Genetics, autoantibodies and clinical features in understanding and predicting rheumatoid arthritis

Helm-van Mil, A.H.M. van der

Chapter 12

The invasiveness of fibroblast-like synoviocytes is an individual patient characteristic associated with the rate of joint destruction in patients with rheumatoid arthritis

A.H.M. van der Helm - van Mil*
T.C.A. Tolboom*
R.G.H.H. Nelissen
F.C. Breedveld
R.E.M. Toes
T.W.J. Huizinga

* Both authors contributed equally to this study

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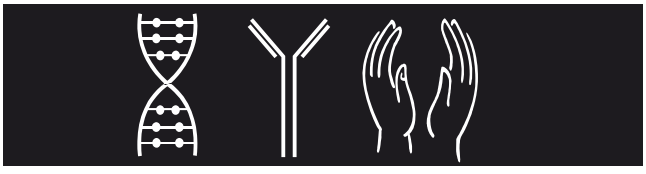


ABSTRACT

Objectives. Rheumatoid arthritis (RA) is characterized by inflammation and destruction of synovial joints. Fibroblast-like synoviocytes (FLS) harvested from synovial tissue from patients with RA can invade normal human cartilage in severe combined immunodeficiency disease (SCID) mice and matrigel in vitro. This study was undertaken to investigate the association of these in-vitro characteristics with disease characteristics in patients with RA.

Methods. Synovial tissue samples of 72 RA and 50 OA patients were obtained; from 7 patients with RA samples of different joints were collected. The FLS invasiveness in Matrigel matrix was studied; the intra-individual and inter-individual differences were compared. Of the patients with the FLS that exhibit the most extreme differences in in-vitro ingrowth (most and least invasive FLS) the X-rays of hand and feet were collected and read according to the Sharp-van der Heijde method to determine the relationship between in vitro invasion data and estimated yearly joint damage progression.

Results. FLS from patients with RA were more invasive than FLS from patients with OA ($P < 0.001$). The mean intra-individual variation in FLS invasion was much less than the mean inter-individual variation (mean ± SD 1067 ± 926 and 3845 ± 2367 for intra-individual and inter-individual variation, respectively; $P = 0.035$), which shows that the level of FLS invasion is a patient characteristic. The mean ± SEM Sharp score on radiographs of the hands or feet divided by the disease duration was 4.4 ± 1.1 units per year of disease duration in patients with the least invasive FLS ($n = 9$), which was much lower compared with the 21.8 ± 3.1 units per year of disease duration in patients with the most invasive FLS ($n = 9$) ($P < 0.001$).

Conclusions. The ex vivo invasive behaviour of FLS from patients with RA is associated with rate of joint destruction and is a patient characteristic given the much smaller intra-individual than inter-individual variation.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease which predominantly targets the synovial joints ultimately leading to joint destruction. The destructive process is suggested to be mediated, at least in part, by fibroblast-like synoviocytes (FLS) from the synovium, because in a severe combined immunodeficiency disease (SCID) mouse co-implementation model it was demonstrated that FLS of patients with RA attach to and invade in normal cartilage (1). Moreover, many groups have observed that in RA, FLS show characteristics of transformed cells like anchorage independent growth (2), insensitivity to apoptosis, and increased proliferation. Processes that are associated with the FLS-change from normal to aggressive behaviour are phosphorylation of the signal transducer and activator of transcription (STAT3) protein and elevated levels of the pro-oncogene c-myc (3). Whether these features are non causal associations or a causal factor is not yet known. FLS in culture express large amounts of proteases which can degrade extracellular matrix components such as collagens. One family of proteases that is expressed by FLS are the matrix metalloproteinases (MMPs). FLS express MMP-1, -3, -9 and -10 and expression of these MMPs correlate with invasion (4). Other protease families that are expressed in RA FLS are the cathepsins and ADAMs (a disintegrin and metalloproteinase). FLS from patients with RA express several oncogenes at higher levels than FLS from normal controls. Oncogenes that are upregulated in RA are c-myc (11), Ras (5) and p53 (6,7). These data have led to the suggestion that aspects of behaviour of FLS in RA resemble malignant tissue. However whether the transformed behaviour is of relevance for the disease characteristics of RA and if so for which characteristics has not been studied. The present study investigates the association of in vitro characteristics of FLS with disease characteristics in RA patients. We particular adressed the questions 1) whether the degree of FLS invasion is comparable in different joints of the same patient, i.e. is the FLS invasiveness a characteristic occurring at multiple joints of the same patient or rather a random process, and 2) whether the degree of in vitro invasion is correlated with the degree of radiological destruction.

