Neutrophil leukocyte-mediated inflammatory reactions in the periosteum and synovial membrane

Petra Hartmann, MD

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Institute of Surgical Research, University of Szeged

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1. INTRODUCTION

1.1. Periosteal and synovial membrane damage in the clinical practice

A tourniquet is often applied in both acute traumatological and elective orthopedic interventions when a bloodless operating field is required. This maneuver subjects all tissues of the extremity to ischaemia-reperfusion (IR)-related changes. IR and reduced blood flow conditions during acute trauma surgery critically influence bone healing mostly by affecting major functions of the periosteum. Impaired periosteal perfusion following fractures frequently results in delayed fracture repair and a non-union or manifest pseudarthrosis. Apart from traumatological interventions, elective orthopedic operations also frequently subject the limbs to iatrogenic IR injury. The application of a tourniquet most often occurs during arthroscopy, but limb hypoperfusion-reperfusion is a frequent consequence of the radical resection of tumors, reconstructions and autotransplantation via vessel anastomoses.

The periosteum has been shown to possess high ischemic sensitivity, but the IR-induced microcirculatory reactions in the synovium have not yet been clarified. Integrity and major functions of the synovial membrane are mostly compromised in arthritis situations. The most common forms of arthritis are osteoarthritis, rheumatoid arthritis and gout, but the remaining forms (juvenile idiopathic arthritis, psoriatic arthritis, ankylosing spondylitis, systemic lupus erythematosus, scleroderma, fibromyalgia and chronic widespread pain) all display a high incidence. Although rheumatic diseases are diverse in etiology all such conditions result in common symptoms including different levels of pain, swelling and joint stiffness. In arthritic disorders, the synovial membrane is a major target in both the initiation and propagation of inflammation, where microcirculatory changes make an important contribution to the pathogenesis. Likewise, attenuation of the inflammatory reactions taking place in the synovial membrane forms the basis of current, up-to-date therapeutic approaches.

In the present studies, neutrophil leukocyte-mediated inflammatory reactions in the periosteum and synovial membrane were examined and compared in IR and arthritis.

1.2. The potential role of C-afferent innervation in the mechanism of ischemic preconditioning (IPC)

Leukocyte-endothelial cell interactions are decisive factors in the sequelae of IR injuries. The number of recruited polymorphonuclear cells (PMNs) and the extent of tissue damage during IR are inter-related phenomena. Intravital videomicroscopy (IVM) is an excellent approach for the visualization and quantification of the interactions between PMNs and the endothelial lining.

During IPC, brief ischemia-reperfusion periods are applied prior to a longer ischemia.

Neurogenic mechanisms have been suggested to be involved in mediating both the local and the remote consequences of IPC. This assumption is based on observations that the release of some neuropeptides can be detected at the site of preconditioning, and these pass into the blood stream and contribute to the transduction of the preconditioning stimulus to remote organs. Calcitonin gene-related peptide (CGRP), a major transmitter of capsaicin-sensitive sensory nerves, has already been addressed as a mediator of the preconditioning induced by brief ischemia. In clinical studies, IPC proved to be associated with the local release of CGRP. The first aim of the present work was to investigate the involvement of these neurogenic processes, with particular emphasis on the role of CGRP in mediating the microcirculatory effects of limb IPC.

1.3. Leukocyte-driven inflammatory reactions in arthritis. Biological and anti-inflammatory effects of endogenous phosphatidylcholine (PC)

PMNs have a crucial role in the initiation and maintenance of arthritic disorders. It has also been found that diminution of PMN infiltration in the synovial membrane directly influences the severity of inflammation. Therefore we employed a standardized, PMN-driven inflammatory model of experimental arthritis.

PC is the most frequent membrane component in the body. It has been shown, however, that inflammation is associated with physical membrane defects which results in PC degradation and the exhaustion of endogenous PC sources. This observation suggests that PC supplementation may be beneficial in various diseases. PC is in important component of the synovial fluid and has a boundary lubrication function under severe loading conditions. Moreover, the composition of the synovial fluid of an osteoarthritic joint is altered considerably, which leads to a reduced concentration of molecules responsible for lubrication. It can also be hypothesized that PC supplementation may restore the normal PC content, which may contribute to the preservation of the epithelial part of the synovial barrier, causing a lesser degree of articular swelling. Hence, we set out to examine the PMN functions in the synovial membrane in response to arthritis and also after PC therapy.

