Endocytosis pathways in endothelium: how many?

Radu V. Stan

Department of Pathology and Immunology and Microbiology, Angiogenesis Research Center, Dartmouth Medical School, Lebanon, New Hampshire

VASCULAR ENDOTHELIUM is a cellular monolayer with the organization of a simple squamous epithelium that lines the entire cardiovascular system and constitutes a regulatable barrier between blood and tissues (9, 17). By its location, it is easily accessible to blood-borne drugs or imaging agents, which would target the endothelium itself or be destined to transport to the underlying tissues (13).

For a drug or imaging molecule/complex to be targeted to a specific vascular bed, it requires binding to a molecule expressed specifically on the surface of the endothelial cells (EC) in that territory, in either normal or pathological conditions. If uptake is desired, the surface molecule should undergo endocytosis, a term that describes the process of uptake of material into cells (2, 10). If the drug molecule/complex requires delivery to the underlying tissue, it can reach it by either locally manipulating the paracellular pathway or taking advantage of one of the transcellular pathways described (13). The latter are either pores that cut across EC, such as fenestrae, vesiculo vacuolar organelles, and transendothelial channels (specific to the EC in select vascular beds but rather poorly described in terms of components and regulation), or are vesicular in nature (17, 18). The vesicular pathway or transcytosis is common to all epithelia (to which endothelium belongs) and is defined as the process by which large molecules are transported across cellular barriers in membrane-bounded vesicular carriers (21). It encompasses the uptake of the molecule at one endothelial front by endocytosis, transport across the cell via vesicular carriers, and exocytosis at the opposite front. From these, it is readily clear that endothelial endocytosis is one of the critical steps to be reckoned with in the rational design of drug targeting.

Endocytosis can occur via diverse mechanisms, which could be divided into two classes: phagocytosis and pinocytosis (reviewed in Refs. 2, 5, and 10). Phagocytosis (in Greek “to eat”) refers to internalization of particulates and occurs rarely in EC. Pinocytosis (in Greek “to drink”) defines the internalization of soluble molecules as bulk or via interactions with their surface receptors. To date, there are several internalization mechanisms that have been described to participate in pinocytosis: clathrin-mediated uptake (CME), caveolae-mediated uptake (CavME), clathrin- and caveolae-independent internalization (CLIC), and macropinocytosis (occurs rarely in quiescent endothelium in situ, if ever). There are several clathrin- and caveolae-independent uptake pathways or CLIC pathways (5) with respect to their dependence on dynamin, a large GTPase involved in the fission of vesicles from the plasma membrane (8). One pathway is dynamin independent (CLIC) (5), has the ability to internalize large molecular weight ligands, and has been shown to mediate the uptake of glycosylphosphatidylinositol-linked proteins (4, 16). Another pathway is dynamin dependent (CLIC-d) and was described for the IL-2R receptor in immune cells (6). Both these pathways depend on membrane cholesterol and on the integrity of membrane microdomains called lipid rafts.

In EC, besides CME and CavME, CLIC was shown to operate for the internalization of proteoglycans and bound growth factors such as FGF-2 (20). Another nonclathrin noncaveolar pathway has been shown to mediate the transport of IgG via the Fc receptor (1, 19). It is not clear whether this pathway is novel or not as no CLIC or CLIC-d markers have been used. A clathrin- and caveolin-1-independent pathway was reported in the case of platelet/endothelial cell adhesion molecule-1 (PECAM-1) constitutive endocytosis and recycling (7), but a formal investigation of its nature was not done.

In past work (11, 12, 14, 15), the authors of the report from Muro et al., the current article in focus (Ref. 13a, see p. L809 in this issue), described a novel endocytosis pathway in EC that they called the “cell adhesion molecule-mediated endocytosis pathway” (CAM-ME). The salient features of this pathway are:

1) It is induced by cross-linking of either adhesion molecules ICAM-1 (CD54) or PECAM-1 (CD31) by multivalent ligands (i.e., nanocarriers coated with antibodies). The ligands have to be multimeric and within certain sizes (i.e., 100–300 nm) in order for endocytosis to be initiated. Directly labeled “monomeric” antibodies against either PECAM-1 or ICAM-1 did not induce internalization, although a basal internalization rate exists for each of these adhesion molecules (3, 7). There seems to be no correspondence between the surface density of either ICAM-1 or PECAM-1 and the rate of internalization.

2) The internalization does not depend on either CME (i.e., no colocalization with clathrin, endocytosis not inhibited by cytosol acidification or monodansyl cadaverine) or CavME (i.e., no colocalization with caveolin-1 or cholera toxin B, no inhibition of uptake by filipin and genistein).

3) For endocytosis to occur, there is a requirement for the activation of dynamin-2 obviated by the inhibitory effect of dominant negative dynamin-2 K44A mutants.

4) Inhibitors (Y-27632) of ROCK (Rho kinase) lower the uptake of clustered ICAM-1, suggesting that the activation of RhoA small GTPase is necessary. RhoA activates NHE1 (15a) and also induces cytoskeletal rearrangements and formation of stress fibers. Actin polymerization is required (i.e., uptake inhibited by latrunculin A and cytochalasin D that sequester monomeric actin or cap short actin filaments, respectively). There is no actin cup formation as in the case of phagocytosis and macropinocytosis.

