Chair's Summary

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Imaging of molecular signatures of specific structures, functions, and pathological alterations in the lung tissue represents an important and challenging goal. This summary outlines general themes of the 24th annual Transatlantic Airway Conference that took place in Lucerne in January 2009 focused on this subject. The following papers presented by the speakers outline specific subjects discussed at the meeting, including identification of candidate molecular signatures, design of imaging probes, and application of specific imaging modalities for visualization of pulmonary molecular signatures.

Noninvasive, real-time visualization of specific signs (markers) of physiologic and pathologic processes in the chest immensely enhances our ability to study, diagnose, and treat lung diseases. Pulmonary imaging, first established using Roentgen's X-rays, has diversified over the course of the last century to include an arsenal of imaging modalities such as X-ray computed tomography (CT), nuclear medicine positron emission tomography (PET) and single photon emission computed tomography (SPECT), optical imaging, and magnetic resonance imaging (MRI). One can now visualize structural information about the chest and lungs in addition to functional and molecular changes of diverse parameters. These include perfusion, ventilation, metabolism, tissue density, cell surface expression markers, and enzyme activity. Undoubtedly, the visual information provided at the structural, functional, and molecular levels by these modalities provides invaluable tools for evaluating the status of normal and diseased lungs in both experimental and clinical settings.

The extraordinary achievements in pulmonary imaging only reinforce the need for a discussion on the visualization of target molecular signatures in the lungs. "Target molecular signatures" are molecules that may serve as specific markers of selected elements of tissue structures, and of physiologic or pathologic processes. Figure 1 provides selected examples of such structures (cells and their compartments, components of extracellular milieu, blood components), functions (cell perturbations, enzymatic activity, ventilation, and perfusion), and abnormalities (metabolic changes, neo-antigens and neo-epitopes, activation and translocation of nuclear factors, and nucleic acid changes).

The imaging of molecular signatures will enhance the precision of differential diagnoses of pathologies that are otherwise indistinguishable by current diagnostic tests, and improve prognoses by stratifying the disease stage and the responsiveness to treatment. With imaging technologies we can ultimately improve the management of lung diseases and usher in a new type of "individualized medicine." Imaging molecular signatures in small animal models assists in studying the mecha-

nisms of pulmonary physiology and pathology. Furthermore, small animal imaging can guide the translational aspects of basic bench research by validating new therapeutics, diagnostics, or animal models of human disease. It is thus appropriate and timely that the organizers of the conference defined its topic as "Imaging Pulmonary Pathology and Target Molecular Signatures."

Identification and visualization of target molecular signatures requires efforts in diverse biomedical areas using numerous strategies (Figure 2). This subject transcends boundaries of many disciplines in biomedical, engineering, chemical, bioengineering, computer, and physical sciences. The conference brought together experts from many of these areas, both clinicians and basic researchers, to educate each other, redefine the current status of the subject, and establish new interdisciplinary collaborations. The meeting focused on three topics: (1) identification of the pulmonary target molecular signatures, (2) design of imaging probes and their delivery to the targets, and (3) imaging modalities for visualization of pulmonary target molecular signatures. However, for the sake of focus (or "in the interest of time"), some aspects of the challenges of the field (i.e., probe design, chemistry of isotope/emitter introduction onto probe, development of instrumentation for imaging, and image analysis) were rather tangentially discussed at the meeting.

IDENTIFICATION OF MOLECULAR SIGNATURES AND EXPERIMENTAL APPROACHES FOR THEIR IMAGING

In his introductory lecture, Dr. Piwnica-Worms provided an overview of optical and radioisotope imaging of molecules and their interactions in living cells and laboratory animals, and gave examples of their applications in the pulmonary imaging (*see* article by Dothager and Piwnica-Worms, pages 403–410, this issue). Many speakers throughout the conference provided particular examples of prospective pulmonary target molecular signatures. Both intracellular (e.g., nucleic acids, enzymes, and their substrates) and extracellular (e.g., components of the cellular milieu as well as membrane proteins, sugars, and phospholipids) molecules may serve as molecular targets and signatures for imaging (1, 2). Drugs, drug carriers, and other nanometer- and micron-scale objects (e.g., environmental pollutants and particles) delivered to the lungs via airway or vascular routes, and the physiologic effects caused by these interventions, also represent important targets for visualization (3–5) (*see* also articles by Dolovich, pages 477–485, and Zhang and colleagues, pages 450–457, this issue).

