Targeting and Imaging Signature Caveolar Molecules in Lungs

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A major goal of molecular medicine is to target imaging agents or therapeutic compounds to a single organ. Targeting imaging agents to a single organ could facilitate the high-resolution, *in vivo* imaging of molecular events. In addition, genetic and acquired diseases primary to a single organ, such as cystic fibrosis, tuberculosis, lung cancer, pulmonary fibrosis, pulmonary hypertension, and acute respiratory distress syndrome, could be specifically targeted in the lung. By targeting and concentrating imaging agents or therapeutics to the lungs, deleterious side effects can be avoided with greater efficacy at much lower dosages. Pathologic changes can be identified earlier and followed over time. In addition, therapeutics that have been abandoned due to toxicities may find renewed utility when coupled with specific targeting agents such as antibodies. To achieve these goals, distinct molecular signatures must be found for each organ or disease-state.

Keywords: vascular endothelial cells; whole-body imaging; proteomics; intravital microscopy; transcytosis

MOLECULAR TARGETING

Most current targeted drugs are directed against specific proteins expressed by tissue parenchymal cells located deep within tissue—for example, function-altering antibodies or targeting antibodies linked to toxic agents. These treatments are very effective *in vitro*, where individual tissue cells are directly in contact with the media, and drugs, including antibodies, have the opportunity to bind their targets. However, the vast majority of these treatments have failed *in vivo*, where drug targeting is much more complex. Movement from the blood into tissue and even solid tumors is significantly limited *in vivo* by the vascular endothelium, a monolayer of attenuate endothelial cells that line all blood vessels (1). Poor delivery can prevent many carrier systems, including antibodies, nanoparticles, and gene vectors, from reaching their target tissue where they can be effective. The larger carriers are often rapidly scavenged from the circulating blood by the reticulo-endothelial system (i.e., liver and spleen), further preventing efficient binding and access *in vivo*.

Small drugs such as chemotherapeutics present an alternative to relatively large antibodies and nanoparticles because they can much more readily enter nearly all tissues. As a result, these agents are rapidly diluted, cleared from the blood, and excreted. Many of these drugs also lack specificity for any single organ or tissue. Their pervasive access can lead to high backgrounds and poor signal to noise during imaging and extensive side effects after systemic treatment. Chemotherapeutics, for example, act

by killing cells that rapidly divide. They are effective against cancer cells, as well as other healthy cells that divide rapidly, such as those in bone marrow, the digestive tract, and hair follicles, which leads to many of the well-known side effects of these drugs.

For both large and small molecules, it appears that only a small proportion of the injected dose actually reaches the inside of diseased tissue and even most tumors where they can be effective. Thus, higher doses must be administered to reach effective levels within the diseased tissue, often leading to severe systemic side effects (2). Diseases of the lung could benefit greatly from improved specific delivery and penetration into the diseased tissue. For example, many patients with lung cancer or pneumonia suffer greatly because little chemotherapy gets into the diseased tissue. To bypass these problems, accessible new targets and ways to penetrate into a specific tissue such as a solid tumor are needed.

VASCULAR ENDOTHELIUM

The inner surface of blood vessels are lined by the endothelium, a thin layer of endothelial cells that help to control the permeability of blood vessels and also plays an important role in vasoregulation, coagulation, inflammation, and tissue metabolism, growth, survival, repair, and overall organ homeostasis and function. Disruption of the vascular endothelium and its normal barrier function can rapidly lead to tissue edema, hypoxia, pathology, and even organ death (3, 4).

The vascular endothelium offers a promising alternative target to tissue cells themselves. The vascular endothelium is in direct contact with the blood and is, therefore, inherently accessible to probes or antibodies that are injected intravenously. In addition, endothelial cells are highly adapted to meet the needs of the underlying tissue and the microenvironment of the tissue surrounding the blood vessels significantly influences the phenotype of the endothelial cells (5–11). Defining the vascular proteome may uncover tissue- or disease-specific molecules that may be useful as targets for site-directed delivery of drugs, genes, or imaging agents.

PATHWAYS ACROSS THE ENDOTHELIUM

Sinusoidal endothelium (liver, spleen, and bone marrow) and fenestrated endothelium (kidneys, endocrine glands, and intestines) are relatively permissive barriers with large intracellular gaps or transcellular openings that allow the relatively rapid exchange of molecules from the blood into and out of tissue. Most tissues, including the lungs, have continuous endothelium. This is the most restrictive barrier with endothelial cells linked by intercellular junctions with various degrees of tightness (12).

Transport across the endothelial cell barrier is essential to support the underlying tissue and may provide pathways that can be exploited to target blood-borne molecules to tissue. Small molecules, water, and solutes can often be passively transported across the endothelial barrier by diffusion and convection through intracellular junctions, fenestrae, or trans-endothelial channels (13). Larger molecules may require active transport to move into or across endothelial cells.

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Many endothelial cells exhibit two distinct forms of active transport: clathrin-coated vesicles and caveolae. Caveolae are 60-nm flask-shaped invaginations found at the plasma membrane in most continuous endothelia that may be involved in endocytosis and transcytosis (14–20). Caveolae are abundant; *in vivo*, they can occupy up to 50 to 70% of the endothelial cell plasma membrane (21–23). Caveolins are structural coat proteins that oligomerize around the bulb of caveolae and appear necessary for caveolae formation *in vitro* and *in vivo* (24–30).

