Oxaceprol, an atypical inhibitor of inflammation, reduces leukocyte adherence in mouse antigen-induced arthritis

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ABSTRACT – Oxaceprol (N-acetyl-L-hydroxyproline), an atypical inhibitor of inflammation, is an established drug for joint disease without serious side-effects. Recent studies have emphasized that oxaceprol has an effect on the microcirculation. Since the exact mechanism of action remains unclear, the aim of our study was to investigate the leukocyte-endothelial cell interactions in oxaceprol-treated mice with antigen-induced arthritis (AiA) using intravital microscopy. In our study, Balb/c mice were allocated to 4 groups (n 7, 8, 8, 8): 2 control groups with saline or oxaceprol and 2 groups of arthritic animals which received saline or oxaceprol (100 mg/kg twice a day intraperitoneally). The severity of arthritis was quantified by the transverse knee joint diameter. For the intravital fluorescence microscopy measurements on day 10 after inducing arthritis, the patella tendon was partly resected to visualize the intraarticular synovial tissue of the knee joint. The number of rolling and adherent leukocytes as well as RBC velocity and functional capillary density (FCD) were quantified in synovial microvessels. Furthermore, leukocyte infiltration was determined in the histological sections with an established score.

No significant changes in mean arterial blood pressure or functional capillary density were found in any of the groups. However, the leukocyte rolling fraction and number of leukocytes adherent to the endothelium were increased in postcapillary venules of the synovium in arthritic animals (0.16 to 0.31, 78 cells/mm^2 to 220 cells/mm^2). In animals with AiA treated with oxaceprol, leukocyte adherence and swelling were significantly reduced in comparison to the arthritic animals treated with saline. Furthermore, the histological score showed less leukocyte infiltration in the oxaceprol-treated arthritic animals. Thus, oxaceprol reduces leukocyte adherence in vivo and leukocyte infiltration in mouse AiA, indicating an effect on synovial microcirculation.

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Oxaceprol (N-acetyl-L-hydroxyproline) is used to treat osteoarthrosis and rheumatoid arthritis. Its clinical efficacy is similar to the classical non-steroidal anti-inflammatory drugs (NSAID) ibuprofen and diclofenac for relief of pain (Franz 1992, Steinbach and Bauer 1995, Bauer et al. 1999). However, unlike these NSAIDs, it causes a low incidence of gastrointestinal side-effects since it does not inhibit prostaglandin synthesis (Ionac et al. 1996). Previous in vitro studies have shown that oxaceprol reduced leukocyte adherence to cultured endothelial cells (Hallmann et al. 1996). Furthermore, in an experimental arthritis model, it has been shown to reduce leukocyte extravasation (Ionac et al. 1996). In a previous in vivo study, we found that oxaceprol reduced leukocyte adherence to postcapillary venules during ischemia/reperfusion in the dorsal skinfold chamber of the awake Syrian golden hamster (Harris et al. 1998). It is well known that leukocyte adhesion and subsequent extravasation play a major role in rheumatoid arthritis and to a lesser extent in osteoarthrosis (Sewell and Trentham 1993). Since the specific mechanisms of action of oxaceprol are still not understood, we hypothesized that it might reduce leukocyte accumulation in mouse antigen-induced arthritis (AiA).

We studied the in vivo effects of oxaceprol on

the microcirculation of synovial tissue and clinical activity during antigen-induced arthritis in Balb/c mice.

Methods

Animals

Female Balb/c mice (20 g body weight) were obtained from Charles River Wiga, Sulzbach, Germany. Before the experiment, they were housed in groups of 10 animals and after induction of the arthritis, they were caged individually. Before starting the experiment, they were assigned to the vehicle-treated control group or the Oxaceprol-treated group.

Animal drop-out

40 animals were used for the study. Of these, 9 had to be excluded due to complications during surgical preparation. They were randomly assigned to the control (n 7), control with oxaceprol (n 8) or AiA with saline (n 8) or AiA with Oxaceprol group (n 8).

Test drug oxaceprol

The drug was obtained from CHEPHASAAR company, Chem.-pharm. Fabrik GmbH, St. Ingbert, Germany and stored at room temperature. It was administered intraperitoneally twice daily in a dose of 100 mg/kg body weight dissolved in 200 mL saline starting on the day of arthritis induction until the intravital microscopic measurements were made on day 8 after AiA induction. Control animals received equivalent volumes of saline (0.9% NaCl).

