CONCISE REPORT

Overexpression of transcripts containing LINE-1 in the synovia of patients with rheumatoid arthritis

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Objective: To identify novel diagnostic markers by comparing gene expression in rheumatoid (RA) and reactive arthritis (ReA) synovium.
Methods: Synovial biopsy specimens were obtained by needle arthroscopy from the knees of 10 patients with either RA or ReA. RNA was isolated from the biopsy specimens and cDNA synthesised for analysis using a customised cDNA macroarray. Confirmatory analysis was performed using in situ hybridisation on a second set of synovial samples.
Results: Two unique transcripts (ReXS1 and fibronectin) were consistently more abundant in ReA and three homologous transcripts were more abundant in RA. The latter all mapped within long interspersed nucleotide elements (LINE-1), that form one of the families of repetitive sequences in the human genome.
Conclusions: The abundance of transcripts containing LINE-1 in the RA synovium may be an epiphenomenon or may have pathogenic significance. Further work is required to determine the identity of the full length transcript(s) before its use as a diagnostic marker in RA can be assessed.

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the synovium of uncertain pathogenesis that results in joint damage and subsequent disability. It is now generally accepted that most patients with RA should receive effective treatment early in the course of their illness.[1] Thus, modern imaging modalities have demonstrated that cartilage and bone damage occur in the earliest stages of the disease and, once present, are currently irreversible.[2] Many patients who present with an acute synovitis do not have RA but one of several other possible diagnoses, including reactive arthritis (ReA). Some of these other illnesses require less aggressive management and others will resolve without treatment, but currently there are no reliable diagnostic markers that allow discrimination at presentation. In recent years a number of methodologies have been developed that permit a comparison between complex tissues without prior expectations of the differences to be found. We have therefore used one of these approaches, cDNA macroarray analysis, for a comparison of RA with ReA in an attempt to identify such markers.

PATIENTS AND METHODS

Patients

The cohorts used in this study initially presented to the Early Arthritis Clinic and are depicted in table 1. Patients with RA all fulfilled the 1987 American College of Rheumatology criteria at a subsequent visit.[3] Patients with ReA were selected from their recent history of a bacterial infection that might be the trigger for joint inflammation. Ultimately, all the patients with ReA achieved remission, whereas all patients with RA developed chronic, persistent synovitis. After ethical approval and the patient's informed consent had been received, synovial biopsy specimens were obtained from an inflamed knee under direct vision by needle arthroscopy.[4] At the time of biopsy, the patients had disease duration of less than one year, and none had received disease modifying antirheumatic drugs or corticosteroids. Synovial biopsy specimens were divided, one specimen being snap frozen in liquid nitrogen and stored at –80°C, and the other was fixed in 4% paraformaldehyde and embedded in paraffin wax.

Table 1 Patient cohorts used in this study. Age, disease duration, and synovitis visual analogue score (VAS) at the time of arthroscopy are depicted. Synovitis VAS provides an indication of the degree of inflammation

		At arthroscopy		
Patient	Sex	Age (years)	Disease duration (weeks)	VAS (0–100)
Cohort1				
RA1	M	50	52	46
RA2	F	33	40	51
RA3	M	62	28	83
RA4	M	67	24	36
RA5	M	22	24	94
ReA1	M	27	10	33
ReA2	M	21	8	32
ReA3	M	29	12	60
ReA4	M	16	3	25
ReA5	M	27	24	29
Cohort2				
RA6	F	53	48	42
RA7	M	60	42	92
RA8	M	53	52	65
ReA6	M	25	10	46
ReA7	M	34	14	31
ReA8	M	29	8	70
ReA9	M	22	12	46

Construction and analysis of cDNA macroarrays

The cDNA macroarrays were constructed and analysed as described previously.[5] Briefly, 1–3 μg RNA extracted from synovial biopsy specimens was Dnase I (Roche) treated and used to synthesise cDNA with the Capfinder system (Clontech). Equal amounts of cDNA as determined by spectrophotometry were

Abbreviations: BLAST, basic local alignment search tool; LINE-1, long interspersed nucleotide elements; PCR, polymerase chain reaction; RA, rheumatoid arthritis; ReA, reactive arthritis.