1.4. Tissue-specific characteristics of the synovial membrane in inflammatory states

Joints act as compartments of the body because they are protected from the local manifestation of systemic, antigen-dependent disorders (*e.g.* infections). A further important aspect of this special barrier function is the limited penetration of certain drugs into the joint cavity. Interestingly, adhesion molecules are expressed not only on the endothelial cells, but also on interstitial cells. Hence, joints possess numerous special features, some of which provide increased protection against inflammatory reactions affecting the extremities. Since

the susceptibility of the synovial membrane to IR has not yet been assessed, we aimed at investigating the simultaneous microvascular inflammatory responses of the tibial periosteum and the synovium of the knee joint in response to a standardized challenge.

2. MAIN GOALS OF THE STUDIES

Our primary goal was to develop a standardized rodent model for examination of the PMN-driven inflammatory reactions in the synovial membrane in monoarthritis, using IVM. To this end, we employed a new method which allows direct IVM observations at the inner surface of the joint cavity. We also used IVM to characterize the *in vivo* microcirculatory alterations in the periosteum in a rat model of hindlimb IR. We investigated the nature and the time course of PMN accumulation in the periosteum and the expression of intercellular adhesion molecule-1 (ICAM-1).

- In our monoarthritis model, we set out to ascertain the anti-inflammatory properties of PC by performing detailed microcirculatory and functional analyses relative to diclofenac sodium, a non-steroidal anti-inflammatory drug commonly used to treat knee joint inflammation.
- In the hindlimb IR, our aim was to reveal the possible involvement of neurogenic factors in the protective effects of limb IPC in the periosteum, with particular emphasis on the role of endogenous CGRP in the process. In line with this, we investigated whether a CGRP agonist treatment human calcitonin gene-related peptide (hCGRP), a competitive antagonism of the CGRP receptor (by $CGRP_{8-37}$), or selective depletion of the chemosensitive C-fibers by the capsaicin analog resiniferatoxin (RTX), influences the IPC-induced microcirculatory reactions in a rat model of experimental limb IR.
- Additionally, we set out to characterize the parallel microvascular inflammatory reactions of the tibial periosteum and the synovium of the knee joint in response to a standardized IR challenge. With this aim, we investigated cell-to-cell interactions, and hemorheological and biochemical consequences of IR in both compartments. We compared these changes with those seen in C/K-induced knee osteoarthritis, a classical model of local sterile inflammation known to be mediated by PMNs.

3. MATERIALS AND METHODS

3.1.1. Arthritis induction

The animals were anesthetized with intraperitoneal (ip) ketamine (50 mg kg^{-1}) and xylazine (12 mg kg^{-1}), the skin over the knee was disinfected with povidone iodide, and

arthritis was then induced with a single intraarticular injection of 75 µl of a mixture of 2% λ-carrageenan– 4% kaolin (C/K) in saline. The contralateral knees were injected with saline.

3.1.2. Preparation of the synovial membrane of the knee joint and the tibial periosteum in rats for intravital microscopic examinations

A novel model was developed for the investigation of the microcirculation in the synovial tissue of the rat knee joint 6 h after the arthritis induction. Under pentobarbital anesthesia (ip 45 mg kg^{-1}), the synovial membrane over the medial condyle of the proximal tibia was exposed by opening the joint cavity through cutting the tendon of the quadriceps femoris. For the examination of the tibial periosteum, the gracilis posterior muscle was cut through and the periosteum of the medial surface of the right tibia was exposed.

3.1.3. Intravital videomicroscopy (IVM)

Microcirculation of the synovia and the periosteum were visualized by fluorescent IVM, fluorescein isothiocyanate being used to label the erythrocytes (0.2 ml), and rhodamine-6G (0.2%, 0.1 ml iv) to stain the leukocytes. The IVM images were recorded with a video camera attached to an S-VHS video recorder and a personal computer.

