

Intravital Microscopy for the Study of the Microcirculation in Various Disease States

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Abstract

The study of the microcirculation by intravital microscopy represents a sophisticated research tool to analyse complex biological interactions and disease mechanisms as well as to develop and test novel prophylactic and therapeutic approaches aimed at the prevention or attenuation of manifestation of disease-associated microvascular disorders and cellular dysfunction. This may include pathogenesis of atherosclerosis and thrombosis, fibrosis and cirrhosis as well as hypertension, diabetes and tumorigenesis. In addition, using the microscopic technique, circulatory and cellular disorders in surgical diseases and procedures, such as shock and resuscitation, ischaemia/reperfusion and transplantation, trauma, sepsis and inflammation, as well as burn injury and wound healing, may be analysed. With the background of the increasing knowledge of molecular and cellular mechanisms of disease evaluated in vitro, the technique of intravital microscopy ideally allows to bridge over from those in vitro observations to test their potential relevance in vivo.

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Historical Aspects

It is more than 150 years ago when the first detailed description of intravital microscopy was given by Waller, demonstrating in the frog tongue the passage of leukocytes (at that time interpreted as “mucous and pus globules”) through microvessels.¹ Later, the mesentery was acknowledged as the most suitable object for the *in vivo* study of microvascular morphology and function, probably due to its ease of exposure and its high translucency.² While *in vivo* morphology and function of the microcirculation, including flow characteristics in arterioles, capillaries and venules, may be still analysed from the microvasculature of the mesentery, the study of the role of the microcirculation in distinct diseases requires the direct approach to the organs of manifestation. Accordingly, almost all organs have been investigated to elucidate the specific microcirculation by intravital microscopy. These include the liver³⁻⁵ and the pancreas,⁶⁻⁹ as well as the spleen,^{10,11} the intestine,¹² the kidney¹³ and the lung.^{14,15} The major drawback of all of these tissue preparations, in particular those giving access to intraabdominal and intrathoracic organs, was that they were acute in nature, and thus did not allow for chronic and repetitive experiments.

To overcome this drawback, Sandison¹⁶ was the first,

describing a chamber in the ear of the rabbit, which allows repeated analysis of the microvasculature over time. During the following decades, several modifications were performed, including the chamber implantation into the skinfold of normal, nude, and severe compromised immunodeficient mice,¹⁷⁻¹⁹ hamsters,²⁰ and rats,²¹ into the cheek pouch of the hamster,²² and even in the skin of men.²³ Apart from the advantage that in those chronic preparations the microcirculation can be studied in the awake animal, thus avoiding the effects of anaesthetic drugs, the chamber design also enables transplant of foreign tissue and materials, allowing for the study of angiogenesis of benign and malignant tissue, rejection of allogeneic and xenogeneic grafts as well as incorporation of synthetic and biosynthetic materials.¹

With the additional use of fluorescent markers, the spectrum of intravital fluorescence microscopy could be widened from the transillumination approach to epi-illumination techniques, enabling the study of solid organs, such as brain²⁴ and heart.²⁵ Moreover, the use of appropriate fluorescent dyes and cellular staining techniques made it possible to study apart from haemodynamics in the microcirculation-cellular and molecular aspects, i.e. leukocyte-endothelial cell interaction,¹

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platelet-leukocyte and platelet-endothelial cell interaction,^{26,27} macrophage activation/phagocytosis,²⁸ pericyte function,²⁹ transcellular transport,³⁰ leukocyte-associated oxygen radical production,³¹ mitochondrial redox state,^{32,33} and cell membrane damage.³⁰ Thus, the technique of intravital fluorescence microscopy may, today, represent an ideal tool to elucidate *in vivo* the mechanisms involved in the pathogenesis of a variety of diseases.¹ As a consequence, a considerable number of reports were published during the last decade, contributing to the understanding of the mechanisms of tumorigenesis, fibrosis and cirrhosis, atherosclerosis and thrombosis, inflammation, shock and sepsis, ischaemia/reperfusion and transplant rejection as well as pancreatitis, wound healing and burn injury.

