7

Innate Mechanisms of Synovitis – Fibrin Deposits Contribute to Invasion

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1. Introduction

1.1 Synovitis and the chicken or the egg dilemma

The studies approaching pathogenesis of rheumatoid arthritis shifted over the years to show that non-immune factors could precede activation of immune cells and were therefore targetable (Firestein & Zvaifler, 1990). In opposition to the classic model, in which an initial challenge to the immune system would over time lead to the autoimmune attack of joints, it was suggested that early mechanisms of disease induction were to be found inside joints. It was observed in vitro, that cells from the joints of patients with rheumatoid arthritis spontaneously produced several cytokines (Brennan et al., 1989a). Further studies would confirm the role of tumour necrosis factor alpha (TNFα) as a master cytokine, since its inhibition led to a drop in levels of the other soluble mediators (Brennan et al., 1989b), as well as reduction in the expression of HLA-DR molecules (Haworth et al., 1991). Interestingly, TNFα was not lymphocyte restricted, but rather pleiotropic. Moreover, it was shown that its principal sources in the arthritic joint were resident macrophages and fibroblast-like cells. This new paradigm was followed by the successful introduction of anti-cytokine therapies, which have totally changed the clinical picture of RA.

Indeed, the rheumatoid lesion at joints is quite unique, and probably sufficient to define the disease. It is characterized by the development of synovitis, a tumour-like transformation of the synovial tissue (Arend, 1997). On one hand, synovitis leads to joint destruction and disability, and on the other it provides a stronghold for spreading the inflammatory process.

1.2 Role of synovial fibroblasts in synovitis

1.2.1 Invasive features of synovial fibroblasts

There is not a uniform theory to explain how synovitis develops. However, one of the major features of synovitis is the acquisition of invasiveness of synovial fibroblasts. It could be said that rheumatoid synovial fibroblasts exhibit features of transformed cells, but unlike these, they do not show genetic aberrations. Rather, it seems that different activation processes are not correctly balanced by regulatory mechanisms in these cells. In this regard, it is typical of rheumatoid synovial fibroblasts to constitutively express growth factors, adhesion

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molecules and proteases, which participate in inflammation and in the destruction of joint tissues (Pap et al., 2005).

One particularly altered regulatory mechanism in rheumatoid synovial fibroblasts is cell growth. In healthy individuals, the synovial membrane is formed by a limiting row of cells, the synovial intima, which overlays connective tissue in an epithelium-like fashion. In rheumatoid arthritis, the intimal layer is characteristically disarranged and hyperplastic (Tarner et al., 2005). Along with the infiltration by leukocytes, there is an increase in the density of synovial fibroblasts. However, the latter does not derive from a high proliferation rate, but from cell longevity. These cells are able to survive in adverse conditions, such as hypoxia and loss of matrix anchorage. Defective apoptosis might therefore be a critical process in acquisition of invasiveness, and different works have shed light on this, describing specific alterations in the regulation of death mechanisms in rheumatoid arthritis (Takami et al., 2003; Jüngel et al., 2006). But on the whole it could be said that the apoptotic machinery of rheumatoid synovial fibroblasts is not impaired, and most of its deregulation could be due to the influence of signals from the inflamed environment (Kammouni et al., 2007).

How rheumatoid synovial cells become invasive brings back the chicken or the egg dilemma. Are they mere effectors activated by immune cells or is it the activation of these cells by innate mechanisms what helps to trespass the barrier of peripheral tolerance, at the same time conferring them with aggressive features? Increasing evidence is rising supporting that local factors associated to inflammation can shape the phenotype of rheumatoid synovial fibroblasts. An intriguing feature of rheumatoid synovitis is local hypoxia (Stevens et al., 1991). The formation of new vessels is characteristic of synovitis, but still the density of blood vessels is insufficient to supply the overgrown tissue, so consequently there are focal areas of ischemia inside the inflamed joint. It has been shown that reduced oxygen leads to an activation of hypoxia sensitive elements, orchestrated by hypoxia induced transcription factor-1 (HIF-1). Apparently, there is a low threshold for the induction of the hypoxia program in rheumatoid arthritis, probably resulting from the stabilisation of HIF-1 by inflammatory cytokines. The vascular endothelium growth factor (VEGF) is one of the molecules induced by HIF-1 thought to play a prominent role in the acquisition of invasiveness by rheumatoid synovial fibroblasts (Distler et al., 2004).

1.2.2 Hurdles in the study of rheumatoid synovial fibroblasts' features

Synovitis is a non-conventional lesion, and it is difficult to put it into experimental coordinates in order to dissect its pathogenic mechanisms. Nonetheless, in primary cultures, rheumatoid synovial fibroblasts but not synovial fibroblasts from osteoarthritic or healthy joints, are able to maintain an activated phenotype after several passages. This rare feature suggests that cells carry with them a stable imprinting of the in vivo circumstances. However, in vitro studies have frequently failed to identify or consistently replicate mechanisms associated to acquisition of invasiveness by rheumatoid synovial fibroblasts. A possible explanation is that cells from the same joint can exhibit a heterogeneous spectrum of phenotypes (Kasperkovitz et al., 2005). In large synovial specimens, an alternation can be observed between overgrown sprouts of tissue (macroscopic villi) and normal-appearing areas, a finding that suggests that the aggressive transformation of synovial fibroblasts is focal. Studies based on primary cultures, as well as on high throughput techniques, can miss the features of small but critical subpopulations of cells.