3.1.4. Nociceptive tests

Mechanical hyperalgesia was quantified by the von Frey test, while hyperalgesia was detected by the paw withdrawal test, applying a Hargreaves apparatus.

3.1.5. Knee joint swelling

Joint inflammation was characterized by the increase in the diameter of the joints 48 h after C/K injection. The diameters were measured with a caliper square.

3.1.6. Histological analysis of PMN infiltration and immunohistochemical analysis of ICAM-1 expression

Tissue samples taken from the synovium and the periosteum were embedded in paraffin, and were either stained with hematoxylin-eosin (to characterize inflammation by the number of infiltrating PMNs) or subjected to immunohistochemical analysis for tissue ICAM-1 expression.

3.1.7. Immunofluorescence detection of the periosteal localization of transient receptor potential vanilloid type-1 (TRPV1) and CGRP

TRPV1 receptor and CGRP peptide immunoreactivity was demonstrated according to a modified method of Baiou *et al.* (J Comp Neurol. 2007;503(2):334-47)

3.1.8. Xanthine oxidoreductase (XOR) and myeloperoxidase (MPO) activity activity

The activity of XOR was determined in the ultrafiltered supernatant of homogenized tissue samples by fluorometric kinetic assay based on the conversion of pterine to

isoxanthopterine in the presence (total XOR) or absence (XO activity) of the electron acceptor methylene blue.

Tissue MPO activity was measured in synovium and periosteum biopsies by the method of Kuebler *et al.* (Int J Microcirc Clin Exp. 1996;16(2):89-97)

3.2. Experimental protocols

3.2.1. Study I.

In this study, the possible involvement of neurogenic factors in the protective effects of limb IPC in the periosteum were investigated, with particular emphasis on the role of endogenous CGRP in the process. The experiments were performed in two major series, where the animals were allotted into one or other of the following experimental groups. In the first group, the periosteal microcirculatory responses to 60-min total limb ischemia followed by a 180-min reperfusion period were examined (IR, n=6). Complete hindlimb ischemia was induced by placing a tourniquet around the proximal femur, with simultaneous occlusion of the femoral artery with a miniclip. The occlusions were then released (t = 0 min), and the periosteal microcirculation was observed via IVM at 60, 120 and 180 min during the reperfusion phase. In further experiments, the CGRP agonist hCGRP (0.3 μg kg^{-1} iv over 5 min (n=6)) or IPC (2x10-min IR) was applied prior to the IR insult. IPC was performed in three subgroups, in which the animals received the CGRP antagonist $CGRP_{8-37}$ (30 μg kg^{-1} h^{-1} iv during a 3-h reperfusion), the capsaicin analog RTX, which selectively depletes the chemosensitive afferents (15 μg kg^{-1} sc diluted with saline from a stock solution dissolved in ethanol; injections were repeated three times, every second day, ending 14 days before the experiments), or vehicle (saline) (n = 6-8). The effect of the systemic RTX treatment on the sensory functions of the chemosensitive primary sensory neurons was confirmed by the abolition of the eye-wipe reaction induced by the intraconjunctival instillation of 50 μl of 0.1% capsaicin solution.

In the second series of experiments, we investigated the consequences of $CGRP_{8-37}$ and RTX treatment on the periosteal inflammatory reactions (n=6-7) after limb IR without IPC to determine the direct effects of the compounds on the postischemic microcirculation.

3.2.2. Study II.

These experiments were performed to develop a standardized rodent model of PMN-driven monoarthritis, and to assess the anti-inflammatory properties of PC by performing detailed nociceptive and morphological analyses of the knee joint and IVM investigation of the synovium, in two experimental series. In the first series, the animals participated in

functional nociceptive tests reflecting the degree of inflammation, while in the second series IVM examinations of the knee joint were performed. The animals in the first group (n=8) were pretreated orally with PC solution (1,2-diacylglycero-3-phosphocholine in 5% glucose) in a dose of 150 mg kg^{-1} (1 ml kg^{-1}) through a plastic gastric tube 4 h before arthritis induction (t = -4 h), and the gavage was repeated 8 h later (t = 4 h). The typical fatty acid composition for PC was 20% palmitic acid, 5% stearic acid, 10% oleic acid, 60% linoleic acid and 5% linolenic acid). A further two groups were gavaged with identical volumes (1 ml kg^{-1}) of diclofenac sodium (0.5 mg kg^{-1}) or saline at t = -4 and 4 h (n=6 and n=8, respectively). On days 2 and 3, the same oral treatments were repeated twice daily.