Tumorigenesis

With the background that the process of angiogenesis and tumour vascularisation plays a pivotal role in tumorigenesis,^{34,35} the study of new vessel formation remains of particular interest in tumour biology-oriented experimental research.³⁶ During the last two decades, the use of chamber models in the cheek pouch of hamsters, the ear of rabbits, and the skinfold of hamsters, mice and rats, allowed for the selective intravital microscopic study of tumour angiogenesis and vascularization,³⁷⁻⁴³ microvascular perfusion heterogeneity,⁴⁴ vascular permeability,^{45,46} extravascular diffusion,^{47,48} leukocyte-endothelial cell interaction,^{49,50} interstitial transport⁵¹ as well as tumour microvessel pressure⁵² and tumour oxygenation.⁵³ Furthermore; the intravital microscopic approach gave access to evaluate the beneficial effect of different treatment modalities, including hyperthermia,^{54,55} irradiation⁵⁶ and haemato-porphyrin derivate photoradiation,⁵⁷ but also anti-angiogenic approaches using antibodies⁵⁸ and small molecules^{58a} directed against specific growth factors and their receptors. With the transfer of the chamber model to immunoincompetent animals, such as athymic and severe compromised immunodeficient mice,^{18,19,59,60} the spectrum of types of tumours available for study was markedly widened, including in particular human tumours. In addition, recent developments with specific fluorescent markers provide now the basis to study the role of tumour angiogenesis and vascularisation by intravital microscopy at a subcellular and molecular level, including quantification of local pH,⁶¹ intracellular calcium,⁶² growth factor gene expression⁶³ and, most interesting for the development of novel therapeutic regimens, specific antibody binding.⁶⁴

Atherosclerosis

Although macrovessels, which are the primary target for the manifestation of atherosclerotic plaques, cannot

be studied by intravital microscopy, there are several approaches which may help to elucidate the pathogenesis during the early stages of experimental atherogenesis. Using the ear chamber in dietary-induced hyperlipidaemic rabbits, Asano et al⁶⁵ were able to demonstrate, with the help of intravital microscopy, a marked acceleration of intravascular adhesiveness of white blood cells, an increased aggregability of red blood cells, as well as a swarming of foamy macrophages in the connective tissue space concurrently with systemic hyperlipidaemia. In addition, in both rabbits fed with a cholesterol-supplemented chow as well as Watanabe heritable hyperlipidaemic rabbits,⁶⁶ the same authors demonstrated by *in vivo* microscopic imaging using polarised light that during development of atherosclerosis, lipid-laden macrophages became visible in the connective tissue space, transforming concurrently to rectangular crystals, supporting that the intravital microscopic observation of the metamorphosis of "microcirculatory" macrophages may serve as an early indicator for the development of atherosclerosis.⁶⁷

A further approach to study early events in the development of atherosclerosis is the *in vivo* analysis of the response to oxidised low density lipoproteins, using microscopic techniques and a chronically instrumented animal model, such as the dorsal skinfold chamber in hamsters.⁶⁸ Using this approach, Lehr and coworkers^{69,70} could demonstrate the importance of oxygen free radicals and leukocyte-endothelial cell interactions in both arterioles and postcapillary venules, involving leukotrienes⁷¹ as well as P-selectin,⁷² the beta-2 integrin CD11b/CD18⁷³ and the receptor for platelet activating factor.⁷⁴ This knowledge is based on intravital microscopic experiments, demonstrating successful inhibition of oxidised low density lipoprotein-induced leukocyte adherence by counteracting the function of the specific inflammatory mediators, adhesion molecules and receptors. Additional experiments gave clear evidence that a similar protection of microvascular inflammatory response is achieved by treatment with vitamin C⁷⁵ or dietary fish oil,⁷⁶ suggesting those regimens as beneficial tools to prevent manifestation of vascular injury in atherosclerosis.

Hypertension

Apart from specific methods to induce systemic hypertension (one-kidney, one-clip hypertension;⁷⁷ salt-induced hypertension⁷⁸), the availability of spontaneously hypertensive animals facilitates the study of the microcirculation in this particular disease. In fact, a variety of investigations using the cremaster and the mesentery preparation have demonstrated that systemic hypertension is associated with microvessel rarefaction,⁷⁹⁻⁸³ increased microvascular tone and small artery resistance,^{81,84-86} impairment of nitric oxide,

histamine, vasoactive intestinal peptide, acetylcholine, serotonin, and sodium nitroprusside-induced vasodilation,^{78,82,87-89} hyperresponsiveness to angiotensin I, cooling, and oxygen-induced vasoconstriction,⁹⁰⁻⁹² enhanced production of oxygen free radicals in the microvasculature,^{93,94} glucocorticoid-induced suppression of P-selectin-mediated leukocyte-endothelial cell interaction,⁹⁵⁻⁹⁷ and predisposition for thrombus formation.⁹⁸ Thus, there are adequate models and sufficient knowledge on hypertension-induced microcirculatory disorders to perform sophisticated *in vivo* studies for the elucidation of the effectiveness of novel anti-hypertensive treatment modalities. The fact that there is also a non-invasive *in vivo* microscopical approach to the cutaneous microcirculation in humans,⁹⁹ the characteristics of hypertension-induced microvascular disorders as well as the effect of anti-hypertensive therapy can similarly be studied in men.¹⁰⁰⁻¹⁰⁴