In order to overcome these limitations, elegant strategies have been employed for the study of invasiveness of rheumatoid synovial fibroblasts. An interesting approach is to specifically look at areas where synovium invades cartilage and bone, the so-called cartilage-pannus junction (CPJ), looking for selective expression of molecules conferring invasiveness (Benito et al., 2004). From this type of study we have learned that cells located in the invasive fronts are mostly macrophages and fibroblasts. Both the up-regulation of anti-apoptotic factors, such as sentrin 1 and the Fas associated death domain-like interleukin 1 converting enzyme inhibitory protein (FLIP), and the expression of protooncogenes, have been found in these areas (Franz et al., 2000; Schedel et al., 2002). On the other hand, studies looking into CPJs are necessarily carried out in specimens obtained at the time of joint replacement surgery. Therefore, we need to be cautious at drawing conclusions, because these samples could reflect longstanding instead of active disease.

The invasive process has been studied in vitro in a transwell system with Matrigel, a method that was designed for the study of metastasis (Tolboom et al., 2002). More recently, a matrix-associated transepithelial resistance invasion (MATRIN) assay was developed to measure the rate of invasiveness from the breakdown of the electrical resistance generated by an epithelial monolayer (Wunrau et al., 2009). This system provides a means of directly assessing the participation of a particular factor in the ability of cells to scatter through the matrix. Also interesting are several three-dimensional co-culture systems in which minced, artificially generated, or native cartilage is put into contact with different subsets of cells that are present at the CPJ.

The experimental severe cellular immunodeficient (SCID) mouse co-implantation model allows the study of mechanisms of invasion in vivo. In this model, human cartilage and rheumatoid synovial fibroblasts are engrafted in nude mice, that is, in an immune independent environment. Several works using this model have shown the ability of rheumatoid synovial fibroblasts to migrate and to destroy cartilage in the absence of immune cell concurrence (Müller-Ladner et al., 1996). On the other hand, some mechanisms of destruction taking place in rheumatoid arthritis might not show up in the SCID mouse model, as the latter does not provide an inflammatory microenvironment comparable to synovitis (Jüngel et al, 2010).

1.2.3 The family of metalloproteinases and related molecules

There are probably several mechanisms accounting for cartilage and bone destruction in rheumatoid arthritis, and different cells and mediators can be involved. Activation of osteoclasts through the RANK ligand system stands as the principal pathway of bone erosion. In this sense, synovial fibroblasts, among other cell types, have an undeniable role as inductors of osteoclast differentiation and maturation in arthritic joints (Kim et al., 2007). But there is also much evidence of the direct ability of synovial fibroblasts to penetrate the adjacent joint structures as a result of their production of various proteases.

Metalloproteinases (MMPs) are a family of Zn^{2+} binding endoproteinases able to degrade the connective tissue. They are synthesized as precursors and cleaved at the N-terminus to their active forms. There is a considerable overlap of functions between them, and some of them are known to trigger the activation of others, as well as interact with additional proteases to generate proteolytic cascades in certain systems.

Of all MMPs, the interstitial collagenase, MMP-1, and stromelysin 1, or MMP-3, are the best characterized in the setting of synovitis. MMP-3 is able to degrade the most abundant extracellular components of the synovial tissue, including fibronectin and laminin, as well as

collagens I and III. In addition, it activates MMP-1, which not only shares some of MMP-3 cleavage targets, but is also able to degrade collagen II, the principal component of articular cartilage. Plasma levels of both proteases have been found to be increased in patients with rheumatoid arthritis (Manicourt et al., 1995). Moreover, they correlated with disease activity, while intra-joint concentrations of the enzymes increased in parallel with the degree of joint inflammation (Ishiguro et al., 1996). Plasma levels of MMP-3 are currently regarded as a surrogate marker of severity, a fact that reflects its relevance as a mediator of joint destruction in rheumatoid arthritis. With immune-detection techniques, MMP-1 and -3 show a patchy distribution throughout the inflamed synovial tissue. Both molecules are consistently found at CPJs, where a diffuse immune-reactivity has been described (Tetlow & Woolley, 1995). Their pattern of distribution has confirmed synovial fibroblasts as the major source of these molecules in the joint.

Also of interest are the group of membrane-anchored (MT-) MMPs, which are bound to integrin chains, and, upon activation, digest pericellular matrix. Of this family, MT1-MMP (also MMP-14), which is over-expressed in rheumatoid synovial fibroblasts, is thought to confer to these cells some of their invasive potential (Yamanaka et al., 2000). In this regard, experiments carried out in the SCID mouse co-implantation model have shown the participation of MMP-1 and MT1-MMP in the degradation of cartilage by rheumatoid synovial fibroblasts (Rutkauskaite et al., 2004; Rutkauskaite et al., 2005). Based on this evidence, the pathway of MMPs has been for a while a promising area of research for therapeutics, not only in rheumatoid arthritis but also in metastatic tumours. The members of the family of tissue inhibitor of metalloproteinases (TIMPs), which act as natural regulators of MMPs, appeared as ideal candidates to develop anti-invasive compounds. In the SCID mouse model, TIMP-1 and -3 over-expressing mutants were able to slow the invasive process (van der Laan et al., 2003). However, less convincing results have been drawn so far in therapeutic experimental approaches and clinical trials.

In summary, it appears that synovial fibroblasts are the main effectors of destruction, a fact that could be considered natural. Fibroblasts are in charge of connective tissue remodelling, both under physiologic conditions and in disease. Production of proteases allows them to migrate through the matrix and restore the injured site in wound healing processes (Woessner, 1991). The same mechanisms take place during invasive processes. In this regard, rheumatoid synovial fibroblasts have been compared to tumour-associated stromal cells, which are non-neoplastic fibroblasts that contribute to metastatic growth by the production of MMPs (Hotary et al., 2003). Interestingly, the presence of MMPs at the synovial tissue is not related to the stage of the disease, and in fact the proteases can be abundant in early synovitis (Katrib et al., 2001). Therefore, fibroblast activation is not necessarily a consequence of longstanding disease, but could be one of the distinguishing processes between non-progressive disease and rheumatoid arthritis.

