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Estimating structural alterations in animal models of lung emphysema. Is there a gold standard?

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SUMMARY

Chronic obstructive pulmonary disease (COPD) is one of the most common lung diseases. The major component of COPD, which affects the gas-exchanging parenchyma of the lung, emphysema, is characterized by destruction of alveolar septae leading to loss of functional surface, loss of alveoli and enlargement of remaining distal airspaces. These microstructural alterations can be modeled in animals and can be measured using stereological methods applied to imaging datasets. Many animal models of emphysema exist, but most of them are insufficiently characterized with respect to the underlying nature (e.g. destructive or developmental) and the degree of the structural alterations. The most popular parameter for assessment of emphysematous alterations, mean linear intercept length, has severe limitations. It can, therefore, not be recommended. Better design-based stereological alternatives exist but are less often applied, such as total volumes of parenchymal compartments (alveolar airspace, alveolar duct airspace, alveolar septum), total alveolar surface area, total alveolar number and mean alveolar size and its size variation. A prerequisite is the use of appropriate fixation, sampling, and specimen processing protocols. This article reviews the challenges of stereologic assessment of emphysematous alterations in the lung and illustrates possible strategies.

1. Introduction

Each day, a human being inhales and exhales more than 10,000 l of air in to and out of the lung. The functional capacity of the lung as a gas exchanger is directly related to its fine structure. Around 85–90% of the lung consists of parenchyma (alveolar region), the remainder constitutes the conducting part for gas and blood. Efficient oxygen uptake requires a large surface area and a thin diffusion barrier. The design of the mammalian lung is optimized in that respect. Alterations in lung structure, e.g. a decrease in alveolar surface area or an increase in air–blood barrier thickness, are pathological hallmarks of common lung diseases – emphysema and fibrosis, respectively.

Biomedical research directed toward a better understanding of the pathogenesis of these diseases and the development of therapeutic strategies requires reliable animal models. In experimental studies, different groups (e.g. control, untreated disease, treated disease) are compared regarding specific outcome parameters. If the histopathological features are the centerpiece of the disease, it is essential to assess the structural alterations quantitatively, thereby adding “hard numbers” to “nice images” to allow for valid statistical analysis.

This brief overview aims to present the current methods of quantifying the structural alterations in animal models of emphysema. The reasons for choosing this topic are twofold: (1) Within the field of lung morphometry, applications to experimental studies in emphysema research are by far the most common ones. (2) Although general standards for lung morphometry based on unbiased stereological principles exist (Hsia et al., 2010), current practice regarding their application to emphysema models often lags behind. By discussing the strengths and weaknesses of the various protocols and parameters that can be used to characterize lung architecture in emphysema, we hope to contribute to better scientific practice in this field.

2. Emphysema and how we model it

Chronic obstructive lung disease (COPD) is one of the most common lung diseases and among the leading causes of disease and death, thus representing a major global health burden (Murray and

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Lopez, 2013). The leading clinical symptom of COPD is chronic airflow limitation, which presents as sputum production, cough and dyspnea, but this is difficult to mirror in animal models. However, two distinct pathological entities can be distinguished in COPD: one affects the conducting airways (chronic bronchitis, functionally characterized by increased airway resistance) whereas the other affects the gas exchange parenchyma (emphysema, functionally characterized by decreased elastic recoil) (Hogg and Timens, 2009; Decramer et al., 2012; Tuder and Petrache, 2012; Holloway and Donnelly, 2013). According to the American Thoracic Society, emphysema can be defined as "abnormal, permanent enlargement of the airspaces distal to the terminal bronchiole, accompanied by destruction of their walls" (American Thoracic Society, 1995). This widely accepted definition can in principle be fulfilled in animal models. Moreover, it can be measured by microscopy-based stereology. Actually, quantitative assessment of micro-structure is the only way to reliably demonstrate the presence of emphysematous alterations – and their prevention or correction by therapeutic interventions.