Thrombosis

The process of microvascular thrombus formation has been studied by intravital microscopy in arterioles and venules of various tissues, including the mesentery¹⁰⁵ and the cremaster muscle of rodents,^{106,107} the cheek pouch of hamsters,¹⁰⁸ the ear of normal¹⁰⁹ or hairless mice,¹¹⁰ as well as the dorsal skin in a chronically instrumented chamber preparation¹¹¹ or the brain using the access via a cranial window.¹¹² In these models the thrombus is induced photochemically by laser or normal light, using appropriate wavelength together with specific dyes, such as bengal or fluorescein isothiocyanate.^{107,110,112} Experimental studies could clearly show that thrombus formation depends significantly on the presence of ADP, as ADP-utilising enzymes successfully inhibit thrombosis.¹⁰⁵ Further, there is evidence that nitric oxide and cyclooxygenase products are involved in photoactivation-induced microvascular thrombus formation,¹⁰⁷ and that stimuli, such as ischaemia or interleukin-2, can also induce intravascular platelet aggregation and thrombi.^{109,113} For control of the suitability of potential therapeutic regimens, intravital microscopic studies demonstrated effective inhibition of thrombosis by heparin,¹⁰⁶ hirudin,^{114,115} lidocaine,¹⁰⁸ pentoxifylline,¹¹⁶ and a glycoprotein Ib inhibitor.¹¹⁴ Apart from the process of thrombus formation, intravital microscopy also allows for the study of microembolism as shown in the cremaster muscle after different types of upstream interventions, such as vascular anastomosis¹¹⁷⁻¹²⁰ and vascular graft implantation.^{106,121} In addition, the microscopic approach enables the investigation of thrombus formation at sites of microvascular anastomoses,¹²² which has been suggested as a simple clinical tool for the early diagnosis of thrombosis at the site of microvascular anastomosis of free flap transfers.¹²³

Diabetes

To evaluate microcirculatory disorders in diabetes mellitus, experiments may include obese/ hyperglycaemic animals¹²⁴ or animals in which the disease state is induced by application of alloxan or streptozotocin.¹²⁵⁻¹²⁷ Virtually all preparations valid for intravital microscopy can be studied. The application of streptozotocin leads to severe microcirculatory disturbances within pancreatic islets, which can be prevented by the oxygen radical scavenger superoxide dismutase.¹²⁸ Moreover, previous experiments using intravital microscopy have demonstrated in diabetic animals increased microvascular permeability,¹²⁹⁻¹³¹ increased interactions between leukocytes and venular endothelium,¹³⁰ impairment of vasodilatory response to insulin¹²⁶ and acetylcholin,^{130,132} decreased renal arteriolar constriction due to angiotensin II and norepinephrine,^{127,133} and failure of coronary microvascular dilatation as a response to coronary stenosis.¹²⁵ Analysis of therapeutic regimens for the treatment of diabetes revealed that for example sulfonylureas, such as gliclazide or glibenclamide, are able to reduce histamine-induced microvascular permeability.¹³⁴ Advantages of the techniques available are: (i) the microcirculation in diabetes mellitus, similarly as in arteriosclerosis and hypertension, can also be studied in men,^{99,135} and (ii) individual microvascular response to treatment does not only include specific medical treatment, but also pancreatic or islet transplantation both in the experimental setup¹³⁶⁻¹³⁸ and under clinical conditions.^{139,140}

Fibrosis and Cirrhosis

Beside specific molecular aspects of fibrogenesis, vascular modifications during the development of cirrhosis have fully been recognised and interpreted in that microvascular changes in the liver are not the mere consequence of fibrosis and nodule formation, but dominate the entire development of the pathological condition.¹⁴¹ Still, there is little information on microvascular derangements during the process of liver fibrosis and cirrhosis. Most studies on the microvasculature have been performed by using corrosion casts and scanning electron microscopy,¹⁴²⁻¹⁴⁶ a technique which is limited to the evaluation of changes in the micro-angioarchitecture, but does not enable to analyse dynamic alterations of microvascular function and its close interrelation with cellular and molecular derangements. Using intravital fluorescence microscopy, Sherman et al¹⁴⁷ have been the first to characterise "fast sinusoids" as remaining capillary blood vessels with increased blood flow velocity during sinusoidal rarefaction. Later on, Vollmar and coworkers¹⁴⁸ reported from intravital microscopic studies sinusoidal free space around dilated postsinusoidal venules with Ito cell migration, substituting occurrence of autofluorescent

collagen deposition, and despite the reduction of sinusoidal density an increase of vascular lumen caused by the formation of shunting vessels bypassing the sinusoids (Fig. 1). Moreover, the use of high molecular fluorescent tracers showed during the process of fibrogenesis a progressive delay of transsinusoidal exchange, which is associated with lymph microvessel expansion and function.¹⁴⁹ Further studies have to elucidate the targets, allowing inhibition of these microvascular alterations, which then may be potentially used to counteract the process of fibrogenesis.

