Imaging inflammatory plasma leakage in vivo

Ellinor Kenne; Lennart Lindbom
Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden

Summary
Increased vascular permeability and consequential plasma leakage from postcapillary venules is a cardinal sign of inflammation. Although the movement of plasma constituents from the vasculature to the affected tissue aids in clearing the inflammatory stimulus, excessive plasma extravasation can lead to hospitalisation or death in cases such as influenza-induced pneumonia, burns, or brain injury. The use of intravital imaging has significantly contributed to the understanding of the mechanisms controlling the vascular permeability alterations that occur during inflammation. Today, intravital imaging can be performed using optical and non-optical techniques. Optical techniques, which are generally used in experimental settings, include traditional intravital fluorescence microscopy and near-infrared fluorescence imaging. Magnetic resonance (MRI) and radioisotopic imaging are used mainly in the clinical setting, but are increasingly used in experimental work, and can detect plasma leakage without optics. Although these methods are all able to visualise inflammatory plasma leakage in vivo, the spatial and temporal resolution differs between the techniques. In addition, they vary with regards to invasiveness and availability. This overview discusses the use of imaging techniques in the visualisation of inflammatory plasma leakage.

Keywords
Inflammation, imaging, endothelial cells

Introduction
The overt clinical signs of inflammation occur as a result of adaptive changes in the microcirculation, namely redness and heat due to arteriolar dilatation and increased local blood flow, combined with swelling of the tissue due to increased vascular permeability and consequent plasma leakage. The decrease in endothelial barrier function that occurs during acute inflammation is designed to maximise the movement of plasma constituents and white blood cells out of the circulation to the site of injury or infection. A controlled increase in vascular permeability aids in clearing the inflammatory stimulus as the exudate contains plasma proteins, e.g. complement factors and immunoglobulins, which are necessary for effective elimination of the harmful agent. Also, components contributing to resolution of the inflammatory response and healing of the tissue come with the exudate. However, inflammatory oedema of both infectious and non-infectious origin may seriously affect organ function and in cases such as influenza-induced pneumonia or sepsis-induced lung injury, excessive plasma extravasation can lead to hospitalisation or death. Other examples where inflammatory oedema contributes to critical illness are in the sequelae of traumatic brain injury or tissue infarction.

The barrier between the blood and extravascular tissue consists of the endothelium with the highly organised glycocalyx network covering the luminal surface, and the basement membrane on the basolateral side. The glycocalyx is composed of negatively charged glycosaminoglycans and contributes to barrier function through electrostatic exclusion of plasma proteins at the endothelial cell surface (1). Although modification of glycocalyx composition and basement membrane protein assembly may influence the barrier function of the vessel wall, the primary mechanism behind increased vascular permeability and oedema formation in inflammation is endothelial cell contraction leading to paracellular gaps through which plasma macromolecules can pass (2). Many inflammatory mediators of different origin such as thrombin, bradykinin, histamine, and cysteinyl leukotrienes, act on endothelial cell surface receptors that signal to the cytoskeletal filaments causing them to contract (2). Other factors generated in inflammatory processes like oxygen free radicals and neutrophil granule products can similarly make the endothelial lining more permissive to passage of macromolecules (3).

Fluid exchange across the endothelium is determined by the Starling principle, which states that the net filtration rate depends on the hydrostatic and colloid osmotic pressures in the intra- and extravascular compartments as well as the permeability of the vessel wall. Inflammatory oedema arises as a consequence of plasma protein extravasation and an increase in colloid osmotic pressure in the extravascular tissue which will draw fluid from the interstitial fluid.
Imaging vascular permeability

Increased microvascular permeability leads to an enhancement of macromolecular transfer across the endothelium, and imaging techniques take advantage of this by visualising the efflux of macromolecules thus providing information about endothelial barrier function. Imaging of permeability changes in inflammation is based on intravenous injection of a tracer compound that is large enough not to leak out from the vasculature under normal conditions but will do so in inflamed tissue. Such compound is typically in the molecular size range of 70 to 150 kDa and owing to the central role of albumin in controlling transcapillary fluid flux, dye-conjugated forms of the plasma protein are frequently utilised for this purpose. The principle of imaging inflammatory plasma leakage dates back to the classical Miles assay where extravasation of Evans blue dye-conjugated albumin in Guinea-pig skin was visually assessed from the extravascular compartment (4). This usually gives a protein-rich exudate compared with the transudate formed in response to e.g. a rise in intravascular hydrostatic pressure which is normally low in protein content. The plasma protein extravasation in inflammation occurs predominantly via a paracellular route, which is regulated by the interplay of adhesive forces between adjacent endothelial cells and the counteradhesive forces generated by endothelial actomyosin contraction (2). The endothelial cells are held together by two different types of junctional protein complexes; adherens junctions (AJs) and tight junctions (TJs), whose occurrence differs depending on the vascular bed. AJs are thought to maintain structural integrity of the junctions whereas TJs are secondary and appear mainly in larger blood vessels and as part of the blood-brain barrier (BBB) where permeability is more tightly regulated (5). The BBB constitutes a special form of endothelium that serves to protect the brain tissue and is characterised by comparatively low permeability for most solutes. Loss of BBB integrity may result in a vasogenic cerebral oedema associated with plasma extravasation, whereas cytotoxic oedema is unrelated to BBB function and caused primarily by intracellular swelling due to cellular injury (6).