Specific probes recognizing molecular targets or their specific microenvironment include nucleic acids, peptides, enzyme substrates, aptamers, sugars, metabolic probes, and a variety of affinity ligands including antibodies and their fragments. Probes can be labeled with specific agents that permit optical (luminescence, fluorescence, or near infrared), nuclear medicine (SPECT or PET isotopes), or MRI (e.g., gadolinium) imaging (for overviews, *see* articles by Ntziachristos, pages 416–418, Divgi, pages 464–468, Blankenberg, pages 469–476, and Kauczor and colleagues, pages 458–463, this issue). In an optimal scenario, these probes are activated locally as a result of interaction with the target or accessory molecules. Specific protease cleavage of

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an activation peptide releasing a quencher from the probe provides an example of such local activation. Models using transfection of cells and animals, and generation of mutant animals expressing reporter genes activated locally in response to physiologic or pathologic changes, allow proof-of-principle of real-time noninvasive pulmonary imaging of intracellular and secreted molecular signatures using optical probes (e.g., luciferin, quantum-dots) or radiolabeled probes (e.g., [^{18}F]FHBG or [^{18}F]FIAU PET) (2, 6). Prospective target molecular signatures include a diverse array of enzymes (kinases, proteases), nuclear factors and intracellular mediators, membrane proteins, growth factors, hormones and receptors, integrins, adhesion molecules, and phospholipids (*see* Dothager and Piwnica-Worms, Ntziachristos, Divgi, and Blankenberg, this issue). Experimental imaging of these molecules in animal models would assist in understanding pulmonary physiology and the mechanisms of pathology (7–9). Some of these approaches, in particular those employing PET/SPECT or MRI, can be translated into clinical practice, for monitoring lung-directed gene therapies via probe imaging of reporter transgene products (2, 10, 11).

Strategies employed for identification and recognition of intra- and extracellular molecular signatures range from standard low-throughput immunoassays (e.g., fluorescence-activated cell sorter) to high-throughput screenings including functional proteomics and genomics, as well as the combinatorial tools of phage display and aptamers. In particular, using phage display libraries against lab animal specimens and samples from terminally ill patients has identified a series of peptides with affinity to specific determinants in diverse regions of normal and pathologically altered vasculature, including pulmonary endothelium and endothelium in lung tumors (12). These peptides are used for targeting agents to the site of interest, and are thus useful as diagnostic probes for imaging or for guiding local therapeutics interventions, such as destruction of target cells (13). Affinity-capturing and proteomics approaches have helped to identify the molecular targets of phage display homing peptides. This goal is especially challenging in the case of nonprotein targets, such as specific phospholipids (*see* article by Giordano and colleagues, pages 411–415, this issue).

Examples of prospective pulmonary target molecular signatures include aminopeptidase P (a marker of normal pulmonary endothelium), aminopeptidase N (a marker of tumor endothelium), cell adhesion molecules expressed by pulmonary airway epithelium (intercellular adhesion molecule [ICAM]-1) and

endothelium at the sites of inflammation (ICAM-1, vascular cell adhesion molecule [VCAM]-1, selectins), and receptors for growth factors expressed in the sites of tumor angiogenesis (e.g., VEGF-receptor) (12, 14–16). Animal and pilot human studies have provided proof-of-principle of molecular imaging of these molecules in the lungs. For instance, SPECT-isotope-labeled antibodies to angiotensin-converting enzyme (ACE), a transmembrane glycoprotein normally expressed in endothelium, provided images of the pulmonary vasculature and revealed pathologic changes in the lungs in animal models of acute lung injury and human patients with sarcoidosis (17). More examples of vascular pulmonary targeting are provided by Giordano and colleagues, Massey and Schnitzer (pages 419–430), and Ntziachristos in this issue.