In the second series, identical treatment protocols were used, except that IVM examinations of the knee joints were started 6 h after arthritis induction (2 h after the second oral treatment with PC (n=8), diclofenac sodium (n=6) or saline (n=8). At the end of the experiments, tissue samples from the synovium were taken for histological assessment.

3.2.3. Study III.

These experiments were conducted to compare the microvascular inflammatory reactions of the tibial periosteum and the synovium of the knee joint in response to a standardized IR challenge. The experiments were performed in two major series, with the animals allotted to one or other of the following experimental groups. In the first group, the periosteal and synovial microcirculatory responses to 60-min total limb ischemia followed by a 180-min reperfusion period were examined (IR, n=6). In the second series, arthritis was induced in anesthetized animals with a single intra-articular injection of 75 µl of the C/K mixture in saline. IVM examinations of the knee joints were started 6 h after arthritis induction. Tissue specimens for immunohistochemical analysis and biochemical determinations were taken at the end of the experiments. Tissue biopsies were fixed in buffered formalin, and biochemical samples were stored at -20 ^{o}C.

4. RESULTS

4.1. The effects of IPC on the postischemic inflammatory reactions of the periosteum. The involvement of sensory nerve activation in the effects of IPC

4.1.1. Macro- and microcirculatory changes

Reperfusion after 60-min limb ischemia was not associated with significant changes in femoral artery blood flow. When IR was preceded by IPC, however, the postischemic femoral blood flow was significantly higher than the preischemic values throughout the examination period. Both CGRP receptor antagonism and sensory denervation with RTX reversed the

elevation in femoral blood flow during reperfusion after IPC+IR. Exogenous CGRP treatment followed by IR evoked effects similar to those seen after IPC+IR. The periosteal capillary RBCV in the IR group decreased during reperfusion in comparison with the preischemic value and no recovery was observed during the examination period. In this respect, IPC+IR caused a temporal restoration to the control value (at 60 min of reperfusion). The RBCV during reperfusion after $CGRP_{8-37}$ and RTX pretreatment combined with IPC was similar to that observed in IR animals. No decrease in postischemic RBCV was seen, however, when hCGRP was administered before ischemia. When $CGRP_{8-37}$ and RTX pretreatment was combined with IR without IPC (Series 2), both the femoral blood flow and the periosteal capillary RBCV were similar to those observed in vehicle-treated IR animals.

4.1.2. Changes in primary and secondary intravascular leukocyte activation and in periosteal ICAM-1 expression

The extent of leukocyte rolling in the periosteal postcapillary venules was approximately doubled in the IR group during the reperfusion phase. When limb IR was preceded by IPC, no significant increase in postischemic rolling was observed as compared with the baseline value. A rise in rolling leukocytes similar to that seen with IR was evident in the presence of CGRP receptor antagonism with $CGRP_{8-37}$, except for the moderately reduced values at the end of the examination period. Two weeks after RTX treatment, a significantly higher rolling PMN fraction was observed in the examined structures, with a subsequent postischemic rise similar to that seen after IR alone. In contrast, administration of the CGRP analog at the corresponding time point as in the IPC protocol (i.e. prior to the ischemia) resulted in significantly reduced interactions in comparison with that of IR. IR produced a significant, approximately 3-fold increase in the number of leukocytes showing firm adhesion (sticking) to the walls of postcapillary venules. The number of sticking leukocytes was significantly reduced if IR was preceded by IPC. This effect of IPC was reversed by both CGRP antagonism and depletion of the chemosensitive afferent nerves with RTX. The hCGRP pretreatment exerted a protective effect similar to that seen with IPC. The data from the second series of the experiments showed that $CGRP_{8-37}$ or RTX alone did not influence the IR-induced leukocyte rolling or adhesion. RTX caused a significant rise in the baseline values of rolling PMNs. In the IPC+IR and hCGRP group, a significantly lower ICAM-1 expression in the periosteal venules was observed than in the IR group. In the $CGRP_{8-37}$- and RTX-treated IPC+IR animals, the ICAM-1 values observed were similar to those seen in the IR group, and similar changes were evident when $CGRP_{8-37}$ and RTX treatment was applied in the presence of IR.