Optical imaging

In experimental settings, the most commonly used method to image permeability changes is using optical imaging, represented by intravital fluorescence microscopy in the visible light region and more recently by NIRF imaging.

Intravital fluorescence microscopy

Fluorescence microscopy is based on the excitation of a fluorophore at one wavelength which results in light emission of a different wavelength. Light filtering then enables direct microscopic viewing or observation via an image intensifier can also be used. Intravital microscopy of exteriorised tissue as a tool for assessment of permeability changes in inflammation has been used since the 1970s (9). With the use of a fluorescent plasma marker it was then possible to visualise plasma extravasation in real time. This technique allows for detailed investigation of alteration of vascular permeability at single vessel level (Fig. 1A). The investigated tissue in an anaesthetised animal is exteriorised to allow microscopic observation, and vascular permeability changes can be examined by intravenous injection of a fluorophore-conjugated plasma marker such as fluorescein isothiocyanate (FITC)-labelled dextran or albumin (10). This technique has contributed much to our knowledge about the activity profile of important inflammatory mediators such as bradykinin, histamine and the leukotrienes (9, 11–13). Most important, the temporal and spatial characteristics of plasma exudation in the microcirculation in response to inflammatory mediators have become apparent with this technique, for example the strict localisation of leaky sites to the postcapillary venular region (10).

With the use of intravital microscopy of exteriorised tissue, it is important to maintain physiological conditions with respect to temperature, extracellular fluid composition, pH, etc. and to minimise inflammation resulting from the preparative surgery (14). A major drawback to intravital microscopy is that it is time-consuming because of delicate surgery and that the technique allows study of only one object at a time. In order to obtain clear optical images fluorescence microscopy is limited to thin tissues, preferably only one vessel layer thick. The hamster cheek pouch preparation has served as rule model in this respect (14). Other tissues used include the rat mesentery, and the rat and mouse cremaster muscle (13, 15, 16). Superficial vessels of whole organs, such as the pial vasculature of the brain, have also been used (17). However, observation of thicker tissues not only results in blurred images but also involves problems with quenching the fluorescence. These problems can be overcome in part using in vivo confocal microscopy, however at the expense of time resolution. Up until now only few studies exploring vascular permeability changes in inflammation have been undertaken with this refined microscopic imaging technique (18, 19). Further improvement is achieved using multi-photon fluorescence microscopy (20, 21). This technique allows deeper penetration into the tissue with less phototoxicity, but the method is...
not yet well established for intravital imaging of permeability changes.

**Near-infrared fluorescence imaging**

Near-infrared fluorescence (NIRF) imaging allows for visualisation with penetration up to several centimeters in the tissue making this method suitable for imaging in small animals and potentially in humans (22). Light in the near-infrared range of 700–900 nm is not absorbed or scattered by endogenous molecules, such as water, oxyhaemoglobin or melanin. The distribution of a fluorescent probe injected into the animal can thus be visualised upon whole body illumination and excitation with a light source (usually laser) in combination with a highly sensitive detection unit. Although NIRF is a method that is commonly used for optical imaging of tumours, it can also be used in the detection of inflammatory vascular leakage (Fig. 1B). Several variations of NIRF imaging devices are commercially available and a number of dyes of different wavelengths have been developed (23). The cyanine dye Cy5.5, which has an excitation/emission maxima close to the near-infrared region, is frequently used for in vivo imaging. However, the excitation/emission spectra of IRDye 800CW is more appropriate for in vivo use and has been found to have a greater signal-to-background ratio (24, 27).