Antigen-induced arthritis

Arthritis was induced in mice using Brackertz et al.'s method (1977). On days –21 and –14, they were first immunized with a subcutaneous injection of 100 μg of methylated bovine serum albumin (mBSA) (Sigma, Deisenhofen, Germany), dissolved in 50 μL of saline. The mixture was supplemented with complete Freund's adjuvant (CFA) (Sigma) and 2 mg/mL of heat-killed Mycobacterium tuberculosis strain H37RA (Difco, Augsburg, Germany). In accord with the protocol, we gave additional intraperitoneal injections of 2×10^9 heat-killed Bordetella pertussis (Institute of Microbiology, Berlin, Germany) on days –21 and –14. Finally, arthritis was induced on day 0 by injecting of 100 μg of mBSA dissolved in 50 μL saline into the left knee joint. Control groups underwent the same procedure, but only received equivalent volumes of saline. The severity of antigen-induced arthritis was determined with the clinical and histological score described below.

Clinical assessment

Joint swelling was determined by measuring the transverse diameter of the knee joint with caliper in units of 0.1 mm.

Surgical preparation

Intravital fluorescence microscopy was performed in the synovial microcirculation of the mouse knee. This model permits intravital study of microhemodynamic parameters, including vessel diameter and red cell velocity in the microvascular network of the synovial microcirculation consisting of arterioles, capillaries, and postcapillary venules contained in the observation window, and quantitative assessment of functional capillary density and leukocyte-endothelial cell interactions (Veihelmann et al. 1998).

For the surgical preparation, anesthesia was induced by inhalation of isoflurane 1.2% and a combination of O_2/N_2O. The mean arterial blood pressure was determined with an arterial catheter inserted into the tail artery and connected to a pressure transducer. The microsurgical procedure was performed as described elsewhere (Veihelmann et al. 1998). Animals were kept on a heating pad to stabilize body temperature, which was controlled by a rectal probe. The left hind limb was placed on a stage with the knee slightly flexed. Immobilization of the extremity was secured by fixation with silicon. After a 1-cm incision distal to the patellar tendon, we made a partial skin resection. The patellar tendon was carefully mobilized and partly resected. Then the intraarticular synovial tissue of the knee joint was visualized. After superfusion with 2 mL of sterile saline, a cover glass was placed on the knee capsule and the intravital microscope was directed onto the synovium (Veihelmann et al. 1998). Special care was taken to avoid any constraints on the preparation. The ani-

mals were killed with 10 mg of pentobarbital intravenously after intravital microscopy.

Experiment protocol

The microscopic set-up has been described in detail elsewhere (Harris et al. 1997). A 20-fold water immersion objective was used to select 2–4 regions of interest in each animal. These 4 regions contained postcapillary venules (18–35 μm in diam.) or capillary areas for the measurement of FCD or both. To measure the leukocyte-endothelial cell interactions, the fluorescent marker rhodamine 6G (Sigma) was injected intravenously in a single bolus of 0.15 mg/kg immediately before the measurement. Rhodamine epiillumination was achieved with a 150 Watt variable HBO mercury lamp in conjunction with Zeiss filter set 15 (BP 546/12, FT 580, LP 590). The FITC measurements were made using a variable 12 V, 100 W halogen light source and the Zeiss filter set 09 (band pass (BP) 450–490, Farbteiler (FT) 510, long pass (LP) 520, Zeiss). Measurements of vessel diameter, venular RBC velocity and FCD were made after a bolus injection of the in vivo fluorescent plasma marker FITC-dextran (mol mass 150 kDa; 15 mg/kg body wt i.v.) (Sigma). The microscopic images were captured with a CLD camera and recorded on S-VHS videotape using both filter blocks consecutively. Data analysis was performed off-line using a computer-assisted microcirculation analysis system (Zeintl et al. 1989).

Microcirculatory parameters

From the Rhodamine-stained video images, the leukocyte endothelial cell interactions were quantified. Leukocytes interacting with the endothelium were classified as rolling or adherent cells. Rolling leukocytes were defined as cells that intermittently interact with the endothelial surface and pass through the vessel visibly slower than red blood cells in the centerline stream. To account for vessels of various diameters, flows and leukocyte fluxes, rolling leukocytes were divided by the total number of leukocytes passing through a plane on the vessel segment during the 30-second observation time (calculated as rolling cells/rolling + non-adherent cells to be independent from thetotal leukocyte count). Adherent leukocytes are defined as those leukocytes that remain attached to the same location on the endothelial surface for the entire observation period of 30 seconds (expressed as cells/mm^2). The endothelial cell surface was calculated from diameter and length (200 (m) of the vessel segment under investigation. We determined the vessel diameter, RBC velocity and FCD from the FITC images. RBC centerline velocity in postcapillary venules was assessed with Cap-Image and expressed as mm/second (Zeintl et al. 1989). FCD is defined as the length of RBC-perfused capillaries in the observation area (expressed as cm/cm^2).