MATERIALS AND METHODS

Patients and synovium

Synovial tissue was obtained from 122 patients (72 with RA and 49 with OA) at joint replacement surgery or synovectomy. 69% of the patients with RA were female and the mean age was 60 years ± 14. The samples were obtained from knee (53 patients), elbow (17 patients), shoulder (12 patients), hip (26 patients), ankle (7 patients), wrist (4 patients) and foot (1 patient). All patients with RA met the criteria of the American College of Rheumatology. Tissue was harvested by an orthopaedic surgeon and collected in ster-

ile phosphate buffered saline (PBS). Connective tissue and fat were removed and tissue was digested with collagenase IA (1 mg/ml; Sigma, St Louis, MO, USA) for at least two hours at 37°C. Cells were separated from the tissue using a 200 mm filter (NPBI, Emmer-Compascuum, The Netherlands) and cultured in 75 cm2 culture flasks (Cellstar, Greiner, Alphen aan de Rijn, The Netherlands) with Iscove's modified Dulbecco's medium (IMDM; Biowitthaker, Verviers, Belgium) supplemented with glutamax (GibcoBRL, Paisley, UK), penicillin and streptomycin (Boehringer Mannheim, Germany), and 10% fetal calf serum (FCS; GibcoBRL, Paisley, UK) at 37°C and 5% CO2. When the cells had grown to confluence they were detached with 0.25% trypsin and split in a 1:3 ratio. For invasive growth analysis passage 1 or 2 FLS were used. Light microscopy and Giemsa staining indicated that more than 95% of cells were FLS. As a control for the possible contamination of macrophages in the cell source, we reasoned that after several passages the macrophages will be gone. Thus the invasiveness of FLS from several patients was tested for several passages. This revealed stable invasiveness for several passages. Moreover, FACS staining of a randomly chosen subset of the samples revealed absence of CD14-positive cells.

In vitro invasion assay

Invasiveness of FLS was measured as described previously (4). Briefly, Transwells (6.5 mm diameter, 8.0 mm pore width; Costar, Cambridge, NY, USA) were coated with paraffin to avoid meniscus formation. Hereafter, the transwells were pre-incubated with 100 ml IMDM for 30 minutes at 37ºC. Transwells were coated overnight with 100 ml of 0.375 mg/ml Matrigel (Matrigel basement membrane matrix; Becton Dickinson, USA) in IMDM under sterile conditions in a laminar flow cabinet. The next day the Matrigel-coated wells were incubated with 100 ml IMDM for 1 hour at 37ºC. Cells were harvested as described above and after removal of the medium, 200 ml of 100,000 FLS/ml in IMDM was seeded in the inner compartment of the transwell system. In the outer compartment, 900 ml IMDM/10% FCS/10% human serum was pipetted and the cells were incubated for 3 days at 37ºC and 5% CO_2. After 3 days, the cells were fixed with 2% glutaraldehyde in PBS for 30 minutes at room temperature. After removal of the glutaraldehyde and subsequent washing with PBS, the cells were stained with a crystal violet solution for 30 minutes at room temperature. The cells were thoroughly washed with PBS and the cells that did not invade through the transwell membrane were removed together with the matrix by cleaning the inner wells of the transwell system with a cotton bud. The number of cells that had grown through the matrix and the transwell membrane were counted under a light microscope. All experiments were carried out in duplicate.

Radiological destruction and FLS characteristics

To compare the association between the degree of FLS invasiveness and joint destruction, the radiographs of hands and feet of the patients with the most invasive FLS (9 patients)

and least invasive FLS (9 patients) were scored according to the Sharp/van der Heijde method (8). The person that scored the radiographs was unaware of the clinical data and study question. The total erosion and joint space narrowing scores, as well as the total Sharp-van der Heijde scores, were divided by the disease duration from date of diagnosis to determine the radiological progression per year (9).

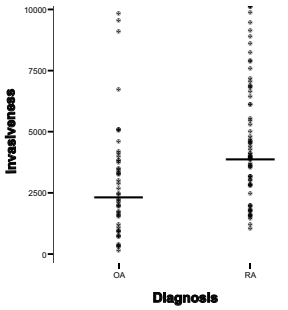
Statistical analysis

Differences in invasiveness of FLS between patients, differences between intra-individual and inter-individual variation and the radiological scores of the patients with the most and less invasive disease were compared with the Mann-Whitney test.

RESULTS

FLS were isolated from these tissues and cultured. When the cells had grown to confluency, the cells were harvested and tested for invasiveness as described before (4). RA FLS were significantly more invasive than OA FLS in this assay ($p< 0.001$ mean ± SD 2884±2326 and 4573±2502 for OA and RA respectively; Figure 1). These results are in line with previous studies (1;4).

Then it was studied whether the invasiveness of FLS from different joints operated at different times exhibit the same invasive characteristics. From 7 patients with RA, two



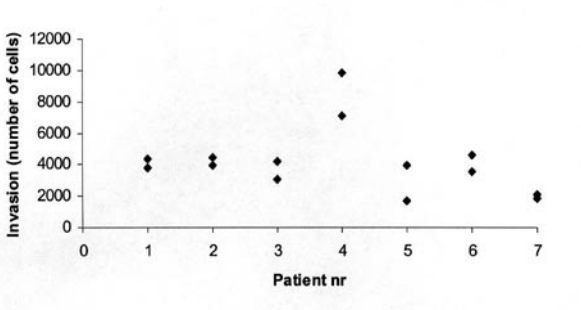


Figure 1 **Figure 2**

Figure 1. In vitro invasiveness of FLS from patients with RA (72) and OA (50). Each dot represents one individual. Invasiveness was measured using a transwell system coated with matrigel. The number of invasive cells was determined by counting the number of cell under the filter.

