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Inflammation Research

Endothelial iNOS versus platelet iNOS: Responsibility for the platelet/leukocyte endothelial cell interaction in murine antigen induced arthritis *in vivo*

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Abstract. *Objective.* Since an increase of platelet-endothelial cell interactions has been observed in mice with Antigen-induced-Arthritis (AiA) as well as an increase of NO expression, the aim of our study was to investigate in vivo the influence of NO, especially the platelet and endothelial inducible NO Synthase, on the platelet- and leukocyte endothelial cell interaction.
Material and Methods. C57/Bl6 mice and iNOS deficient mice were disposed in 6 groups (each=7). After induction of AiA, rolling and adherent fluorescence labelled platelets and leukocytes were investigated by intravital microscopy (IVM) on day 8 after AiA. Rank SUM Test and ANOVA on ranks have been performed regarding the data.
Results. All arthritic mice presented an increase in platelet and leukocyte interaction with the endothelium compared to control groups. The arthritic iNOS deficient mice showed a more intense interaction of platelets and leukocytes with the endothelium in comparison with the wild-type arthritic mice. The group using arthritic wild-type recipient and iNOS deficient donor mice showed an increase in cell-interactions, leading to an endothelial effect, compared to the group using iNOS deficient arthritic recipient and wild-type donor mice.
Conclusion. The IVM data lead to an anti-inflammatory effect of NO, since NO followed an increase in platelet- and leukocyte- endothelial cell interaction in iNOS deficient mice with AiA. In addition, we have shown for the first time in vivo that platelet NO produced by iNOS seems to have a minor influence on the leukocyte induced tissue damage in contrast to endothelial iNOS. Therefore, selective platelet inhibition would not interfere with the protective effect of NO.

Key words: Platelets – Nitric oxide (NO) – Arthritis – Mouse – Intravital microscopy

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease whose causes have not yet been solved so far. It is a progressive issue leading to the final outcome of the disease, such as deformity, disability and joint destruction [1, 2]. So far it is known that the endothelium cell damage is responsible for the destruction and the progression of the disease. The role of leukocytes, aggravating the inflammation, has been investigated *in vivo* in recent years [3]. The role of platelets is not yet fully understood. But it is known that platelets when they are activated, release proinflammatory mediators [4–9] and have an interaction with leukocyte activation that might cause further damage [10–12]. Furthermore, we have recently shown an increasing interaction of platelets in Antigen-induced Arthritis (AiA) *in vivo* [13].

A chronic inflammatory disease, as the RA, is known to be related with an increased production of nitric oxide (NO). NO is synthesized from arginine in a reaction catalyzed by NO synthase (NOS), of which several isoenzymes exist. The two major isoencymes known are the inducible (iNOS) and the constitutive form (cNOS). The cNOS is mainly expressed in neuronal cells, partly in endothelial cells, and produces continuously few amounts of NO. The other form, the iNOS, is induced by proinflammatory cytokines and bacterial products [14] and is expressed in different cell types, such as platelets, neutrophils, endothelial cells, macrophages, chondrocytes and synovial fibroblasts. A few hours after stimulation the maximum of NO is produced by iNOS [15–17]. The effect of NO is not yet fully clear, so far there have been shown proinflammatory, toxic, as well as anti-inflammatory effects [18–20].

In earlier studies, the inhibition of NOS enhanced leukocyte-endothelial cell interactions and migration of activated leukocytes into inflamed tissue [21–23]. Also Veihelmann et al was able to prove similar results, using selective versus non-selective iNOS/NOS inhibition [24, 25].

In combination with prostacyclin, NO also regulates the adhesion and aggregation of activated platelets [26, 27]. In vitro research proved a reduction in "shape change" of platelets, as well as the expression of adhesion molecules, e. g. GPIIb/IIIa, P-selectin, by thrombin and U-46619 activated platelets [28]. The secretion reaction of platelets is regulated by NO, too [29, 30]. A reduction of the agonist-induced increase of the intracellular calcium concentration by NO might be responsible for that. The recruitment and activation of platelets is not only regulated by endothelial cells, but also by activated platelets and their own NO production [31, 32].