Histology

After killing the animal, the knee joint was removed and fixed with 8% paraformaldehyde. After fixation in paraformaldehyde 8% at pH 7.2 over 12 hours, the joints were incubated in 20% EDTA at pH 7.2 for 3 days at room temperature to decalcify the bone. Samples were washed with phosphate buffer saline (PBS) and dehydrated with an automatic dehydrator model. After embedding in paraffin, the joint was sliced into 3 μm-thick sections that were stained with hematoxylin and eosin.

To evaluate the severity of the arthritis and leukocyte infiltration, we used the histogical score introduced by Brackertz et al. (1977): 0 = normal knee joint, 1 = normal synovium with occasional mononuclear cells, 2 = two or more synovial lining cells and perivascular infiltrates of leukocytes, 3 = marked hyperplasia of synovium and dense infiltration of leucocytes (not only perivascular), 4 = synovitis, pannus formation, and cartilage/subchondral bone erosions. In each animal, two cuts were performed and in each section, the number of infiltrated leukocytes was counted in 5 fields.

Statistics

For differences within the group, we used Kruskal-Wallis analysis followed by an all pairwise multiple comparison procedure. The level of significance was set at $p < 0.05$.

All experiments complied with the current laws of the German government.

Results

Microcirculation and clinical analysis

No significant change in the mean arterial blood

Table 1. Macro- and microhemodynamic parameters

Parameter	Control (n 7)	Control + Oxa (n 8)	AiA (n 8)	AiA + Oxa (n 8)
MAP (mm Hg)	77 (7)	75 (4)	70 (7)	69 (4)
Venular diameter (mm)	22 (2.1)	25 (2.3)	27 (1.9)	25 (3.1)
FCD (cm/cm^2)	244 (39)	241 (14)	262 (42)	264 (13)
RBC velocity (mm/sec)	0.8 (0.02)	0.72 (0.03)	0.47 (0.07) [a]	0.54 (0.1)

Figures in parentheses are SEM. MAP mean arterial pressure, AiA antigen-induced arthritis, Oxa oxaceprol, FCD functional capillary density, RBC red blood cell. Diameter, FCD, and RBC velocity were assessed using a computer-assisted microcirculation analysis system. P-values < 0.05 were considered significant.
[a] $p < 0.05$ vs. control

Table 2. Swelling and leukocyte-endothelial cell interactions

Parameter	Control (n 7)	Control + Oxa (n 8)	AiA (n 8)	AiA + Oxa (n 8)
▲ Diameter LKJ (mm)	0.03 (0.02)	0.03 (0.03)	0.57 (0.04) [a]	0.1 (0.02) [ab]
Leukocyte rolling fraction	0.18 (0.02)	0.17 (0.04)	0.31 (0.03) [a]	0.23 (0.03)
Leukocyte adhesion (cells/mm^{-2})	87 (12)	108 (24)	211 (31) [a]	98 (18) [b]

Figures in parentheses are SEM. AiA antigen-induced arthritis, Oxa oxaceprol, ▲ Diameter LKJ – change in left knee joint diameter given as increase (mm). Data are given as mean. P-values < 0.05 were considered significant.
[a] $p < 0.05$ vs. control
[b] $p < 0.05$ vs. AiA.

pressure occurred during the entire course of the experiment in the 4 groups. Moreover, the venular diameters and FCD were similar in the 4 groups (Table 1). However, in the AiA group, we found a significant reduction in venular RBC velocity ($p = 0.03$), compared to the control values and the values of the AiA group treated with oxaceprol (Table 1). In the AiA group, the rolling fraction increased significantly more than in the controls ($p = 0.01$, Table 2). The number of leukocytes adherent to the endothelium was significantly greater in the arthritic animals than in the control groups and the AiA group treated with oxaceprol ($p = 0.002$, Table 2). Treatment with oxaceprol tended to reduce leukocyte rolling and significantly reducted leukocyte adherence ($p = 0.004$) similar to the values of those in the control groups. The treatment also caused a significant reduction in swelling, compared to the AiA group without treatment ($p = 0.001$, Table 2).