4.1.3. The effects of sensory nerve depletion of periosteal TRPV1 and CGRP expression

In periosteal tissue samples obtained from control animals, numerous nerve fibers and fiber bundles exhibited TRPV1 and CGRP immunoreactivity. The perivascular nerve fibers were frequently associated with small blood vessels which could be identified as arteries and arterioles. These fibers exhibited an almost complete overlap of the TRPV1 and CGRP immunoreactivities, suggesting their colocalization in periosteal sensory nerves.

4.2. Consequences of C/K-induced knee joint inflammation. The effects of diclophenac and PC on the consequences of arthritis induced by C/K

4.2.1. Changes in secondary hyperalgesic reactions of the hindlimb and knee joint swelling

In the first series, the extent of inflammation was estimated by means of functional tests 24 h after arthritis induction. The mechanical touch sensitivity was considerably increased in response to arthritis, as the C/K-injected limbs responded to a lower level of trigger (24.2±4.1 g mm^{-2}) than the saline-injected control limbs (77.7±5.1 g mm^{-2}) in animals receiving the saline vehicle. This parameter was significantly diminished in response to diclofenac and PC treatments, albeit complete restoration was not achieved. The thermal nociceptive latency was also significantly decreased in the saline-treated group (from the control level of 13.1±0.5 s to 7.2±0.7 s in the injured leg), and diclofenac and PC treatments exerted similar protective effects to that seen with the von Frey test. The changes in knee cross-section furnish a direct and objective measure of joint inflammation. The cross-sectional area in the C/K-injected knees was ~ 35% larger than that in the contralateral knees 48 h after the challenge, but was significantly reduced, by ~ 20%, by diclofenac and PC treatments; in the case of diclofenac, complete restoration to the level for the saline-injected knees was achieved.

4.2.2. Microcirculatory changes

In the second experimental series, the microcirculatory consequences of the joint inflammation were quantified via IVM, and the leukocyte-endothelial interactions (rolling and sticking) in the postcapillary venules of the synovial membrane were determined. The data relating to the rolling fraction of the PMNs in the postcapillary synovial venules exhibited a large degree of dispersion and no baseline differences could be observed between the C/K- and saline-injected knees or the groups which participated in the treatment protocols (data not shown). However, the injection of C/K was accompanied by a statistically significant, ~ 6-fold increase in PMN leukocyte adherence (sticking) to the endothelial layer as compared with the control side. This reaction was considerably reduced by PC (by ~ 40%, $p < 0.05$), but

was only moderately ameliorated by diclofenac treatment (by ~ 22%).

The RBCV in the capillaries of the synovial membrane was very high in both the control and the C/K-injected legs (1374±75 and 1111±89 μm s^{-1}, respectively) of the saline-treated animals. Neither diclofenac nor PC treatment influenced this parameter (1238±120 and 1150±129 μm s^{-1}, respectively).

4.2.3. Histomorphometric changes

Histomorphometric analysis revealed a definite increase in the tissue accumulation of PMNs, as evidenced by the increase in the number of infiltrating granulocytes in the perivascular regions at the end of the experimental protocol in each group. Likewise, immunohistochemical analysis indicated a considerable increase in the ICAM-1 immunoreactivity positivity in the venules, but not in the arterioles of the synovial membrane. Diclofenac administration did not influence these changes, whereas oral PC treatment significantly reduced the increases in both parameters.

4.3. Differences between periosteal and synovial microvascular inflammatory reactions in response to limb IR

4.3.1. Microhemodynamic changes

The baseline RBCVs in the periosteum were significantly lower than those in the synovial membrane. In response to IR, the RBCV decreased only in the periosteum by 180 min of reperfusion. The RBCVs in the capillaries of the synovial membrane did not change substantially in response to the IR insult, but were reduced in the limbs injected with C/K.