Based on experimental and clinical studies, many molecular mediators and events have been attributed to the pathogenesis of emphysema, such as inflammation induced by noxious agents (e.g. smoke), protase–antiprotease or oxidant–antioxidant imbalances, alterations of interstitial tissue homeostasis (e.g. by TGF-β), autoimmune processes (e.g. auto-antibodies against endothelial cells), apoptotic cell death (e.g. by VEGF blockade), or accelerated aging (e.g. by telomere shortening) (for review, see Shapiro and Ingenito, 2005; Barnes, 2008; Taraseviciene-Stewart and Voelkel, 2008; Cosio et al., 2009; MacNee and Tuder, 2009; Sethi et al., 2009; Tuder and Petrache, 2012; Holloway and Donnelly, 2013). Despite the progress made in the last years, we are still far from a clear and comprehensive understanding from which successful therapeutic concepts could be deducted, and more research in this field is certainly necessary. This research requires the modeling of emphysema in experimental animals. A large variety of such animal models have been developed in various species ranging from non-human primates via dogs, pigs, sheep, rabbits, guinea pigs and rats to mice (see Martin and Tamaoka, 2006; Brusselle et al., 2006; Plopper and Hyde, 2008; Chapman, 2008; Abraham, 2008). However, although the mouse – particularly due to the technical advances in manipulating its genome – has become very popular, specific differences in lung structure, function and immunology between humans and mice have to be taken into consideration (Parent, 1992; Irvin and Bates, 2003; Mestas and Hughes, 2004; Wright et al., 2008; Gharib et al., 2010). Even within mice, different strains exhibit different sensitivities to the development of emphysema (Guerassimov et al., 2004). To induce emphysema in mice, several models exist. These include not only exposure models like pancreatic elastase instillation or long-term smoke inhalation, but also genetic models (Dawkins and Stockley, 2001; Shapiro, 2007; Wright et al., 2008; Baron et al., 2012). But, how well are these genetic models really characterized? In general, the benefits of stereology for quantitative morphological phenotyping of genetically altered organisms have not yet been fully exploited (Lucocq, 2007). In particular, the important distinction between true destruction in a normally developed lung (which fulfills the criteria for emphysema) vs. impaired lung development (reduced or delayed alveolarization, which does not fulfill these criteria) is not addressed in most studies. This would actually require something which is usually not done: a comprehensive phenotype analysis at different developmental stages (Fehrenbach, 2002/2003, 2006). Are, therefore, conditional transgenic mice, in which specific genes are only activated or inactivated in the lung after normal postnatal development has been completed, the solution? In principle they are, however, it turned out that the reverse tetracyclin-transactivator (rtTA) system, when expressed specifically in the lung either by nonciliated (Clara) bronchiolar epithelial cells (via CCSP-rtTA) or by alveolar epithelial type II cells (via SP-C-rtTA), may, per se, lead to emphysema-like alterations (Sisson et al., 2006; Morimoto and Kopan, 2009). A similar problem was noted in an SP-C-Cre mouse line (Jeannotte et al., 2011). These findings of off-target effects led to a "toxic alert" in the field, emphasizing that extreme caution is necessary when interpreting data obtained in these existing models and that new and better models need to be developed (Whitsett and Perl, 2006; Perl et al., 2009; Rawlins and Perl, 2012).

3. Microscopy-based quantitative assessment of emphysema: prerequisites

It is beyond the scope of this article to provide a comprehensive introduction to the principles of stereology. This information is available in the literature (Howard and Reed, 2005; Baddeley and Vedel Jensen, 2005). General information about applications of stereology in lung research has also been provided (Ochs, 2006; Weibel et al., 2007; Knudsen and Ochs, 2011; Mühlfeld et al., 2013; Schneider and Ochs, 2013; Ochs and Mühlfeld, 2013; Mühlfeld and Ochs, 2013). Global standards for quantitative assessment of lung structure have been published as an official research policy statement of the American Thoracic Society and the European Respiratory Society (Hsia et al., 2010). But how do these standards apply to the characterization of emphysematous alterations (and their prevention and treatment) in animal models of human disease? Some practical points of particular relevance for emphysema assessment are therefore listed in Table 1 and discussed below.

A proper *study design* is of outmost importance. After a thorough qualitative analysis, the appropriate parameters that best characterize the alterations in quantitative terms (based on the first-order stereological parameters volume, surface area, length, and number) are chosen. In a small pilot study, the stereological sampling and analysis design is determined (how many animals per group, how many samples per animal, how many sections per sample, how many fields of view per section at what magnification, what test system). The overall guiding principle is the use of unbiased sampling and measurement principles which are essential to obtain accurate (valid) data. This is critical because bias (systematic error) cannot be detected in the final data, nor can it be decreased by increasing the number of measurements. In contrast, the precision (reproducibility) of the data can easily be controlled because it can be calculated from the data and, if necessary, increased by including more samples and measurements. This is of particular importance as heterogenous alterations in emphysema may require a higher sampling effort than usual (i.e. more counts on more samples per animal) to achieve a sufficient precision, especially in fully back-crossed inbred mouse strains where biological variation between individuals is low (see Ochs, 2006 and the discussion of the "do more, less well" principle below).

Proper *specimen preparation* is also essential, because the quality of the material determines the quality of the measurements. Lung fixation and further processing of sampled tissue blocks (postfixation, dehydration, embedding) should yield consistent and reproducible results by preserving the cellular architecture as well as the in vivo tissue dimensions as closely as possible. Although a "gold standard of physiological lung fixation" does not exist (a fixed lung is by definition no longer "physiological"), conditional "silver standards" have been defined (Hsia et al., 2010; Mühlfeld et al., 2013). These protocols have been tested in a variety of species and fulfill the criteria of consistent and homogeneous fixation of the whole lung with minimal tissue deformation which are essential for unbiased sampling for stereological analysis (compare Weibel et al., 1982). A particular problem in emphysema research is the