As NIRF allows for whole body visualisation it can be used to locate sites of permeability increases in various inflammatory conditions. NIRF has been used in the in vivo imaging of arthritis where intravenous injection of a fluorochrome led to an increased

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**Figure 1: Representative pictures of intravital imaging techniques.**

A) Intravital microscopy of the hamster cheek pouch before (pre) and after (post) chemoattractant stimulation to induce neutrophil-dependent plasma leakage in post-capillary venules. Hamsters were given FITC-dextran (Mw 150,000) intravenously as a plasma marker. B) NIRF images of mouse brain 4–8 h (A) and 8–12 h (B) following middle cerebral artery occlusion (MCAO). NIRF-BSA was given intravenously as a macromolecular tracer. MCAO resulted in enhanced permeability in the affected hemisphere 4–8 h, but not 8–12 h after reperfusion. Reproduced with permission from Klohs et al., J Neurosci Methods 2009, Elsevier (27).

C) Transmission (top set) and pulmonary transcapillary escape rate (PTCER) PET images of a normal subject and a patient with acute respiratory distress syndrome (ARDS). The increase in permeability in the ARDS patient is apparent in the PTCER image (53). Reproduced with permission from Harris et al., J Appl Physiol 2007, Am Physiol Soc (35).

D) MR images of rat lung following repeated administration of ovalbumin to induce lung inflammation. Images were collected before and after administration of a contrast agent (Pre-Gd and Post-Gd, respectively), and the difference between the images indicates increased vascular permeability. Reproduced with permission from Tigani et al., Am J Physiol 2007, Am Physiol Soc (46).
signal from the arthritic joint (25). This was, in part, attributed to the binding of the fluorochrome to albumin. To decrease background signal from the small fluorochromes, they can be conjugated to albumin, allowing detection of plasma extravasation (26, 27). Leakage of NIRF fluorochrome-conjugated albumin has been found to correlate with the extravasation of Evan’s blue, which traditionally has been used to assess permeability alterations (27). A recent study has shown that near-infrared fluorochromes can be encapsulated into nanoprobes of 100–200 nm in diameter (28). Nanoprobes are approved for clinical use and mice do not present with any side effects after intravenous injection of the nanoprobes. The nanoprobes accumulate in arthritic joints 24 hours after intravenous injection as an indication of increased vascular permeability and treatment against arthritis decrease the fluorescence signal to that of non-arthritic joints (28).

Non-optical imaging

Permeability increases resulting from inflammation can also be visualised using non-optical techniques. These include magnetic resonance (MRI) and radioisotopic imaging and are used both in experimental research and in the clinical setting; however, primarily in the latter so far.

Radioisotopic imaging

With the use of nuclear medicine and radiopharmaceuticals, it is possible to visualise permeability alterations in vivo using a gamma camera. Vascular permeability is measured as the movement of radiolabelled proteins from the blood to the extravascular tissue (29). Images are acquired by the detection of emitted photons outside the subject (30). Positron emission tomography (PET) imaging utilises positron-emitting isotope labeled tracers (Fig. 1C). The annihilation reaction between a positron, which is emitted by the isotope, and a nearby electron in the tissue produces two high energy photons. The photons are detected to create an image of the tissue (31). The radionuclide used in single photon emission computed tomography (SPECT) emits a single gamma ray upon radioactive decay. An external camera captures the gamma rays in multiple angles and, based on mathematical formulation, these images are reconstructed to create a three-dimensional image (32).

Barrier function of the pulmonary endothelium is normally quantified as the pulmonary transcapillary escape rate (PTCER) (29). Increases in PTCER are seen with several lung disorders (such as acute pneumonia, active interstitial lung disease, and acute respiratory distress syndrome), and are therefore an index of functional lung injury. PTCER has been used in several studies (33, 34) to investigate lung injury following ischaemia. The protein tracers that have been used in PET imaging of PTCER are 68Ga-transferrin and 11C-methylalbumin (35). Markers used for PET/SPECT imaging of blood brain barrier function have comparatively low molecular weights, are non-lipid and unable to cross the intact BBB under normal conditions (36). Increased tracer uptake to the parenchyma after acute stroke as determined by SPECT was shown to correlate with the clinical outcome (37, 38). In addition, micro-SPECT was used to determine alterations in BBB function in an experimental model in rats (39). Although the positron-emitting compounds generally have short half-lives, which reduces the radiation exposure to the subjects (35), a major drawback with this method is the exposure to radioactivity. Furthermore, as PET lacks accurate spatial and temporal resolution and anatomical information, it is usually combined with computed tomography (CT) or MRI (31).

