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## Innovations in fluorescent microangiography: seeing is believing!

Many common pulmonary diseases are characterized by remodeling of the pulmonary microvasculature via capillary loss, arteriolar thickening, or smooth muscle hyperplasia. While the fact that these changes occur is well recognized, progress in mechanistic understanding has been impeded by a lack of adequate microvascular imaging techniques.

In this issue of *Lab Invest*, **Dutly *et al***[1] (p. 409) present a novel approach for three-dimensional analysis of the pulmonary microvasculature. This approach resolves previous limitations of approaches relying on tissue sections, which do not allow tracing of individual blood vessels; barium sulfate-gelatin infusions, which require perfusion pressures far greater than those present *in vivo*; and corrosion casting which cannot identify active vascular remodeling. Dutly *et al* perfused the pulmonary and systemic (bronchial) circulations of rats with differently colored fluorescent polystyrene microspheres suspended in liquid agarose. To achieve physiologically relevant conditions, the viscosity of the perfusate was similar to the viscosity of blood in the microvasculature, physiologically relevant perfusion pressures were used, and the animal was ventilated throughout the process. After the vessels were filled, the agarose was solidified by intratracheal injection of chilled paraformaldehyde and 200 $\mu$m sections were prepared. These thick sections were suitable for nuclear labeling and some immunostains.

The sections were analyzed by confocal miscroscopy and three-dimensional reconstructions were created. These allowed the authors to obtain images detailing the branching of pulmonary and bronchial vessels throughout the lung. A representative image appears on the cover of this issue of *Lab Invest* and three-dimensional rotating movies are available for download at nature.com/labinvest/3700399. To assess vessel number, density, and length the authors created a skeletonized frame representation of the vessels and analyzed it quantitatively. As a demonstration of the utility of this approach, lungs of normal rats were compared to those of rats with experimentally induced pulmonary arterial hypertension. Diseased lungs showed a profound loss of vasculature and arteriolar wall thickening. It should therefore be obvious that this new approach has great potential in studies of vascular remodeling in lung disease as well as in studies of other organs.

Despite these advances, this new technique is not without imperfections. The morphometric skeletonization method described in this study may not be ideal for quantification, as only one optical section was utilized in the evaluation. Moreover, the skeletonized image may not faithfully represent the branching pattern. Further optimization of the technique may also be needed to increase the applicability of this system to other models and organ systems. Nonetheless, this fluorescent microangiographic approach does generate remarkable images that provide a new view of the pulmonary vasculature. While the quantitative analytical methods are not yet perfect, such limitations are inherent in any work at the leading edge of technical advances. Thus, we expect a bright future for this novel fluorescent technique.

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## MCP-1: the missing link between NO deficiency and EPO-induced cardiovascular dysfunction

Erythropoietin (EPO) is becoming widely used in the clinics to treat anemia, which occurs in almost all dialysis-dependent end stage renal disease patients. The beneficial effects of EPO may be counterbalanced by its contribution to atherosclerosis, which is a major morbidity and mortality factor in chronic renal disease (CRD) patients. It has been suggested that the atherogenic effects of EPO are mediated through nitric oxide (NO), but the mechanisms by which it occurs are largely unknown. EPO reduces NO availability, the consequences of which may be compounded in EPO-treated CRD patients by a relative NO deficiency created by CRD itself. NO inhibits vascular smooth muscle cell proliferation and migration, which may be how its deficiency promotes cardiovascular dysfunction. The study presented by **Desai *et al***[1] (p. 369) confirms that one of the mechanisms by which EPO contributes to cardiovascular pathology is an increased proliferation of endothelial cells and vascular smooth muscle cells. This study also directly links EPO to NO production by showing that EPO reduces eNOS message and protein expression. A more novel result reported in this study is that EPO induces endothelial cells to produce monocyte chemoattracttant protein-1

(MCP-1), a chemokine that orchestrates the recruitment, adhesion, and transendothelial migration of monocytes during atherogenesis. The MCP-1 induction was reversed by exposure to NO donor, firmly establishing NO deficiency as the trigger for this chemokine induction. This study therefore adds a significant piece to the puzzle by demonstrating that EPO perturbs the equilibrium between the pro- and antiatherogenic activities of MCP-1 and NO, respectively. The discovery of a link between EPO treatment and MCP-1 induction brings EPO closer to a well-characterized pathway in cardiovascular pathology, which has important translational potentials to improve outcomes for CRD patients.