5) Pharmacological inhibition of Src by radicicol or PKC inhibition by either bisindoylmaleimide I or by H7 lowered the uptake of nanocarriers coated with either anti-ICAM-1 or anti-PECAM-1.

6) Nocodazole disruption of microtubules or phosphatidylinositol 3-kinase inhibition by wortmannin have no effect on CAM-ME, which add to the differences with micropinocytosis.

Address for reprint requests and other correspondence: R. V. Stan, Dartmouth Medical School, Dept. of Pathology, HB 7600, Borwell 502W, 1 Medical Center Dr., Hanover, NH 03755-0651 (e-mail: Radu.V.Stan@Dartmouth.edu).
By these features, the CAM-ME closely resembles CLIC-d described by Lamaze et al. (6) described for IL-2 receptor in immune cells with the difference that CAM-ME is induced, whereas CLIC-d seems to be constitutive. Another point is that CLIC-d seems to depend on lipid rafts, whereas CAM-ME does not (i.e., not inhibited by filipin). A formal possibility still exists that a constitutive pathway such as CLIC-d could be entered by a different receptor upon activation by large multimeric ligands (that signal ligand size, perhaps). The assessment of the colocalization of IL-2 receptor with ICAM-1 or PECAM-1 cross-linked by antibody-coated nanocarriers should resolve this matter.

Upon endocytosis, the cross-linked ICAM-1 or PECAM-1 form endosomes that will fuse in ~30–60 min to the early endosome antigen (EEA)-1 containing early endosomes. From here, ICAM-1 is segregated from the nanocarriers and recycled to the cell surface via rab11-sorting endosomes, whereas the nanocarriers are transported to the lysosomes.

The work by Muro et al. (13a) reported in the current article in focus clearly furthers our knowledge of the molecular requirements for the CAM-ME as well as the endocytic trafficking of the internalized ligands. Upon clustering by multivalent ligands as an initiating step, ICAM-1 colocalizes and associates with Na\(^{+}/H^{+}\) exchanger ion channel NHE1. The activity of NHE1 is required for endocytosis of liganded ICAM-1 but not for clustering as shown by both NHE1 knockdown by small interfering RNA (siRNA) as well as pharmacological inhibition by amiloride and EIPA (a more potent derivative of amiloride). In light of specificity problems inherent with most pharmacological inhibitors, the siRNA experiment is most convincing, although it is not clear whether several individual siRNA (preferable) or a mixture of pre-screened siRNA were used to demonstrate the effect. The endocytosis step also depends on Ca\(^{2+}\) entry and calmodulin (previously demonstrated to bind NHE1) as shown by pharmacological inhibitors such as BAPTA and thapsigargin. An interesting question would be to see what is the mechanism of Ca\(^{2+}\) entry (i.e., transient receptor potential cation channels?) involved in this process.

After endocytosis to an NHE1-positive, poorly described, early compartment, ICAM-1 and the ligand travel to the EEA-1-positive classic early endosomes. Here, ICAM-1 dissociates from the ligand presumably due to the low pH and is sorted to the rab11-positive sorting endosomes from where it is recycled to the cell surface. The multimeric ligands (in this case, the antibody-coated nanocarriers) are transported to the lysosomes for degradation.

Another important point in this paper is the discovery of a “monensin switch” by which the fate of the nanocarriers can be modulated to increase their lifetime within the endothelium. The authors (13a) report that monensin prevents the transport of the nanocarriers to the lysosomes. Early and recycling endosome acidification is regulated in part by Na\(^{+}/H^{+}\) exchanger NHE6. A possible explanation for the effect is that monensin increases the activity of NHE6, creating the conditions of a gain of function situation. The fact that NHE6 is involved is demonstrated by a loss of function experiment achieved by the NHE6 siRNA knockdown, which prevents the transport of nanocarriers to the lysosomes. Another loss of function experiment is based on the finding that PKC activates intracellular NHE channels. PKC inhibition by H7 results in a lack of NHE activation and activation of transport correlating the siRNA experiment, notwithstanding the much broader serine/threonine kinase inhibition activity of H7 at the concentration used. In consequence, upon monensin treatment, the pH of the early endosomes increases, resulting in continuous association of ICAM-1 with the nanocarriers and the transport of the whole complex to the recycling endosomes and from there to the cell surface. The authors examine this hypothesis but conclude that there is more to the monensin/endoosomal acidification effect since chloroquine, another inhibitor of endosomal acidification, does not prevent the transport of nanocarriers to the lysosomes. They propose that the unpressured Na\(^{+}/H^{+}\) exchange by monensin may secondarily lead to Cl\(^{-}\) influx to the endosomal lumen to maintain ion balance, accompanied by H\(_{2}\)O influx and endosomal engorgement favoring exocytosis. All in all, the net effect of monensin treatment is the extended lifetime of the drugs administered via nanocarriers, which may improve drug efficacy.

The precise mechanism of CAM-ME is far from being clear as is the nature of the early endosomes involved in uptake and the intermediate relays, if any, to the classic EEA1-positive early endosomes. Many questions remain with respect to its relationship to other endocytosis pathways in EC and whether cell-type variations exist or not. A hurdle in all this might be the lack of a standardized nomenclature and criteria for each pathway due to insufficient data on each of them. A systematic comparison of the pathways (i.e., comprehensive use of markers) so far described should shed light on this. The future looks bright as better and more specific tools are built and more data become available.

REFERENCES


