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Signals emanating from leukocyte-endothelium interactions during inflammation

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ABSTRACT

Leukocyte trafficking throughout the vasculature is a crucial step for the development of innate and adaptive immunity (1). The coordinate function of adhesion receptors, cytoskeleton and signaling molecules in both cellular types is fundamental during leukocyte extravasation. Hence, the correct integration of "outside-in" and "inside-out" signals in leukocytes and endothelium during each stage of the process is critical to allow the completion of the so-called "multi-step paradigm" (leukocyte tethering and rolling involving selectins and their ligands, followed by leukocyte firm adhesion and crawling mediated by integrins and their endothelial counter-receptors and the subsequent diapedesis also mediated by junctional proteins) (2, 3). This review focuses on the signaling pathways triggered during the extravasation that allow leukocytes to efficiently migrate towards the inflammatory foci to exert their effector functions.

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RESUMEN

Señalización derivada de la interacción leucocito-endotelio durante la inflamación

El tráfico leucocitario a través de la vasculatura es un paso crucial para el desarrollo de la inmunidad innata y adaptativa. El funcionamiento coordinado de los receptores de adhesión, el citoesqueleto y las moléculas señalizadoras tanto en el leucocito como en el endotelio es fundamental durante el proceso de extravasación. Así, la correcta integración de las señales del exterior hacia el interior y del interior hacia el exterior en ambos tipos celulares durante cada etapa del proceso es crítica para la consecución del llamado "paradigma multi-secuencial" (el rodamiento de los leucocitos en el que están implicadas las selectinas y sus ligandos, seguido de la adhesión firme mediada por las integrinas leucocitarias y sus contra-receptores endoteliales, así como el subsiguiente paso de diapedesis en el que también están implicadas moléculas típicas de uniones intercelulares). Esta revisión se centra en las vías de señalización que se desencadenan durante la extravasación que permiten a los leucocitos migrar de manera eficiente hacia los focos inflamatorios donde ejercen sus funciones efectoras.

Palabras clave: Inflamación.- Adhesión leucocitaria.- Migración transendotelial.- Señalización mediada por receptores de adhesión.

ROLLING MEDIATORS AS SIGNALING RECEPTORS

Free-flowing leukocytes contact with and adhere to the vascular wall under shear forces to initiate an inflammatory response or to migrate into a secondary lymphoid organ (homing). Leukocyte tethering and rolling on activated endothelial cells are the first steps of the sequential process of extravasation, followed by the firm adhesion and transendothelial migration of leukocytes (4). These initial contacts are largely mediated by selectins and their ligands.

Selectins (P-, E- and L-selectin) are cell adhesion molecules that predominantly mediate the initial interactions of leukocytes with endothelium. They are type I transmembrane glycoproteins that bind to sialylated carbohydrate moieties present on ligand molecules in a

calcium-dependent manner. Although selectins and their ligands tend to interact with a variable affinity, their rapid association and dissociation rates mediate transient contacts between leukocytes and endothelium ("tethering") (5, 6). Tethering results in the slowing of leukocytes in the bloodstream and their rolling on the surface of endothelium, which favors subsequent interactions with endothelial cells mediated by integrins and their ligands, increasing the adhesiveness of leukocytes, that leads to their final arrest on the vessel wall (7).

