Relationship between angiogenesis and inflammation in experimental arthritis

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ABSTRACT. *Background.* **Angiogenesis is involved in rheumatoid arthritis (RA) leading to leucocyte recruitment and inflammation in the synovium. Furthermore, synovial inflammation itself further potentiates endothelial proliferation and angiogenesis. In this study, we aimed at evaluating the reciprocal relationship between synovial inflammation and angiogenesis in a RA model, namely collagen-induced arthritis (CIA).** *Methods.* **CIA was induced by immunization of DBA/1 mice with collagen type II in adjuvant. Endothelial cells were detected using a GSL-1 lectin-specific immunohistochemical staining on knee joint sections. Angiogenesis, clinical scores and histological signs of arthritis were evaluated from the induction of CIA until the end of the experiment. Angiogenesis was quantified by counting both the isolated endothelial cells and vessels stained on each section. To evaluate the effect of increased angiogenesis on CIA, VEGF gene transfer was performed using an adeno-associated virus encoding VEGF (AAV-VEGF), by intra-muscular or intra-articular injection in mice with CIA. Results. We showed an increase in synovial angiogenesis from day 6 to day 55 after CIA induction, and, moreover, joint vascularization and clinical scores of arthritis were correlated ($p < 0.0001$, $r = 0.61$). Vascularization and histological scores were also correlated ($p = 0.0006$, $r = 0.51$). Systemic VEGF overexpression in mice with CIA was followed by an aggravation of arthritis as compared to AAV-lacZ control group ($p < 0.0001$). In contrast, there was no difference in clinical scores between control mice and mice injected within the knee with AAV-VEGF, even if joint vascularization was higher in this group than in all other groups ($p = 0,05$ versus non-injected group). Intra-articular AAV-VEGF injections induced more severe signs of histological inflammation and bone destruction than AAV-Lac Z or no injection.** *Conclusion.* **Angiogenesis and joint inflammation evolve in parallel during collagen-induced arthritis. Furthermore, this work shows that exogenous VEGF can aggravate CIA. It is direct evidence that the increase in joint vascularization leads to an exacerbation of arthritis. Taken together, these results emphasize the role of angiogenesis in inflammatory arthritis. It also suggests an early involvement of angiogenesis in joint inflammation.**

Keywords: angiogenesis, arthritis, inflammation, VEGF

Rheumatoid arthritis (RA) is a chronic and destructive disease characterised by an inflammatory, erosive synovitis. The clinical presentation is heterogeneous, with a wide spectrum of age-of-onset, degree of joint involvement, and severity. Progressive destruction of the joint including cartilage, bone and soft tissue, produce the characteristic deformities of RA.

In RA, the synovium is inflamed and infiltrated by leucocytes. Hyperplasia of synovial cells and inflammatory cells compose the pannus, which is responsible for the local destruction of the joint [1].

Angiogenesis is defined as the development of new capillaries from preexisting blood vessels [2]. It occurs during growth and the female reproductive cycle, in repair after injury, in diabetic retinopathy, in cancer and in chronic inflammatory diseases such as RA. Angiogenesis is an important and early step in the pathogenesis of RA since it participates in the initiation and perpetuation of the disease [3]. Formation of new blood vessels within the synovium ensures the development and persistence of the pannus by increasing the supply of nutrients, cytokines and inflammatory cells to the synovial membrane. Vascular endothelial growth factor (VEGF) is considered to be the main

proangiogenic factor. It is produced by many cell types (fibroblasts, lymphocytes, neutrophils, macrophages...) and induces neovascularization in vivo and stimulates endothelial cell proliferation in vitro. Moreover, it is able to increase vascular permeability and stimulates leukocyte extravasation into inflammatory processes [4]. A role for VEGF has been shown in RA and experimental models. Synovial cells can produce high levels of VEGF [5]. VEGF mRNA is strongly expressed in RA synovium but not in osteoarthritis synovium [6] while the isoform VEGF165 is only detected in RA synovia [7]. In patients with active RA, an increased circulating VEGF level is correlated with disease activity and with radiographic changes [8].

Interestingly, it was previously shown that anti-rheumatic drugs usually used to treat RA (methotrexate, salazopyrine, gold, cyclosporine, glucocorticoids) have anti-angiogenic effects which are independent from their immunosuppressive action. Recently, the treatment of RA patients with TNF inhibitors, such as infliximab [9] or etanercept [10], has been linked to lower serum VEGF levels. This reinforces the concept that angiogenesis could play a major role in the development and the perpetuation of RA.