4.3.2. Microcirculatory leukocyte activation

No baseline differences were observed between the periosteum and the synovium in PMN rolling and adherence (sticking) to the endothelial layer of the postcapillary venules. Reperfusion after 60-min total limb ischemia was accompanied by a statistically significant (~ 160%) increase in PMN rolling and a more than 2-fold increase in PMN sticking in the periosteal venules. Significant increases were not observed in these parameters in the synovium at 180 min of reperfusion. The rolling and adherence attained significantly higher values in response to C/K injection.

4.3.3. Changes in XOR activity and MPO activity

Significant increases in XOR activity were observed in response to all of the challenges in all structures. The highest degree of increase was observed in response to C/K injection. IR caused similar extents of increase in this parameter in the synovium and the periosteum. Significant increases in MPO levels were not detected in response to IR in the periosteum or the synovium. In the C/K-treated limbs, however, the synovial MPO activity

was ~ 10-fold higher than in the contralateral limbs.

4.3.4. Tissue ICAM-1 expression

In response to IR, considerable increases in ICAM-1 immunoreactivity were observed in the venules of the periosteum, but not in those of the synovium. Injection of the joint cavity with C/K resulted in an increase in ICAM-1 expression similar to that in the postischemic periosteum.

5. DISCUSSION

5.1. Effects of different therapeutic interventions on the PMN-endothelial interactions

Study I demonstrated that the local inflammatory consequences of limb IR, as evidenced by the enhanced leukocyte-endothelial interactions in the periosteum, could be ameliorated effectively by IPC. The major finding of this study is that the protective effects of IPC against IR-induced inflammatory injury are mediated by sensory nerves which express the TRPV1 receptor and contain CGRP. The contributions of chemosensitive C-fiber afferents and the sensory neuropeptide CGRP to this phenomenon are supported by the observations that the protective effects of IPC were eliminated by administration of the CGRP antagonist $CGRP_{8-37}$ or by the RTX-induced depletion of C-fiber afferents. Moreover, systemic application of a CGRP analog produced decreases in leukocyte-endothelial cell interactions similar to those seen after IPC. These findings collectively suggest that activation of the chemosenstive C-fiber afferents and the release of CGRP from the activated nerve terminals during the induction of IPC may reduce the inflammatory reactions produced by IR.

In our models of limb IR and knee C/K arthritis, the local injury was manifested in significant increases in the primary and secondary forms of PMN-endothelial interactions (rolling and firm adherence) in the postcapillary venules of the periosteum and the synovium. Because of the crucial roles of PMN reactions in the early phase of inflammation, we set out to examine the effects of PC on leukocyte activation in the synovial membrane itself. IVM is a powerful tool for the on-line visualization of microcirculatory events which may be coupled to structural, histological analyses in the synovial tissue. In previous studies, however, the synovium was observed without opening of the joint capsule and this technique permitted visualization only of Hoffa's fatty body, intra-articular fatty tissue containing synovial cells on the interior surface of the joint. The methodology presented by our study allowed direct visualization of the synovial membrane. Injection of carrageenan resulted in an inflammatory response which was characterized by increases in primary and secondary leukocyte-

endothelial interactions. Considerable differences between groups occurred only in the number of sticking leukocytes, depending on the treatment applied. Specifically, although diclofenac obviously reduced the symptoms of arthritis (swelling and nociceptive reactions), only PC supplementation ameliorated the microcirculatory inflammatory reactions significantly. Histomorphometric analysis of leukocyte recruitment in the synovial tissue supported the IVM results: C/K injection induced a marked increase in the number of PMNs in the perivascular region of the synovium, which was moderated by PC supplementation. However, administration of diclofenac did not modify the perivascular infiltration of leukocytes significantly, suggesting that the anti-inflammatory effect of the NSAID compound could not be mediated by factors other than influencing the recruitment of PMNs in this case.