Since, there have been no *in vivo* studies so far, which demonstrate the admission of NO on the platelet-endothelial cell interaction in the synovial microcirculation, the aim of our present study was to investigate *in vivo*, the influence of NO by differentiation between platelet and endothelial iNOS on the platelet- and leukocyte-endothelial-cell interactions. iNOS deficient platelets were used of an inbred donor gathered by cardiac punction in recipient wild-type mice and vice versa.

For achieving these results we used a model for intravital microscopic analysis of the mouse synovial microvasculature in association with transgenic iNOS deficient mice [33].

Materials and methods

Animals

Female inbread C57/Bl6 mice and B6.129P2-*Nos2*^{tm1Lau}/J (iNOS deficient) (Charles River, Sulzfeld, Germany) weighting 18–24 g were used for the experiments. The experiments were approved and performed according to the German legislation for the protection of animals. The animals were kept in an air-conditioned environment with 12 h light/dark cycles, housed in single cages and fed laboratory chow (Ssniff, Spezialdiäten, Soest, Germany) and water ad libitum. Before starting the experiments, they were randomly assigned to controls without AiA either containing C57/Bl6 or iNOS-deficient animals or to identically assembled experimental groups with AiA (n = 7). In addition to those groups, we used two groups using either C57/Bl6 or iNOS deficient AiA animals with opposite blood donor to differentiate between endothelial and platelet involvement. To asses the severity of AiA, joint swelling was determined daily until day 8 by measuring the transverse diameter of the knee joint using a calliper in units of 0.1 mm. Intravital microscopy as well as tissue sampling were performed on day 8 after induction of arthritis in all groups.

Antigen-induced Arthritis (AiA)

On days –21 and –14 prior to the induction of arthritis mice were immunized by a subcutaneous injection in the left flank of 100 µg of methylated bovine serum albumin (mBSA) (Sigma, Taufkirchen, Germany), dissolved in 50 µl of Freund's complete adjuvant (Sigma, Taufkirchen, Germany) and supplemented with 2 mg/ml heat-killed Mycobacterium tuberculosis strain H37RA (Difco, Augsburg, Germany) and an additional intraperitoneal injection of 2×10^9 heat killed Bordetella pertussis (Institute of Microbiology, Berlin, Germany) as previously described by Brackertz et al. [34]. Finally, arthritis was induced on day 0 by injection of 100 µg mBSA in 50 µl saline into the left knee joint using a sterile 33-gauge micro canola. Control animals underwent the same procedure but received equivalent volumes of saline into the knee joint.

Surgical preparation

Mice were anesthetized by inhalation of isoflurane 1,5 % (Forence, Abbott, Wiesbaden, Germany) and a mixture of $0_2/N_2O$, using a vaporizer (Dräger, Lübeck, Germany). Arterial and venous catheters were implanted into the tail. The mean arterial blood pressure was measured using the arterial catheter. Animals were kept on a heating pad to stabilize body temperature. The left hind limb was placed on a stage with the knee slightly flexed. Through a 1 cm incision distal to the patellar tendon, the tendon was carefully mobilized, cut transversally, and elevated to provide visual access to the intraarticular synovial tissue as described elsewhere [3]. At the end of the experiment, animals were sacrificed with an intravenous injection of 10 mg pentobarbital (Nembutal, Sanofi, Hannover, Germany). The knee joints were then removed for histology and immunostaining.

Blood sampling and platelet preparation

For intravital fluorescence microscopy, separation and ex vivo fluorescent labelling of platelets was based on a previously described protocol [35]. One millilitre blood from a donor mouse was harvested by cardiac puncture and collected in polypropylene tubes containing 0.2 ml volume of 38 mmol/l citratic acid/75 mmol/l trisodium citrate/100 mmol/l dextrose, 15 µl prostaglandin E1 (PGE1) (Sigma, Taufkirchen, Germany) and 0.5 ml Dulbecco phosphate-buffered saline (D-PBS) (PAN Systems, Aidenbach, Germany). Blood was centrifuged at 900 U/min for 10 min. Platelet-rich plasma was gently transferred to a fresh tube containing 1.5 ml D-PBS, 0.4 ml volume of 38 mmol/l citratic acid/75 mmol/l trisodium citrate/100 mmol/l dextrose and 50 µl PGE1. The fluorescent marker aminoreactive succinmidylester carboxyfluorescein-diacetat (CFDA-SE) (MW 535, Molecular Probes, Eugene, OR) was added to label platelets *in vitro*, and the blood was centrifuged at 3,000 U/min for 10 min. The platelet pellet was resuspended in 0.3 ml D-PBS. The purity of the sample and platelet concentration was determined before infusion using a Coulter A^{c}T Counter (Coulter Corp, Miami, FL). A total of 100×10^6 fluorescently labelled platelets were transfused via the lateral tail vein, corresponding to approximately 10 % of all circulating platelets [36], shortly before starting IVM.