Angiogenesis inhibitors are able to modulate disease in RA animal models. For example, TNP-470 reduces severity in rat collagen-induced arthritis [11], and in KRN-NOD mice [12]. More specific anti-angiogenesis drugs have been shown to inhibit CIA [13]. The delivery of soluble VEGF receptor 1 by gene therapy or VEGF antibody [14] significantly suppressed disease activity in CIA [15]. Conversely, the VEGF RII is ineffective in treating KRN-NOD mice [16]. Others anti-angiogenic cytokines, have been used to inihibit arthritis and bone destruction, for example by blocking the angiopoitin receptor Tie2 [17, 18]. Eventually, the angiogenesis inhibitor protease-activated kringles 1-5, which is related to angiostatin, blocks synovial neovascularization and reduces joint inflammation and destruction [19]. Another angiogenesis inhibitor, thrombospondin 1 has been used in CIA model in rats [20]. Intra-articular administration of an adenoviral vector encoding thrombospondin 1, significantly ameliorated the course of CIA, accompanied by reduction of synovial hypertrophy and fewer blood vessels. Decreased angiogenesis and arthritic disease have also been observed in rabbits treated with intra-articular administration of $\alpha v\beta 3$ antagonists [21].

All these studies suggest that angiogenesis is involved in RA, since it leads to leucocyte recruitment and inflammation in the synovium. Furthermore, synovial inflammation itself further potentiates endothelial proliferation and angiogenesis. However, the reciprocal relationship between synovial inflammation and angiogenesis is not well defined.

Abbreviations:

IM	intramuscularly
IA	intra-articularly
CII/CFA	Collagen type II emulsified in complete Freund's adjuvant
IFA	incomplete Freund adjuvant

In this study, we aimed to evaluate this point in a mouse model for RA, namely collagen-induced arthritis (CIA). We show that angiogenesis within the joint occurs very early during CIA development and that there is a correlation between angiogenesis and joint inflammation. Interestingly, we demonstrate a critical role for VEGF in arthritis since VEGF-induced neovascularization in mice with CIA, leads to increased clinical and histological signs of inflammation.

MATERIALS AND METHODS

Induction and assessment of arthritis

Male DBA/1 mice (5-7 weeks old) were purchased from Harlan Olac (Bicester, UK). Arthritis was induced with native bovine collagen type II (CII) (Chondrex, Morwell Diagnostics, Switzerland) as previously described [22]. Mice were injected subcutaneously in the tail with 100 µg of CII emulsified in Freund's adjuvant (DIFCO, France). On day 21, mice were boosted with a subcutaneous injection of CII in incomplete Freund's adjuvant (DIFCO, France). Mice were monitored for evidence of arthritis using a blind procedure. Clinical severity of arthritis in each joint or group of joint (toes, tarsus, and ankle) was scored as follows: 0, normal; 1, erythema; 2, swelling; 3, intense swelling; and 4, deformity and ankylosis or necrosis. These scores were summed to obtain the arthritic score; the mean arthritic score on each clinical observation day and in each group was used to evaluate CIA severity. For clinical scores, an analysis of variance method (ANOVA test) was used.

For histological analysis, legs were dissected free, and processed for histological examination [23]. The severity of synovial proliferation and the severity of inflammatory cell infiltration were each scored using a four-point scale (0-3, where 0 is normal and 3, severe) [24].

Adeno-associated viral vectors

The AAV used in this study contained VEGF 164 cDNA or LacZ cDNA, whose expression was driven by the CMV promoter. The vectors were provided by the Production Service Unit of Genethon III, which is part of the Gene Vector Production Network (GVPN, http://www.genethon.fr/gvpn). The methods used for AAV-VEGF and AAV-LacZ vector construction, production, purification, and titration have been described in detail elsewhere [25].