CAVEOLAE AS TRANSPORT VESICLES

Caveolae were first identified in 1953 (20). Since then, researchers have debated over whether these membrane invaginations might play a role in transport (31). Evidence collected in the last two decades overwhelmingly favors a role as dynamic vesicular carriers. Caveolae contain proteins classically associated with vesicular transport, including vesicular SNAP receptors (v-SNAREs), N-ethylmaleimide-sensitive factor (NSF), soluble NSF attachment protein (SNAP), and GTPases. Electron microscopy of ultrathin section of rat lung confirmed that vesicle-associated membrane protein (VAMP) was localized within caveolae (32). These proteins are associated with caveolae under basal conditions; no further recruitment is needed, suggesting that rapid transport might be possible (33). Much like other vesicular pathways, caveolae-mediated endocytosis is sensitive to N-ethylmaleimide (NEM), a thioalkylating agent that inhibits the fusion of vesicles to target membranes (34, 35). Caveolae have also been functionally implicated in active transport of select ligands and even virus to specific locations within the cell (36–43).

More recently, isolated endothelial cell plasma membranes have been used to show that caveolae can bud and form free vesicles in the presence of GTP and ATP (44). GTP hydrolysis was needed for this budding. GTP γ S, a nonhydrolyzable form of GTP, cannot induce budding and actually inhibits GTP-induced caveolar budding (45). This was confirmed on cultured cells, where electron microscopy showed that stimulation with GTP led to the loss of 78% of surface caveolae (45). GTP likely acts by stimulating the GTPase dynamin. Dynamin is necessary for caveolar fission; mutant forms of dynamin prevented caveolar fission (44, 46). Western analysis showed that dynamin is highly enriched in caveolae, and electron microscopy localized dynamin to the neck of caveolae, where it may act as a “pinchase” to mediate the final steps of vesicle budding (44, 46). Thus, key molecular machinery for budding, docking, and fusion are clearly present in caveolae. This caveolae-mediated internalization has been confirmed in hepatocytes (47), which have readily apparent caveolae in cell culture but few to no caveolae *in vivo*.

METHODS OF IDENTIFYING TISSUE-SPECIFIC MARKERS

To be effective, molecular targeting depends on the specific and unique expression of target proteins in different tissues and disease states. Multiple methods have been developed over the years to identify differential gene and protein expression, as summarized in Table 1. Monoclonal antibodies are one early method to investigate tissue-specific protein expression. Antibodies against unknown proteins can be generated by directly injecting target tissue, cells, or even tissue subfractions as an immunogen (48–52). Monoclonal antibodies can then be used to screen expression in cells and tissue as well as to immunoprecipitate the protein of interest for sequencing and identification. This can be a slow and laborious process, but produces robust antibodies.

Phage display libraries are another way to discover novel targets. George Smith first suggested that bacteriophages could be used to display proteins or antibody fragments in 1985 (53). Because the approach can use a large, random library, it is unbiased, and unknown targets can be identified. In a process called panning, phage that bind to a target (antibody, protein, or even tissue) are repeatedly isolated and amplified. Phage can be purified and used as probes themselves to isolate the binding partner for identification. Phage can also be injected intravenously, to circulate and presumably to bind a single protein at the endothelial surface (54, 55). They can then be isolated from each organ or tissue of interest. However, the liver and spleen rapidly scavenge phage from the blood, mostly before they have a chance to circulate through each organ and bind sufficiently (56). In addition, short peptides can lack specificity and may bind a large range of proteins in a multitude of organs, requiring additional *ex vivo* validation. These problems can be partially overcome by avoiding direct *in vivo* panning but rather screening antibody phage libraries on key membranes and then creating antibody-like fusion proteins, which unlike the phage, are not rapidly removed from the circulating blood and can indeed immunotarget successfully *in vivo* (57). Phage display libraries have revealed some promising targets, but currently this method may not yet be optimally suited for the high-throughput needed to comprehensively map protein expression and identify tissue-specific proteins.

Genomics approaches can be used to comprehensively define expression patterns and identify differences among samples in a relatively rapid manner. These approaches have identified unique gene expression on vascular endothelial cells derived from different tissues, from angiogenic tissue, and from tumor tissue (58, 59). Large changes are needed to detect differences between tissues. Unfortunately, changes in gene expression do not always correlate with changes in protein expression, nor does genomic data offer information about the location of a protein. Obviously, the location of a protein affects its accessibility and can also affect its function. Post-translational modifications make distinct proteins that cannot be assessed through genomic analysis and can also alter protein location and function. To truly identify accessible, tissue-specific targets, protein expression itself must be characterized at a very large-scale level that comprehensively identifies tens of thousands of proteins.

Two-dimensional (2D) gels are one simple and rapid way to visualize differences between tissues. In these gels, mixtures of proteins are separated by two distinct properties, providing better separation between proteins (60). Spots that are unique to one tissue can be excised and identified by mass spectrometry (61, 62). For these approaches, successful identification of proteins requires that the proteins migrate onto the gel. Many proteins simply do not separate well on such gels and can be underrepresented or lost altogether. Mass spectrometry-based techniques can rapidly identify large numbers of proteins based on the presence of digested peptides. Highly complex samples are difficult to separate, and low abundance proteins can be lost (63). Protein arrays offer a third alternative to identify differences between tissues. These use antibodies or peptides to identify the proteins present in the sample but are limited by affinity of the probes and the complexity of the sample (64).

