)	$CD4^+$ T cells, NK cells	33, 34
JAM	endothelial cells, epithelial cells	10, 24
VE-cadherin/catenin complex	endothelial cells	11

na[21]. The distribution of endothelial CD31 has been shown to be important for transmigration. $CD31^+$ naive T cells spontaneously migrate through monolayers of CD31-transfected murine fibroblasts, where CD31 is concentrated at the intercellular junctions[40]. In contrast, unless a chemotactic gradient is created, T cells do not migrate through ICAM-1-transfected cells on which ICAM-1 is uniformly distributed. Thus, T cells may transmigrate in response to adhesion molecules on endothelial cells which show polarized distribution with an increasing gradient torward the endothelial junctions. In the case of transfected CD31, a gradient of CD31 on fibroblasts might induce haptotactic movement (i.e. movement along a gradient of molecules bound to substrates) of $CD31^+$ T cells via the CD31 homophilic interaction. A more recent study using CD31-deficient mice showed that CD31 does not play a critical role in leukocyte transmigration *in vivo* and *in vitro*[12] because there is no difference in an inflammatory response between CD31 knockout and wild-type mice. The results suggest that CD31 is involved in the transbasement membrane migration. However, there may be different mechanisms in the mutant mice that compensate for the lack of CD31.

Junctional adhesion molecule (JAM), a member of the immunoglobulin superfamily, has been reported as a molecule involved in transmigration which is concentrated at the intercellular junctions of endothelial cells[24]. The molecule is codistributed with tight junction components, such as occludin, at the apical region of the junction. It appears that JAM not only promotes cell-cell adhesion, but also modulates monocyte transmigration, because anti-JAM antibodies inhibit spontaneous and cytokine-induced transmigration of monocytes without affecting their adhesion to endothelial cells *in vitro*. In mice with experimental meningitis, intravenous injection of the antibodies significantly inhibits leukocyte recruitment into the brain parenchyma as well as blood-brain-barrier permeability[10]. Interestingly, the combined treatment of TNF-α and IFN-γ to endothelial cells reduces the concentrated distribution of JAM at intercellular junctions without changes in the amount of JAM on the endothelial surface[31]. Similar modulation by cytokines is also observed in CD31 expression on human endothelial cells[36]. While treatment of either TNF-α or IFN-γ to endothelial cell monolayers increases leukocyte transmigration, the combined treatment with them decreases it, regardless of an increase in endothelial adhesion of leukocytes[35]. Cytokine-induced redistribution of JAM or CD31 may explain the paradoxical finding of the combination effect.

Since $CD31^-CD26^{hi}$ memory T cells predominantly migrate across HUVEC monolayers in our culture system, CD31 is unlikely to be involved in post-adhesive T cell transmigration. Recently, we reported the anti-4C8 mAb that inhibits 80–90% of T cell transmigration without interfering with adhesion to HUVEC monolayers[26]. The antigen recognized by anti-4C8 mAb is an 80 kDa molecule that is expressed on lymphocytes, monocytes, NK cells and eosinophils, but not on neutrophils or HUVEC. Electron microscopy revealed that adherent T cells are arrested at the junction between neighboring endothelial cells in the presence of anti-4C8 mAb, suggesting that the ligand for the 4C8 antigen may be present at the junctional level. Molecules that directly mediate the process of transmigration across HUVEC monolayers should transduce signals to selectively stimulate cell motility of $CD26^{hi}$ T cells. Indeed, lamellipodia and filopodia characteristic for motile cells were induced in about 15% of T cells when incubated on anti-4C8 mAb-immobilized plastic wells without any additional stimulation. Cross-linking of the 4C8 antigen induced actin polymerization in $CD26^{hi}$ T cells, required for the formation of membrane raffles. Moreover, anti-4C8 mAb impregnated in collagen gels predominantly stimulated migration of $CD26^{hi}$ T cells into the gels, whereas anti-LFA-1 mAb failed to produce such an effect on unstimulated T cells. The observation indicates that the 4C8 antigen is therefore a candidate for mediating selective transmigration of resting $CD26^{hi}$ T cells across HUVEC monolayers. However, it is unclear why the signals through the antigen are transduced in $CD26^{hi}$ T cells in spite of its high expression on all $CD3^+$ T cells. As activated, but not resting, T cells move randomly on substrates with immobilized anti-LFA-1 mAb, the activation state of T cells may be important for successful signal transduction via the 4C8 antigen. Since the expression of CD26 is up-regulated with cell activation, $CD26^{hi}$ T cells are thought to be in an activated state to some extent compared with other cells. The relative activation state might modulate signal transduction via the 4C8 antigen.