AAV-mediated reporter gene transfer into mice joints

Six mice were injected with 3×10^7 AAV-Lac Z infectious particles (i.p.) into the right knee. Two mice were killed every week during the 3 weeks following injection. The legs were dissected and incubated in 10% formol for 2 hours. Then, 30 µl of X-gal staining buffer (potassium ferricyanide, potassium ferrocyanide and magnesium chloride and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside 1 mg/mL) (Invitrogen) were injected intra-articularly into the dissected legs, which were then incubated with X-gal staining buffer overnight at 37°C. They were processed for histological study as described above. The slides were counterstained with nuclear fast red and lac Z activity was detected by optical microscopy.
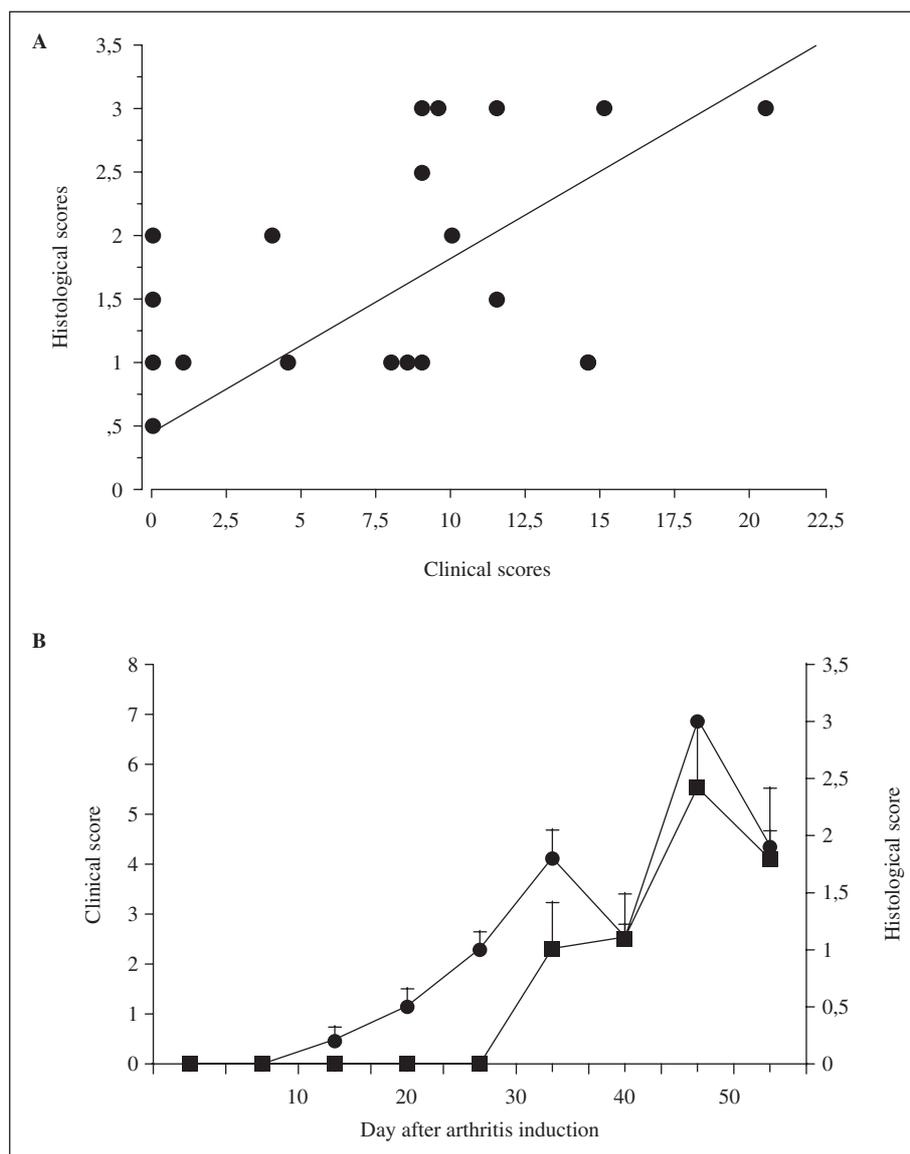
Figure 1

A) Correlation between clinical and histological scores. CIA was induced with CII/CFA at day 0. Five mice were sacrificed each week from day 0 to day 59. Each point represents an individual mouse; r = 0.74 p < 0.0001 (Spearman correlation test). **B**) Evolution of clinical and histological scores during the course of CIA. Arthritis was induced at day 0. Clinical scores (closed squares) were evaluated twice a week as described in the material and method section. For histological scores (closed circles), five mice were killed each week and legs were processed as described. Each point are the mean score ± SEM on a given day (n = 5).

Determination of VEGF levels

VEGF164 (mVEGF) was measured in the sera or in cell culture supernatants using commercially available ELISA systems according to manufacturer's instructions (Duoset, R&D Systems).

Quantitative RT-PCR on VEGF 164 in synovial tissue

The knee joints of mice injected with 4.3×10^8 ip AAV-VEGF were dissected out 3 weeks after injection under a magnifying glass, and synovial tissue was taken.