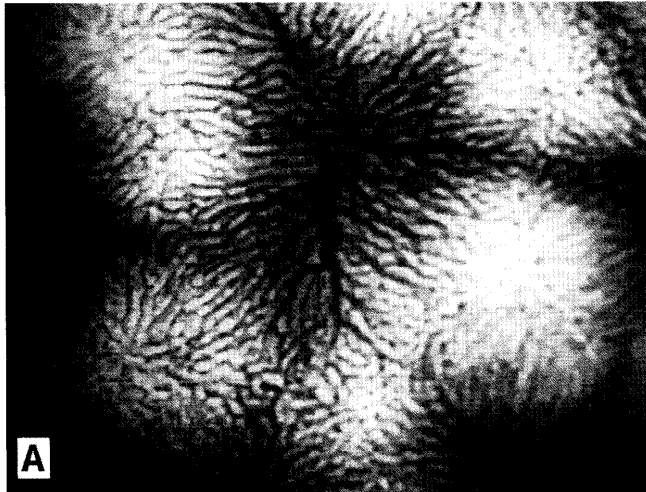


Fig. 1A.

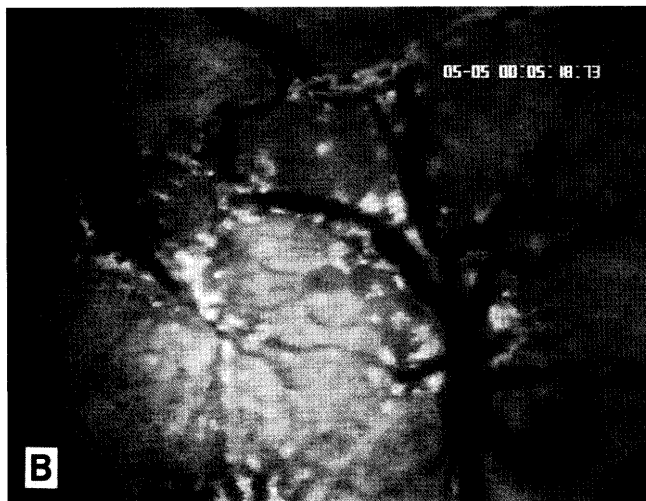


Fig. 1B

Fig. 1. Intravital microscopy of normal (A) and cirrhotic rat liver (B; twelve weeks of CCl₄-exposure) after contrast enhancement with sodium fluorescein. Note the cirrhosis-associated reduction of sinusoidal density, but the increase of vascular lumen caused by the formation of shunting vessels bypassing the sinusoids and enlargement of postsinusoidal venules. Collagen deposition is most prominent in the perivenular space. (Magnification $\times 110$)

Inflammation

Microcirculatory recruitment of white blood cells is the essential event, promoting the contact of both specific and non-specific immunocompetent cells with

the microvascular endothelial lining. While Atherton and Born in 1972^{150,151} have already studied in detail kinetics of white blood cell flow behaviour within microvessels, including rolling and firm adhesion to endothelium, the molecular mechanisms of these pathophysiological processes were identified only recently. Using intravital microscopy, von Andrian and co-workers^{152,153} were first to demonstrate *in vivo* that the process of leukocyte adhesion in microvessels follows a two-step sequence, i.e. rolling and adherence, which are mediated by distinct adhesion molecules.¹⁵⁴⁻¹⁵⁶ Today a considerable number of intravital microscopic studies using both monoclonal antibodies and gene-targeted animals have clarified in detail individual roles of L-, P-, and E-selectin for leukocyte rolling, as well as beta-2 integrins on leukocytes (LFA-1, Mac-1) and ICAM-1 and VCAM-1 on endothelial cells for firm adherence.¹⁵⁷⁻¹⁶³ In fact, this knowledge provides now the basis for the development of novel therapeutic strategies to minimise overwhelming leukocyte-induced tissue injury in inflammation. Moreover, most recent studies brought evidence that in the process of leukocyte-endothelial cell interaction within the microvasculature, additional cells may be involved, such as platelets, functioning as carriers of the white blood cells to direct them specifically to endothelial sites of interest.^{26,72,164}

Shock/Resuscitation

Microcirculatory disorders are known to play a crucial role in the manifestation of shock/resuscitation-induced tissue injury. Using intravital microscopy, several studies have demonstrated deterioration of nutritive (capillary) blood flow after bleeding to a MAP of around 40 mmHg without complete recovery after resuscitation, even in case of whole blood or 4-fold crystalloid fluid replacement.¹⁶⁵⁻¹⁶⁷ Nutritive perfusion may be improved using colloid or hypertonic hyperoncotic solutions, as shown in the liver and pancreatic microcirculation.¹⁶⁷⁻¹⁷⁰ These types of resuscitation may also reduce heterogeneity of microvascular perfusion and improve tissue oxygenation, which are well known to be characteristic features of the microcirculatory disorders in shock.¹⁶⁶ Apart from nutritive perfusion failure, shock/resuscitation is also associated with an inflammatory response (reflow-paradox), as shown by the increased number of leukocytes adhering in capillaries/ sinusoids and postcapillary/postsinusoidal venules of the microvasculature. This inflammatory response has been shown to be attenuated by using specific colloid/ hypertonic solutions,¹⁶⁷⁻¹⁶⁹ or by counteracting inflammatory mediators and adhesion molecules, such as TNF- α ,^{171,172} interleukin-1,¹⁷³ platelet activating factor,¹⁷⁴ leukotrienes,¹⁷⁵ or the CD18 leukocyte glycoprotein.¹⁷⁶⁻¹⁷⁸ Apart from these results, intravital microscopic analyses revealed distinct mechanisms, which are active to counteract shock-induced microvascular perfusion