A couple of other molecules involved in the transmigration process of leukocytes have been reported. NKRP1A, a member of the C-type lectin family, termed NK1.1 in the mouse, is expressed on about 15% of human $CD4^+$ T cells as well as NK cells. According to a report using HUVEC monolayers, 70–85% of migrated T cells are positive for NKRP1A[33]. IL-12 enhances the NKRP1A expression and transmigratory capacity of Vδ2 T cell clones from multiple sclerosis patients as well as healthy subjects[34]. Anti-NKRP1A mAb significantly reduces transmigration of peripheral blood T cells and IL-12-stimulated Vδ2 T cell clones, but the

mAb also suppresses T cell adhesion to HUVEC monolayers[33, 34]. CD47 is a 50 kDa membrane protein of the immunogloburin superfamily which is known as integrin-associated protein (IAP) with a broad tissue distribution: leukocytes, endothelial cells and epithelial cells[6, 22]. IAP is involved in the adhesion of neutrophils and calcium fluxes of endothelial cells in response to plating on extracellular matrix proteins containing Arg--Gly-Asp. Recent reports have shown that the molecule participates in the process of transendothelial and transepithelial migration of neutrophils[8, 32]. The transmigration is inhibited by either treatment of neutrophils or epithelial/endothelial cells with blocking antibodies against CD47. Although how CD47 mediates the process remains to be defined, CD47 appears not to function via homophilic adhesive interactions between neutrophils and epithelial cells.

Opening of Endothelial Junctions Triggered by Leukocyte Adhesion

Endothelium is firmly sealed by occluding junctions to prevent unnecessary passage of circulating cells and plasma constituents from blood into extravascular tissues. The junctions are composed of at least four types of adhesive structures: tight junctions (TJ), gap junctions (GJ), adherens junctions (AJ), and syndesmos[9]. Each of the structures is formed by homotypically bound molecules, which are the complex of cadherins and catenins at AJ and occludin and claudin at TJ. TJ are well developed in the brain microvessels and in large arteries, where the permeability is strictly controlled[37]. On the other hand, although AJ are observed in all types of the endothelium, they are a major component of the junctional organelles, especially in postcapillary venules, where active transmigration of blood leukocytes occurs. AJ consist of a transmembrane adhesive protein specific for the endothelium, namely vascular endothelial (VE)-cadherin. The cadherin has $[Ca^{2+}]$ i binding motifs in the extracellular domains and the ability to interact with the cytoskeletal proteins in the cytoplasmic domains.

It is an interesting issue how leukocytes bound to the endothlium pass through the sealed junctions comprising adhesion proteins such as cadherins. In this regard, there have been reports providing evidence that leukocyte adherence diminishes the endothelial barrier function. Adhesive interaction via ICAM-1 or VCAM between neutrophils and endothelial cells increases the concentration of intracellular $[Ca^{2+}]$ i in endothelial cells[18]. Pretreatment of endothelial cells with an agent blocking the $[Ca^{2+}]$ i rise abolishes neutrophil transmigration, but not adhesion. Integrin-dependent attachment of neutrophils to TNF-activated endothelial cells induces AJ disassembly, in which catenins and plakoglobins dissociate from the VE-cadherin/catenin complex within only minutes[11]. This disorganization appears to cause the opening of the endothelial junctions, resulting in an increase in neutrophil transmigration as well as endothelial permeability in inflammatory sites. However, it is unlikely that endothelial cells completely part from each other, since CD31-mediated cell contact remains unchanged. Although this phenomenon is specific for neutrophils, signals transduced by lymphocyte adhesion also generate reorganization of endothelial cytoskeleton[1, 13]. Cross-linking of endothelial ICAM-1 and coculture of lymphocytes and endothelial cells induce actin polymerization to form stress fiber in endothelial cells, and provoke phosphorylation of actin-related proteins, focal adhesion kinase, paxillin and $p130^{Cas}$, through activation of the small G protein Rho. When endothelial cell monolayers were pretreated with cytochalacin D, an inhibitor of actin polymerization, lymphocyte transmigration is inhibited without reduction of adhesion. Endothelial adhesion of lymphocytes may result in the opening of the junctional structures through triggering organic changes in the actin-based cytoskeleton, so that lymphocytes easily penetrate into the junctions.

Conclusions

Transendothelial migration of T cells is a more complicated phenomenon than the adhesive processes to endothelial cells. It is unlikely that the integrin-dependent adhesion merely causes the transmigration in response to a gradient of chemoattractants. At least three additional events appear to be required for the directional movement of adherent T cells from the apical endothelial surface to underneath: 1) polarization of the T cells in cell shape and redistribution of chemokine receptors, 2) interactions between adherent T cells and molecules bound to the endothelial surface to stimulate cell motility, and 3) co-ordination with endothelial cells to open the junctions. Recent reports suggest that the interaction of cell surface molecules between the two cell types and soluble factors such as RANTES participate in these events. Particularly, adhesive proteins at the junctions are thought to play an important role in the transmigration of leukocytes. It could be considered that T cells and endothelial cells transmit signals to each other via such proteins while

passing through the narrow junctions, whereby motile activity is promoted in migrating T cells. The amount and distribution of the junctional proteins possibly influence transmigration. Although it is largely unknown which molecules act in transmigration, the precise mechanisms may be elucidated in the near future.

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