Total mRNA were then isolated from the synovial tissue using 1 mL of Tri Reagent (Euromedex) according to the manufacturer's instructions, in a final volume of 20 µl H_2O DEPC. cDNA synthesis was then preformed from total RNA with the SuperScriptIII[TM] RNase H-reverse transcriptase (In Vitrogen) according to the manufacturer's instructions, in a total volume of 20 µL. PCR reactions were performed in a volume of 15 µL containing oligo-nucleotide primers (0.4µM) (reporter gene actin: sense primer AGAGGGAAATCGTGCGTGAC and anti-sense primer CAATAGTGATGACCTGGCCGT; VEGF164: sense primer: GCCAGCACATAGAGAGAATGAGC and anti-sense primer: CAAGGCTCACAGTGATTTTCT GG), dNTP (in Vitrogen) and Fast Start DNA master plus SYBR Green (Roche Molecular Biochemicals) containing Taq DNA polymerase, reaction buffer and the double strand DNA-specific fluorescent dye SYBR Green I. DNA was amplified with the Light Cycler [TM] in a two-step procedure: i) for VEGF: denaturation at 95°C for 10 minutes and 45 cycles with denaturation at 95°C for 10s, annealing at 63°C for 5s and extension at 72°C for 8s; ii) for actin: denaturation at 95°C for 8 minutes and 45 cycles with denaturation at 95°C for 10s, annealing at 62°C for 5s and extension at 72°C for 8 s. Acquisition of the fluores-
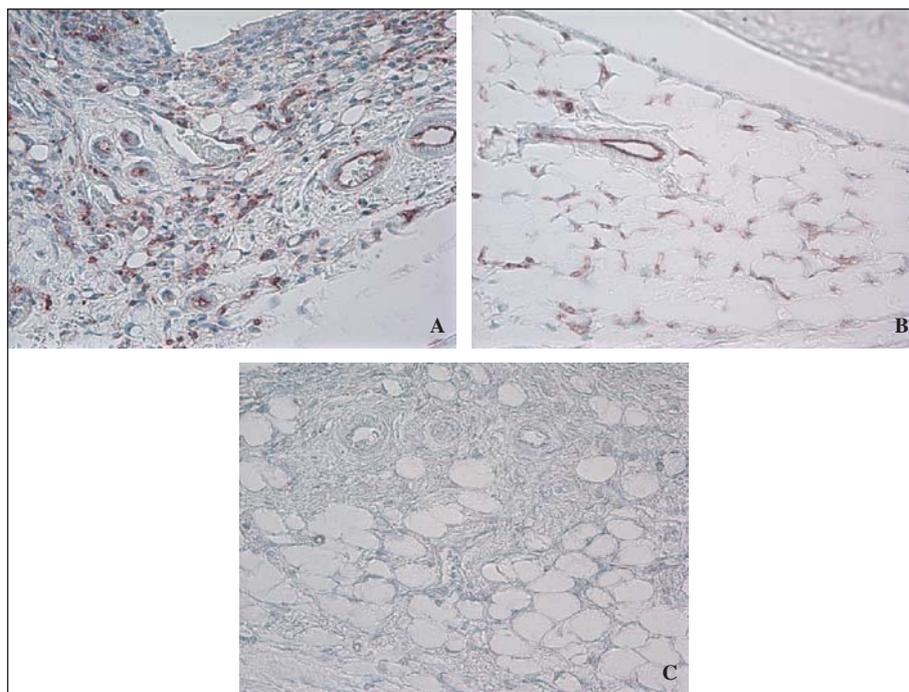
Figure 2
Example of GSL-1 lectin immunohistochemical staining of vessels in the knee joint. **A**) Arthritic joint from mice induced for CIA since 7 weeks. **B**) Non-arthritic joint. **C**) control section: no staining is observed without GSL-1. Magnification x400.

cence signal from the samples was carried out at the end of the elongation step. Relative VEGF164 transcripts values were calculated with RealQuant software (Roche Molecular) and expressed in arbitrary units.

AAV-VEGF injection in mice with CIA

Three weeks before induction of CIA, groups of 10 mice were injected intra-articularly (6×10^7 ip) or intramuscularly (2.5×10^8 ip) with AAV VEGF or AAV Lac Z. These amounts were the highest we could inject given i) the limited volume of each injection, ii) the limited concentration of the initial batch of AAV-Lac Z, iii) the need to inject the same amount of AAV-VEGF as AAV-Lac Z. A control group (n = 10) did not receive any treatment. Arthritis was induced as described previously. Mice were killed 59 days after induction of CIA and the paws were dissected and processed for histological studies as described previously.

Quantification of angiogenesis within the joint

Endothelial cells were detected using a GSL-1 lectin immunohistochemical staining with the steptavidin-biotin-peroxidase complex method. Knee joint slides were deparaffinized in xylene and dehydrated through serially diluted ethanol solutions down to distilled water. After blocking endogeneous peroxidase activity in blocking solutions for 1 hour, the slides were pretreated with blocking serum and then incubated with GSL-1 isolectin B4 (20 µg/mL) for one hour at room temperature. Then, the slides were incubated with goat antibody to GSL-1 isolectin B4 (10 µg/mL) for one hour, washed, and incubated with biotinylated rabbit antigoat immunoglobulins (4 µg/mL) for 30 min in moist chamber at room temperature. The samples were incubated with streptavidin-biotin-peroxidase for 10 min using diaminobenzidine tertrahy-

drochloride as the chromogen. Between each step, the slides were washed three times for 5 min with TBS. They were counterstained by incubation with hemalun for 40 sec and mounted with Glycergel. All reagents were obtained from Dako (Trappes, France) apart from GSL-1 and antibody against GSL-1 which were obtained from Vector Laboratories (Burlingamme, CA, USA).