failure, i.e. arteriolar vasomotion and capillary flowmotion.¹⁷⁹ Although these studies nicely show that the capillary flowmotion directly depends on the arteriolar flowmotion,¹⁷⁹ the regulatory mechanisms remain to be determined.

Ischaemia-reperfusion/Transplantation

Ischaemia/reperfusion, which has to be considered an inflammatory disease,¹⁵⁵ challenges a considerable number of disciplines in clinical medicine, including surgery, traumatology, orthopaedics, and transplantation, but also cardiology and neurology with the clinical conditions in myocardial infarction and stroke. Early experimental studies have demonstrated that the microcirculation represents a major target in manifestation of reperfusion injury,^{180,181} characterised on the one hand by the “no-reflow” phenomenon, prolonging tissue ischaemia,¹⁸² and on the other hand by an inflammatory leukocytic response, which takes primary place in postcapillary venules by their interaction with the microvascular endothelium.¹⁸³ Since the manifestation of this leukocytic response requires reperfusion, the microvascular reperfusion event, which should be beneficial to the tissue due to reintroduction of oxygen, paradoxically contributes to the aggravation of injury (“reflow-paradox”¹⁸³). Intravital microscopic studies in various tissues have shown that the ischaemia/reperfusion-induced leukocyte-endothelial interaction involves distinct adhesion molecules, including selectins on both the endothelial and the leukocytic surface, as well as leukocytic β_2 -integrins (mainly CD11b/CD18) and endothelial ICAM-1.¹⁸⁴⁻¹⁸⁹ In addition, using the *in vivo* microscopic approach to analyse the microvasculature, those studies have also shown that a variety of mediators are involved in triggering and aggravating the leukocytic response, and thus tissue injury, including oxygen radicals, leukotrienes, thromboxane, and platelet activating factor.^{181,183,190-194} Recent experimental investigations have further extended this knowledge by elucidating the role of the nitric oxide-endothelin system.¹⁹⁵⁻¹⁹⁹

Although reperfusion injury in organ transplantation may be different to what is known in warm ischaemia, since the transplants undergo prolonged ischaemia times by storage in distinct preservation solutions,^{28,200} analysis of the microcirculation of transplanted gastrointestinal organs, i.e. liver,²⁰¹⁻²⁰³ intestine,^{204,205} and pancreas,^{137,138} revealed quite similar microvascular characteristics of ischaemia/reperfusion injury, including microvascular perfusion failure and leukocyte-endothelial cell interaction. Thus, recent research has focussed on specific tools to counteract manifestation of microvascular injury after cold ischaemia by using distinct preservation^{206,207} or rinse solutions,²⁰⁸⁻²¹¹ by pharmacologic conditioning,^{212,213} by modifying the operative technique,^{214,215} or

by modulating the action of the individual mediators potentially involved in the induction of the microcirculatory deteriorations.^{138,203,208}

In contrast to vascularised organ transplants, successful transplantation of islets of Langerhans require the process of angiogenesis and vascularisation, which can be monitored and studied by the use of intravital microscopy.²¹⁶⁻²¹⁸ Strikingly, these studies demonstrate that the avascular, freely transplanted islets, and even pseudo-islets, develop a new microvasculature, similar to that observed in pancreatic islets *in situ*,^{219,220} although the endothelium of the newly formed microvessels has been shown to originate from the vasculature of the host tissue.²²¹ Cryopreservation of the isolated islets applied before transplantation does not alter the process of vascularisation,²²² as does not hyperglycaemic conditions of the recipient.¹³⁶ In contrast, immunosuppressive agents, such as cyclosporine, deoxyspergualin or RS61443 inhibit the vascularisation process,²²³⁻²²⁶ while the anti-oxidative compound vitamin E has been shown to effectively reduce graft rejection without affecting vascularisation.²²⁷