REDUCING COMPLEXITY BY FOCUSING ON ENDOTHELIAL CELLS

Each of the above methods is limited by the complexity of the starting sample. Though it is clearly important to define the proteins at the endothelial cell surface, these cells are only

TABLE 1. METHODS OF IDENTIFYING PROTEINS AT THE SURFACE OF ENDOTHELIAL CELLS

Method	Advantages	Disadvantages
Methods of identifying proteins		
Antibody-based	Creates topological map of protein expression	Slow and laborious, depends on quality antibodies, can be difficult to identify unknown antigens
Phage display	Unbiased, unknown targets can be identified, applicable <i>in vivo</i> and <i>in vitro</i> , large libraries can be screened	Phage are rapidly scavenged by the liver <i>in vivo</i> , short peptides lack specificity, not high-throughput
Genomics	Rapid, high throughput, nonbiased, many genomes are nearly complete for straightforward identification of genes	Large changes needed, protein expression does not always correlate with protein expression, large number of "hits" require laborious validation
Two-dimensional gels	Simple, rapid, separates complex samples. Can be directly used to show differences between tissues.	Large amounts of material are needed, some proteins do not migrate into gel
Mass spectrometry	Rapid, high throughput, nonbiased, requires small amounts of sample	Limited by complexity of sample, low abundance proteins can be lost, large number of hits can require extensive validation
Protein arrays	Rapid, sensitive, large-scale screen	Biased, requires specific probes for each protein
Methods for reducing complexity		
Cultured cells	Large amounts of material, readily accessible	Cultured cells rapidly lose <i>in vivo</i> phenotype
Lectin analysis	Reveals differences in glycoproteins, can be applied <i>in vitro</i> or <i>in vivo</i>	Only applicable for specific glycoproteins
Radiolabeling	Verifies proteins at the surface of endothelial cells	Requires large amounts of radionuclides, no way to purify proteins, small radionuclides readily permeate into tissue
Biotinylation	Labels proteins at the cell surface, strong interaction between avidin and biotin can be used to purify proteins for identification	Difficult to control the degree of biotinylation, small molecules can permeate into endothelial cells and tissue
Silica nanoparticles	Selectively label surface proteins, do not enter into tissue, entire luminal surface and its caveolae can be isolated	Technically complicated, small amounts of tissue are obtained

a tiny part of any whole tissue homogenate. Even highly enriched endothelial cell proteins can be missed when the total organ homogenate is analyzed (65). Studying endothelial cells requires a reliable method to isolate these cells from the entire tissue. Several methods are described in Table 1.

Endothelial cells were first isolated and successfully cultured in the 1970s (66, 67). This allowed tremendous insights into the molecular components and functions of these cells and showed that they were rapidly responsive to vasoactive cytokines, shear stress, and inflammatory compounds (4, 68–70).

Numerous chemical techniques were developed in the late 1980s and early 1990s to label surface proteins of endothelial cells. Because proteins at the surface of endothelial cells *in vitro* and *in vivo* are directly exposed to the media or to blood, they also can be labeled by reagents added to the media, perfused through the vasculature, or injected intravenously (71–74). Different lectins can bind to different sets of surface glycoproteins, which has been used to compare different segments of vasculature (75), different organs (72), and *in vitro* versus *in situ* protein expression (74). Radio-iodination of surface compounds *in vitro* identified albumin-binding proteins (37, 76). *In situ* radiolabeling is inefficient and requires high amounts of Iodine-125, often exceeding 10 mCi, making this process difficult (74). Moreover, the small radionuclides can readily cross the vascular wall to enter the tissue and radiolabel other nonendothelial cells. Radiolabeling is most often used to verify the presence of known proteins, as there is no simple way to separate and identify radiolabeled proteins, significantly limiting the utility of this approach.

Biotinylation can chemically label proteins for rapid purification. It can label proteins at the surface of vascular endothelial cells, especially cells grown in cultures in which conditions can be well controlled (71). The strong interaction between biotin and avidin can be used to purify the biotinylated proteins, which can then be identified with mass spectrometry (77, 78) or antibodies (79–83). This method has been used to identify proteins at the endothelial cell surface (77, 84) and cell junctions

(79), to determine differences between luminal and abluminal surfaces of cells (82, 85), to identify signaling components present at the cell surface (83), and to determine the location of proteins at different membrane subdomains (80). *In vivo* biotinylation identifies a unique pattern of proteins from the whole tissue homogenate, showing that this method isolates a subset of proteins (77, 78, 84, 86). These studies provide a significant advancement toward vascular mapping *in vivo*; however, it is difficult to control the degree and the location of biotinylation, especially *in vivo*. Biotin may not have equal access to all parts of the cell surface. All proteins may not be biotinylated similarly. Biotinylation reagents are quite small. They can readily enter almost all tissues and even cross lipid membranes to get inside cells. They can readily permeate throughout a tissue to label and ultimately help identify perivascular proteins inside the surrounding tissue and even possibly within endothelial cells (77). Using polar or charged biotinylation reduces entry inside cells, but access into tissue beyond the endothelial cell surface remains. This somewhat pervasive access and labeling may be appropriate for identifying candidate targets accessible to small targeting agents, but can greatly complicate identification of tissue-specific endothelial cell surface proteins useful for direct targeting of antibodies, proteins, nanoparticles, and gene vectors.

Colloidal silica particles offer a promising alternative to more permeable reagents. When perfused through the vasculature *in vivo*, these nanoparticles are too large to penetrate into the tissue and thus can quite selectively coat the endothelial cell surface. After cross-linking and tissue homogenization, the dense silica-coated luminal endothelial cell plasma membranes are easy to separate from other components of the tissue, and even other components of the endothelial cells (87). The endothelial plasma membrane can be further subfractionated to study functional microdomains such as caveolae (87). The silica nanoparticles are too large to readily enter into the caveolae. Caveolae can be separated mechanically from the luminal membrane by shear stress or the addition of GTP and then

isolated by buoyant density centrifugation (19, 44, 87). Electron microscopy of the isolated caveolae shows a homogenous population of appropriately sized, 60- to 80-nm vesicles. Western analysis shows that this isolate is enriched for caveolae markers such as caveolin but depleted of markers for other subcellular organelles. In addition, magnetic immunobeads labeled with caveolin antibodies showed that the population was highly pure (> 95%) (88).