For each mouse, three non-serial sections from each knee were studied. For each section, five pictures were taken at low magnification (x400, Olympus) using SpotAdvanced® (Diagnostic Instruments Inc.) software. The area of picture that was not in the synovium was subtracted from the total area. Any GSL-1 stained cell or group of cells with a lumen was considered as an individual vessel. Synovial vascular density was calculated as follows: each vessel in the synovium was counted; the number of vessels was divided by the synovium area.

Statistical analysis

All statistics were performed using the Statview 5.0 software. Results were compared using Student's t test. For correlation, we used the Spearman test. For repeated measures (clinical scores) we used an ANOVA test. Differences were considered significant when $p < 0.05$.

RESULTS

Progression of synovial angiogenesis during CIA: relation with time, clinical and histological parameters

To characterize the progression of angiogenesis during the arthritic process, we evaluated the vascularization within the knee joint of mice with CIA using a quantitative method. The experimental protocol consisted of inducing

CIA in DBA/1 mice, and then evaluating clinical (2-3 times a week) and histological signs of arthritis (groups of 5 mice were sacrificed every week from week 1 to week 8). *Figure 1A* shows that clinical and histological scores were strongly correlated (r = 0.74; p < 0.0001, Spearman correlation test). Interestingly, the onset of histological signs of arthritis occurred earlier than clinical symptoms *(figure 1B)*.

To study synovial vascularization during the course of CIA, histological sections of the knees were prepared from each of the 5 mice sacrificed every week, and staining of endothelial cells was performed by immunohistochemistry with gsl-1. *Figure 2* shows a representative example of immunohistochemical staining of the synovium from an arthritic, inflammatory knee joint *(figure 2A)* and from a normal joint *(figure 2B)*. Assessing the synovial vascular density, we found that vascularization increased over time, from day 0 to day 55 post-arthritis induction (r = 0.548 p = 0.0006; Spearman correlation test) *(figure 3A)*. The links between angiogenesis and arthritis parameters were then studied. We observed a statistically significant correlation between joint vascularization and clinical scores of arthritis (r = 0.53; p < 0.0001) *(figure 3B)*. A significant correlation was also shown between vascularization and the histological score for inflammation and destruction (r = 0.575; p < 0.0001) *(figure 3C)*.

Effect of VEGF administration on arthritis parameters and on synovial vascularization

The effect of VEGF administration within the joint on CIA was studied using a gene transfer method to obtain stable intra-articular levels of VEGF. Therefore, we injected the viral vector AAV encoding the VEGF gene into mice knee. To ensure that this strategy allowed synovial expression of the transgene, we checked for the expression of the reporter gene Lac Z, after injection into the joint. One, two or three weeks after the injection of AAV-Lac Z, each leg was processed as described in the Material & methods section. Lac Z expression was observed in synovial and subsynovial cells *(figure 4)*, two or three weeks after AAV-Lac Z injection (no expression was found in mice after one week).

To assess that VEGF expression could be detected within the joint after intra-articular injection of AAV-VEGF, three DBA/1 mice were injected with AAV-VEGF into both knee joints. One mouse received the same amount of AAV-LacZ into both knees. Taking into account the difficulty of detecting VEGF protein in a very small volume of synovial fluid by ELISA, the expression of VEGF was evaluated by quantifying VEGF mRNA by realtime, quantitative RT-PCR in synovial tissue 3 weeks after the AAV-VEGF injection. Levels of VEGF mRNA were almost twice as high in mice that had received AAV-VEGF as compared to control mice (4.50 ± 1.99 *versus* 2.8 ± 1.05, arbitrary units ± sem, respectively).