Trauma/Sepsis

There is little knowledge on the direct influence of trauma on the microcirculation, which is probably due to the lack of adequate experimental models. However, the few studies which have analysed the microcirculation after trauma by intravital microscopy demonstrate, apart from a compromise of capillary perfusion,²²⁸ recruitment of leukocytes within the microvasculature with adhesion to endothelium of both arterioles²²⁹ and venules.²²⁸ The inflammatory response can be observed in brain^{230,231} as well as in peripheral tissues,^{228,229,232} and is probably mediated by P-selectin and intercellular adhesion molecule-1 (ICAM-1²³³). New models with standardised trauma induction which allow for the analysis of the microcirculation with intravital microscopy may, in future, provide more detailed insights in the manifestation of trauma-induced microvascular disorders.²³⁴ This also holds true for the special case of compartment syndrome, in which a recent study demonstrated that the mechanism of microcirculatory disturbances is probably not due to arteriolar vasospasm or a critical closing pressure but rather due to a decrease in arteriovenous pressure gradient.²³⁵

Although it is widely accepted that the sepsis/endotoxin-induced sequence of events is primarily vascular in origin, the underlying mechanisms and the exact role of endotoxins have not been clearly established, and hypotheses range from vasomotor instability, vasoconstriction and vasodilation, hypo- and hyperreactivity to catecholamines, to disturbances in blood barriers and increased microvascular permeability.²³⁶ In fact, both endotoxin-induced arterial constriction and dilation have

been reported, and Cryer et al^{237,238} have demonstrated even a differential arteriolar response during live *E. coli* bacteraemia-induced high cardiac output (hyperdynamic) phase of sepsis, i.e. constriction of large (A1) arterioles but dilation of small (A3, A4) arterioles. The most detailed description of the microcirculatory response to endotoxin was given by Brånemark and Urbaschek.²³⁹ Using *in vivo* microscopic techniques, these authors reported arterial constriction and venular dilation 10 minutes after endotoxin administration with additional luminal narrowing of the arterioles due to swelling of pericytes and endothelial cells.²³⁹ However, initial arterial vasoconstriction is followed within 10 to 15 minutes by degranulation of mast cells and subsequent loss of arterial tone.^{239,240} These observations are in agreement with previous studies of Zweifach et al²⁴¹ demonstrating dose-dependent induction of both an endotoxin-induced increase and a decrease in vascular reactivity to catecholamines, culminating in a complete absence of vessel reactivity. It has been advocated that secondary arterial vasodilation comes as a result of tissue release of several vasoactive mediators, including histamine, serotonin, and bradykinin,²⁴² but also of prostanoids^{237,242} and endothelium-derived relaxing factor (nitric oxide).^{243,244}

Besides alteration of local vasomotor control, endotoxins have been demonstrated to induce microcirculatory disorders by capillary engorgement, endothelial swelling and intravascular hemoconcentration.²⁴⁵ *In vivo* microscopic studies of the liver revealed significant perfusion failure of individual sinusoids upon endotoxin exposure,^{246,247} probably due to swelling of endothelial and Kupffer cells. Impairment of nutritional flow results in tissue hypoxia,²⁴⁸ depletion of energy stores,²⁴⁹ and, consequently, damage of parenchymal

cells. In parallel with the deterioration of nutritional flow in septic state,²⁵⁰ *in vivo* microscopy revealed endotoxin-induced accumulation of leukocytes within the microvasculature, and their adherence to the endothelial lining, in particular of postcapillary venules (Fig. 2).^{246,247} The extent of microcirculatory disturbances, which cannot be prevented by anti-oxidants²⁵¹ or monoclonal antibodies directed against one single adhesion molecule (ICAM-1²⁵²), seems to be dependent upon: (i) the numbers of leukocytes interacting with the endothelial surface, and tissue sessile macrophages, (ii) state of activation of these cells, and (iii) release of their potentially toxic mediators, and may be modified by distinct modulation of Kupffer cell and leukocytic response.^{253,254} Aggregation and adhesion of platelets with formation of microthrombi, and adhesion of lymphocytes to macrophages (Kupffer cells) have further been suggested to contribute to the breakdown of the microcirculation in endotoxic shock.²⁴⁶

Pancreatitis

During the last decade, the microcirculation has been proposed to be a primary target in the manifestation of pancreatitis.²⁵⁵ With the use of high resolution *in vivo* fluorescence microscopy, detailed quantitative analyses of the pancreatic microcirculation have demonstrated a marked reduction of (i) the number of perfused capillaries and (ii) microvascular haemoglobin saturation, both in proportion to the severity of the disease.²⁵⁵⁻²⁵⁸ Moreover, within the pancreatic gland, microvascular perfusion pattern was characterised by local heterogeneity due to focal necrosis, with pronounced impairment of nutritive perfusion in perinecrotic, but less impeded capillary blood flow in non-necrotic areas.²⁵⁹ In parallel, capillary erythrocyte velocity was found predominantly reduced



Fig. 2A.



Fig. 2B.