Adequate functionality of fluorescently labelled platelets has been evaluated by flow cytometric analysis (FACSort flow cytometer; Becton Dickinson; Heidelberg; Germany) before and following *in vitro* activation by phorbol-mystrate-acetate (PMA), (Sigma, Taufkirchen, Germany).

Intravital fluorescence microscopy

The microscopic setup has been described in detail previously [37]. An intravital microscope (Zeiss, Oberkochen, Germany) equipped with a 20 fold water immersion objective (total magnification 432 fold: Zeiss, Jena, Germany) was used to visualize the synovial microcirculation. Four to six nonoverlapping regions of interest (ROI) were selected, each containing postcapillary venules, as well as capillaries for the measurement of functional capillary density (FCD). By means of a computer controlled platform which moves step by step driven by the computer, identical vessel segments were reinvestigated with respect to the parameters described above. CFDA-labelled platelets were visualized after intravenously injection using a variable 12 V, 100 W halogen light source and the Zeiss filter set 09 (Band pass [BP] 450–490, dichroic filter [FT] 510, long pass [LP] 520; Zeiss). For *in vivo* labelling of the leukocytes in the next step, the fluorescent marker rhodamine 6G (Sigma, Taufkirchen, Germany) was injected intravenously as a single bolus of 0.15 mg/kg immediately prior to measurement, after examination of platelets. Rhodamine epillumination was achieved using a 150 W variable HBO mercury lamp in conjunction with the Zeiss filter 15 (BP 546/12, FT 580, LP 590). The use of different fluorescence filter sets allowed selective visualization of either CFDA-labelled platelets or leukocytes labelled by Rhodamin 6G. At the end of the experiment, measurements of vessel diameter, venular red blood cell (RBC) velocity and FCD were made using the Zeiss filter set 09 in conjunction with a bolus injection of fluorescein isothiocyanate (FITC)-Dextran (molecular mass 150 kd, 15 mg/kg body weight intravenously; Sigma, Taufkirchen, Germany). Microscopic im-

Table 1. Macro- and microhemodynamic parameters.

Parameter	Controls			
	C57/Bl6		iNOS –/–	
MAP, mm Hg	80 ± 3		82 ± 4	
Vessel diameter (µm)	12.4 ± 0.9		10.7 ± 0.7	
FCD (cm/cm^2)	255.6 ± 14.9		298.1 ± 5.2	
RBC velocity (mm/s)	0.43 ± 0.05		0.58 ± 0.11	
Parameter		AiA		
	iNOS+/+	iNOS–/–	iNOS+/–	iNOS–/+
MAP, mm Hg	85 ± 3	77±5	79 ± 2	82 ± 2
Vessel diameter (µm)	11.9 ± 0.3	9.9 ± 0.2	11.4 ± 1.0	11.8 ± 0.5
FCD (cm/cm^2)	334.7 ± 8.6	279.0 ± 9.3	277.3 ± 6.5	313.9 ± 7.7
RBC velocity (mm/s)	0.38 ± 0.04	0.5 ± 0.04	0.49 ± 0.08	0.25 ± 0.01

MAP = mean arterial pressure; FCD = functional capillary density; RBC = red blood cell. Diameter, FCD and RBC velocity were assessed by computer- assisted image analysis. Data are given as means ±SEM.

ages were acquired and recorded on videotape. Data analysis was performed off-line using a computer-assisted analysis system (CAP-Image, Dr. Zeintl, Heidelberg, Germany) [38].

Microcirculatory parameters

The following microcirculatory parameters were assessed using intravital microscopy: Diameters of venules (in micrometers), functional capillary density (FCD), defined as the length of red blood cell perfused capillaries respectively vessels within the observation area (expressed as cm/cm^2) and centreline red blood cell velocity (RBC) (millimetres per second) were determined. Platelets and leukocytes were classified according to their interaction with the endothelial cell lining as free flowing, rolling or adherent cells [39]. Rolling platelets or leukocytes were defined as cells crossing an imaginary perpendicular through the vessel under study at a velocity significantly lower then the centreline velocity in the microvessel. They were determined as the fraction of all platelets or leukocytes passing a predefined vessel segment within an observation interval of 30 s. Adherent cells were defined as cells that did not move or detach from the endothelial lining in each vessel segment within an observation period of 30 s. Adherence was quantified as number of cells per square millimetre of endothelial surface, calculated from the diameter and length of the vessel segment observed (1/mm^2).