Systemic (intra-muscular) or local (intra-articular) injections of AAV-VEGF were then performed in mice. Control groups received AAV-Lac Z (either intra-articular or intra-muscular) or no injection. Three weeks later, CIA was induced and clinical evaluation was performed twice a week. All mice were killed on day 59 for histological evaluation and quantification of vascularization. *Figure 5*
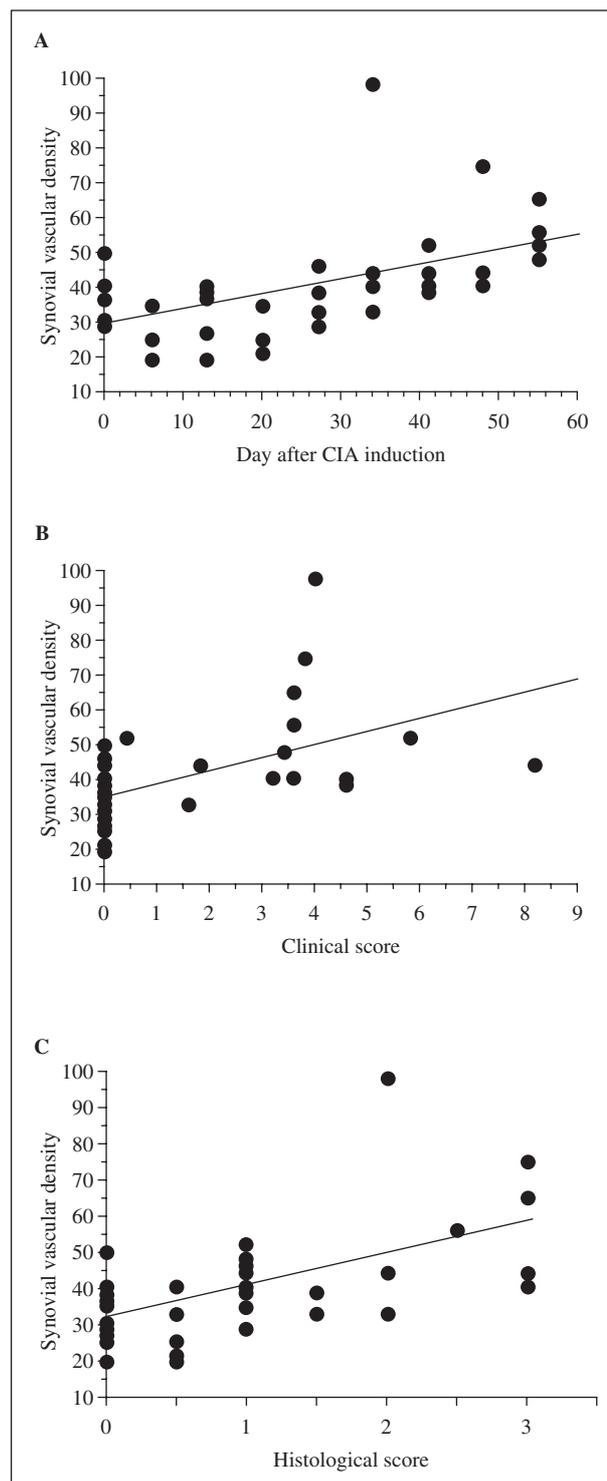

Figure 3
Evolution of synovial vascular density during evolution of CIA and correlation with clinical or histological scores. CIA was induced with CII/CFA at day 0. Five mice were sacrificed each week from day 0 to day 59. After GSL-1 immunohistochemical staining, three slides per leg were taken and five pictures of synovium per slide were numerized. The number of vessels in the knee synovium was counted for each picture. **A**) Increased vascular synovial density during CIA. r = 0.548 p = 0.0006. **B**) Correlation between synovial vascular density and clinical score; r = 0.53 p < 0.0001. **C**) Correlation between synovial vascular density and histological score; r = 0,575 p < 0,0001. Each point represents synovial vascular density for an individual mouse (Spearman correlation test).

shows that overexpression of VEGF induced by gene transfer via intra-muscular injection, was followed by an aggravation of clinical arthritis as compared to the AAV-LacZ control group ($p < 0.0001$, ANOVA test). No significant difference in clinical scores was observed between control mice and mice injected into the knee with AAV-VEGF (data not shown).

Synovial vascular density was higher in the AAV-VEGF administered by intra-articular injection than in other groups ($p = 0.05$ versus the non-injected group; t test) *(figure 6)*.

Histological inflammation and bone destruction were increased in the group that received intra-articular AAV-VEGF as compared to the control groups (no injection and intra-articular injection of AAV-LacZ) *(table 1)*, although this increase was not statistically significant. Mice receiving AAV-VEGF by injection did not show any increase in histological scores.

DISCUSSION

Angiogenesis is an essential step in the development of arthritis. In this study, we showed that synovial vascularization increases over time following arthritis induction. Vascularization was also correlated with severity of joint inflammation, assessed either by clinical or histological scoring.

Few studies have focused on synovial angiogenesis in animal models of arthritis. Early endothelial cell proliferation was shown to predict the persistence of inflammation in the model of carrageenan synovitis in the rat, whereas synovitis in the absence of angiogenesis was followed by resolution of inflammation [26].

Vascularization within synovial tissue results from a competition between angiogenic and anti-angiogenic factors. All these factors are expressed, but previous studies using immunohistological techniques have shown that they are localized on separate microscopic foci within chronically inflamed human synovium [26].