It has been demonstrated that L-selectin activates multiple signaling pathways involved in the reorganization of the actin cytoskeleton, such as the MAPK cascade (8), the tyrosine kinase $p56^{lck}$ and Ras (9) or the Rho GTPase Rac2 (10). In this regard, it has been described that neutrophils from $Rac2^{-/-}$ mice show deficient actin polymerization and L-selectin-mediated rolling (11). On the other hand, the best characterized selectin ligand named PSGL-1 (P-selectin glycoprotein ligand-1) activates the MAPK pathway (12), and acts as a negative regulator of human hematopoietic progenitor cells (13). In addition, it has been shown that PSGL-1 induces a rapid synthesis of uPAR and different cytokines such as TNF-alpha, IL-8 and MCP-1 in neutrophils, monocytes and T cells (14-17). Moreover, PSGL-1 induces activation of beta-2 integrins and binding to ICAM-1 in neutrophils (18-20). Our group has also described the interaction of PSGL-1 with ERM proteins, which link membrane molecules with the actin cytoskeleton (21, 22). This interaction is of critical importance for the leukocyte activation that occurs before extravasation, because it allows the recruitment of the tyrosine kinase Syk by association to ERM proteins through their phosphorylated ITAM-like motifs. Therefore, after PSGL-1 ligation to P-selectin or E-selectin, Syk conveys rolling-emanating signals to the activation of gene expression programs (23). This phenomenon suggests that the intracellular signals induced through PSGL-1 have a priming effect on leukocyte activation, up-regulating the expression of different molecules further involved in extravasation and effector functions (24) and an unsuspected role inducing tolerogenic functions in dendritic cells (25). Since it has been demonstrated that the cytoplasmic tail of L-selectin also interacts with moesin (26), it is very likely that selectins use a similar strategy to trigger intracellular signaling cascades. In this regard, it has

been shown that E-selectin is dephosphorylated upon endothelial cell interaction with leukocytes, supporting its role as a signal transduction molecule (27). In addition, P-selectin also functions as a signaling receptor, mediating stimulation through its interaction with ligands expressed by leukocytes (28). Finally, it has been recently reported that another E-selectin ligand, CD44, can control rolling velocity and mediate E-selectin-dependent redistribution of PSGL-1 and L-selectin to a major rear pole on slowly rolling leukocytes through p38 signaling to promote firm adhesion (29).

CHEMOKINE MODULATION OF INTEGRIN ACTIVITY

During their rolling, leukocytes are stimulated by chemokines and integrin ligands expressed on the surface of endothelial cells. These outside-in signals induce an important increase in the affinity and/or avidity of leukocyte integrins (inside-out signals) that allows the shear-resistant arrest of these cells and their firm adhesion to activated endothelium.

Leukocytes modify their adhesive properties to be properly adapted to every immune scenario (30). Hence, free-flowing leukocytes maintain their integrins in non-adhesive conformation, to avoid unspecific contacts with non-inflamed vascular walls, but upon encountering localized inflammatory foci, a rapid *in situ* activation of leukocyte integrins by endothelium-displayed activating signals takes place during the transition of rolling to adhesion (31). Therefore, the regulation of integrin adhesiveness is independent of their membrane expression and, during the extravasation process, it is modulated by the chemokines coupled to apical endothelial glycosaminoglycans (32). These chemokines act by signaling through GPCRs and induce an array of "inside-out" signals within fractions of seconds, leading to multiple conformational changes of integrins with important effects on leukocyte adhesion and morphology (33-35). Both the endothelium-immobilized chemokines and the chemokine receptors expressed on leukocytes are concentrated on microvilli to facilitate their interaction. The presence of specific chemokines on different vascular beds contributes to orchestrate

the selective recruitment of leukocyte subsets to inflammation foci or secondary lymphoid organs (26). In addition, chemokines may exert a differential effect on specific integrins within the same microenvironment. Accordingly, it has been described that chemokines can only mediate lymphocyte arrest dependent on VLA-4/VCAM-1 when binding their GPCRs with high affinity and with high relative occupancy, but this signal threshold-dependent effect is not observed with chemokine-stimulated β2 integrins (37).