Histological examination

We found a dense infiltration of leukocytes in the synovium and synovial hyperplasia in the group with AiA (score 3, $p = 0.002$, Table 3). Oxaceprol reduced the extravasation of leukocytes in the synovium (only moderate infiltration and synovial lining cell proliferation), compared to the AiA group using the score of Brackertz et al. (1977) (score 2, Table 3). The difference was significant ($p = 0.003$) in the quantified count of the histological cuts (Table 3).

Discussion

Our findings show that treatment with oxaceprol significantly protects the microvasculature against the disturbances due to AiA. In addition, the reduction in leukocyte adherence also preserves tissue perfusion in mouse AiA.

Table 3. Histological score given as the median and quantitative leukocyte infiltration

Groups	Histological score	Extravasated leukocytes
Control, n 7	0	9 (4)
Control + Oxa, n 8	0	7 (4)
AiA, n 8	3 (1)	100 (18) [a]
AiA + Oxa, n 8	2 (1)	39 (12) [a b]

Figures in parentheses are SEM, AiA antigen-induced arthritis, Oxa oxaceprol. Histological sections graded as: 0 normal knee joint, 1 normal synovium with occasional mononuclear cells, 2 two or more synovial lining cells and perivascular infiltrates of leukocytes, 3 hyperplasia of synovium and dense infiltration, 4 synovitis and pannus formation and cartilage/subchondral bone erosions (Brackertz et al. 1977). Extravasated leukocyte measurements were made in 2 histological cuts in each animal. In each section, we counted the number of infiltrated leukocytes in 5 fields. Data are given as mean (SEM) (given as number per observation field).
P-values < 0.05 were considered significant.
[a] $p < 0.05$ vs. control
[b] $p < 0.05$ vs. AiA.

Antigen-induced arthritis is an established animal model for the study of human rheumatoid arthritis (Brackertz et al. 1977). The fact that AiA is not inducible in nude mice underlines the involvement of T-cells in the pathomechanism of this model which is in agreement with human RA. The main advantage of AiA in comparison to other established animal models is that arthritis is induced in the knee joint which is necessary for our intravital microscopic observations. The knee is the only joint which can be accessed for intravital microscopy without inducing major trauma (Veihelmann et al. 1998). In this study, arthritis was actually induced in the knees, as shown by swelling during the entire observation period. The severity of arthritis was shown by the high histological score. Leukocyte infiltration and pannus formation were seen in almost all animals with AiA. The synovial microcirculation model used in this study has also been found to be a stable preparation for at least 60 minutes (Veihelmann et al. 1998). Thus, the microvascular changes in this study are due to AiA and are not an artifact due to the surgical preparation. In our laboratory, we have established that leukocyte-endothelial cell interactions are significantly enhanced in different phases of AiA (Veihelmann et al. 1999).

Oxaceprol has no effect on local microhemodynamic parameters since there were no differences between the two control groups and the arthritic group treated with oxaceprol in the microvascular diameter, RBC velocity or FCD. This is an important finding since it also means that the shear stress acting on the rolling and adherent leukocytes is the same in the other groups. Thus, as reported by Harris et al. (1998), any differences encountered in the rolling and adherence must be due to changes in the leukocytes or endothelial cells which affect the adhesive forces between the two.

The reduction in RBC velocity in the AiA group is the expected reactive response to the increase in joint pressure due to the swelling. Furthermore, the reduction in swelling and the enhancement of RBC velocity in arthritic animals treated with oxaceprol indicates that the drug may partly preserve tissue perfusion during AiA.

The increase in rolling after AiA was only slightly, but not significantly, attenuated in the AiA group treated with oxaceprol. This was also true of the number of adherent leukocytes, but the difference was significant. This increase in leukocyte rolling and adherence after AiA indicates an expected activation of the leukocytes. Furthermore, this means that oxaceprol affects leukocyte rolling and adherence, although the mechanisms for this are still not understood.

Moreover, treatment with oxaceprol causes a significant reduction in the arthritic swelling of the knee joints. These results provide the first in vivo evidence of this effect of the drug in experimental arthritis.

The reduction in leukocyte adherence helps to protect the integrity of the endothelium to the extent that the macromolecular leakage is reduced, although we can not prove this because synovial capillaries are highly fenestrated (Levick 1995). Thus, the marker FITC-dextran extravasates immediately after application. However, these findings indicate that microvascular perfusion is maintained and that the endothelial barrier remains intact, as shown by the reduction in leukocyte infiltration in the histological sections.