Studying endothelial cells *in vivo* is essential to define relevant molecular targets. Though distinct *in vivo*, once in culture, endothelial cells isolated from different organs differentiate into a more common phenotype. They lose expression of tissue-specific proteins and the number of surface caveolae decreases 30- to 100-fold (5, 89–91). Very recent mass spectrometry analysis shows profound differences in proteins expression *in vitro* versus *in vivo*. Approximately 40% of the proteins expressed *in vivo* are not found *in vitro* (61). In light of this, tissue-specific vascular targets must, at the very least, be validated *in vivo*.

MAPPING THE VASCULAR ENDOTHELIAL CELL PROTEOME

Early attempts in the 1980s and 90s to define the endothelial cell proteome focused on using antibodies and other specific probes to identify individual proteins (as detailed above). These methods provided valuable insight into the functional components of endothelial cells, but such approaches are difficult and necessarily piecemeal. Mass spectrometry-based approaches can provide a much more comprehensive profile of endothelial cell proteins. Initial studies using mass spectrometry-based approaches were done on endothelial cells *in vitro* using 2D gel electrophoresis or protein arrays followed by mass spectrometry analysis to identify proteins (64, 92). Though most cell types, including endothelial cells, are predicted to contain very large numbers of distinct proteins, these studies only identified a handful of proteins (< 60). Clearly, more comprehensive approaches are needed to fully map the vascular endothelial cells, and especially the proteins present at the plasma membrane.

As described above, the luminal surface of endothelial cells can be isolated *in vivo* using a silica-coating technique. Though far simpler than total tissue homogenate, the membrane isolate is still a highly complex mixture of proteins. Recent advances in proteomics techniques have made it possible to analyze complex samples, in part by pairing one- or two-dimensional liquid chromatography (1D- or 2D-LC) with mass spectrometry. 2D-LC separation can greatly improve sensitivity by diminishing peptide co-elution and thus enhancing identification of less abundant peptides not previously detected. Even so, each mass spectrometry measurement only identifies a portion of the proteins present in a sample. Therefore, replicate measurements are absolutely necessary to maximize the ability of a single method to identify proteins. When a sample of endothelial cell plasma membrane was repeatedly analyzed, 7 to 10 replicates were needed to reach 95% analytical completeness (61). After this point, additional replicates failed to identify significant amounts of new proteins. Comprehensive measurement is clearly necessary to define the endothelial cell proteins within a given tissue and to identify differences between tissues.

Using a statistically defined endpoint not only helps eliminate the inherent variance between single mass spectrometry measurements, but also increases the replicability of findings between different laboratories, as well as improves the utility of future mass spectrometry-based analyses (93). This requires heavy instrument time, but more clearly identifies proteins in

a sample. The extra mass spectrometry measurement days may well be time well spent to avoid a long list of candidates that may be temporarily satisfying, yet ultimately overwhelming with few actual true positives.

LUNG VASCULAR ENDOTHELIUM

Our initial large-scale attempt to map the luminal surface of lung vascular endothelial cells using shotgun proteomics with multiple replicate measurements identified 450 distinct proteins (61). As expected, many of these proteins were associated with the plasma membrane. Proteins that peripherally associate with the inner leaflet of the plasma membrane made up 35% of the proteins, while 31% were integral membrane proteins or proteins with lipid anchors, 25% were cytoskeletal or junctional proteins, and 8% were externally bound. Structural and signaling proteins made up more than half of the proteins found associated with the luminal plasma membrane. Trafficking and adhesion proteins, extracellular enzymes, and transporter enzymes were all found in abundance as well (61). It is unlikely that this is the full complement of membrane proteins. This study failed to identify several known endothelial cell markers, including specific enzymes, adhesion molecules, and growth factor receptors, many of which are integral membrane proteins. Indeed, only 15% of the proteins we identified were integral membrane proteins. Many more are expected.

Integral membrane proteins are especially challenging to identify with current large-scale shotgun proteomic methods. They are intrinsically hydrophobic, resistant to extraction and solubilization, and different proteins occur across a wide concentration range. In the most comprehensive profiling of the luminal plasma membrane of lung vascular endothelial cells to date, we have combined multiple mass spectrometry methods, each with multiple replicates to identify 1,833 proteins, including more than 500 lipid-embedded proteins (63). To help preserve lipid-embedded proteins, we used harsh detergents to solubilize proteins and separated these proteins on SDS-PAGE gels. After gel prefractionation, proteins were digested in gel and then analyzed via 1D-LC or 2D-LC paired with mass spectrometry. All methods were compared with the more commonly used shotgun approach, namely gel-free 2D-LC paired with mass spectrometry, which we had used previously. Several benefits of gel prefractionation were seen. First, less sample was needed. For traditional gel-free approaches, 150 µg was used. For each of the gel-based approaches, 40 µg of sample was sufficient. Larger sample volumes did not lead to the identification of greater numbers of proteins. Second, fewer replicates were needed to reach 95% analytical completeness. For gel-free approaches, 7 to 10 replicate measurements were needed, while the gel-based approaches only required 4 to 5 replicates (63).

Most importantly, gel-based approaches provided superior protein identification by identifying greater numbers of proteins, providing better protein coverage, and identifying proteins across a broad dynamic range. Together, the three gel-based methods more than quintupled the protein identifications relative to the gel-free method, accounting for over 98% of the total protein identifications. Gel-based methods identified more peptides per protein, leading to greater protein coverage. The lower limit of meaningful protein identification for gel-free methods was estimated at greater than 2 ng, but each gel-based method has much better sensitivity, down to 0.1 ng. The three gel-based methods could detect proteins over about five orders of magnitude. Though the gel-based methods identified more proteins than the gel-free method, each method identified unique proteins (63).