Small GTPases play a central regulatory role in the integrin activation induced by chemokines, mainly through RhoA activation or/and RhoH inactivation (37-39). It has been also suggested that Rac1 and its GDP/GTP exchanging factors (GEFs) Vav-1 and DOCK2 are involved in the chemokine-mediated activation of integrins in T and B cells (40-42). However, other authors propose that DOCK2 is mainly involved in microvilli collapse, lamellipodium formation, and lateral mobility induced by chemokines (43). On the other hand, the ras-like small GTPase Rap1 can regulate integrin activation through RAPL that binds to the cytoplasmic tail of LFA-1 α chain (44, 45). The important role of Rap1 in integrin activation has been recently underscored by a deficiency in lymphocyte adhesiveness (LAD III), that correlates with a selective impairment in the activation of Rap1 induced by chemokines via CalDAG-GEFI (46, 47). Furthermore, the activation of phosphatidylinositol 3-OH kinase (PI3K) and PKCζ by chemokines is involved in LFA-1 clustering at areas of low ICAM-1 density (33, 37, 38). In addition, the ARF-guanine-nucleotide exchange factor Cytohesin-1 induces LFA-1 activation by direct interaction with its β2 cytoplasmic domain (48, 49). However, there are also negative regulators of integrin activation, such as PKA, H-ras and ILK (integrin-linked kinase) (37, 50, 51). Due to the complexity and timeframes of the signaling mechanisms controlling integrin activation, it is conceivable the existence of preformed compartmentalized protein networks ("signalosomes") in leukocytes encountering endothelial chemokines (37).

INTEGRIN SIGNALING TRIGGERED BY LIGAND BINDING

Upon interaction with their ligands, integrins activate distinct myosin contractility effectors, actin-remodeling GTPases and molecules involved in microtubule network regulation at the leading and trailing edges of motile leukocytes (52, 53). During cell polarization, Cdc42, MLCK, Rac, RAPL, Rap1, mDia, Myosin-IIA and chemokine receptors are redistributed to the cellular front, participating in the formation of exploratory filopodia and in the extension of lamellipodia. In contrast, Rho and ROCK (both involved in trailing edge retraction), the microtubule-organizing center (MTOC) and the adhesion receptors ICAM-1, ICAM-3, CD44 and CD43 move towards the rear pole (54). Interestingly, the redistribution of integrin ligands to the uropod seems to be involved in the recruitment of bystander leukocytes through this cellular structure (55). Hence, the integration of signals generated in both cellular poles leads to a coordinate movement of the leukocyte.

The α4 integrins can bind paxillin upon dephosphorylation of Ser988 in their cytoplasmic domain at the sides and rear pole of the cell, whereas PKA-mediated phosphorylation of these integrins is confined at the leading edge of the cell. Paxillin regulates α4 integrin function (tethering and firm adhesion) in immune cells (56), enhancing their rate of migration and reducing their spreading. In addition, paxillin/α4 interaction down-regulates the formation of focal adhesions, stress fibers and lamellipodia by triggering the activation of different tyrosine kinases such as FAK, Pyk2, Src and Abl (57). The α4/paxillin complex inhibits stable lamellipodia by recruiting an ADP-ribosylation factor GTPase-activating protein (Arf-GAP) that decreases Arf activity, thereby inhibiting Rac, and limiting lamellipodia formation to the cell front (58).

On the other hand, it has been described that LFA-1 and Mac-1 may use the adaptor molecules talin, α-actinin, filamin and 14-3-3 to properly anchor to the actin cytoskeleton (59, 60). Regarding the subcellular localization, the pattern of LFA-1 varies from low expression in the lamellipodia to high expression in the uropod. However, it has been reported that high affinity clustered LFA-1 is restricted to a mid-cell zone, termed the “focal zone”, different from focal adhesions and focal contacts. In addition, talin, properly activated by phosphorylation or PIP_2,

is essential for the formation and stability of the focal zone and for LFA-1-dependent migration (61).

SIGNALS TRIGGERED BY ENDOTHELIAL RECEPTORS UPON INTEGRIN ENGAGEMENT

VCAM-1 and ICAM-1, members of the Ig superfamily, are the two major endothelial adhesion molecules involved in the binding to the main leukocyte integrins VLA-4 and LFA-1, respectively (62, 63). ICAM-1 but not VCAM-1 is expressed at low levels in resting endothelium, and both molecules are induced upon cell activation by pro-inflammatory cytokines such as IL-1 and TNF-α (64, 65).