Immunostaining

Paraffin sections were incubated with 0.1 % Pronase E solution (Merck) and with 1 % goat serum for 20 min to block nonspecific binding, a ploy-clonal rabbit anti-mouse iNOS antibody (Dianova, Hamburg, Germany) at a 1: 100 dilution in PBS, and a biotinylated anti-rabbit IgG antibody (Vectastatin; Vector, Los Angeles, CA) with 1.5 % human serum for 30 min. Slides were incubated with an avidin-biotin complex (Vector) for 30 min. Following incubation with 0.01 % 3-amino-9-ethylcarbazole, sections were counterstained with hemalum (Merck), hydrated, and coverslipped.

Incubations were performed at 24 °C in a humidity chamber, and all washes lasted for 10 min each. Each tissue block was stained with and without the primary antibody to monitor background staining.

Adhesion molecule expression was assessed semiquantitatively by a blind observer, who examined 3 tissue sections each (2 observation fields per section) from 3 individual animals, and scored the findings as followed: -- = no staining, + = weak staining and ++ = strong staining.

Histological assessment

To evaluate the severity of arthritis, histological sections were performed using a standard scoring protocol introduced by Brackertz et al. [34]. After fixation in paraformaldehyde 8 % at pH 7.2 over 12 h, the joints were incubated in 20 % EDTA at pH 7.2 for 3 h to decalcify the bone. Samples were washed in phosphate buffer saline (PBS) and dehydrated with an automatic dehydrator model (Shandon, Frankfurt, Germany). After embedding in paraffin, the joint was sliced into 3 µm-thick sections which were stained with haematoxylin and eosin.

Severity of arthritis was estimated according to the histologic score, following the scoring method of Brackertz et al: 0 = normal knee joint, 1 = normal synovium with occasional mononuclear cells, 2 = 2 or more synovial lining cells and perivascular infiltrates of leukocytes, 3 = hyperplasia of synovium and dense infiltration, 4 = synovitis, pannus formation, and cartilage/subchondral bone erosions.

Statistical Analysis

The data are expressed as mean ±SEM. Statistical significance was tested using a Rank SUM Test (Man-Whitney U test) and a repeated measurements ANOVA on ranks (Friedman´s test). P values < 0.05 were considered significant.

Results

All the groups, no matter if control or AiA group, did not show any sign of lack of care for coat or any unusual behaviour. Throughout the experiment, there was no significant loss of body weight in the AiA groups (C57/Bl6 and iNOS deficient) in comparison to the control groups indicating no severe systemic reaction.

Microhemodynamic parameters

In Tab. 1 are the results from the FITC measurements. All the examined parameters did not show any significant changes after AiA induction neither between the wild-type and the iNOS deficient animals, nor compared to the two control groups.

Knee joint diameter

The results of the knee joint diameter are shown in Figure 1. In both control groups, the knee joint diameter increased on day 1, which is most likely due to the injected volume of saline. On day 2, the diameter returned to baseline values in control groups. In the AiA groups however, the knee joint