Integral membrane proteins are thought to comprise at least 20% of all open reading frames in the genome (94). Protein prefractionation by SDS-PAGE greatly enhanced detection by mass spectrometry analysis. Each gel-based method detected two- to sixfold more integral membrane proteins than the gel-free approach. Even when normalized to the number of soluble proteins identified, gel-based methods identified a greater amount of integral membrane proteins (63). This may be predictable because soluble proteins are far more amenable to in-solution digestion than integral membrane proteins and therefore ideal for analysis with gel-free approaches.

Gel-free mass spectrometry methods usually require that samples be precipitated and resolubilized, often in urea. Many proteins may be lost during this process. To determine where proteins were lost, some samples were simply solubilized in cell lysis buffer. Others were processed further and precipitated, then resolubilized in cell lysis buffer or in urea to determine the relative effects of precipitation and resolubilization. Protein abundance was compared by Western analysis of 64 known proteins. Most soluble proteins were equivalently present, regardless of preparation. Integral membrane proteins and lipid-anchored proteins were often lost during either the precipitation step or the resolubilization step. When samples were prepared as they are for gel-free mass spectrometry, some lipid-embedded proteins decreased by as much as 90%, which can prevent downstream identification by mass spectrometry (63). Thus, increased protein identification by gel-based methods arises in part from the overall reduced sample complexity for each mass spectrometry measurement as afforded by the gel prefractionation and in part from the improved retention and separation of integral membrane and lipid-embedded proteins in the gels for digestion.

QUANTIFICATION AND NORMALIZATION OF MASS SPECTROMETRY DATA

Multiple replicate measurements and even multiple mass spectrometry-based approaches are likely necessary to comprehensively define the proteins present in a sample. Mass spectrometry measurements contain inherent biases and variations. Replicate samples, regardless of the abundance feature used, will usually show variation in protein abundance, which is likely not a reflection of biological change. Proper quantification and normalization is needed to minimize inherent experimental bias and variability so that real changes between distinct samples can be reliably detected.

Currently used quantitative mass spectrometry experiments often rely on chemically (95) or biosynthetically (96) tagged systems. Relative differences in protein expression can then be quantified (97), but comparisons between multiple datasets and with previously existing data are impossible. Newer label-free methods are being developed that eliminate the need for expensive labeling reagents and extra analytical capabilities, and permit comparison between datasets. These rely on mass spectrometry output features of abundance, such as spectral or peptide counts (98–101) or chromatographic peak area or height values (102–105) to determine relative protein abundance. These methods correlate with protein abundance (101–103, 106), but detailed comparative analysis and extensive validation is needed to determine which is more accurate.

We tested various methods to quantify, normalize, and compare complex label-free proteomic data using features intrinsic to typical mass spectrometry measurements such as spectral and peptides counts, fragment ion (ms/ms) intensities (unpublished data). These features are much easier to extract from the mass spectrometry data than peak area measurements

and are much more suitable for quantification when using a low-resolution mass spectrometer. Alone, no single feature successfully normalized replicate mass spectrometry measurements. Replicates of a single liver sample were significantly different. Two published normalization methods that use spectral counts, NSAF (107) and Rsc (108), were also unable to sufficiently normalize the liver data. We incorporated these features and protein length into a single index called SI_N . SI_N was able to successfully normalize mass spectrometry measurements from replicate measurements, from different sample loads, and from different instruments.

The novel SI_N method was validated in several ways. SI_N does not over-normalize the mass spectrometry data, because real differences were still readily detectable. Significant differences could be detected between heart and kidney samples. In addition, densitometry of Coomassie blue-stained gels correlated with mass spectrometry estimates of protein abundance. When the relative enrichment of a given protein found at the lung plasma membrane was compared with the protein abundance in the general tissue homogenate, both densitometry of Western blots and SI_N values from mass spectrometry analysis showed excellent positive correlation. SI_N clearly compensated for experimental and random bias and noise across a wide array of techniques and samples (unpublished data). The ability to use SI_N to accurately determine the relative abundance of thousands of proteins in complex samples is impressive and will hopefully facilitate comparison of data between labs. In addition, by providing a simple and amenable method to quantify and normalize mass spectrometry data, we hope that replicate mass spectrometry measurements and the more comprehensive data they generate will become the norm.

NOVEL CAVEOLAR TARGETS IN LUNG TISSUE

Our lab has used several approaches to map the vascular proteome of the lung and other organs to identify tissue-specific proteins. Initial monoclonal antibodies and phage display antibody libraries were constructed using the isolated luminal surface of endothelial cells as an immunogen (56). These were then screened against Western blots or directly on the isolated endothelial cell membranes. Specific probes were used to identify proteins by immunoprecipitation and mass spectrometry. Endothelial cell membranes isolated from major organs were analyzed on 2D gels to produce high-resolution “maps” of the proteins present in the sample (61, 62). In many cases, antigens that were difficult to detect in the whole tissue homogenate were readily apparent in the isolated plasma membranes, showing that silica-based tissue fractionation revealed an enriched population of proteins. Furthermore, each organ showed a specific and characteristic array of proteins, strongly suggesting that tissue-specific markers could be identified. We used a systems biology approach to identify lung-specific endothelial cell surface proteins. Cells rapidly de-differentiate when removed from their natural environment and cultured. Therefore, we hypothesized that proteins found in cultured endothelial cells might be more universally expressed, while those expressed only *in vivo* might rely on the specific tissue microenvironment for expression and may represent possible tissue-specific targets. We isolated the luminal plasma membrane from lung vascular endothelial cells *in vivo* or in culture and analyzed each sample using replicate mass spectrometry measurements. Of the 450 proteins identified at the luminal surface of lung vascular endothelial cells *in vivo*, only 263 were found at the surface of cultured lung endothelial cells. The other 187 were found only *in vivo*. To focus on proteins that were only expressed *in vivo*, we applied strict identification criteria to rat lung endothelial plasma membranes isolated