To help understand why rheumatoid and not other synovial fibroblasts turn invasive, a revealing study put in relationship the mRNA expression levels of MMPs with local hypoxia. Not only hypoxic cultures resulted in an increase in MMP-1 and MMP-3 transcripts, but also HIF-1α siRNA transfects yielded 50% lower mRNA levels of MMP-3 (Ahn et al., 2008).

Pulling the thread of research coming from invasive neoplasms and stromal cells, additional synovial fibroblast-dependent proteases were discovered at CPJs, showing potent in vitro capacity to destroy bone and cartilage. One of these molecules, that heralds the aggressive behaviour of tumours, is the urokinase type plasmin activator (uPA) (Duffy & Duggan,

2004). While uPA is able to degrade the extracellular matrix, it also activates MMPs and proteoglycanases through the cleavage of their precursors. Several studies have shown that uPA is over-expressed in joints from patients with rheumatoid arthritis, in correlation with disease severity (van der Laan et al., 2000). In spite of this role, uPA could be a double-edged therapeutic sword in rheumatoid arthritis, due to its activity in extra-vascular fibrinolysis, as we discuss in the next section. Interesting evidence was drawn in mice with antigen-induced arthritis, since uPA-deficient animals depicted a more severe phenotype as compared to wild type littermates (Busso et al., 1998). From subsequent studies, it can be concluded that aggressive features mediated by uPA are linked to its cell attachment activity, through the binding of its high affinity receptor, uPAR. New released work has found that the uPA-uPAR pair is a mediator of invasiveness in the SCID mouse co-implantation model (Serratì et al., 2011). In turn, uPAR is part of a larger complex, the urokinase plasminogen activating system (uPAS), formed by its assembly with 4 serin protease inhibitors at the cell surface. Triggering of uPAS is associated to proliferation, adhesion, migration and neoangiogenesis in tumours. These findings point to the complex as a better therapeutic target than the protease itself (Ulisse et al., 2009).

1.3 The role of fibrin in rheumatoid arthritis

1.3.1 Haemostasis activation overflows the fibrinolytic capacity in the joints with rheumatoid arthritis

Since the extra-vascular activation of haemostasis is a characteristic feature of inflammation, fibrin deposition in the inflamed synovial tissue is considered a non-specific event. During inflammation, the exudation of plasma into joints can result in coagulation factors achieving high concentrations at the synovial effusion. In fact, joints affected with osteoarthritis, infections, and trauma, often show fibrin deposits, albeit not as widespread as found in rheumatoid arthritis (Clemmensen et al., 1983). The striking abundance of fibrin in rheumatoid synovial tissues has been attributed to both an increased formation and a low clearance of the clots. As pointed out in different studies, rheumatoid arthritis flares provoke a status of extra-vascular thrombophilia, so that the influx of fibrinogen and its immobilization are high (Carmassi et al., 1996). Fibrin networks are thicker in patients with rheumatoid arthritis than in controls, and presumably more resistant to proteolysis as well (Kwasny-Krochin et al., 2010). This feature along with a reduced fibrinolytic activity can explain the accumulation of fibrin inside rheumatoid joints. Of the two regulatory systems that activate plasmin to degrade fibrin, the tissue plasminogen activator (tPA) is reduced in rheumatoid synovial tissues. Similarly, there is an increased production of the inhibitors of plasminogen activator, PAI-1 and PAI-2, which act by preventing fibrin dissolution through a constitutive pathway (Weinberg et al., 1991; Ronday, et al., 1996).

Local activation of complement is an inflammation-dependent mechanism that can help to stabilize fibrin clots thereby decreasing the fibrinolytic potential of the joint. In particular, we explored some years ago the local production of the regulatory factor C4b-binding protein (C4BP). The protein C-S anticoagulatory system is a principal mechanism for preventing the uncontrolled activation of haemostasis. The beta chain of C4BP binds protein S with high affinity in an equimolecular fashion (Dahlbäck, 1989). Only free protein S is active and the free fraction depends on the availability of C4BP beta. Reduced levels of free protein S are associated with an increased risk of thrombotic events. Interestingly, we showed local production of C4BP beta by rheumatoid synovial fibroblasts, as well as its co-localization with fibrin-rich areas at the synovial tissue (Sánchez-Pernaute et al., 2006). The

beta chain of C4BP has also been found in omen fibroblasts participating in the invasion and resorption of the corpus luteum, therefore indicating that besides its prothrombotic role, the molecule is important in fibroblast-dependent remodelling processes.
In the light of these experimental data, a potential participation of fibrin in synovitis has been argued by different groups including ourselves (Busso & Hamilton, 2002; So et al., 2003; Sánchez-Pernaute et al., 2003b), and several antithrombotic strategies have been tried, proving useful in attenuating the inflammatory process in experimental models of rheumatoid arthritis (Busso et al., 1998; Varisco et al., 2000).

1.3.2 Fibrin as an autoantigen

Fibrin is one of the major substrates for peptidyl deiminases (PAD) inside inflamed joints. These enzymes transform arginine residues into citrulline, and subsequently change the physical properties of the protein. This modification could alter binding sites of plasmin, making the polymer resistant to proteolytic degradation. Moreover, it can also turn the molecule antigenic (Schellekens et al., 1998). This fact was confirmed with the characterization of anti-citrullinated peptide antibodies (ACPA), since they were shown to target epitopes from fibrin in a specific association with rheumatoid arthritis (Masson-Bessiere et al., 2001). Thus, fibrin can been considered a key mediator in the loss of immune tolerance in the disease. Since ACPA antibodies reach higher concentrations inside joints than at the periphery, deposition of fibrin in joints and exposure of its citrullinated form to immune-competent cells should be an early pathogenic event. On the other hand, ACPA can be found in pre-clinical stages in at least half of patients. To explain this contradiction, it has been suggested that the immune system is primed for citrullinated epitopes outside joints, for example in the lung or the oral cavity, with arthritis coming on a second wave (Quirke et al, 2011). We propose a different mechanism. According to recent studies, synovitis can remain asymptomatic during the first stages of the disease. A first mild flare of arthritis could, therefore, be the event during which citrullinated fibrinopeptides are generated inside joints and presented to the central immune system (van de Sande et al., 2011).