As for the simultaneous comparison of the synovial and the periosteal microcirculatory inflammatory reactions induced by IR (Study III), the increases in both the primary and secondary forms of PMN-endothelial interactions (rolling and firm adherence) were confined to the periosteal postcapillary venules, but these IR-induced reactions were virtually missing in the synovial membrane. The causes of tissue-specific ischemic tolerance have not yet been clarified, and many factors should be considered in this phenomenon. These include differences in oxidant-induced injury, ICAM-1 expression in the postcapillary endothelial cells and functional microhemorheology. In our study, C/K-induced osteoarthritis was used as a positive control because it is associated with severe tissue destruction known to be mediated by infiltrating PMNs. The MPO activity data relating to the final step of PMN-endothelial interactions showed that only C/K-induced arthritis was sufficient to bring about significant increases in tissue infiltration by PMNs within the examined time frame. Taken together, our data clearly suggest that PMN-mediated inflammatory reactions are less severe in the IR-challenged synovium than those in the periosteum or in response to arthritis.

5.2. Effects of different therapeutic interventions on the ICAM-1 expression

The increased tissue expression of ICAM-1 parallels the changes in PMN-endothelial interactions in all of the models examined. Among the endothelium-derived compounds, ICAM-1 is constitutively expressed on the endothelial surface and up-regulated in response to a variety of proinflammatory cytokines.

In Study I, the decreased tissue ICAM-1 expression in response to IPC and to exogenous CGRP clearly suggests a protective effect of physiological levels of CGRP on the leukocyte-endothelial cell interactions. Previous findings regarding the role of CGRP in ICAM-1-dependent PMN adhesion are rather controversial. An increased adhesion of PMNs

to endothelial cells can be demonstrated by CGRP *in vitro* and by capsaicin *in vivo* in human volunteers, suggesting a pro-inflammatory effect of TRPV1 stimulation on the endothelial cells. On the other hand, an ameliorating effect of CGRP on the activation of endothelial cells, adhesion molecule CD11b expression (by a cAMP-dependent mechanism) and the superoxide production of PMNs have been demonstrated *in vitro*. It is conceivable that, during the course of the capsaicin-induced neurogenic inflammatory response, the acute and massive release of neuropeptides produces high local tissue neuropeptide concentrations which most probably exceed the amount of mediators released by pathophysiological stimuli (*e.g.* IR injury) *in vivo.* Hence, a careful consideration of TRPV1-positive stimulation is warranted in the different experimental settings using capsaicin-evoked neurogenic inflammation.

In Study II, arthritis was associated with a significant increase in the expression of ICAM-1 on the endothelial cells of the postcapillary venules. The relationship between ICAM-1 and carrageenan-induced inflammation has been clearly proven in other models (such as pleurisy and air pouch inflammation). Inflammatory cytokines increase ICAM-1 expression primarily through the activation of gene transcription by NF-κB binding to the ICAM-1 promoter. The beneficial effect of PC on vascular ICAM-1 formation can be explained by its influence on TNF-α-induced NF-κB transcription. In a supplementary experimental series, we set out to validate the effect of PC on TNF-α-induced NF-κB transcription, and we therefore measured the TNF-α level in the synovial lavage fluid with ELISA. C/K injection resulted in an increase, but, owing to the high data dispersion, the increase was not found to be statistically significant. Interestingly, the lowest TNF-α values were found in the PC-treated animals. On the basis of these novel *in vivo* data, a TNF-α-related effect of PC cannot be excluded, but this issue requires further clarification.

In Study III, IR injury was associated with a significant increase in the amount of ICAM-1 expressed on the surface of the periosteal postcapillary endothelial cells, but not in the synovium. The presence of ICAM-1 on postcapillary endothelial cells in the inflammatory synovial microenvironment has been shown previously and has been proven to be an appropriate marker for the estimation of inflammation. Furthermore, based on previous observations of the inflamed synovium, adhesion molecules are expressed not only on the endothelial, but also on the interstitial cells. Accordingly, the C/K arthritis model provided a broad spectrum of microcirculatory inflammatory reactions, including enhanced ICAM-1 expression, demonstrating the lower microcirculatory vulnerability of the synovial membrane to IR.