The definite reduction in leukocyte infiltration, as judged by the difference in histological score and in the quantitative measurements of the histological sections, suggests that oxaceprol is very

effective in inhibiting leukocyte emigration. Moreover, since the difference in the histological score between 2–3 is considerable, this corresponds to a significant decrease in knee joint diameter. This accords with previous in vivo studies of experimental arthritis which showed a similar effect in rat adjuvant arthritis (Ionac et al. 1996). The mechanism for this effect is not known. It seems likely that the marked reduction in leukocyte adherence is partly responsible for this decrease (Parnham 1999). However, other mechanisms involving the endothelium and adhesion molecules (i.e., PECAM's) are also possible, such as effects on expression of adhesion molecules on leukocytes or on the synovial endothelium. In rheumatoid arthritis, the presence of proinflammatory cytokines such as TNF-α, IFN-γ, and IL-1 can cause activation of leukocytes and endothelial cells. Such activation can lead to the upregulation of various adhesion molecules (Gearing and Newman 1993). Histological studies in experimental arthritis and in arthritic patients have shown that there is an enhanced expression of ICAM-1 and E-selectin on infiltrating mononuclear cells and synovial endothelium (Mojcik and Shevach 1997). In mouse antigen-induced arthritis, we found a phase-dependent expression of various adhesion molecules (ICAM-1, P-selectin and E-selectin) on leukocytes and endothelial cells of the synovium (Veihelmann et al. 1999). More studies must determine whether oxaceprol directly or indirectly affects the expression of specific endothelial or cellular adhesion molecules which inhibit leukocyte infitration into inflamed tissue.

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Bauer H W, Klasser M, von Hanstein K L, Rolinger H, Schladitz G, Henke H D, Gimbel W, Steinbach K. Oxaceprol is as effective as diclofenac in the therapy of osteoarthritis of the knee and hip. Clin Rheumatol 1999; 18: 4-9.

Brackertz D, Mitchell G F, Mackay I R. Antigen-induced arthritis in various strains of mice. Arthritis Rheum 1977; 20: 841-50.

Franz M. Die Behandlung degenerativer Gelenkerkrankungen mit Oxaceprol. Rheuma 1992; 12: 137-41.

Gearing A J H, Newman W. Circulation in disease. Immunol Today 1993; 14: 506-12.

Hallmann R, Zhou X, Brune K, Zimmermann U. Blockade der Leukozytenadhäsion an Gefässendothel. Z Rheumatol 1996; 55: 58.

Harris A G, Hecht R, Peer F, Nolte D, Messmer K. An improved intravital microscopy system. Int J Microcirc Clin Exp 1997; 17: 322-7.

Harris A G, Schropp A, Messmer K. Effects of Oxaceprol on the microcirculation in ischemia/reperfusion injury. Eur J Med Res 1998; 3: 182-8.

Ionac M, Parnham M J, Plauchithiu M, Brune K. Oxaceprol, an atypical inhibitor of inflammation and joint damage. Pharmacol Res 1996; 33: 367-74.

Levick J R. Microvascular architecture and exchange in synovial joints. Microcirculation 1995; 2: 217-33.

Mojcik C F, Shevach E M. Adhesion molecules. Arthritis Rheum 1997; 40: 991-1004.

Parnham M J. Antirheumatic agents and leukocyte recruitment. New light on the mechanism of action of oxaceprol. Biochem Pharmacol 1999; 58: 209-15

Sewell K L, Trentham D E. Pathogenesis of rheumatoid arthritis. Lancet 1993; 341: 283-6.

Steinbach K, Bauer H W. Klinischer Vergleich von Oxaceprol und Diclofenac bei Gonarthrosen und Coxarthrosen. Extracta Orthop 1995; 18: 18-21.

Veihelmann A, Szczesny G, Nolte D, Krombach F, Refior H J, Messmer K. A novel model for the study of synovial microcirculation in the mouse knee joint in vivo. Res Exp Med 1998; 198: 43-54.

Veihelmann A, Harris A G, Krombach F, Schütze E, Refior H J, Messmer K. In vivo assessment of synovial microcirculation and leukocyte-endothelial cell interaction in mouse antigen-induced arthritis. Microcirc 1999; 6: 281-90.

Zeintl H, Sack F-U, Intaglietta M, Messmer K. Computer-assisted leukocyte velocity measurements in intravital microscopy. Int J Microcirc Clin Exp 1989; 8: 293-302.