Caveoli represent an interesting target for molecular imaging and site-specific interventions in the pulmonary biomedicine (15, 18). Transmembrane and surface proteins are concentrated in the caveoli, flask-shape invaginations in the plasmalemma in many cell types, including the pulmonary endothelium. Antibodies, their fragments, and affinity peptides binding to caveolar determinants, such as aminopeptidase P, accumulate in the lungs within minutes of vascular administration, and traverse endothelium via vesicular transcellular transport. These ligands, once labeled with radioisotopes or fluorescent probes, can allow for noninvasive PET imaging of the pulmonary vasculature and invasive optical imaging of extravascular transfer of ligands in lab animals, respectively (*see* Massey and Schnitzer, this issue).

Other examples of prospective extracellular and intracellular target molecular signatures, including phospholipid binding sites and intracellular enzymes typical of apoptotic cells, nuclear factors and signaling enzymes (e.g., kinases and phosphatases), are discussed below and in the papers of the speakers (*see* Ntziachristos, Dothager and Piwnica-Worms, and Blankenberg, this issue).

DESIGN AND DELIVERY OF PROBES FOR IMAGING PULMONARY TARGET MOLECULAR SIGNATURES

Agents for imaging molecular signatures include radioisotopes (PET and SPECT), stable MRI isotopes (e.g., gadolinium), contrast materials (e.g., hyperpolarized gases), and optical reporters (fluorescent, luminescent, and NIR-emitting substances). To achieve molecular imaging, these agents are coupled to

Pulmonary molecular signatures & targets are molecular structures specific for:

Lung structure, mechanistic studies, diagnostics, drug targeting

Figure 1. Pulmonary molecular signatures: targets for imaging. Selected examples of the molecular signatures of the pulmonary structures, function, and pathology (*see* explanations in the text).

Imaging target molecular signatures in lungs

Figure 2. Imaging pulmonary molecular signatures: key elements. Selected examples of approaches, probes, and imaging modalities employed for identification and imaging molecular signatures in the lungs. CT = computer tomography; MRI = magnetic resonance imaging; MS = mass-spectroscopy; PET = positron emission tomography; SPECT = single photon emission CT.

probes recognizing molecular signatures or carriers that facilitate delivery of labeled probes to the target sites (16) (*see also Massey and Schnitzer, Giordano and colleagues, and Zhang and colleagues, this issue*).

The airways are a natural route for delivering imaging probes and their carriers to the lungs, while also allowing for the introduction of imaging tools (e.g., catheters for intrabronchial microscopy [19]). The utility of this route, although attractive for imaging molecular targets in the airways and alveoli, is limited by several factors, including: (1) the heterogeneity of probe distribution in the tissue via ventilation, (2) inaccessibility of small airways for currently available catheters, and (3) difficulties in delivering probes and carriers into and across the alveoli (in part due to barriers of mucus, surfactant, and fibrous tissues, which may further enhance in pathology) (20). However, this is the route for inhalation of hyperpolarized gases for MRI contrast, and it affords excellent structural and functional lung imaging in human and animal studies (21, 22), as well as for imaging the distribution and effects of inhaled ^{11}C -labeled and ^{18}F -labeled drugs using PET in animals and human patients (3) (*see articles by Emami and colleagues, pages 431–438, and Kauczor and colleagues, this issue*).

The vascular route is widely used for the delivery of perfusion and angiography contrast agents and imaging probes, including labeled blood cells, affinity ligands, and nanocarriers directed to surface determinants expressed on quiescent (ACE, aminopeptidase P), activated (cell adhesion molecules), or pathologically altered (aminopeptidase N, VEGF-receptor, PS) endothelial cells in the pulmonary vasculature. Imaging pulmonary vessels provides important information in acute lung injury (ALI), inflammation, and tumor growth, among many other lung maladies (8, 14, 16, 23). Transport pathways across endothelium (e.g., via caveoli) permit delivery of imaging probes into the pulmonary interstitium and alveolocytes (*see Massey and Schnitzer, this issue*).