1.3.3 Fibrin as a scaffold

We focused our studies on "a non-immune" participation of fibrin in the development of synovitis. Since fibrin networks provide binding sites for the migration of cells, in this way facilitating wound healing, we proposed that the synovial tissue might grow through the engulfing of fibrin deposits at the lining surface. In a time-dependent approach, we studied events taking place from the first stages of the disease in antigen induced arthritis, and were able to describe the transition from acute inflammation, to deposition of fibrin clots, and subsequent synovitis-like tissue modifications taking place at fibrin-synovium interfaces (Sánchez Pernaute et al., 2003a). We then proposed that the binding of the free-surface of the lining cells would alter their polarity, thereby changing their resting phenotype into a migrating one, a mechanism that would contribute to invasiveness.

1.3.3.1 Immobilization of fibrin affects cell binding

Soluble fibrinogen turns into solid fibrin through the release of fibrinopeptides A and B, with the rest of its alpha and beta chains remaining mostly unchanged. Therefore, most cell binding sites are shared by the two macromolecules. But it is in our opinion the insolubility of fibrin which accounts for an invasive response of synovial fibroblasts in rheumatoid arthritis. Experiments carried out in macrophages demonstrated that the binding affinity of

cells increases with fibrinogen transformation into fibrin (Shainoff et al., 1990). Considering that blood cells express different alpha beta integrin chains that could bind circulating fibrinogen, the affinity of the soluble molecule needs to be low. In this regard, the shear forces elicited by fibrin networks may have an influence in the avidity towards cell receptors. This mechanism has been recently found to account for activation of colon tumour cells upon engagement of fibrin by the hyaluronate receptor, CD44 (Alves et a., 2009).

The cell binding activity of fibrin networks is further enriched by its cross-linking with different matrix proteins, including collagens, proteoglycans or fibronectin, which also bear a variety of cell binding sites.

Yet to be understood, is why rheumatoid but not non-rheumatoid hosts develop an invasive response to fibrin clots inside joints. It could be simply a matter of magnitude, as we discussed several years ago (Sanchez-Pernaute et al, 2003b). It could also rely on intrinsic features of rheumatoid synovial fibroblasts, that up to date have not been found. Alternatively, it is attractive to speculate that resistance of the clots to plasmin proteolysis in rheumatoid joints makes local macrophages and fibroblasts secrete additional proteases that degrade fibrin through a non-constitutive pathway (Bini et al., 1999). Between these potential fibrin-degrading proteases stands MMP-3, which is also one of the major mediators of joint destruction. In this way, the active process of digestion of the insoluble macromolecule could be regarded as a favourable environment for the destruction of structures nearby.

1.3.4 A potential role of fibrin in the architecture of synovitis

The early works focused on the histopathology of rheumatoid arthritis, described a gradient in fibrin deposition from more abundant and solid-like at the surface, to patchy and reticular in inner areas (Andersen & Gormsen, 1970). Working with specimens from patients undergoing joint replacement surgery we described that fibrin-rich areas of the synovium were organized differently than non-fibrinous regions (Sánchez-Pernaute et al., 2006). With immune-detection techniques we found that both cells and extracellular matrix elements had a differential distribution in fibrinous and non-fibrinous areas, and there were transition zones between them (Figure 1). In this regard, matrix deposition and fibroblast-like synovial cells, as well as vessels, increased in density in fibrin-rich areas, up to an interface with solid-like fibrin deposits at superficial areas, where cells were scant and there were no vessels. Macrophages were clustered in the vicinity of fibrin deposits, although they were also abundant in non-fibrinous regions; but lymphoid aggregates clearly stayed apart (unpublished observations). This particular organization has led us to focus on the interaction between fibrin and synovial fibroblasts aiming to find mechanisms of invasiveness induced by fibrin in these cells.

Studies conducted in synovial specimens are frequently based on small biopsies. These explants constitute a fine way to reflect events taking place in the whole synovial tissue, in particular as regards cell activation and the participation of subtypes of infiltrating leukocytes (Smith et al., 2006). Elegant studies have proved that the pathology of the synovial tissue from biopsies can be employed to measure response to therapy and even unveil specific molecular predictive markers. However, this kind of study rarely describes the features of fibrin-rich areas, probably because in these regions the architecture of the tissue is distorted and difficult to read (as illustrated in figure 1). We believe that these areas are routinely discarded and thus critical components of the synovial pathology might be underestimated.