A

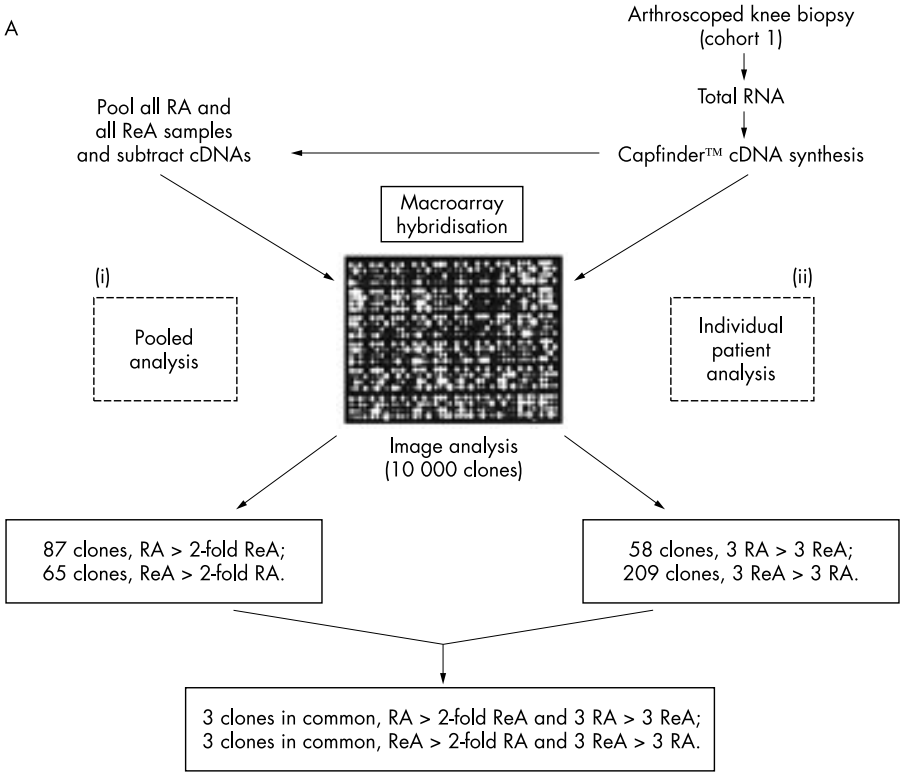

Figure 1. Summary of cDNA macroarray analysis. (A) A comparison of gene expression was carried out initially between (i) pooled, enriched cDNAs (two grids used), and (ii) cDNAs from patients RA1–3 and ReA1–3 (six grids used). The clones that gave consistent differences between RA and ReA samples and were common to both the pooled and individual patient analyses were selected. (B) Sequence analysis of the clones. The number of bases of cloned insert DNA is shown. The six clones (RXS1–3 and ReXS1–3 with accession numbers BU576960–5 respectively) were searched against the GenBank nucleotide database (BLASTNR) as well as the human EST (expressed sequence) database (BLASTEST). The accession numbers of the best matches are depicted. ND depicts not done.

B

Clone ID	bp	Accession no	BLASTNR	BLASTEST
RXS1	132	BU576960	Multiple hits	99% AI862212
RXS2	136	BU576961	Multiple hits	98% AI862212
RXS3	177	BU576962	Multiple hits	95% AI610326
ReXS1	148	BU576963	FLCO165 (AF130094)	BM471802
ReXS2	148	BU576964	FLCO165 (AF130094)	BM471802
ReXS3	256	BU576965	Fibronectin (NM002026)	ND

pooled from each biopsy sample to give two populations of cDNAs representing RA or ReA transcripts. These were subtracted from one another to enrich for specific cDNAs using the polymerase chain reaction (PCR)-Select kit (Clontech). Aliquots of enriched cDNA were Rsa I digested, cloned into a plasmid vector and transformed into *E coli*. Five thousand colonies derived from each subtractive hybridisation were randomly selected for PCR amplification using M13 forward and reverse primers, and the DNA was directly spotted onto nylon membranes. Luciferase cDNA was also spotted to serve as a normalisation control and provide orientation markers. Membranes were hybridised to radiolabelled probes synthesised from the pooled subtractions and the individual patient cDNA. For each hybridisation, 0.5 µg synovial cDNA was added to 0.1 µg luciferase cDNA and the probes were only used if the incorporated counts between experiments were within 10% of each other. The filters were exposed to phosphor screens and the images analysed using ImageQuant (Amersham). For the pooled subtractions, a secondary screen was performed on selected cDNAs as described before.[5] Only clones that were consistently more abundant in both pooled and individual patient analyses were analysed further. These were sequenced using dye terminator chemistry and the resulting sequences searched against the nucleotide databases using the basic local alignment search tool (BLAST) algorithm.[6]