Imaging probes and pro-probes can be transported to their targets using a variety of delivery devices or carriers ranging in size from 20 to 50 nanometers to 0.5 μm (for intravascular route) and 1 to 50 μm (for the airway route and mechanical retention of microspheres injected via intravenous route). Examples of carriers amenable to the high loading of a probe and thereby providing pointed high-signal intensity include liposomes, polymer nanocarriers and dendrimers, quantum dots, and albumin microspheres (16, 24, 25). The chemical composition of the carriers controls the rate of their degradation and probe release. The delivery of carriers to selected cells and intracellular compartments is controlled by the size and shape of the carrier and the affinity to selected cell determinants or “molecular signatures.” Many of these delivery platforms are multifunctional; they can deliver optical, MR, and nuclear medicine imaging probes, possess affinity moieties for specific recognition of targets, and have elements of design facilitating intracellular delivery (*see Zhang and colleagues, this issue*).

Small cell-permeable probes (e.g., glucose derivatives) have favorable pharmacokinetics to deliver to diverse intracellular targets (2, 10, 26). However, specificity in terms of pathologies (e.g., inflammation versus tumor growth) is low, and the background signal in nontarget cells and tissues can be relatively high (27). Affinity ligands of cellular surface determinants provide specific recognition and imaging of the target cells (28). However, these probes are separated from the intracellular targets. Using determinants involved in constitutive (e.g., caveolar determinants) or inducible (e.g., cell adhesion molecules) endocytosis helps to deliver probes into the cells (29, 30). Design of carriers endowed with membrane-permeating features (e.g., coating with membrane-permeating peptides) or

endosomes-escape functions (e.g., using pH-sensitive materials) provides avenues for the cytosolic delivery of imaging probes (3, 25, 31). Conjugation of probes with peptides containing recognition motifs for nuclei, mitochondria, Golgi, and other organelles may even further enhance precision of subcellular delivery of the probe and resultant signal (*see Dothager and Piwnica-Worms and Zhang and colleagues, this issue*).

IMAGING MODALITIES FOR VISUALIZATION OF PULMONARY TARGET MOLECULAR SIGNATURES

Many imaging modalities can be used for visualization of target molecular signatures in the lungs, yet no single modality offers a winning combination of maximal spatial resolution, specificity, selectivity, sensitivity, signal penetration (*see articles by Kauczor and colleagues, Emami and colleagues, Divgi, and Coxson and Lam, pages 439–443, this issue*). Selection of the best modality is further complicated by practical issues of affordability, safety, and availability (e.g., generator versus cyclotron-produced radioisotopes). For example, the excellent structural resolution afforded by MRI (22) is inferior in its sensitivity to other modalities, like PET and SPECT for molecular imaging. The difficulties of labeling protein probes by PET radioisotopes has shifted the utility of immunoimaging from this sensitive imaging modality toward less-sensitive SPECT (28). However, pulmonary imaging is served by a multifaceted arsenal of modalities with constantly improving performance. Examples of newer imaging techniques include multi-row detector X-ray CT (MDCT) and optical coherence tomography (OCT) providing high-quality structural and functional characteristics of the lungs guiding studies of chronic obstructive pulmonary disease (COPD), ALI, fibrosis, and tumors, among other pulmonary maladies (32–35); intrabronchial catheter-based confocal microscopy (CBCM) (19); virtual pinhole PET (VP-PET) and pulmonary MRI with hyperpolarized gases; and others (21, 22, 36). Some techniques (e.g., PET, SPECT, optical imaging) are designed to identify target molecular signatures (2, 28, 31), whereas other modalities (e.g., CT and MRI) provide more of a structural and functional map for localization of these signatures (22, 33, 34). Comparison of data obtained using different modalities (e.g., CT versus OCT, PET versus SPECT) and use of combined modalities (e.g., PET/CT and SPECT/CT, PET/MRI) provide increasingly more precise localization of specific molecular signals in the thoracic structures, permitting a better account for heterogeneity of the readouts throughout the lungs and between subjects (10) (*see Kauczor and colleagues, Emami and colleagues, Divgi, and Coxson and Lam, this issue*).