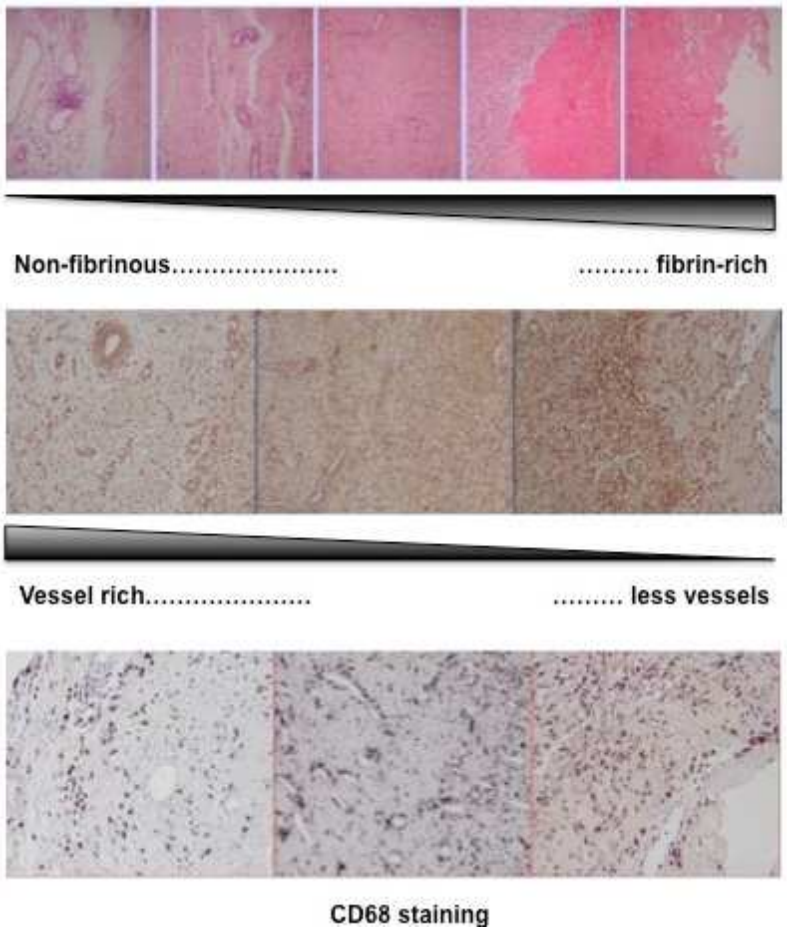

Fig. 1. Stratification of synovitis in relationship to fibrin deposits in a representative rheumatoid synovial tissue

Low-magnification microphotographs of a representative rheumatoid synovial tissue with an overlying fibrin deposit. Left column shows staining of the different tissue regions with haematoxylin-eosin. Middle column shows cell distribution with anti β actin antibodies. On the right side, immune-localization of macrophages with anti CD68 antibodies is shown.

2. Fibrin contributes to the production of MMP-1 and MMP-3 by rheumatoid synovial fibroblasts

In order to test whether deposition of fibrin might trigger the production of MMPs, we investigated the presence of fibrin in synovial tissues from patients with rheumatoid arthritis. We observed a similar pattern of distribution to those of MMP-1 and -3. These findings led us to explore in vitro whether fibrin could activate the production of MMPs by rheumatoid synovial fibroblasts. In these studies, which we next describe, we introduced an

in vitro system that attempts to reproduce the interaction between fibrin and fibroblasts that takes place inside the joint. This model of cell stimulation had originally been developed to study leukocyte migration through vessel walls and is known as in situ fibrin polymerization (Qi & Kreutzer, 1995). In contrast to other types of cultures involving cells and matrix proteins, this approach conserves the shear forces of freshly clotted fibrin networks and exposes cells to the deposits by their apical surface.

2.1 Experimental methods

2.1.1 Obtention and handling of samples

Synovial tissues were obtained during joint replacement surgery from 8 patients with rheumatoid arthritis according to the American College of Rheumatology criteria (Arnett et al., 1988). For histologic studies, the synovial membrane was immediately fixed in formaldehyde, dehydrated in ethanol and embedded in paraffin. When tissues included bone edges, they were decalcified by a 48 hour incubation in formic acid. For in vitro studies, cells were isolated by disruption with 1.5 mg/ml dispase II at 37ºC for 1 hour in agitation, and cultured in 10% fetal calf serum (FCS) enriched Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 50 UI/ml penicillin-streptomycin, 0.2% amphotericin B, and 10 mM HEPES.

2.1.2 Immune-detection techniques

We studied the distribution of fibrin, MMP-1 and MMP-3 with double labelling immune-detection methods. Tissues were rehydrated, blocked with 6% bovine albumin and 3% serum of second antibody hosts, and incubated with the specific antibodies at 10 µg/ml overnight, at 4ºC. Secondary antibodies were applied for 1 hour, at room temperature. As control, tissues were incubated with an isotype IgG from the species of primary antibodies. Development of fibrinogen immune-reactivity was done with peroxidase and Histogreen, using nuclear fast red for counterstaining. Diaminobenzidine was applied after a byotinilated secondary antibody to develop MMP-3, and counterstaining was done with hematoxylin. An alkaline phosphatase labelled antibody was employed to detect MMP-1, and nitroblue tetrazolium with 5-bromo 4-chloro 3-indolyl phosphate was used as substrate for development, plus nuclear fast red as counterstaining. Inhibition of endogenous peroxidase was done with 1% H_2O_2 methanol. Alkaline phosphatase activity was blocked with 5 mM levamisole.

2.1.3 Fibrin-cell cultures

Between passages 4th and 7th cells were grown to confluence at 37ºC in 5% CO_2, starved from serum during 48 hours and exposed to in situ clotted fibrin. Chilled fibrinogen was mixed in 0.5% foetal calf serum enriched DMEM at 1 mg/ml on ice, and 0.75 UI/ml thrombin was added. The mixture was immediately spread on top of the cell monolayers and the cultures were transferred to the incubator to allow formation of fibrin clots.

2.1.4 Gene expression studies

Four different cell cultures were employed. Cells were incubated with fibrin or medium alone for 12 hours. At the end of the incubation period, the clots and supernatants were removed, cells were washed and total RNA was isolated. Following retrotranscription, gene expression studies were done with quantitative PCR (qPCR) using cDNA as templates and

TaqMan primer-probe reagents (MMPs) or SYBR Green techniques (urokinase). Results were analysed following the ΔΔCt method, using the expression of ribosomal 18s as house keeping gene and an untreated culture as reference.