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## The role of $\beta$2 integrin subsets in intestinal disease: a sticky problem

Inflammatory bowel disease is a complex disease involving many genetic and environmental factors, resulting in mucosal immune activation and leukocyte infiltration. Immune downregulation reduces disease activity in humans and animal models. While available approaches to immune downregulation are effective, their lack of specificity leads to infectious complications in a significant number of patients. Thus, increased understanding of the mechanisms of abnormal immune regulation in inflammatory bowel disease may lead to improved immunotherapy with fewer complications.

In this issue of *Lab Invest*, **Abdelbaqi *et al***[1] (p. 380) study the role of $\beta$2 integrins in leukocyte recruitment. The $\beta$2, or CD18, integrin family consists of four different adhesion proteins: LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), p150/95 (CD11c/CD18), and CD11d/CD18, which serve crucial roles in leukocyte recruitment, adhesion, migration, and signaling. $\beta$2 Integrin expression is ubiquitous among leukocytes, as is CD11a expression. In contrast, CD11b expression is limited to granulocytes, monocytes, NK cells, and subsets of B and T lymphocytes. To compare the specific roles of LFA-1 and Mac-1 in immune regulation, Abdelbaqi *et al* took advantage of knockout mice lacking CD11a, CD11b, or CD18. These were studied using the well-characterized colitis induced by dextran sodium sulfate (DSS).

As expected, both CD11a and CD18 knockout mice had markedly reduced neutrophil recruitment and clinical measures of the colitis that developed were less severe than in wild-type mice. This is consistent with a large body of work suggesting that leukocyte recruitment and $\beta$2 integrin signaling are important components of the immune response that leads to colitis. These data therefore support the idea that CD11a- or CD18-blocking antibodies may be of therapeutic use in inflammatory bowel disease.

Remarkably, despite a mild reduction in neutrophil recruitment, CD11b knockout mice had more severe clinical disease than wild-type mice. This is a particularly surprising result, since CD11b/CD18 has been the primary $\beta$2 integrin implicated in neutrophil adhesion and migration to and across endothelium and epithelium. However, given the variety of leukocytes that express CD11b, other cell populations that express CD11b must be considered. For example, the authors suggest that this may reflect loss of function in specific subsets of regulatory lymphocytes. This hypothesis would be consistent with the increased numbers of plasma cells recruited to the lamina propria of DSS-treated CD11b knockout mice as well as a recent report implicating a critical role for $\beta$2 integrins in T-regulatory cell development and function.[2]

While these studies do not explain the role of CD11b in moderating DSS-induced colitis severity, they do serve to remind us that the *in vivo* situation is more complicated than *in vitro* studies might suggest. Knocking out CD11b, the molecule neutrophils use to stick to other cell types, may create unanticipated problems.

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## We are what we smell—not only rotten eggs

The repulsive hydrogen sulfide odor released from cracked rotten eggs is so powerful that the adjacent living space is often vacated. The same odor, however, does not drive people away from hot springs. Simply put, although our noses do not like the smell of hydrogen sulfide, our bodies certainly benefit from soaking in sulfurous thermal water, as it may prevent or reduce skin problems as well as bronchial and pulmonary diseases (characterized by increased inflammatory infiltration by polymorphonuclear neutrophils).

Searching for cellular and molecular mechanisms for the involvement of hydrogen sulfide in inflammatory processes has been a research challenge for many years. Claesson *et al* in 1989 examined the effects of $H_2S$ exposure for 1 h at 1 mM, a concentration sometimes seen within a deeper periodontal pocket, on isolated human polymorphonuclear

leukocytes. They did not find significant changes in phagocytosis and bactericidal capability of these cells with this short treatment.[1] A later study showed that the pretreatment of isolated human granulocytes *in vitro* for 30 min with hydrogen sulfide at 1 mM enhanced cell apoptosis. Valitutti *et al*[2] also reported that mitogen-induced T lymphocyte proliferation and IL-2 production was inhibited in the presence of different percentages of sulfurous thermal water in culture media.[3] These authors suggested $H_2S$ as being the main immunosuppressive element in sulfurous thermal water that acts on cultured peripheral blood mononuclear cells.[3] In these earlier studies, $H_2S$ was merely treated as a toxic metabolite from bacterial or environmental sources.