Fig. 2. Intravital microscopy of hamster striated muscle postcapillary venules of the dorsal skinfold chamber after lipopolysaccharide (LPS)-exposure. Contrast enhancement is achieved by fluorescein-isothiocyanate dextran 150,000 (staining the intravascular space; A) and rhodamine-6G (staining the DNA of white blood cells; B). Note the marked accumulation of leukocytes, which adhere to the endothelial lining of the postcapillary venules. (Magnification $\times 10$)

in perinecrotic tissue, although some alteration of blood cell flow-velocity was also found in macroscopically non-necrotic areas.²⁵⁹

Beside nutritive perfusion failure, acute pancreatitis is characterised by a local and systemic inflammatory response with activation of leukocytes and their enhanced interaction with the microvascular endothelium, as demonstrated by the *in vivo* observation of leukocytes adhering to the postcapillary venular wall initially after induction of bile salt-induced pancreatitis in rats.²⁶⁰ Inhibition of leukocyte adherence and subsequent reduction of acinar necrosis by application of both radical scavengers and leukotriene receptor antagonists²⁶⁰ indicate that these cellular components and humoral mediators play a pivotal role in the mechanisms underlying acute pancreatitis-associated acinar cell injury. The microcirculatory derangements following induction of pancreatitis, namely nutritive perfusion failure and microvascular leukocyte adherence, are similar in nature with microcirculatory events observed subsequently to ischaemia and reperfusion in other organs, such as the liver,^{261,262} intestine,^{204,205} striated muscle^{183,263} and the skin.¹⁸⁰ Thus, opposing the traditional concept of acute pancreatitis as a primary enzymatic injury, the hypothesis that the pathogenesis of acute pancreatitis involves ischaemia-reperfusion-associated events has attracted new attention.²⁶⁴⁻²⁶⁶

Complete ischaemia of the pancreas followed by reperfusion and reoxygenation is classically akin to the situation encountered with pancreas transplantation. Beside the disturbed integrity of structures within the acinar cells, which can clearly be attributed to the ischaemic insult, reperfusion-associated events are thought to account for different, distinct cellular and metabolic responses, including autophagocytosis, acceleration of cellular metabolism and leukocyte activation / reaction, which have the consequence of later acinar cell necrosis.²⁶⁷ Experimental models of pancreatic ischaemia-reperfusion recently confirmed that ischaemia, but in particular, reperfusion, is associated with leukocyte activation and adherence, consecutive microvascular perfusion failure and oedema formation, increased enzyme release and histomorphological alterations, similar as known in acute interstitial pancreatitis.²⁶⁶ The severity of changes was found to be dependent on the duration of both ischaemia and reperfusion.²⁶⁸ Finally, ischaemia of the pancreas of two hours with ensuing reperfusion for five days was capable to induce haemorrhagic-necrotising pancreatitis.²⁶⁹ Despite the fact that-except for-pancreatic transplantation-the originally described model of ischaemia-reperfusion (arterial vessel clamping and reopening)²⁷⁰ does not really simulate conditions of acute pancreatitis in which flow may be reduced but not completely abolished, ischaemia-reperfusion as potential causative factor in the

manifestation of acute pancreatitis is strongly supported by observations of Kusterer and coworkers²⁶⁰ in the rat model of bile salt-induced pancreatitis. With the use of intravital fluorescence microscopy, these authors have demonstrated that pancreatitis is characterised by early arteriolar vasoconstriction with complete cessation of capillary blood perfusion (ischaemia), followed by arteriolar vasodilation with re-establishment of blood flow (reperfusion). In these experiments, increased leukocyte-endothelial cell interactions in postcapillary venules were not observed during vasoconstriction (ischaemia), but were prominent during vasodilation (reperfusion).²⁶⁰ The authors, therefore, interpreted vasoconstriction/vasodilation as a trigger mechanism in the manifestation of acute pancreatitis, mimicking the pathologic insult of ischaemia/reperfusion with the inflammatory response, i.e. leukocyte activation and leukocyte-endothelial cell interaction. Thus, the classical model of bile salt-induced haemorrhagic-necrotising pancreatitis is associated with the pathological sequelae of ischaemia-reperfusion.

Moreover, haemorrhagic hypotension, which is also thought to trigger manifestation of pancreatitis, might not only cause microcirculatory insufficiency, i.e. low flow state with hypoxia, but, as recently shown,¹⁷⁹ induces also arteriolar vasomotion with intermittent capillary perfusion, characterised by alternate cessation and onset of capillary perfusion. Thus, haemorrhagic shock and systemic hypotension are associated with, at least, local/regional ischaemia (shutdown of perfusion) and reperfusion of pancreatic tissue, thereby inducing an inflammatory response with the potential to initiate the process of acute pancreatitis.