Endothelium is no longer considered as a passive barrier during Transendothelial Migration (TEM). In fact, endothelial cells form "docking" structures to firmly attach leukocytes upon the binding of VCAM-1 and ICAM-1 to their respective leukocyte ligands, VLA-4 and LFA-1. These cup-like structures are based on microvilli that emerge from the endothelial apical surface and their essential constituents include endothelial adhesion receptors, together with the actin cytoskeleton, adaptor proteins (ERM, alpha-actinin, vinculin, VASP) and signaling molecules (PIP_2, Rho/ROCK) (66). The relevant role of these structures for leukocyte adhesion and transmigration has been unveiled in experiments under flow conditions (66).

VCAM-1 and ICAM-1 are capable of transducing signals after ligand binding, that cooperate to increase endothelial permeability and facilitate leukocyte transmigration (67, 68). VCAM-1 is involved in the opening of the "endothelial passage" through which leukocytes can extravasate. In this regard, VCAM-1 ligation induces NADPH oxidase activation and the production of reactive oxygen species (ROS) in a Rac-mediated manner, with subsequent activation of matrix metalloproteinases and loss of VE-cadherin-mediated adhesion. This signaling pathway can be blocked by TGFbeta1 and IFNgamma (69-72). On the other hand, cross-linking of both VCAM-1 and ICAM-1 induces a rapid increase in intracellular Ca^{2+} concentration (28, 73). ICAM-1-mediated calcium increase triggers activation of Src and subsequent

phosphorylation of cortactin (73, 74). ICAM-1 is also able to activate RhoA inducing stress fiber formation (75) and phosphorylation of FAK, paxillin and $p130^{Cas}$, which in turn trigger different signaling pathways involving JNK or p38 (76-78). Moreover, ICAM-1 cross-linking stimulate c-fos and rhoA transcription (75). ICAM-1 cross-linking can also induce its own expression as well as that of VCAM-1, as a regulatory mechanism to facilitate leukocyte transendothelial migration (TEM) (79). More recently, it has been described the ICAM-1-mediated tyrosine phosphorylation of VE-cadherin dependent on Src and Pyk-2 activity and required for neutrophil transmigration (80).

INTEGRINS DURING TRANSENDOTHELIAL MIGRATION (TEM)

Once the leukocyte finds a proper site for transmigration, mostly at intercellular junctions, it extends exploratory pseudopodia in between the two adjacent endothelial cells. Subsequently, pseudopodia evolve into a lamella squeezed into the monolayer gap. During this process, LFA-1 is the integrin with a more prominent role. This molecule is rapidly relocalized, forming a ring-like cluster at the leukocyte-endothelial interface, where it interacts with ICAM-1 and, in some cellular models, with JAM-A. When the transmigration process is over, LFA-1 is finally concentrated at the uropod (81, 82). Interestingly, the NADase/ADP-ribosyl cyclase CD157 is localized at endothelial junctions and can associate with β2-integrins, playing an important role in TEM (83).

ENDOTHELIAL SIGNALS CONTRIBUTING TO THE LEUKOCYTE TRANSMIGRATION

Several molecules specifically localized at endothelial intercellular contacts induce signaling pathways to facilitate leukocyte transmigration. This is the case for CD99 that activates in leukocytes and probably in endothelial cells the ERK, JNK and MAPK pathways (84). In addition,

PECAM-1 can trigger "outside-in" signals via PKC and PI-3K leading to activation of alphav-beta3 (85).

CONCLUDING REMARKS

Understanding the complex signaling networks that govern the extravasation process is of critical importance for the identification of novel therapeutical targets in the treatment of chronic inflammatory and autoimmune diseases. Moreover, the development of new non-invasive and localized drug release strategies instead of systemic pharmacological administration will contribute to this aim.

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