Figure 2. In 7 RA patients in vitro invasiveness of FLS was obtained from two different joints. Synovial samples were obtained at different times, and invasiveness was measured using a transwell system coated with matrigel. The number of invasive cells was determined by counting the number of cell under the filter. Tissue samples were obtained from hip (1), shoulder (3), elbow (6), wrist (3), ankle (1), knee (1).

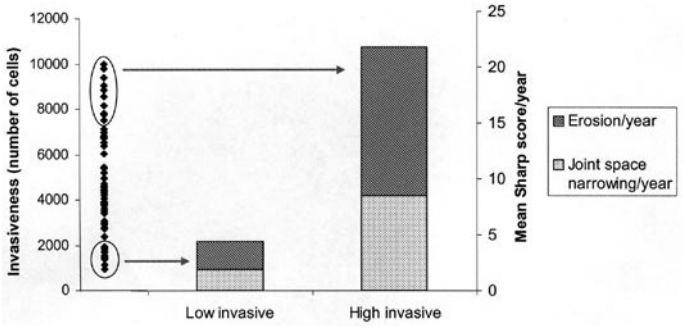


Figure 3. Association between in vitro invasiveness of FLS and estimated yearly radiological progression. From the nine most invasive and the nine least invasive RA patients the radiographs of hand and feet were scored according to the Sharp-van der Heijde method. The total Sharp-van der Heijde scores were divided by disease duration to determine the yearly radiological progression.

different samples were obtained from two different joints. The differences in invasiveness within the different samples of individual patients was significantly less than the differences in invasiveness between patients (p= 0.035; median difference ± SD: 1067 ± 926 and 3845± 2367 for intra- and inter-individual variation respectively). The results are shown in Figure 2. From the group of OA patients, three patients were operated twice. This group was too small to analyse the differences between intra-individual and inter-individual variation. Thus, the variation within patients is smaller than the variation between patients.

Next it was adressed whether the *in vitro* invasiveness of FLS was correlated with radiological joint destruction in RA. It has been published that the estimated yearly progression rate obtained by dividing the Sharp-van der Heijde score of radiographs of hands and feet by the disease duration correlated well with the observed destruction rate in observational studies with multiple measurements (9). Therefore, we assessed the association between invasiveness of FLS and radiological joint destruction. The Sharp-van der Heijde scores from the nine most invasive and the nine least invasive FLS were determined and the estimated yearly progression rates were compared (Figure 3). For the patients with the most invasive FLS the total Sharp-van der Heijde score per year disease duration is 21.8 (SEM 3.1), with an erosion score of 13.3 (SEM 1.7) and a narrowing score of 8.5 (SEM 1.6). For the patients with the least invasive FLS, the total Sharp-van der Heijde score per year disease duration is 4.4 (SEM 1.1), with an erosion score of 2.5 (SEM 0.7) and a narrowing score of 1.9 (SEM 0.5). No difference in disease duration was observed between patients with most and least invasive FLS (15.9 ± 11.5 versus 15.0 ± 7.3 years respectively; p=0.85). These results show a strong association between invasiveness of FLS and radiologically estimated yearly rate of joint destruction (p<0.001).

DISCUSSION

This study shows that in RA the intra-individual variation in FLS invasiveness is much less than the inter-individul variation, indicating that the invasive behaviour is a characteristic of an individual RA patient. Furthermore, this study shows for the first time that the in vitro FLS invasiveness is associated with radiological joint destruction. Patients with the least invasive FLS have significantly lower Sharp-van der Heijde scores per year disease duration compared to the patients with the most invasive FLS. This suggests that the invasive behaviour of FLS is of relevance for the pathogenesis of RA.

The finding of a rather large variation in rate of invasion of FLS between patients implies that the mechanism or processes underlying invasive behaviour differs between individuals. The mechanism that leads to transformation of FLS is not fully understood.

Previous studies have shown a myriad of alterations in the behaviour of FLS in RA. One very striking change in FLS is the expression of oncogenes (10). Oncogenes that are upregulated in RA are c-myc (11 ;12), Ras (5), p53 etc. Inhibition of the Ras pathway reduced expression of MMP-1 and MMP-3. Inhibition of both Ras and c-myc pathways also reduced invasion into normal human cartilage in the SCID mouse coimplantation model (13). And it is demonstrated that down-regulation of p53 influences proliferation and invasion of RA FLS (14;15). Transformation of FLS is different in different individuals, suggesting that a genetic component plays a role.

This study shows that the transformed behaviour of FLS in patients with RA is a patient characteristic and is strongly associated with clinical joint destruction. We used FLS from passages 1 and 2 in this study. Although no macrophages were detected in these samples by light microscopy and Giemsa staining, and no CD14-positive cells were detected in a randomly chosen subset of the samples, we cannot exclude unambiguously that macrophages contaminated the cells. However, the experiments in which FLS from different passages and from different donors were compared yielded similar levels of invasiveness, which is evidence against major artefacts from macrophages. Further research should elucidate the exact mechanism of transformation of FLS and the role of a genetic component.

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