2.1.5 Immunoblot techniques

Confluent cells were incubated with or without fibrin and protein levels of MMPs were measured at several time points. The protein levels of MMP-1 were determined in cells lysates as obtained with Laemmli's solution. Supernatants and fibrin were mixed with 150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% sodium dodecyl sulphate, 1% Triton X-100, 1% sodium deoxycholate and 5 mM ethylenediamine tetraacetic actid, with 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonylfluoride, at 4°C and homogenised. One hundred μl of the homogenates were incubated with the anti MMP-3 antibody for 1 hour at 4°C. Then, protein A/G plus-agarose immunoprecipitation reagent (20 μl) was added, and samples were incubated overnight on a rocking platform at 4°C. Finally, samples were washed and centrifuged to pellet beads with the complexed MMP-3, supernatants were discarded and precipitates were mixed in loading buffer. Protein extracts and immuno-precipitates were resolved in 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose filters. The filters were then blocked with 5% skimmed milk and 0.03% Tween and blotted overnight with the specific antibodies. Horseradish peroxidase-labelled secondary antibodies were applied for 1 hour, at room temperature, and the binding was developed with enhanced chemi-luminescence. Results were expressed as relative increase with regard to baseline. Beta-tubulin levels were used as control of cell protein content, and the Coomassie blue staining method was used in supernatants.

2.1.6 Statistics

Data are expressed as median (range); comparison between conditions was done with the non-parametric Wilcoxon rank test, using SPSS software.

2.1.7 Reagents and probes

Mouse anti human MMP-1 and goat anti human MMP-3 (R&D systems, Basel, Switzerland), goat anti human Fibrinogen (Abcam, Cambridge, UK), donkey anti goat-HRP, goat anti mouse-HRP, rabbit anti goat biotinylated antibodies, 1:500 in IHC, 1:10000 in WB (Jackson ImmunoResearch, LaRoche, Switzerland), goat anti mouse-alkaline phosphatase antibodies 1:40, Histogreen, nuclear fast red, DAB, peroxidase ABComplex (Dako, Zug, Switzerland), NBT-BCIP (Roche), Protein A/G Plus Agarose Immunoprecipitation Reagent (Santa Cruz Biotechnology Inc., CA), ECL chemoluminiscence (Amersham Pharmacia Biotech, Little Chalfont, UK) Fetal calf serum, DMEM, penicillin, streptomycin, HEPES, fungizone (Life Technologies, Basel, Switzerland), dispase (Roche, Reinach, Switzerland), fibrinogen (American Diagnostica inc., Stamford, CT), mouse anti human tubulin, thrombin (Sigma Aldrich, Buchs, Switzerland) MiniRNeasy spin column purification kit, RNase-free DNase set (Qiagen, Basel, Switzerland), SYBR green master mix, ABI Prism 7500 Sequence Detector (Applied Biosystems, Rotkreuz, Switzerland). Primer pairs and probes: MMP 1: 5'-tgtggaccatgccattgaga-3' (fwd), 5'-tctgcttgaccctcagagacc-3' (rev), FAM5'-ccaactctggagtaatgtcacacctctgacattcacc-3'TAMRA (probe); MMP 3: 5'-gggccatcagaggaaatgag-

3′ (fwd), 5′-cacggttggagggaaaccta-3′ (rev), FAM5′-agctggatacccaagaggcatccacac-3′TAMRA (probe); MMP 9: 5′-ggccactactgtgcctttgag-3′ (fwd), 5′-gatggcgtcgaagatgttcac-3′ (rev), FAM5′-ttgcaggcatcgtccaccgg-3′TAMRA (probe); MMP 13: 5′-tcctacaaatctcgcgggaat-3′ (fwd), gcatttctcggagcctctca-3′ (rev), FAM5′-catggagcttgctgcattctccttcag-3′TAMRA (probe); MMP 14: 5′-tggaggagacacccactttga-3′ (fwd), 5′-gccaccaggaagatgtcatttc-3′ (rev), FAM5′-cctgacagtccaaggctcggcaga-3′TAMRA (probe); urokinase: 5′-tgtcagcagccccactactac-3′ (fwd), 5′-cacagcattttggtggtgac-3′ (rev).

2.2 Results

2.2.1 Fibrin and MMPs co-localized in the synovial tissues from patients with rheumatoid arthritis

We studied the distribution of fibrin(ogen) in synovial tissues from 8 patients with rheumatoid arthritis. Three of them included areas of invasion into cartilage and bone. The binding was strong and abundant in all samples, showing either an amorphous or a reticular pattern, as has been described (Andersen & Gormsen, 1970; Clemmensen et al., 1983). Fibrin predominated in the vicinity of the lining layer. Solid-looking deposits were mostly acellular, while more organized material was found in interstitial areas, with cells inside also capturing the antibody. Fibrin immune-reactivity was strong at areas of interface with bone and cartilage.

Next, we studied the distribution of MMP-1 and MMP-3 in serial cuts of the same tissues, alone and in combination with fibrin, using double-staining methods. Immune-reactivity to both MMPs was high in the rheumatoid synovial tissues. Interestingly, MMP-1 predominated inside cells, and MMP-3 was mostly secreted. The binding of both antibodies was high at erosive fronts.

In double-staining studies, interstitial immune-reactivity to both MMP-1 and MMP-3 was associated with fibrin deposits. Furthermore, both proteases co-localized with fibrin at the invasive fronts. Fibroblast-like cells in fibrin-rich areas depicted a strong immune-reactivity to MMP-1 and MMP-3. These findings indicated that fibrin-rich areas were at the same time active sites of protease production.

2.2.2 Gene expression of proteases in rheumatoid synovial fibroblasts exposed to fibrin clots

In view of these observations, we carried out in vitro studies to look into a possible effect of fibrin on the production of proteases by rheumatoid synovial fibroblasts.