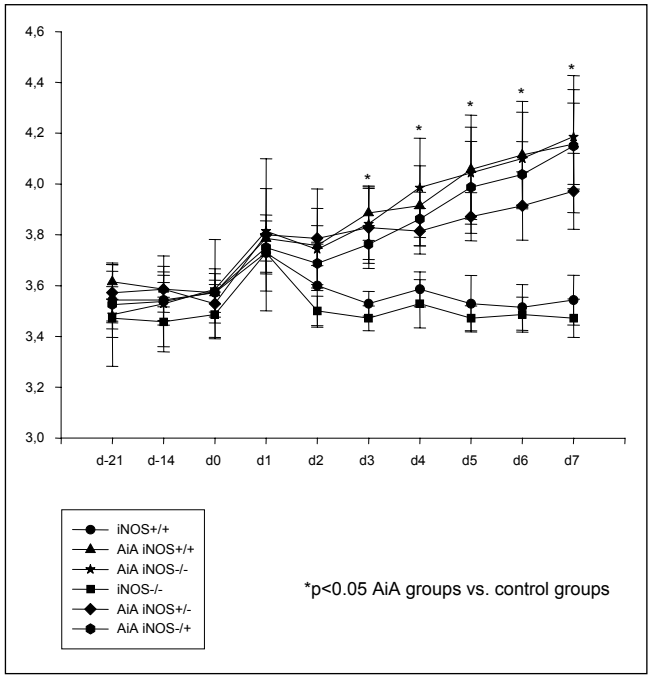

Fig. 1. Changes in the left knee joint diameter over the whole observation period. Dots represent the mean ±SEM of 7 animals per group. * = P < 0.05 AiA groups versus control groups.

diameter showed an increase in significant swelling from day 3 to day 7, compared to the control joints.

Platelet-endothelial cell interaction in vivo

The results for the platelet-endothelial cell interaction are shown in Figure 2. All AiA groups are significantly increased regarding the rolling as well as the adherent fraction compared to the two control groups. Concerning the rolling fraction, the AiA non deficient group stays on a same level with the AiA group using wild-type recipient and iNOS deficient donor mice (AiA iNOS+/+ 0.12 ± 0.01; AiA iNOS +/– 0.12 ± 0.01). The two other AiA groups are significantly increased versus the two AiA groups mentioned above (AiA iNOS–/– 0.16 ± 0.004; AiA iNOS–/+ 0.15 ± 0.01), but there has been no significant change noticed within those groups themselves. Looking at the adherent cells the relations are similar, showing a significant increase in the AiA groups towards the control groups. Only a slight difference between the AiA non deficient and the AiA iNOS+/– group (AiA iNOS+/+ 993.5 ± 33.7 mm^{-2}; AiA iNOS+/– 1062.7 ± 151.2 mm^{-2}) is visible. The arthritic iNOS deficient mice and the arthritic group with deficient recipient and wild-type donor (AiA iNOS–/– 1858.1 ± 188.0 mm^{-2}; AiA iNOS–/+ 1714.7 ± 117.7 mm^{-2}) do not show a significant increase comparing each other, but a significant increase compared to the two other AiA groups, bigger in regard to the rolling fraction.

Leukocyte-endothelial cell interaction in vivo

After AiA in C57/Bl6 and iNOS deficient mice, leukocyte behaviour in the synovial vessels was investigated with intravital microscopy, shown in Figure 3. Rolling and adhesion of leukocytes was only rarely observed in both control groups. But there was a significant increase in the rolling fraction and the adherent cells in AiA groups compared to the control animals. Regarding the rolling fraction there was only a small difference between the groups with different recipient and donor mice (AiA iNOS–/+ 0.15 ± 0.01; AiA iNOS+/– 0.17 ± 0.02) and an increase in the arthritic iNOS deficient mice (AiA iNOS–/– 0.2 ± 0.03) in comparison to the arthritic wild-type animals (AiA iNOS+/+ 0.14 ± 0.01). Looking at the adherent cells, we noticed a bigger range for the AiA groups, in comparison with the results from the platelets. The arthritic iNOS deficient (AiA iNOS–/– 1501.9 ± 293.4 mm^{-2}) and the AiA group using deficient recipient and wild-type donor mice (AiA iNOS–/+ 1552.9 ± 153.6 mm^{-2}) showed a significant increase compared to the wild-type AiA animals (AiA iNOS+/+ 783.9 ± 44.3 mm^{-2}) and the group using wild-type recipient and iNOS deficient donor mice (AiA iNOS+/– 1157.1 ± 97.7 mm^{-2}). Furthermore, we have seen a significant enhancement of the group with endothelial iNOS deficient recipient (AiA iNOS–/+ 1552.9 ± 153.6 mm^{-2}) in regard to the group using platelet iNOS deficient recipient (AiA iNOS+/– 1157.1 ± 97.7 mm^{-2}).