In RA, several studies have shown a higher degree of vascularization than in osteoarthritis or in normal synovial tissue [4, 27]. Moreover, it seems that there are not only more vessels, but above all, the distribution of vessels in the synovium is modified in synovitis [28-30]. In the normal synovium, vascular density is not uniform throughout the synovium and the number of blood vessels is

Table 1
Histological scores of inflammation and articular destruction. Treated mice were injected with AAV-VEGF or AAV-Lac Z three weeks before induction of CIA (day 0). Histological evaluation was performed at day 59 after induction of arthritis. Values are expressed as means ± SEM. There was no statistical difference between groups (t test)

	Inflammation	Destruction
Groups		
IA VEGF	1,9 ± 0,2	1,6 ± 0,3
IA Lac Z	1,5 ± 0,2	1,4 ± 0,2
IM VEGF	1,7 ± 0,2	1,4 ± 0,2
IM Lac Z	1,1 ± 0,3	0,8 ± 0,3
None	1,6 ± 0,4	1,4 ± 0,3

IA: intra-articular; IM: intra-muscular.

typically the greatest close to the synovial surface [31]. In contrast, in RA, there is an increased vascular density in the deeper synovium and there is also a reduced vascular density within the synovial surface. These changes explain that angiogenesis and pockets of hypoxia can coexist in the RA joint.

It is not clear whether synovial angiogenesis occurs before inflammation. The hypothesis of Hirohata *et al.*, suggesting very early angiogenesis in RA [32], showed that angiogenesis was present before synovial proliferation or cellular infiltration. However, this study was performed in only three patients and RA was diagnosed one to two years after arthroscopic synovectomy. In our study, in CIA, histological signs of arthritis occurred before the onset of clinical symptoms. Angiogenesis, as the increase of synovial vessel density, started early during the course of arthritis in this animal model of RA, even if we were unable to show that it preceded histological synovitis.

Our study shows a strong correlation between vascular density and clinical or histological arthritis. These data suggest that angiogenesis contributes to joint damage by increasing the vascularity of the inflammatory pannus, thereby supporting its growth and facilitating inflammatory cell infiltration. Furthermore, various proinflammatory cytokines, such as IL-1, TNF-α, IL-6, IL-8, PlGF are increased in RA and are known to induce VEGF production [9, 33-35]. The direct link between these two phenomena could help to define new targets in the treatment of inflammatory arthritis. It is strengthened by the effectiveness of angiogenesis inhibitors, such as TNP-470, soluble VEGF receptor 1 or VEGF antibodies in several animal models of RA.

In addition, our data show that VEGF administration increased joint vascularization and worsened clinical and histological inflammation signs in CIA. Moreover, VEGF overexpression within the synovium was associated with worsened clinical and histological synovitis. These data are consistent with the study by Yamashita *et al.* in which it was shown that an overexpression of FGF within the arthritic joints [36], in adjuvant arthritis, induced more severe arthritis with an increase in synovial angiogenesis and bone destruction. Interestingly, in this former work, the FGF gene transfer to the non-AIA joint was without effect.

Our study suggests a direct role of angiogenesis, through overexpression of VEGF, in the induction of a more severe arthritis in mice. This result is consistent with findings of others in RA models. For instance, VEGF and its receptors were shown to be expressed within inflamed joints [14], VEGF expression coinciding with an increase in neovascularization. Inhibition of VEGF, directly or by targeting its receptors, leads to a decrease of disease activity in several animal models of RA [13-16, 37, 38].