Thus, we studied five members of the MMP family that are prominent effectors of cartilage and bone destruction in rheumatoid arthritis. This revealed that exposure to in situ clotted fibrin resulted in the up-regulation of the gene expression of MMP-1, MMP-3, and MMP-9 to a variable extent in all cell cultures (Figure 3). The mRNA levels of MMP-1 were 26-fold increased (range: 1,5 to 71; $p < 0.07$), and those of MMP-3 were 27-fold increased (range: 2 to 126; $p < 0.07$). MMP-9 gene expression was increased 7-fold the presence of fibrin (range: 3 to 39; $p < 0.07$), while no differences were found in the gene expression of MMP-13 and MMP-14.

Additionally, we studied urokinase gene expression, since it is the constitutive fibrin-degrading molecule, and a potential mediator of tissue injury in rheumatoid synovial tissues. In our conditions, fibrin did not elicit any changes in urokinase mRNA levels compared to baseline.

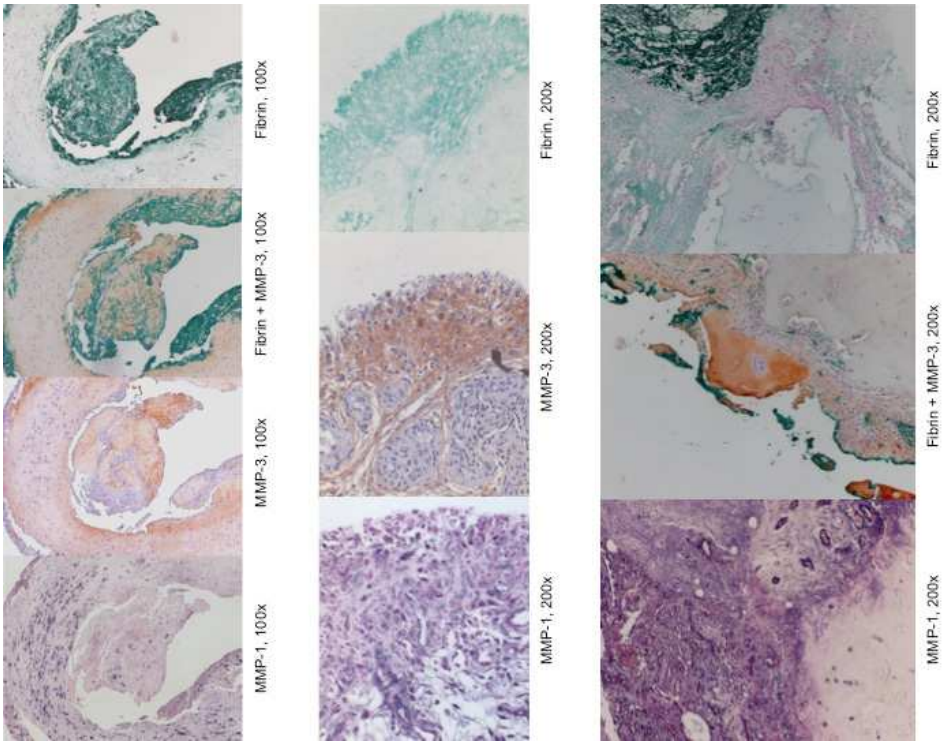

Fig. 2. Immune-staining of fibrin and MMPs in the rheumatoid synovial tissues

Left column shows a low-magnification view of a fibrin-rich area of the synovial tissue, with the different staining techniques. Middle column shows the intimal layer of a fibrin-rich area in detail. Right column depicts the staining of the molecules at areas of invasion into bone and cartilage. Fibrin is shown in green (with red counterstaining), MMP-3 is shown in brown (and counterstained in blue), and MMP-1 is shown in blue (with red counterstaining).

2.2.3 Fibrin increased the production of MMP-1 by rheumatoid synovial fibroblasts

Six different cell cultures were employed in immunoblot experiments. In all cases, treatment with fibrin increased MMP-1 production. In our conditions, both the proenzime and the active form of MMP-1 increased between 18 h and 24 h in rheumatoid synovial fibroblasts treated with fibrin (Figure 3). At 24 h, the active enzyme increased to 4-fold as compared to untreated cells (range: 1,5 to 6; $p < 0.03$). On the other hand, we could not detect MMP-3 in cell lysates, but it was abundantly found in supernatants from cell cultures. Upon stimulation with fibrin, baseline levels of MMP-3 in supernatants were found increased only in half of the cell lines tested (ns) (Figure 3).

2.3 Discussion and future research

Our studies illustrate how deposition of fibrin can contribute to the invasive process in rheumatoid arthritis. Previous works had described the distribution of MMP-1 and MMP-3 in the rheumatoid synovial tissue. In the same way, fibrin distribution in rheumatoid joints

was well known. A novel finding is that all three followed a similar pattern of distribution. We could also confirm their abundance at CPJs, although we cannot draw conclusions as regards the impact of these finding in the erosive tendency.

Our studies suggest that fibrin triggers the transcription of several MMPs and the production of MMP-1. However, the study of MMPs is complex. These molecules are secreted in the form of zymogens, and need in situ activation through the cleavage of an N-terminus peptide. This post-translational modification probably constitutes a major check-point for the regulation of MMPs. Additionally, to find out what the global matrix turn-over could be, it would have been desirable to assess levels of TIMPs. TIMPs are the natural regulators of MMPs, an effect carried out through an equimolecular binding and inactivation. Both chondrocytes and synovial fibroblasts can produce TIMPs. In this regard, the balance between TIMPs and MMPs is decisive for the outcome (Martel-Pelletier et al., 1994). Functional studies, such as substrate-based zymography, are usually performed to demonstrate active proteolysis.