Magnetic resonance imaging

MRI uses a powerful magnetic field to excite hydrogen atoms and measures the time it takes for these atoms to return to their relaxed state. The difference in relaxation time between tissues gives rise to the contrast in the MRI image. Water, for example, which is abundant in interstitial oedema, has a high molecular mobility which yields a long relaxation time. Contrast agents can be used to either increase or decrease the signal intensity of the image in the tissue or organ in which the agent has accumulated (40). The marker gadolinium diethylene triamine pentaacetic acid (Gd-DTPA) is often used as a contrast agent in the assessment of vascular permeability. It has a molecular mass of 550 Da (41).

Visualisation of permeability changes in the brain (41–43) and lung (44–47) using MRI is performed in both experimental and clinical settings (►Fig. 1D). Water content in affected tissues can be measured using non-contrast based imaging and combined with the use of Gd-DTPA it is possible to measure plasma leakage. As Gd-DTPA is a relatively small molecule, experiments using gadolinium-conjugated bovine serum albumin (Gd-BSA) have been performed (48). This reveals more information with respect to macromolecular permeability. Gd-DTPA was found to be more widely distributed than Gd-BSA in the brain following cerebral ischaemia. Additionally, Gd-BSA extravasation was associated with higher tissue water content as measured by non-contrast based imaging (41). MRI has been used to determine leakage in the lung before and after the injection of a contrast agent. The difference in signal was used to determine the amount of plasma leakage (46).

Methodological considerations

In studying the inflammatory process, a major advantage of intravital imaging techniques is to be able to directly visualise plasma extravasation in a living subject. This allows for the collection of valuable data not possible to retrieve by other techniques. However, as with all other methods, there are limitations to intravital imaging that need to be considered when utilising these techniques (►Table 1).

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Resolution versus invasiveness

As the resolution of an image increases it will provide more information about the mechanistic basis of inflammatory permeability alterations will increase. However, enhanced resolution is often obtained using very invasive techniques. Traditional intravital microscopy usually involves invasive surgery as it requires exteriorisation of the tissue to be observed and the animal is commonly sacrificed following the experiment. In addition, the surgical procedures and imaging collection can be time-consuming resulting in a low throughput compared to less invasive methods. On the other hand, it is generally less expensive and available without need for advanced equipment. Furthermore, intravital microscopy allows for the visualisation of physiological processes with high spatial and temporal resolution and permits visualisation at the single vessel level which for example makes it possible to determine where in the vascular tree the leakage occurs. With a resolution in the range of 1 μm intravital microscopy is superior compared to other imaging methods.

Although it may be possible to obtain similar information using more advanced equipment, e.g. MRI or PET, such detailed data have not been reported in inflammatory vascular imaging. For MRI and PET, the resolution varies greatly between clinical and animal scanners as well as for the size of the animal. Scanners that are dedicated to small animal research are superior with regard to resolution (31). The low resolution, relative to intravital microscopy, of NIRF, MRI and PET is balanced by the lack of invasiveness in these techniques. These methods allow for whole body scans and measurements can be performed repeatedly over days, weeks or months. Radiation-mediated methods, however, might limit longitudinal studies, especially in patients. Repeated image collection allows for the visualisation of both the oedema formation and its resolution, yielding additional important information. These methods are also available to use in the clinical setting although NIRF imaging techniques currently can only penetrate a couple of centimeters in human tissue. Another advantage of using whole organ visualisation, as with NIRF, MRI and PET, is that it provides information about the collective response of a whole tissue. In intravital fluorescence microscopy the trade-off to the high spatial resolution is that the whole tissue response is difficult to detect.

Quantification of plasma leakage

A major drawback of all methods of intravital imaging is the difficulty to quantify the plasma leakage from the images obtained. This is in contrast to those methods that directly assess plasma exudation through collecting the exudate or extraction of a plasma marker from the tissue. However, it has been shown that detected fluorescence intensity in intravital microscopic images correlate well with the actual concentration of extravasated plasma marker (49). Images can be analysed for intensity of a colour or light, size of a stained area or a ratio between staining outside and inside the vessel. There is no consensus to the methods used for quantification among the different types of intravital imaging, and the risks for bias are therefore larger compared to quantitative methods. Analyses of the images should therefore preferably be blinded and strict parameters as to how the images should be evaluated need to be employed. If possible, in the experimental setting intravital imaging techniques should be complemented with other methods that can measure permeability more directly.

