Targeting Endothelial Cell Surface Receptors: Novel Mechanisms of Microvascular Endothelial Barrier Transport

Asrar B. Malik

University of Illinois College of Medicine, Chicago, IL 60612, USA

Role of 60kD Albumin-binding Protein in Activating Transcytosis in Endothelial Cells

The delivery of novel biological therapeutic agents across the vascular endothelial barrier (such as monoclonal antibodies targeting specific receptors; e.g., anti-epithelial derived growth factor receptor antibody) represents a major challenge in drug delivery. The situation becomes even more complex in protein delivery across the much more restrictive blood-brain barrier. In studying endothelial barrier function, we have identified new mechanisms of delivery of biologic therapeutics.1-3 Maximum anti-tumor effects or other protective effects of the specific protein thus can be achieved at doses below the maximum tolerated dose. In this review, I summarize below some of key recent development in this area with emphasis on our work.

We and others have investigated the characteristics and function of gp60,4-12 an endothelial cell membrane 60-kDa albumin-binding protein localized in caveolae (2, 5, 6).2,5,6 Gp60 is a crucial protein found on endothelial plasma membrane involved in mediating the flux of albumin across the endothelial barrier by a process termed “receptor-mediated albumin transcytosis”.2,5,6 We have identified the key mechanisms of its activation in regulating endothelial permeability to proteins.5,6 Gp60 organization on the endothelial cell surface is punctate as shown by immunofluorescence using an anti-gp60 antibody conjugated with bifunctional, N-hydroxysuccinimidy fluorophore (Cy3).5 Also gp60 was shown to co-localize with caveolae, the plasma membrane invaginations rich in endothelial cells.6 Addition of a secondary antibody to anti-gp60 antibody-treated endothelial cells induced cross-linking of gp60 and resulted in cell surface gp60 clustering or activation.5 This resulted in 3-fold increase in the endothelial cell uptake (or endocytosis) of albumin and the luminal-to-abluminal permeability (or transcytosis) of albumin.5 Importantly, another protein tracer, horseradish peroxidase, was also transported across the endothelial barrier by the engagement of the albumin-mediated transcytosis machinery.5 Thus, these studies showed that macromolecules are transported across the endothelium by a “piggy-back” mechanism in the activation of gp60 (as shown in Fig. 1).

In other studies we used the water-soluble styryl pyridinium dye N-(triethylaminopropyl)-4-(p-dibutylaminostyryl) pyridinium dibromide (FM 1-43) to quantify caveolae-mediated vesicle trafficking across the endothelial barrier by confocal and digital fluorescence microscopy.13 FM 1-43 and fluorescently labeled anti-gp60 antibody were co-localized in endocytic vesicles within 5 min after gp60 activa-
These caveolae-derived vesicles then migrated to the basolateral surface via transcytosis where they released FM 1-43, the fluid phase styryl probe. Also the activation of cell-surface gp60 by cross-linking (as described above) increased trans endothelial albumin permeability. Caveolin-I and gp60 were shown to co-localize in these vesicles indicating the caveolar origin of the vesicles. Importantly, Src kinase phosphorylation of caveolin-1 was required for the activation of transcytosis and delivery of proteins across the endothelial barrier. Vesicle formation induced by gp60 and migration of vesicles to the basolateral membrane required the interaction of gp60 with caveolin-1 and this was followed by the activation of the Src kinase regulating the signaling of transcytosis. These findings indicate that activation of gp60 secondary to Src activation stimulates transcytosis of proteins across the endothelial cell monolayer. We have demonstrated that this pathway can be exploited for the delivery of protein therapeutics across the vessel wall endothelial barrier (and even possibly the blood-brain barrier). We have demonstrated that this pathway can be exploited for the delivery of protein therapeutics across the vessel wall endothelial barrier (and even possibly the blood-brain barrier).