In situ hybridisation and immunohistology

For the preparation of RXS1 riboprobes, the PCR was carried out with RXS1 plasmid DNA as template and primers RXS1T7F (dGACTGATAATACGACTCACTATAGGGCGACGCGG GGTGGGGGGAGGG) with RXS1R (dCATGTGCACAATGT-GCAGG) and RXS1F (dCGCGGGGTGGGGGGAGGG) with RXS1T7R (dGACTGATAATACGACTCACTATAGGGCGACATGT GCACAATGTGCAGG). The PCR products were in vitro transcribed with DIG labelled dUTP and T7 RNA polymerase to create sense and antisense riboprobes respectively.[7] For in situ hybridisation analysis, 4 µm tissue sections were incubated with 10 ng riboprobe followed by anti-DIG-alkaline phosphatase (Roche). Sections were counterstained with nuclear fast red. For immunohistological analysis, tissue sections were incubated with monoclonal murine antibodies against human CD3 (Novocastro) or CD68 (Dako). The sections were counterstained with haematoxylin.

Results and discussion

We have performed a comparative gene expression analysis on synovial biopsy specimens taken from patients with RA and ReA. By studying tissue from the site of the inflammatory lesion, we aimed at identifying molecules that were pathologically significant and of potential diagnostic use. Figure 1A shows a schematic diagram for the cDNA macroarray analysis.