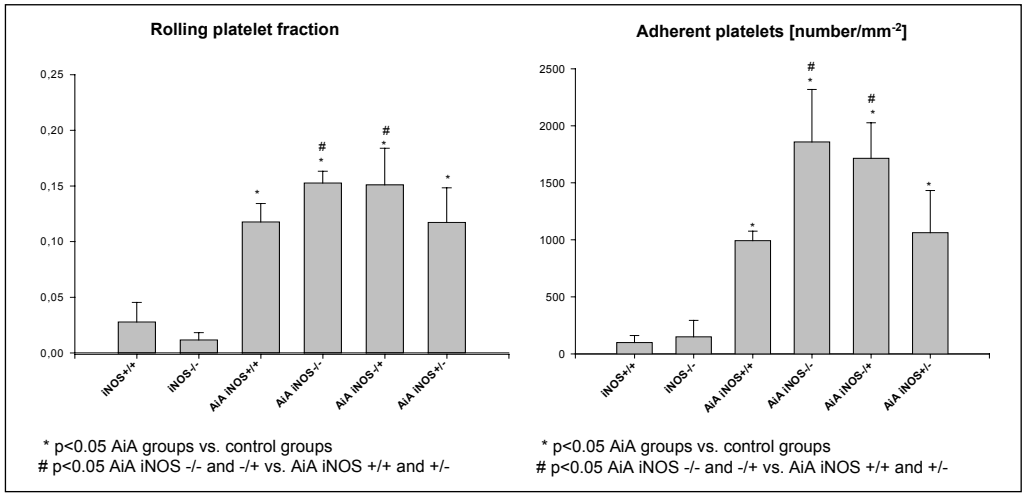

Fig. 2A, B. Fraction of rolling platelets (given as the number of rolling platelets over the sum of the rolling and non adherent platelets) (A) and number of platelets adherent (mm^{-2}) (B) to the endothelium in postcapillary venules in the synovium of the mouse knee joint. * = P < 0.05 AiA groups versus control groups. # = p < 0.05 iNOS deficient arthritic recipient mice versus C57/Bl 6 arthritic recipient mice. Data are given as mean ±SEM (n = 7).

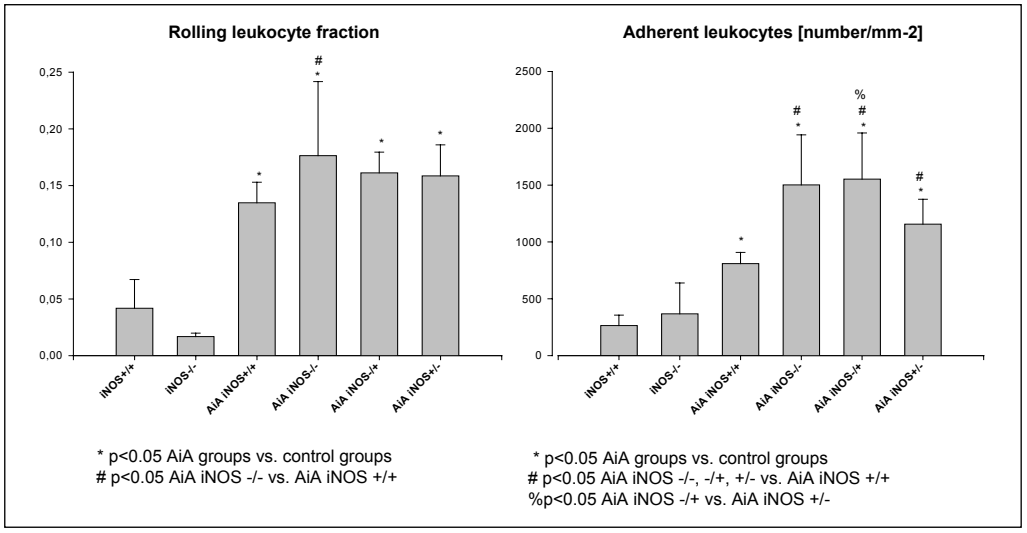

Fig. 3 A, B. Fraction of rolling leukocytes (given as the number of rolling leukocytes over the sum of the rolling and non adherent leukocytes) (A) and number of leukocytes adherent (mm^{-2}) (B) to the endothelium in postcapillary venules in the synovium of the mouse knee joint. * = P < 0.05 AiA groups versus control groups. (A) # = p < 0.05 iNOS deficient arthritic mice versus C57/Bl6 arthritic mice; (B) # = p < 0.05 iNOS deficient arthritic recipient mice versus C57/Bl 6 arthritic recipient mice. %p < 0.05 AiA iNOS deficient recipient and wild-type donor versus AiA wild-type recipient and iNOS deficient donor. Data are given as mean ±SEM (n = 6–7).