The article by **Rinaldi *et al***[4] (p. 391) in this issue of Laboratory Investigation reports the effect of $H_2S$ on apoptotic status of human neutrophils. Using NaHS as a $H_2S$ donor, the authors found that NaHS promoted the survival of cultured granulocytes in a dose-dependent (EC50, 0.5 mM) and time-dependent (6–24 h) manner under stress conditions. On the other hand, $H_2S$ enhanced lymphocyte death but had no effects on eosinophils. The delayed onset of apoptosis of granulocytes in the presence of $H_2S$ was mediated by the inhibition of caspase three cleavage and p38 MAPK phosphorylation. Thus, complex regulatory mechanisms for differential apoptosis among different cell types are revealed as previous studies have shown increased apoptosis of smooth muscle cells and inhibited HEK-293 cell proliferation by endogenous and exogenous $H_2S$. Furthermore, the study by Rinaldi *et al*[4] speaks of broader clinical implications, especially respiratory problems such as chronic obstructive pulmonary disease, by providing molecular mechanisms for the cellular effect of $H_2S$. Together with its demonstrated effect on reducing mucous hypersecretion associated with bronchitis, the effect of $H_2S$ on cellular apoptosis may initially contribute to acute inflammatory and bactericidal defense and subsequently prevent the development and accelerate the resolution of chronic inflammatory processes. In this scheme, $H_2S$-releasing compounds or the manipulation of endogenous $H_2S$ production may offer novel avenues for prevention and/or treatment of chronic inflammation and respiratory diseases.

Our bodies in fact produce the 'rotten-egg gas'[5] and we are exactly what we smell. $H_2S$ levels in the circulation have been reported to be 10–50 $\mu$M in rats and 10–100 $\mu$M in humans. Usually, the tissue level of endogenously produced $H_2S$ is higher than that in circulation. The endogenous concentration of $H_2S$ in rat, human, and bovine brain tissues is in the range of 50–160 $\mu$M. In this context, one missing piece in the aforementioned studies[1–4] is the involvement of endogenous $H_2S$ in the modulation of polymorphonuclear leukocyte survival. Filling this knowledge gap will not only guide the usage of $H_2S$ as a therapeutic agent, it may also decode cellular mechanisms for pathogenic development and prognosis of inflammation. Several approaches should be taken to tackle the interaction of endogenous $H_2S$ with polymorphonuclear leukocytes. Exogenously applied $H_2S$ should be kept within a biologically relevant range, after taking existing endogenous $H_2S$ into account. Endogenous production of $H_2S$ should be increased or decreased by manipulating the expression and activity of $H_2S$-generating enzymes.[5] Apoptotic status and proliferation of polymorphonuclear leukocytes under these conditions would bear more biological or physiological meanings.

It is also imperative that future studies on the effect of $H_2S$ on inflammatory infiltration at cellular level should proceed beyond *in vitro* experimentation and extend to the levels in tissues as well as in whole animals. Ultimately, the regulation of endogenous production of $H_2S$ in human inflammatory pathologies and the means to interfere with this process should be explored.

Evidence has been accumulating at an accelerated speed in recent years, documenting the signaling importance of endogenous $H_2S$ as the third gasotransmitter, together with nitric oxide and carbon monoxide.[5] Integrated trans-disciplinary researches, including those from environmental biology, human physiology and pharmacology, will help improve our basic understanding of $H_2S$ biology and facilitate its wide applications. In the near future, we may be able to answer some fundamental questions such as why we are not smelly (given that we are what we smell), whether it is good or not so good to be smelly, and how to control the releasing of smelly hydrogen sulfide from our cells and our bodies.

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