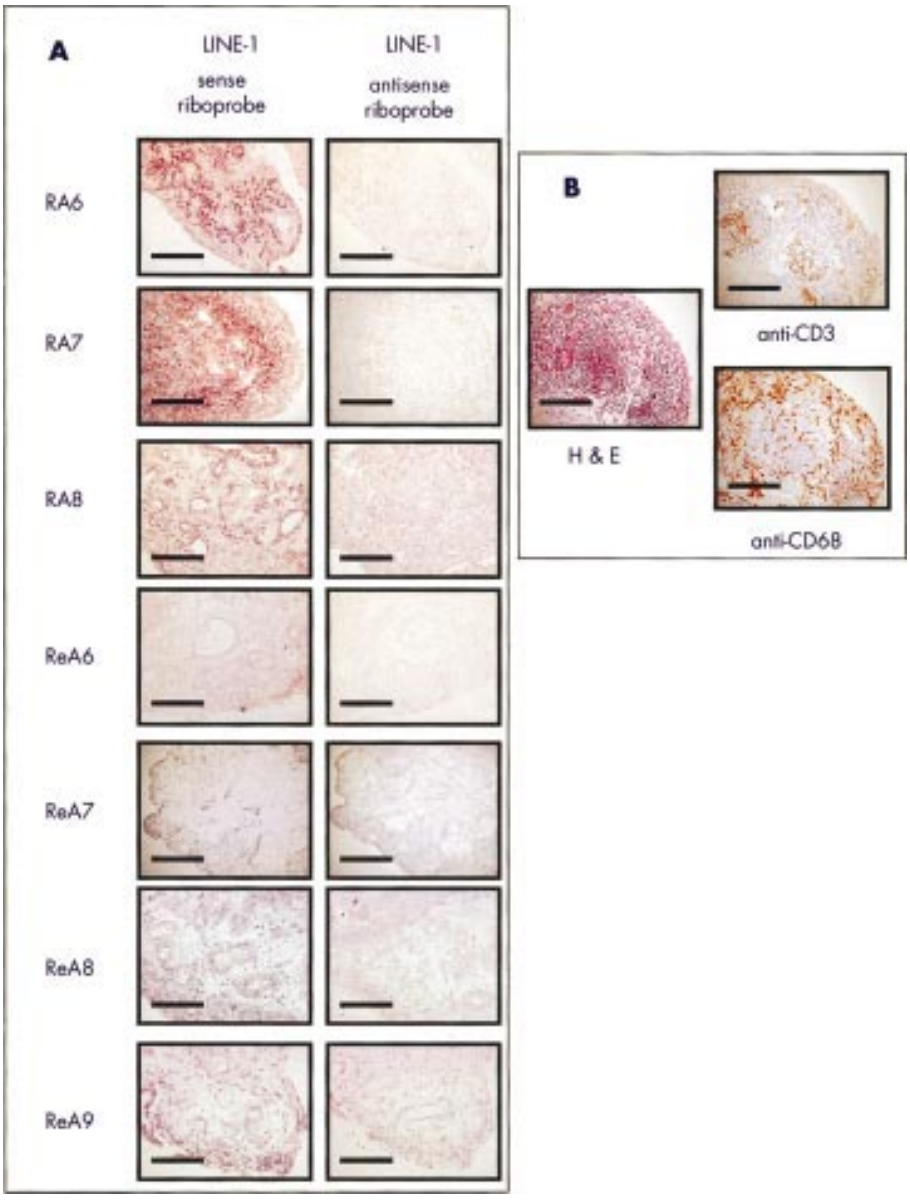

Figure 2 In situ hybridisation and immunohistology. (A) RXS1 in situ hybridisation. Serial sections of inflamed synovium from patients RA6–RA8 and ReA6–ReA9 were analysed using LINE-1 sense and antisense riboprobes. The images depicted are ×200 magnification and the bar marker represents 50 μm. Note that RA6 and RA7 gave intense sublining layer staining for the LINE-1 sense riboprobe. (B) Patient RA7 immunohistology. Serial sections were analysed for general histology (haematoxylin and eosin), T cells (anti-CD3), and macrophages (anti-CD68), and the staining compared with the in situ hybridisation pattern. Standard histology depicts a lymphoid follicle and coincides with surrounding cells that give predominant in situ hybridisation staining with the LINE-1 sense riboprobe.

High resolution software was used to highlight transcripts in the pooled, subtracted cDNA that were at least twofold more abundant in one type of synovitis than the other. After a secondary hybridisation screen 95% of these were confirmed; 87 gave higher expression in RA, and 65 in ReA. Because we aimed at identifying consistent changes in gene expression, we also analysed unsubtracted cDNA prepared from biopsy specimens of three cases of RA (RA1-RA3) and ReA (ReA1-ReA3) in a similar manner. Fifty eight clones demonstrated consistent overexpression in all three RA samples and 209 clones in all three ReA samples. Overall, 6 clones (RXS1–3 and ReXS1–3) were common to both pooled and individual patient analyses. It is noteworthy that only a few genes achieved the rigorous criteria of demonstrating differential expression in both pooled and individual patient analyses. This is likely to reflect heterogeneity of the RA and ReA patient groups, secondary to genetic or pathogenic factors.

The recombinant inserts of the six plasmid clones were analysed by sequencing and matched against the nucleotide databases (fig 1B). The sequences are deposited in the EST database at the NCBI (http://www.ncbi.nlm.nih.gov) as accession numbers BU576960–BU576965. The three clones whose corresponding transcripts were consistently up regulated in ReA included ReXS3, a 256 bp fragment corresponding to part of the 3′-untranslated region of the fibronectin gene. Previously, fibronectin mRNA has been found to be up regulated in RA compared with osteoarthritis synovium.[8] However, there are at least 30 fibronectin variants that arise from alternative splicing[9] and post-translational modifications,[10] and so the ultimate outcome of fibronectin expression will depend on the particular isoform present. ReXS1 and ReXS2 clones were identified as identical expressed sequences in the database. This new transcript is being analysed for its functional relevance to inflammatory

disease. Clones RXS1, RXS2, and RXS3 correspond to a part of transcripts that were consistently more abundant in RA and may encode "persistence factors" in chronic inflammatory arthritis. Nucleotide sequence analysis showed that these clones were more than 90% identical to each other. When the sequences for the RA clones were searched against the databases, transcribed sequences that contain part of the 3′-end of long interspersed nucleotide elements (LINE-1) were identified.

To confirm the results of the macroarray analysis, we synthesised LINE-1 sense and antisense riboprobes that contain RXS1 and performed in situ hybridisation analysis on serial sections of synovium (fig 2A). There was intense sublining layer staining in the sections taken from the patients with RA. In comparison there was slight staining in one RA and all the ReA sections tested. It was noteworthy that the LINE-1 sense riboprobe gave the predominant staining, suggesting that the hybridising transcript is on the antisense strand of LINE-1. Of note, a previous study demonstrated LINE-1 sense transcripts within RA synovium and a potential role in cell signalling.[11] The discrepancy may reflect the different riboprobes used between studies; we focused on the 3′-untranslated region of LINE-1, whereas the latter study analysed LINE-1 reverse transcriptase (ORF2). Nevertheless, the biology of such repetitive sequences is incompletely understood and the presence of reverse transcriptase in full length LINE-1 may provide a means for the production of the antisense transcripts.[12]

To try to establish the cell(s) that gave rise to the in situ hybridisation results in RA synovium, serial sections were stained as depicted in fig 2B. A lymphoid follicle is present and the in situ hybridisation staining is most prominent around the periphery of this follicle, although not clearly localised to either T cells or macrophages. The demonstration that antisense LINE-1 transcripts lie close to lymphoid follicles in the RA synovium reinforces the potential pathogenic significance.[13] We are in the process of trying to identify the corresponding full length transcript, although this work is rendered difficult by the multiple copies of LINE-1 sequences within the human genome. LINE-1 antisense transcripts are capable of transposition integration in the genome by the process of L1 retrotransposition.[12] This can lead to gene knockout,[14] or create hybrid proteins in somatic cells[15] that might lead to autoimmunity and might be incorporated into new hypotheses for RA pathogenesis.

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