In RA, serum VEGF levels have been shown to be higher than in osteoarthritis or in normal subjects and are correlated to disease activity [8, 9, 39-41]. Moreover, cells expressing VEGF have been localized to the RA synovial lining layer, subsynovial macrophages, fibroblasts surrounding microvessels and vascular smooth-muscle cells [39, 42-44]. Others proangiogenic factors, such as angiopoietin 1 and 2 and their receptor Tie-2, are also expressed in RA synovial tissue [18, 45-49] and could be of interest as specific targets in the disease.
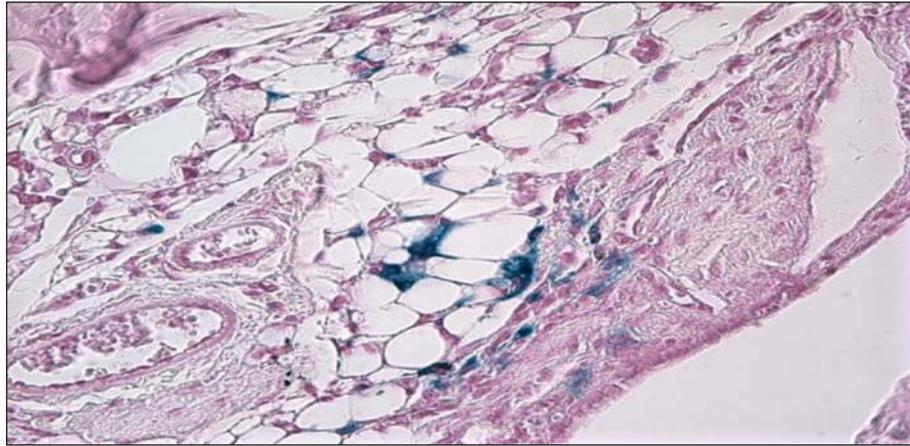
Figure 4
Lac Z expression in joint, three weeks after intra-articular injection of AAV-Lac Z into the knee. X-gal staining and nuclear fast red counterstain. Magnification x 100.
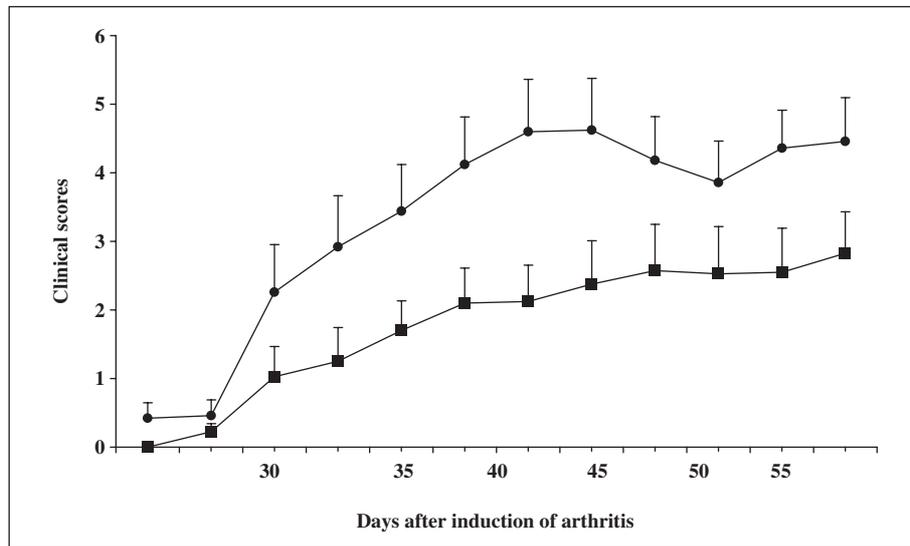

Figure 5
Aggravation of clinical arthritis induced by overexpression of VEGF. AAV-VEGF (closed circles) or AAV-Lac Z (closed squares) were injected intramuscularly, three weeks before induction of CIA (day 0). Clinical scores were evaluated twice a week from day 21; p < 0.0001; ANOVA test.
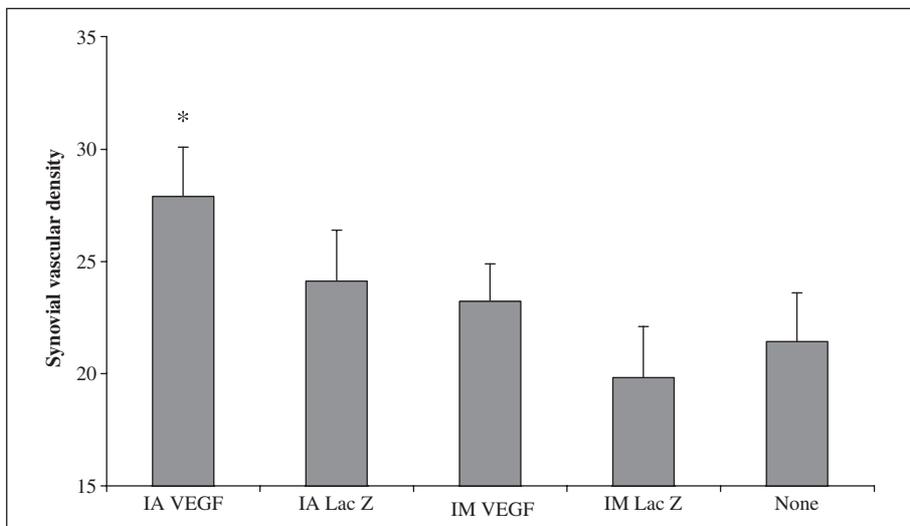

Figure 6
Synovial vascular density in arthritic mice treated with AAV-VEGF or AAV-Lac Z injected three weeks before induction of CIA (day 0). Number of joint vessels was quantified as described previously after immunohistochemistry by GSL-1. Values are expressed as means ± SEM.* p < 0.05 *versus* non-injected group (Student's t test).

Our results contribute to emphasise the role of synovial angiogenesis, and especially for VEGF, in chronic arthritis. Data from animal models have contributed to the idea that VEGF-induced angiogenesis plays an important role in the aetiology of arthritis and maintenance of synovitis. The inhibition of VEGF-mediated effects should be explored in RA, as has already been done in cancer. These data suggest that approaches targeting VEGF and neovascularization could be an exciting, future therapeutic possibility for human RA.

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