New technological developments constantly expand the utility of imaging modalities. Classical structural imaging tools of CT and MRI are increasingly being used for functional imaging. Functional impedance tomography using dynamic CT or imaging of local patterns of ventilation, pH, Po_2 , and perfusion using polarized gas contrasted MRI are aiding in the characterization of emphysema, COPD, and inflammation (8, 21, 36). The shallow penetration of optical signals limits utility of high-resolution optical methods to molecular imaging in small rodents. Yet, recent animal studies have revealed the potential utility of quantitative transluminal and fluorescence molecular tomography for multicolor visualization of pulmonary maladies including inflammation, angiogenesis, and tumors (37). Intrabronchial microscopy extends the utility of optical imaging to molecular diagnostics of pulmonary maladies including COPD, asthma, fibrosis, emphysema, tumors, and inflammation (19). In addition to imaging lung microstructures using the innate fluorescence of collagen and macrophages, intrabronchial microscopy allows high-resolution imaging of laser-ignited probes delivered to the lungs via circulation or airways. Simultaneous with imaging is the ability to sample the bronchoalveolar

fluid and mucus, and obtain a microbiopsy of tissue (see article by Thiberville and colleagues, pages 444–449, this issue).

Examples of attempts to image specific markers of pulmonary pathologies in animals and humans include imaging of apoptosis, inflammation, tumors, fibrosis and sarcoidosis, COPD, emphysema, and ALI/ARDS. In most cases, these modalities provided structural and functional imaging; only some cases involved imaging of identified molecular signatures. For example, both cell surface and intracellular molecular signatures of apoptosis are being visualized in animal models using ^{99m}Tc - or ^{18}F -labeled annexin V in SPECT and PET, respectively, and ^{18}F -labeled caspase inhibitor in PET (38). Both SPECT and PET are employed for imaging fibrosis and sarcoidosis in patients using ^{111}In -octreotide (SPECT-fibrosis) (39) and ^{18}F -FDG (PET-sarcoidosis) (40). Cellular specificity and pharmacokinetics of these probes remain to be fully characterized, to understand whether this approach provides imaging of a specific molecular signature, or more generic pathologic changes such as enhanced tissue uptake, typical for ^{18}F -FDG-PET in many localized pathologic processes. ^{18}F -FDG-PET also affords high-resolution imaging of lung tumors and inflammation in animal models and human patients using enhanced uptake of ^{18}F -FDG. More specifically targeted affinity probes for imaging lung tumors, including antibodies, have been largely utilized by SPECT (28, 41). However, a team led by the late Dan Schuster recently visualized pulmonary inflammation in animals using ^{64}Cu -labeled polymer nanocarriers targeted to ICAM-1 with PET (16).

CHALLENGES AND PERSPECTIVES

Imaging of pulmonary molecular signatures is transitioning from the neonatal stage to infancy. There is still a long way to go before this strategy finds broad clinical utility. Visualization of specific molecular events will require an increase in spatial resolution of imaging modalities, up to nanometer scale, as is currently available with invasive microscopy methods, while retaining high sensitivity and tissue penetration of the signal, as is observed with nuclear medicine imaging. Parameters critical for imaging target molecular signatures include: (1) signal specificity (i.e., target/nontarget signal ratio—e.g., ratio between signals in a tumor and in a normal tissue, provided by labeled antibody); (2) signal selectivity (i.e., targeted/nontargeted probe signal ratio—e.g., ratio between signals in a tumor provided by labeled antibody and control IgG); (3) signal penetration through tissue, sensitivity, and spatial resolution of its acquisition; and (4) a method's availability and safety (including potential side effects of delivery of probes to the target). Enhancing the signal specificity by combining multiple features is the key. An ideal imaging strategy would employ the following: (1) facilitation of probe delivery to the target by local pathologic factors (e.g., enhanced vascular permeability in inflammation); (2) recognition of specific surface determinants on the target cell (e.g., cell adhesion molecules expressed on cell surface in inflammation or phospholipids on surface of the apoptotic cells); (3) employment of internalization mechanisms typical for the target cells (e.g., endocytosis via cell adhesion molecules or caveoli) to trap the signal; and (4) delivery of imaging probes to specific intracellular target molecular signatures that can elicit signal emission by cleavage, intercalation, phosphorylation, or other site-specific modification. Visualization of molecular events using optimally tuned modalities and targeted probes will enhance the experimental and clinical utility of these imaging technologies and usher in a new age in imaging pulmonary pathology and molecular target signatures in the lungs.

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