in vivo, while low-stringency criteria were applied to membranes from cultured endothelial cells. Only endothelial cell surface proteins that extend into the blood are readily accessible to circulating antibodies. By analyzing the sequences of identified proteins, we identified 11 differentially expressed proteins likely to have domains outside the cell. To confirm organ specificity, we used specific antibodies to determine the presence of proteins in various organs and in culture. Consistent with the mass spectrometry findings, all 11 proteins were enriched in lung endothelial plasma membranes but were not found at the surface of cultured lung endothelial cells. *In vivo*, many of these proteins were expressed in other organs, including heart, kidney, and liver. Two proteins, aminopeptidase P (APP) and OX-45, were detected in endothelial cells only from lung. Unfortunately, OX-45 is also expressed on various leukocytes (109). Thus, radiolabeled antibodies against OX-45 showed little targeting to the lung but instead remained in the blood (61).

MEANS TO VALIDATION

Successful molecular targeting likely depends on several factors. Tissue-specific probes must be specific for a single organ and their target proteins must be accessible. The first step in validation of possible targeting systems is to verify the specificity of the probe. Analyzing Western blots of the endothelial cell surface membranes should show one band of the appropriate weight.

Western analysis can be used initially to test tissue specificity and localization of the target protein. To verify restricted tissue expression, the probe can be tested against proteins isolated from many different organs (62). In addition, the localization of the target protein can be assessed by analyzing whole tissue homogenates as well as different subfractions (110). In this way, the presence of the protein at the endothelial cell surface or within caveolae can be determined. Localization of the probe to a type of cell or even sublocation within the cell can be further verified in tissue slices by using immunohistochemistry, immunofluorescence, and electron microscopy (110).

One of the most vital questions to answer is whether the protein target is accessible to intravenously injected probes. The classical way to determine this is to inject radiolabeled probes. At various times after intravenous injection, major tissue types can be dissected and analyzed for radioactivity. This allows a quantitative assessment of the tissue distribution of the probe and also an approximate time course (111). Immunospecificity and localization of the probe can be compared using different indices (51, 56, 62, 110). This is especially relevant for antibody-based targeting system, because specific antibodies can be compared with control IgG. Comparing antibody accumulation in a target organ for a specific antibody versus a control antibody can give an immunospecificity index. Comparing organ accumulation to antibody remaining in blood can give a localization index. Specific localization can be calculated by comparing localization of the specific antibody to the localization of the control antibody. Although robust and quantitative, such analyses are highly invasive and static, requiring the killing of many animals at each time point.

Noninvasive *in vivo* imaging may be the new gold standard for *in vivo* targeting of probes. New methodologies such as γ -scintigraphic (112), positron emission tomography (PET) (113), and luminescence imaging (114) provide visual as well as quantitative analysis of probe targeting. These methods are noninvasive and can be used over time in the same animal. This makes these methods the most objective, dynamic, and comprehensive tools to date. Rapid dynamic imaging can create a true time course with far fewer animals than those needed for

standard biodistribution studies. In addition, minor tissues that are difficult to isolate can also be studied with *in vivo* imaging. The above methods can provide important quantitative data for assessing targeting grossly at the whole body and organ level but not so much at the cell or subcellular level.

Traditionally, the final validation of successful endothelial cell targeting involves intravenous injection of the probe. At set times after injection, tissue is excised and the probe is localized with immunohistochemistry, immunofluorescence, and/or electron microscopy. This can provide valuable confirmation of the ability of antibody to target the endothelial cell surface, to be transcytosed across the endothelial cell barrier, and to penetrate deep into the tissue; however, these are static methods and can only provide "snapshots" over time. Using this approach for initial validation can be problematic. For example, simply showing tissue histology with probe signal in one tissue but not another does not constitute validation of tissue-specific targeting. A proper titration curve determining signal range and sensitivity of the tissue staining assay is mandatory, but not usually performed, to ascertain the dynamic range of the assay and the degree of specificity in targeting. Immunohistochemistry usually does not have a large dynamic range. Thus, it is not surprising that even only two- to threefold higher expression in a tissue can render immunostaining that appears specific but rather is false-negative for many other tissues. To minimize this problem, the signal should be maximally developed (even to saturation) in the highest expressing tissue before stopping the reaction in other tissues. Then, other tissue can be declared negative within the dynamic range of the tissue. It is fairly clear that using more than one method to validate seems most wise.

Continuous, dynamic, noninvasive imaging in live animals is clearly the desired standard. Intravital microscopy is a valuable tool for visualizing, at the level of light microscopy, specific organ endothelial cell targeting and processing of candidate antibody probes (i.e., transendothelial transport by caveolae with interstitial accumulation). Especially when paired with tissue growing in dorsal skinfold chambers, intravital microscopy can present a high-resolution, dynamic view of endothelial cell targeting as well as the movement of probes into tissue, ultimately to better assess tissue/organ targeting, uptake, and accumulation in live animals (110).

LUNG-SPECIFIC TARGETING *IN VIVO*

Several proteins, including angiotensin-converting enzyme (ACE) (115), platelet/endothelial cell adhesion molecule (PECAM) (116, 117), and intercellular adhesion molecule (ICAM) (117), have been used to specifically target the lung and to deliver therapeutic compounds *in vivo*. Many of these proteins are not lung specific and instead rely on the extensive lung vasculature and the lung receiving the vast majority of cardiac output for specific targeting. A partial list of pulmonary markers can be found in Table 2. In addition, without a means to bypass the endothelial barrier, many of these antibodies and their cargo will remain bound at the endothelial cell surface and unable to reach cells within the underlying tissue, which is often the therapeutic target. Though these proteins can effectively retarget intravenously injected probes, proteins with restricted lung expression and that provide a portal into underlying tissue might expand the utility of vascular targeting.