Wound Healing

There is a general agreement that appropriate wound healing requires the process of angiogenesis and vascularisation of the newly formed tissue. In fact, due to the mechanically altered tissue (wound induction) there are only few models available which allow the study of the microcirculation during wound healing by intravital microscopy. The probably most reliable model is the wound model in the ear of the hairless mouse,²⁷¹ which has the major advantage that wound closure is not affected by contraction, a mechanism which is present in almost all other wound models in loose skin animals, and which requires a distinct device for allowing microcirculatory analysis of new vessel formation in granulation tissue.²⁷² Using the ear of the hairless mouse, quantitative analysis of neovascularisation can be performed during the entire process of wound healing by repeated intravital microscopic analysis,²⁷¹ and induction of ischaemia to the ear or diabetes to the animal may include evaluation of critically endangered healing processes. Using this model of wound healing and intravital microscopy, recent studies have demon-

strated a role of VEGF in the healing process,⁶³ and modulation of wound healing by application of hyperbaric oxygen²⁷³ as well as basic fibroblast growth factor²⁷⁴ and platelet-derived growth factor.²⁷⁵

Burn Injury

It was as early as 1967 when Herbert J Robb²⁷⁶ first reported on microvascular derangements in burn injury by studying the microcirculation with intravital microscopy. Characteristics found were arteriolar and venular dilation, platelet deposition—first at the venular but later also at the arteriolar endothelium—and, finally, intravascular thrombosis and stasis of blood flow. While these experiments have been performed in the mesentery and small intestine, later studies, in order to more closely mimic burn injury in patients, have used skeletal muscle²⁷⁷ and in particular chronic models with peripheral tissues, such as the hamster dorsal skinfold²⁷⁸⁻²⁸⁰ and the ear of normal and hairless mice.^{281,282} With the aim to further elucidate the role of microvascular derangements in burn injury, Eriksson et al²⁸³ demonstrated the importance of the inflammatory response, as visualised *in vivo* by marked leukocyte adherence and transmigration, while others showed significant capillary perfusion failure and confirmed that platelet thromboembolism is the major cause of microvascular nutritive dysfunction.²⁸²

Flap Surgery

Although major improvements have been achieved in flap surgery by technical developments and increased surgical experience, there is still a considerable number of skin flaps as well as osteomyocutaneous flaps, which fail due to derangements of microvascular perfusion. This brought major interest to develop appropriate models, allowing for the study of the microcirculation by the use of intravital microscopy. Since flap failure represents rather a chronic than an acute process, chronic models seem to be more appropriate to undertake *in vivo* studies on the microvascular mechanisms of flap failure. Such chronic model can be achieved by using the ear of the hairless mouse²⁸⁴ after incision at its base, leaving only one of the three neurovascular bundles for its blood supply.²⁸⁵ Using this model, Barker et al²⁸⁶ demonstrated that normovolaemic haemodilution is effective to ameliorate microcirculation and thus skin flap necrosis. Similar protection of the microcirculation has been achieved by the use of growth factors²⁸⁷ or distinct vasoactive substances.^{288,289} Since free flap transfer gains more and more attraction in clinical practice, in particular for covering large defects by osteomyocutaneous flaps, and deterioration of the microcirculation in endangered flaps may vary between the different tissues involved, the development of models allowing the intravital micro-

scopic study of the microcirculation of the different/individual tissues, i.e. skin, subcutis, muscle and periosteum/bone, have been suggested to be appropriate for future experimental investigations in free flap research.²⁹⁰

Material Implantation

With the increasing interest for bioengineered foreign tissues, there will be a need to study the microcirculation after foreign material implantation, including the analysis of the inflammatory response as well as the evaluation of new vessel formation. To study these objectives, the use of chronic chamber models may be ideal, since they not only allow for repeated microscopic analysis over time periods of up to four weeks, but also for simultaneous implantation of foreign materials into the fine striated muscle and subcutaneous tissue. Previous experiments including synthetic, bio-synthetic and solely biological materials have demonstrated that engraftment and neoangiogenesis of vascular prosthesis are dependent on material porosity, but in particular on material composition.²⁹¹⁻²⁹³ Moreover, ongoing studies indicate distinct leukocytic responses depending on the implant material, probably allowing assess to appropriate inertness of foreign materials for clinical use.

Conclusion

The study of the microcirculation by intravital microscopy represents a sophisticated research tool to analyse complex biological interactions and disease mechanisms as well as to develop and test novel prophylactic and therapeutic approaches aimed at the prevention or attenuation of manifestation of disease-associated microvascular disorders and cellular dysfunction. With the background of the increasing knowledge of molecular and cellular mechanisms of disease evaluated *in vitro*, the technique also ideally allows to bridge over from those *in vitro* observations to test their potential relevance *in vivo*.¹ Since the technique of intravital microscopy can be applied also in men (at least for the study of the skin microcirculation), results from experimental studies may be directly transferred to clinical practice, in particular for the study of disease mechanisms and effectivity of treatment in chronic ischaemia, arteriosclerosis, diabetes and hypertension.

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