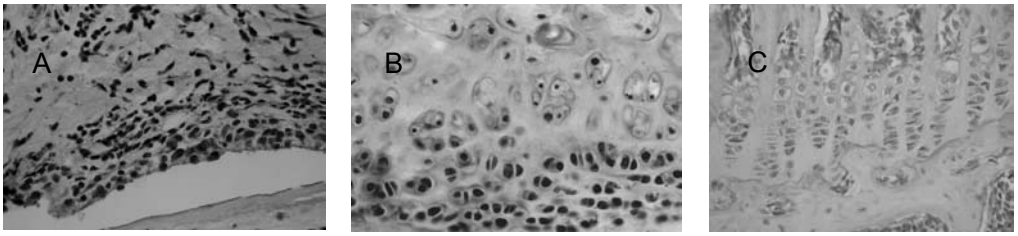

Fig. 4A, B, C: Synovial tissue samples of the examined knee joint with dark stained iNOS. A marked synovial fibroblasts in an animal of the C57/Bl6 strain with AiA and B marked chondrocytes in an arthritic animal. No iNOS expression in an AiA iNOS deficient animal (C).

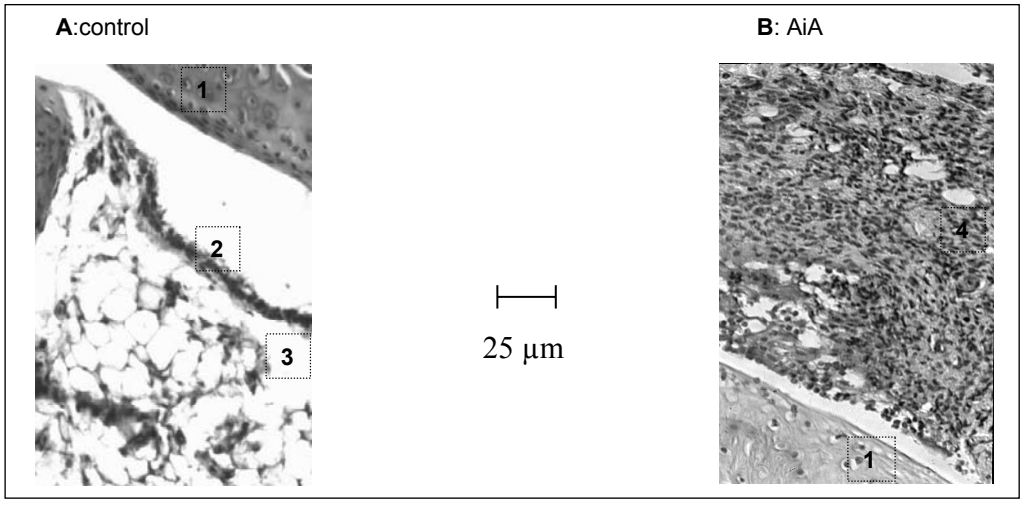

Fig. 5A, B. Histological section of the mouse knee joint with the synovial cell layer; A: control animal, B: AiA animal. Histological pictures sagital through the mouse knee joint (Hematoxylin-Eosin, magnification ×25) Legend: 1 = cartillage; 2 = synovial cell layer; 3 = subsynovium; 4 = subsynovium with strong leukoyte infiltration

Immunostaining

iNOS expression in C57/Bl6 mice with induced arthritis was enhanced as shown in Figure 4A, B compared to the iNOS expression in arthritic iNOS deficient mice Figure 4C. The control group using iNOS+/+ animals showed only weak iNOS expression and no expression in the deficient control group.

Histological score

Compared to the AiA groups, both control groups showed no arthritic changes. The AiA groups on the other hand showed reactions normal for arthritis, from perivascular infiltrates of leukocytes up to hyperplasia of synovium and some even synovitis and pannus formation as well as bone erosions, as shown in Tab. 2 and Figure 5.

Discussion

Platelets seem to be involved in the pathogenesis and the maintenance of rheumatoid arthritis, but the exact procedure is not solved so far. Previous studies with intravital microscopy have proved an increase of platelet-endothelial cell interaction in AiA *in vivo* [13]. Knowing that the function of

Table 2. Histological score by Brackertz et al.: changes in the synovial morphology.