Aminopeptidase P (APP) may fulfill both these needs. Electron microscopy showed that APP antibodies specifically immunolabel caveolae in vascular lung endothelium and can target colloidal gold nanoparticles to caveolae after pulmonary artery perfusion *in situ* (51, 110). Little to no labeling of caveolae was seen with control antibodies. To assess the specificity and

TABLE 2. MARKERS OF NORMAL AND PATHOLOGICAL PULMONARY ENDOTHELIUM

Markers	Lung	Tumor	Other Tissue
Lung endothelial markers			
ACE	Highly enriched	Present	Heart, kidney, liver
APP	Highly enriched	Not expressed	None
Aquaporin 1	Present	Present	Heart, kidney
Carbonic anhydrase	Highly enriched	Not expressed	Heart, kidney, liver
DDP4	Enriched	Enriched	Liver
ECE	Present	Present	Heart, kidney, liver
OX-45	Highly enriched	Not expressed	None
PV-1	Highly enriched	Highly enriched	Heart, kidney, liver
RAGE	Present	Present	Heart, liver
STR	Present	Present	Heart, kidney, liver
TM	Present	Present	Heart, kidney, liver
Tumor endothelial markers			
AnnA1	None	Highly enriched	None
AnnA8	Low	Highly enriched	None
APN	Present	Enriched	Heart
C-CAM	Enriched	Present	Liver
Endoglin	Present	Present	Heart, liver
EphrinA5	Present	Present	Heart, kidney, liver
EphrinA7	Low	Present	Heart, kidney, liver
MPO	Low	Enriched	Heart
Neuropilin nucleolin	Low	Present	Heart, liver
TfnR	Present	Enriched	Heart
Tie2	Low	Enriched	Heart, liver
VEGFR1	Low	Highly enriched	Kidney
VEGFR2	Low	Present	Heart, kidney, liver
VitDBP	None	Enriched	Liver

rapidity of lung targeting of APP antibodies in live animals, we injected rats with radiolabeled APP or control antibodies via the tail vein (62, 110). Whole-body γ -scintigraphic imaging revealed that APP antibodies could rapidly target the lungs. The lung silhouette was first discernible 10 seconds after injection, and the heart could be seen as a dark, signal-free shadow. In contrast, control IgG was detected throughout the entire animal and exhibited little to no specific organ targeting. SPECT-CT imaging revealed that antibodies against APP produced the highest-intensity lung image, which remained essentially unchanged from 30 minutes (Figure 1) through 48 hours. We also tested several other antibodies against proteins that show relative lung enrichment, but are not found in caveolae.

Consistent with reports in the literature, CD34, ACE, podocalyxin, and PECAM antibodies also specifically targeted the lung, though at lower levels than APP, and the lung-specific signal quickly dissipated after 1 hour. Signal from radiolabeled ACE antibodies was distributed through the thoracic cavity, consistent with ACE expression in both lung and heart luminal endothelial cell membranes. Podocalyxin and PECAM antibodies also targeted other organs, including kidney and liver (62, 110). To dynamically follow antibody targeting in real time at high resolution, we engrafted rat lung tissue into dorsal skin fold window chambers on nude mice (110). The lung tissue revascularized within 1 to 2 weeks. After tail vein injection of fluorophore-conjugated antibody, the rat lung tissue was moni-

Figure 1. SPECT-CT imaging of aminopeptidase P antibodies shows antibody accumulation in the lungs *in vivo*. Radiolabeled antibodies were injected intravenously, and SPECT-CT images were acquired 30 minutes later.

Figure 2. Overview of caveolae-dependent trafficking of antibodies. (a) Antibodies targeted against caveolae (anti-APP, light blue) are rapidly transported into the lung tissue, antibodies that bind outside of caveolae (anti-CD34, yellow) accumulate at the cell surface, and non-specific IgG molecules (black) stay in the blood. (b) When caveolin-1 protein is decreased, aminopeptidase P antibodies remain at the cell surface, implicating caveolae in their transendothelial transport. (c) According to this model, aminopeptidase P antibodies are specific for lung endothelial cells and do not accumulate in blood vessels from other tissues. Reprinted by permission from Reference 119.

tored continuously with intravital microscopy. Within 10 seconds, APP antibodies bound to the lung endothelium, but did not bind to vasculature outside of the engrafted rat lung tissue or to engrafted rat heart or liver tissue. This shows that engrafted tissue retained both its species- and tissue-specific markers, even when growing in a mouse host. APP antibodies bound to the vascular endothelium within the lung tissue in approximately 10 seconds. Lower-resolution images showed that the entire engrafted lung tissue was fluorescent within 10 minutes. APP antibody appeared to be actively pumped into the lung tissue, even acting against a concentration gradient. Control IgG did not block binding and transcytosis of APP antibodies. IgG also did not bind the endothelium or extravasate in the tissue, even when much higher concentrations were used. Targets that were accessible to the blood and enriched in caveolae were clearly necessary to see rapid transcytosis. The caveolin antibody did not bind to the lung endothelium and was not transcytosed. This is not surprising, because caveolin is inaccessible to the blood. Antibodies against CD34 rapidly bound the lung microvessel surface but did not cross the endothelial cell barrier, even when higher amounts were used and imaging was extended to 30 minutes. Caveolae themselves appear necessary to transport antibodies across the endothelial cell barrier and into tissue. We used lentivirally expressed short hairpin RNAs against the caveolin-1 gene to lower the amount of caveolae. APP antibodies could still bind to the lung microvessel surface in this tissue, but the antibody was not transcytosed or pumped into the lung tissue (110). Figure 2 provides a schematic summary of events at endothelial cell surface. This cartoon illustrates caveolae-mediated trafficking of antibodies and comes from

a News and Views articles written about our 2007 Nature Biotechnology paper documenting the pumping ability of caveolae to enable antibodies to target and penetrate specifically into the lung after intravenous injection.