5.3. Possible role of microhemorheological/perfusion changes in PMN-endothelial interactions

Microvascular injury is often accompanied by an increased heterogeneity of tissue perfusion, a reduction in functional capillary density and deteriorations in microvascular velocity and red blood cell deformability. It has also been recognized that expression levels of adhesion molecules and the consequent extent of leukocyte-endothelial interactions are strongly influenced by microhemodynamic parameters. As such, perfusion changes and secondary shear stress *per se* influence PMN activation and adhesive interactions by changing the dynamics and half-lives of molecular bonds. Alterations in microvascular perfusion are typical manifestations of the postischemic injury in the periosteum; hence, reduced microvascular velocities may influence the adhesion molecule expressions. In contrast, a higher velocity of PMNs results in higher degree of shear stress along the vessel walls, which reduces the possibility of firm adhesion to the endothelial cells.

Study I targeted the role of CGRP in the beneficial effects of limb IPC. CGRP is known to be one of the most potent vasodilator in the body and the main source of the circulating CGRP is the spillover release of CGRP from the perivascular nerve terminals. Blood vessels in all vascular beds are surrounded by a rich perivascular capsaicin-sensitive innervation, and receptors for CGRP have been identified in the media and intima of resistance vessels. A local vasodilatory effect of exogenous CGRP has also been shown, but the application of its specific antagonist $CGRP_{8-37}$ was without any effect. Hence, the role of endogenous CGRP in regulating the regional hemodynamics under resting conditions was reported to be rather unlikely. Furthermore, the postischemic macro- and microhemodynamic changes, and the anti-inflammatory effects of CGRP are probably independent of its presence in the (micro)circulation in view of the rather short half-life (~10 min) of the peptide. These data suggest that IPC and CGRP bring about their alleviating effect on cell-to-cell interaction not by affecting microhemodynamic changes, but rather by influencing adhesion molecule expressions directly.

C/K-induced local inflammation led to pronounced alterations in PMN-endothelial interactions, but not in RBCV values. PC did not influence microvascular velocities either. Hence, again, PC may potentially directly modulate adhesion molecule expressions and its action on PMN-endothelial interaction does not seem to be based on microhemodynamic differences. Clarification of the exact mechanism of its action requires further investigations.

Study III, however, provides clear evidence that the periosteum and the synovium are characterized by different microvascular velocities. Specifically, the velocities in the synovial

membrane were considerably higher than those measured in the periosteum, even under resting circumstances. Furthermore, the IR affected microcirculatory velocities in the periosteum, but not in the synovium. The impact of these findings cannot be accurately judged because, owing to methodological limitations, these studies did not provide data on velocities in the venular side. It is likely, however, that higher velocities in the capillaries are most probably accompanied by higher perfusions in the venules where PMN-endothelial interactions take place. The relatively high microvascular velocities most probably have potential anti-inflammatory consequences. Consequently, we propose that a higher synovial microvascular velocity and a lesser degree of deterioration in this parameter may be responsible, at least in part, for lower PMN-endothelial interactions in the postischemic microvasculature of the synovial membrane. This assumption is supported by the notion that C/K induces not only a deterioration in RBCV, but also significant increases in both primary and secondary PMN-endothelial interactions in the synovial membrane. Further studies are required to assess the potential link between microhemorheological and adhesion molecule expression changes in the synovium and other tissue types.

6. SUMMARY OF NEW FINDINGS

- Osteoarthritis is accompanied by PMN-driven inflammatory reactions in the synovial membrane, which can be quantified appropriately by our new method using IVM.
- Limb IPC reverses the periosteal microcirculatory inflammatory reactions in which CGRP, released from the chemosensitive afferent nerves, plays a decisive role. This protection is manifested in an amelioration of leukocyte-endothelial interactions and endothelium-derived adhesion molecule expression. Controlled activation of chemosensitive afferent nerves by IPC may provide potential therapeutic benefits against IR-induced PMN reactions in the periosteum.
- Limb IR induces different microcirculatory consequences in the periosteum and the synovium. A postischemic deterioration of the microvascular perfusion, activation of PMN-endothelial interactions and an increased adhesion molecule expression occur in the periosteum, but these reactions are less intense in the synovium. The higher baseline microcirculatory velocities and a lesser degree of postischemic perfusion deficit in the synovium may explain the lower susceptibility of the synovial microcirculation to IR injury.
- Similarly to diclofenac, exogenous PC, ameliorates knee joint inflammation, adhesion and the tissue accumulation of PMNs; the potential therapeutic benefit provided by PC may

result from the reduction of PMN-mediated inflammatory pathways.

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