**Identification of Myeloperoxidase-derived Peptide Regulating Protein Transcytosis Across the Vascular Barrier**

Another series of studies further advanced the concept of target delivery of therapeutic proteins. We identified the crucial role of an amino acid sequence of myeloperoxidase (MPO) in binding to albumin with high affinity. The binding was shown to be a requirement for the normally high transport of MPO seen across the endothelium. A unique sequence was identified using matrix-assisted laser desorption/ionization (MALDI) analysis of 80- and 60-kDa proteins purified from human lung tissue. These proteins were shown to be the MPO light and MPO heavy chains, respectively. A peptide (RLATE LKSLN PRWDG ERLYQ...
EARKI VGAMVC) corresponding to the MPO-heavy chain (residues 425-454) demonstrated high-affinity binding (in the nanomolar range) to human serum albumin. Replacement of the positively charged residues, R and K, with G prevented the binding of albumin to the peptide, indicating a charge dependent interaction. We observed that albumin increased the binding of an iodinated-MPO tracer to lung microvascular endothelial cells by 2-fold as well as the rate of transendothelial flux of the MPO tracer in vessels; thus indicating the crucial importance of albumin binding to MPO in mediating the transendothelial transport of MPO. Moreover, excess amount of the peptide sequence prevented the interaction between MPO and albumin. Disruption of caveolae with cyclodextrin also prevented the albumin-induced increase in transendothelial flux of MPO indicating the critical involvement of caveolae in this transport mechanism. We observed by confocal imaging that albumin induced the rapid internalization of MPO and its co-localization with albumin-labeled vesicles. MPO was shown to co-localize with the caveolae markers cholera toxin subunit Band caveolin-I in the endocytosed vesicles. Thus, transcytosis of MPO by caveolae induced by its charge-dependent interaction with albumin is an important means of delivering MPO to the subendothelial space. Moreover, the identified albumin binding peptide sequence of MPO (with its high affinity albumin binding sites) is of great potential clinical significance in the delivery protein drugs across the vascular endothelial barrier in therapeutically relevant dosages (as shown in Fig. 2).

Clinical Relevance of gp60 and MPO-derived Albumin Binding Peptide to Molecular Therapy and Drug Targeting

The findings described above have shown the potential exploiting of transcytosis of proteins...
by caveolar trafficking in endothelial cells for the delivery of biological therapeutics across the microvascular barrier. In this context, gp60, the albumin binding protein, is crucial in the mechanism of albumin transport and along with other proteins conjugated to albumin. Gp60 receptor-mediated transcytosis can be exploited for the transport of therapeutically-active agents which do not normally pass through the endothelial barrier.14

Another important clinically relevant drug targeting advance described in the above studies is the discovery of the unique MPO-derived peptide that binds with high affinity to albumin such that albumin-MPO peptide complex can then dock unto gp60 to activate transcytosis.4 This approach is also useful for facilitating the delivery of biologics via the trafficking of caveolae across the vascular barrier.2,3 The identified albumin docking peptide (ADP) sequence of MPO1 can be conjugated to therapeutic proteins such as anticancer monoclonal antibodies and injected iv. Thereby ADP-therapeutic protein binds to circulating albumin in a high affinity manner and is transported across the vascular barrier. Thus ADP may be important not only in the delivery of therapeutic proteins but also delivery of diagnostic agents or markers of disease processes to track the efficacy of therapy (e.g., a labeled monoclonal antibody which binds to a receptor marker of a disease). This novel approach has great potential for cardiovascular, cancer, inflammatory, and autoimmune diseases. It may be of value for the treatment of a disease of the CNS (e.g., Alzheimer's Disease, Parkinson's Disease, multiple sclerosis, and amyotrophic lateral sclerosis, and a CNS neoplasia) in which the transcytosis of ADP may facilitate the transport of the therapeutic agent across the blood-brain barrier.

Acknowledgements
This study was supported by NIH grants P01HL060678, P01HL077806, R01HL045638, and T32HL07829.

References