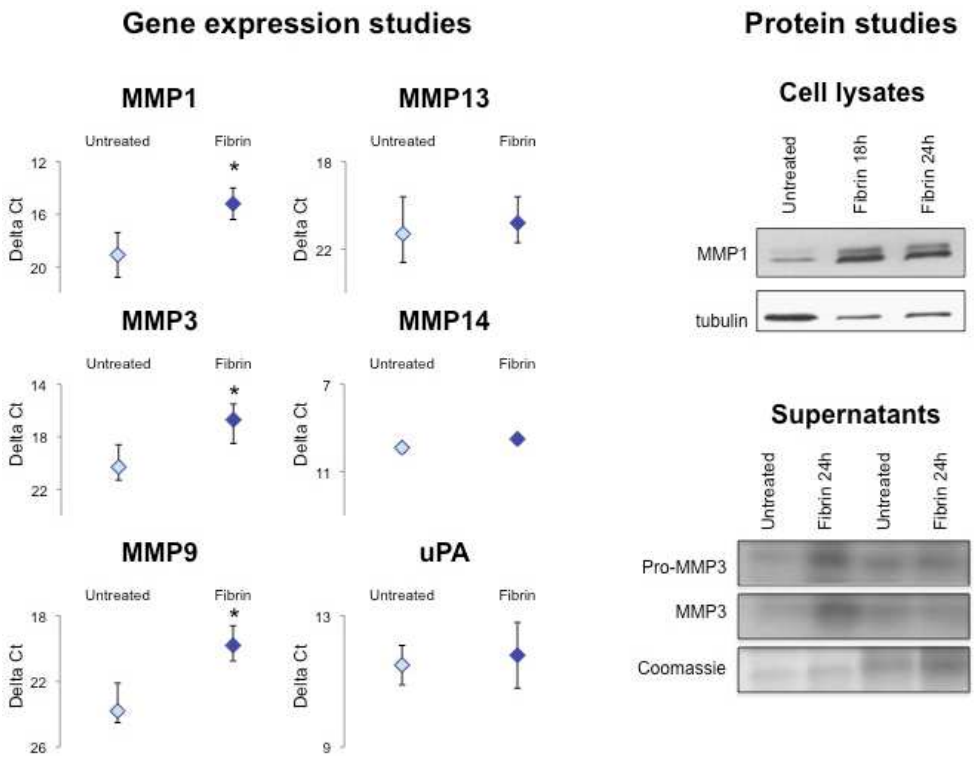

Fig. 3. Expression of MMPs in rheumatoid synovial fibroblasts exposed to fibrin

Graphs on the left show changes in baseline level expression of the mentioned genes (median ± SEM), in ΔCt values using 18s as house keeping gene (the lower ΔCt values, the higher gene levels are). On the right, representative immunoblots from different experiments are shown. Detection of MMP-1 was done in cell lysates and MMP-3 was

studied in the extracellular fraction. Both antibodies detected both the zymogen (upper band) and the active protease (lower band).

Nevetheless, it was interesting to find that three MMPs previously correlated to the invasive potential of rheumatoid synovial fibroblasts, were up-regulated by fibrin at the mRNA level (Tolboom et al., 2002). Due to its insolubility, fibrin might be degraded in a non-constitutive way by MMP-3 secreted by surrounding cells (Bini et al., 1999). On the balance of the evidence, we believe that fibrin-rich regions should not be considered a result of long-standing inflammation, but a site for active destruction.

In agreement with previous studies, most of the synovial fibroblast cultures that we employed in our studies did not constitutively express MMP-13 as assessed with qPCR techniques, and only in some was it induced after exposure to fibrin (Moore et al., 2000). In the production of this, as well as the other proteases tested, there seemed to be a high variability between patients. In fact, our studies suggested the existence of two subsets according to their response to fibrin. Approximately half of the cultures strongly reacted with the up-regulation of MMPs, while the other half showed mild or absent response. We believe that this is another example of the heterogeneous character of rheumatoid arthritis.

Although there was no regulation of MT1-MMP at the mRNA level by fibrin, an interesting finding drawn by our experiments was the high expression of MT1-MMP by unstimulated rheumatoid synovial cells, pointing to the prominent role of the protease in the activity of synovial fibroblasts as already suggested (Miller et al., 2009).

In summary, using a novel culture system for the study of fibrin interaction with synovial cells, we could show induction of proteases putatively associated to invasiveness, that were further localized at fibrin-rich areas in the synovial tissues.

3. Conclusion

In this chapter, we give an overview of the role of fibrin in the pathogenesis of synovitis. In doing so, we have neither solved the paradigm about what starts first nor discovered what triggers the invasive behaviour of rheumatoid synovial fibroblasts. Instead we provide some lines of thinking supporting the "inside – out pathway" as it has recently been named (Schett & Firestein, 2010).

The "fibrin pathway" has been little explored in rheumatoid arthritis therapeutics. The balance between clotting and dissolution of fibrin is a candidate process to target that could help to retard joint destruction.

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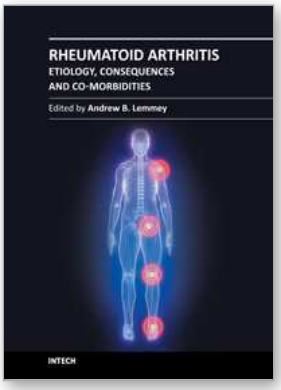

Rheumatoid Arthritis - Etiology, Consequences and Co-Morbidities
Edited by Dr. Andrew Lemmey

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The purpose of this book is to provide up-to-date, interesting, and thought-provoking perspectives on various aspects of research into current and potential treatments for rheumatoid arthritis (RA). This book features 16 chapters, with contributions from numerous countries (e.g. UK, USA, Japan, Sweden, Spain, Ireland, Poland, Norway), including chapters from internationally recognized leaders in rheumatology research. It is anticipated that Rheumatoid Arthritis - Etiology, Consequences and Co-Morbidities will provide both a useful reference and source of potential areas of investigation for research scientists working in the field of RA and other inflammatory arthropathies.

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