Groups	Histological score				
	0	1	2	3	4
C57/Bl6 control	6	1	0	0	0
iNOS deficient control	7	0	0	0	0
C57/Bl6 AiA	0	0	1	5	1
iNOS deficient AiA	0	0	1	4	2

AiA = antigen-induced arthritis, histological sections graded as: 0 = normal knee joint; 1 = normal synovium with occasional mononuclear cells; 2 = two and 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).

NO has proved different outcomes [20–22], the influence of NO has never been examined in this correlation.

Therefore, we investigated for the first time in an *in vivo* study the influence of NO on the platelet- and leukocyte-endothelial cell interactions in AiA and afterwards, we examined if the effects seen are related to the platelet or the endothelial iNOS.

Throughout all the groups, we were able to prove that all arthritic animals showed a significant increase in rolling and adherent platelets as well as leukocytes compared to the two control groups, which reflects the expected results regarding previous studies.

INOS deficient arthritic mice showed an increase in intravital parameters, respectively in rolling fraction and in adherence of platelets and leukocytes, versus wild-type mice. Similar results, being controversial discussed in literature so far, have been shown in segmental intestinal ischemia/ reperfusion studies [40], leading to the conclusion of NO having an anti-inflammatory effect, also proved in studies using selective and non-selective NOS inhibition [41].

In addition, we added two arthritic groups using animals from two strains with different recipient and donor mice and vice versa. Comparing the results of these groups with the two other AiA groups and with themselves, it was possible to differ between endothelial and the platelet iNOS. Since the outcomes of arthritic iNOS deficient recipient mice and wild-type donor mice were increased in comparison to the arthritic C57/Bl6 mice and were on the same level as the iNOS deficient arthritic animals, we assume that the endothelial iNOS is mainly involved in the platelet and leukocyte cell interactions in AiA.

The group with arthritic wild-type recipient mice and iNOS deficient donor mice was on a lower level than the iNOS deficient arthritic mice and the iNOS deficient recipient and wild-type donor animals, and on the same level or rather slightly increased to the arthritic wild-type mice, leading to the conclusion that the platelet iNOS plays a minor role in the maintenance and the amount of the inflammation in the tissue.

The iNOS expresses, after stimulation, up to ten times more NO than the two constitutive forms of NOS. The regulating factor for the iNOS expression is the availability of substrate and co-substrate for the limit of NO production [42–44]. A possible reason why the platelet iNOS did not show results up to the level of the endothelial iNOS might be, due to the fact that in our research we only resuspended a certain amount of labelled platelets back into the recipient mouse. Though, the smaller percentage of fluorescently labelled platelets could be an explanation for the minor influence. Thinking of that possibility might also be an explanation for the lower results being seen regarding the leukocytes. Following those results, it seems obvious that the platelet-leukoycte endothelial-cell interactions raise and fall depending on each other in the same direction. By assuming that the endothelial iNOS has a protective character, there is no contradiction in inhibiting platelets and/or leukocytes and the existence of endothelial iNOS.

Our histological results showed the same amount of inflammation in all AiA animals, no matter if iNOS deficient or wild-type animals. The immunhistological samples proved the purity of the iNOS deficient mice. As expected, the iNOS expression in wild-type arthritic mice was enhanced and not present in iNOS deficient AiA animals.

The knee joint diameter does not show an additional increase in the diameter in the AiA iNOS deficient mice compared to the wild-type AiA group, indicating no further clinical enhancement depending on NO. This might be due to the fact that knee joint swelling is only possible up to a certain maximum. Differences in inflammation are better visible in intravital microscopy than in clinical parameters, because they are more detailed and offer a greater range for measurements.

Stability of our model was monitored by microscopy, cardiovascular and behavioural parameters – there were no significant changes between the different groups regarding macro- and microhemodynamic parameters. By using FACS analysis, we made sure that it was still possible to activate platelets after labelling, independent of iNOS deficiency or wild-type animals.

In summary, we provide the evidence that the absence of iNOS leads to an increase in platelet-and leukocyte endothelial-cell interaction and therefore a possible increase in tissue damage which leads to the conclusion of an anti-inflammatory effect depending on the presence of NO. In addition, we conclude that the endothelial iNOS seems to play the major role leading to the increased interactions and the results described above and the platelet iNOS only a subordinate part.

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