Choice of macromolecular marker

Optical imaging is in some cases limited by high background signals resulting from autofluorescence (excitation of endogenous

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Table 1: Comparison of intravital imaging techniques.

<table>
<thead>
<tr>
<th>Imaging technique</th>
<th>Practical resolution</th>
<th>Penetration depth</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intravital microscopy</td>
<td>μm</td>
<td>μm – mm</td>
<td>High temporal and spatial resolution</td>
<td>Invasive and time consuming</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Allows for repeated measurements</td>
<td>Limited penetration depth</td>
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<td></td>
<td></td>
<td></td>
<td>High signal to background ratio</td>
<td></td>
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<tr>
<td>Near-infrared fluorescence</td>
<td>mm</td>
<td>cm</td>
<td>Non-invasive</td>
<td>Low resolution</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Simple and rapid acquisition of images</td>
<td>Limited penetration depth</td>
</tr>
<tr>
<td>Radioisotopic imaging</td>
<td>mm – cm</td>
<td>No limit</td>
<td>Non-invasive</td>
<td>Exposure to radiation</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Established method for clinical use</td>
<td>Expensive and complex</td>
</tr>
<tr>
<td>Magnetic resonance imaging</td>
<td>mm</td>
<td>No limit</td>
<td>Allows for repeated measurements</td>
<td>Low resolution</td>
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<td></td>
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<td>Provides anatomical information</td>
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<tr>
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<td>Established method for clinical use</td>
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compounds), non-specific binding and scattered excitation light leakage through optical filters, which results in the erroneous collection of fluorescent light. Therefore, the choice of fluorophore should be made carefully as this may affect the signal to background ratio. The size of the macromolecular marker is also an important factor to consider as a marker with a low molecular weight, which easily diffuses over the endothelial barrier might give a high background. On the other hand, a marker that is too large might not be able to diffuse even though the endothelial barrier is disrupted, which can be the case especially in the BBB. A small marker will also be cleared more rapidly through the kidney, limiting long term measurements. Therefore, the marker used should depend on the time-course of the experiment, the tightness of the endothelial barrier in the investigated tissue and the expected response in vascular permeability caused by the inflammatory stimulus.

One problem with intravenous administration of a fluorescent dye or radioisotopic marker and the quantification of extravasated dye in inflammatory conditions is that the amount of dye within the vessels is much higher than that outside which might contaminate the signal from the extravascular tissue. This can be avoided by injecting two dyes with different emission wavelengths simultaneously, one with a lower molecular mass which can cross the vascular wall and one with a higher molecular mass that cannot. By subtracting the fluorescence of the dye inside the vessel from that which can pass through the endothelial barrier, it is possible to image the extravasated dye correctly (50, 51).

Additional considerations

It needs to be pointed out that visualisation of macromolecular efflux from the vasculature may not accurately reflect a corresponding change in vascular permeability. Plasma leakage is much more dependent also on the perfusion level of the tissue and both vasodilatation and vasoconstriction may occur in the inflammatory process. The amplifying effect of vasodilator mediators on inflammatory oedema formation caused by permeability-increasing agents is well established (52). The contribution to plasma protein leak because of a change in blood perfusion or intravascular pressure is not accounted for by most imaging techniques. Additionally, in experimental research, the animal must be anaesthetised during the examination, whether it depends on single tissue or whole body scans. As blood pressure alterations due to anaesthesia can affect plasma leakage, it is important to maintain proper anaesthetic levels during the experiment.

Conclusion

The use of intravital imaging in the study of permeability changes in inflammation has provided much knowledge about the mechanisms controlling endothelial barrier function and it can also be used in the clinical setting for the diagnosis of various disease conditions. However, the field is still evolving with the introduction of more advanced technology. Several new methods for intravital imaging of vascular permeability have been utilised, in particular in the field of cancer research as tumors present with leaky vessels. Their potential use in studies of vascular function in inflammation, on the other hand, is still not fully exploited and advances in this area are to be expected in the near future.

References


Abbreviations

AJs: adherens junctions; ARDS: acute respiratory distress syndrome; BBB: blood-brain barrier; BSA: bovine serum albumin; CT: computed tomography; DTPA: diethylenetriamine pentaacetic acid; FITC: fluorescein isothiocyanate; Gd: gadolinium; MCAO: middle cerebral artery occlusion; MRI: magnetic resonance imaging; NIF: near-infrared fluorescence; PET: positron emission tomography; PTCER: pulmonary transcapillary escape rate; SPECT: single photon emission computed tomography; TJs: tight junctions.