TARGETING LUNG TUMORS

Clinical utility depends on finding specific endothelial markers for disease states. Endothelial cells are extremely sensitive to the tissue environment and can rapidly change when removed from their normal environment (61, 62). Less drastic changes, such as inflammation and disease, may also alter tissue enough for changes to be reflected in the surrounding vasculature. Caveolae are found at the luminal surface of most endothelium and may facilitate transport to many different organs and even solid tumors, thus providing a universal pathway that can be exploited to deliver drugs or imaging agents to diseased, as well as healthy tissue.

When injected into rats bearing multiple tumors in the lungs, APP antibodies did not traffic to the tumors but instead targeted the surrounding normal lung tissue (62). When the luminal endothelial surface of vasculature from lung tumors was analyzed by Western analysis and with mass spectrometry, APP expression was lost. Tumor vascular endothelium expressed a distinct array of proteins, clearly different from healthy lungs, suggesting that the solid tumors might form a distinct type of tissue that influences protein expression in its neovasculature. Several possible markers for tumor endothelium are listed in Table 2. We again applied a subtractive proteomic approach and mass spectrometric analysis of these plasma membranes

Figure 3. (a and b) Tumor-bearing rats were intravenously injected with ^{125}I -AnnA1 antibodies and imaged 4 hours later. Tumor-bearing lungs were excised for imaging. (c) Tumors, circled in yellow, could be seen to overlap with (d) hot spots from planar imaging. Reprinted by permission from Reference 62.

and identified 15 proteins that were differentially expressed. As expected, several known tumor angiogenesis markers were up-regulated, including vascular endothelial growth factor (VEGF) receptors-1 and -2, Tie2, aminopeptidase-N, endoglin, carcino-embryonic antigen-related cell adhesion molecule 1, and neuropilin-1. Previously unknown tumor-induced vascular proteins (annexin A1, annexin A8, ephrin A5, ephrin A7, myeloperoxidase, nucleolin, transferrin receptor, and vitamin D-binding protein) were also detected. Most of the proteins (12 of 15) were much more enriched in endothelial cell plasma membranes isolated from the tumors in the lung versus normal lung tissue. Unfortunately, almost all of these proteins exist in the healthy tissue of one or more major organs, though at lower levels. One surprising result was the induction of a novel protein at the endothelial cell surface. AnnexinA1 (AnnA1) is normally found intracellularly; however, AnnA1 could be detected at the luminal surface of endothelial cells taken from tumor tissue. This protein was specific for tumor endothelium and did not appear in the endothelium of normal organs. Immunohistochemistry showed that AnnA1 was also found in vascular endothelium of human solid tumors of the prostate, liver, breast, and lung, but not matched normal tissue (62).

To test whether AnnA1 was expressed on the luminal surface of endothelial cells and thus accessible to circulating antibodies, we performed whole-body imaging using radio-labeled monoclonal antibodies. Gamma-scintigraphic planar imaging of rats with tumors captured 4 hours after injection showed a distinct focus of radioactivity in the lung and little signal elsewhere (Figure 3). No targeting of normal organs was seen. Even lung targeting was absent in tumor-free rats. Non-targeting control IgG did not show detectable selective tumor uptake. When the lungs were excised and imaged, the radioactive hot spots labeled by AnnA1 antibodies corresponded to visible tumors (Figure 3). Surprisingly, 80% of the animals treated with radiolabeled AnnA1 antibodies survived 8 days or longer, while all IgG-treated and untreated rats died within 7 days. This increased survival is striking because in this model, many animals die within 2 to 4 days of treatment and thus may lack sufficient time to benefit from the treatment. A single injection of antibody caused significant remission even in advanced disease (62).

FUTURE DIRECTIONS

The rapid targeting and transcytosis shown above goes well beyond traditional vascular targeting and exploits a natural pathway to deliver imaging agents or therapeutic compounds across the endothelial barrier, actually concentrating them in tissue where they can be effective. By delivering these agents to specific sites, harmful side effects can be decreased (110, 118). In addition, active pumping of antibodies and conjugated molecules across the endothelial cell layer and into tissue may prevent degradation (110), further increasing therapeutic potential.

Lung-specific antibodies can be used to target many drugs to the lungs and may also be effective against cancer and other infectious lung diseases such as pneumonia and influenza. Targeting caveolar proteins could also direct imaging agents, nanoparticles, or even genetic material to a specific organ or disease state. Targeting imaging agents to the lung is clearly beneficial for early disease detection, but is also likely to facilitate molecular imaging of lung function *in vivo*. Caveolae are rich in signaling molecules, and targeted small molecules, siRNAs, peptides, or nanoparticles could be used to activate or inhibit specific pathways. Thus, pumping agents into a specific tissue may finally allow the goals of molecular targeting to be

realized as well as offer new windows into the function of organs *in vivo*.

This system could also be used to target drugs to any single organ with a specific, blood-accessible biomarker. To fulfill this goal, the vascular endothelial cell proteome of all major organs must be comprehensively mapped and targets must be validated. The benefits of targeting caveolae are just becoming clear. To truly exploit this pathway, we must understand its limitations. Are there size constraints? How are molecules processed and where do they go? Ultimately, targeted therapies may have a tremendous impact by increasing the efficacy of drug therapies while decreasing dose, treatment length, and side effects as well as allowing powerful *in vivo* imaging of both healthy and diseased tissue.

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