THE POTENTIAL ROLE OF

HUMAN ENDOGENOUS RETROVIRUS K10 IN THE PATHOGENESIS OF

RHEUMATOID ARTHRITIS: A PRELIMINARY STUDY

Extended Report

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Abstract

Objective: To examine whether human endogenous retrovirus K10 is associated with autoimmune rheumatic disease. In this study we developed a novel multiplex reverse transcription polymerase chain reaction (RT-PCR) system to investigate HERV-K10 mRNA expression in rheumatoid arthritis (RA) patients.

Methods: 40 patients with RA, 17 with osteoarthritis (OA) and 27 healthy individuals were recruited and total RNA was extracted from peripheral blood mononuclear cells (PBMCs) and analysed using multiplex RT-PCR for the level of HERV-K10 gag mRNA expression. Southern blot and DNA sequencing confirmed the authenticity of PCR products.

Results: Using histidyl tRNA synthetase (HtRNAS) gene as a housekeeping gene in the optimised multiplex RT-PCR we demonstrated a significantly higher level of HERV-K10 gag mRNA expression in RA patients compared with OA (p value = 0.01) and healthy individuals (p value = 0.02).

Conclusion: The results of the multiplex RT-PCR showed an enhanced mRNA expression of the HERV-K10 gag region in patients with RA in comparison to patients with OA and healthy individuals, which could contribute to the pathogenesis of RA.

Introduction

The aetiology of most autoimmune diseases such as RA has yet to be established, but indirect evidence indicates that a trigger operating on the background of hormonal and genetic predisposing factors may be involved. Human endogenous retroviruses (HERVs) have been suggested as potential etiological agents for certain autoimmune disorders, in particular the rheumatic diseases [1-3]. The human genome contains many types of retrovirus-like elements [4]. HERVs represent footprints of previous retroviral infection and have been coined ‘fossil viruses’. They are transmitted vertically through the germ-line and thus inherited by successive generations in a Mendelian manner. Full length HERVs share the LTR-gag-pol-env-LTR structure of infectious retroviruses. HERVs incorporate reverse transcriptase into their replicative cycle and may code for retrovirus-like particles, but are generally not infectious; in fact, most sequences are defective due to accumulation of mutations, frame shifts and deletions. About 8% of the human genome is derived from HERV sequences [5]. Over time, HERVs have been subjected to repeated amplification and transposition events giving rise to single- and multi-copy proviruses that are distributed within the DNA of all cells. Over 26 families of HERVs have been identified during the past two decades [5]. Whilst many are defective through the accumulation of mutations, deletions and termination signals within coding sequences, a limited number retain the potential to produce viral products and indeed form viral particles (HERV-K). Furthermore, some HERVs have been implicated in certain autoimmune diseases and cancer; hence endogenous viruses may have a potential role in the aetiology and pathology of disease. Mechanisms whereby HERVs could influence autoimmunity include molecular mimicry i.e. HERVs sharing amino acids common to host proteins, superantigen motifs which bypass the normal major histocompatibility complex (MHC) restrictive process of T cell stimulation, aberrant expression of antigens and the presence of neo-antigens perhaps as a result of HERV and/or exogenous viral combinations [6-8].

Previous investigations of rheumatic patients have demonstrated antibodies to HIV-1 and HTLV-
I/-II retroviral products in patient serum [9, 10]. One such study by Nelson et al. [11] showed a significant association of anti-retroviral antibodies in patients with RA, systemic lupus erythematosus (SLE) and polymyositis (PM) and yet PCR failed to detect exogenous retroviruses when using specific oligonucleotide primers. To explain this paradox, HERVs, which possess regions of sequence homology to exogenous retroviruses, have been suggested as potential aetiological triggers of disease [6, 12, 13].

Retroviral antigens have also been detected at the site of disease: 45% of RA synovial sections were shown to be positive using antibodies to HTLV-I p24 and p19 and retrovirus-like particles have been observed in a human T cell line co-cultured with salivary gland tissue from a primary Sjogrens Syndrome patient [14]. Despite patients in these studies partially seroconverting to HIV/HTLV, again no evidence of past or active infection by either virus was detected. This may suggest the presence of an unknown exogenous or endogenous retrovirus with homology to HIV/HTLV.

This ambiguity in the serological evidence indicated a need to clarify the role of putative retroviral agents in the pathogenesis of rheumatological diseases. To date there have been few reports on the detection of retroviral agents in rheumatic diseases using direct molecular approaches such as the PCR. Additionally to our knowledge no simple quantitative comparison has been performed on the level of HERV mRNA expression in health and disease states. Consequently, we sought to develop a novel multiplex RT-PCR system to identify possible candidate agents in patients with RA.

Methods:

Patients and Controls: The RA patients and the disease control group were selected from the rheumatology clinic at Heartlands Hospital in Birmingham and New Cross Hospital in Wolverhampton. The normal healthy donors (NHDs) were a volunteer group made up of hospital workers.

Blood samples were taken from 40 RA patients of an average age of 61 years (age range of 34-85) and of average disease duration equating 75 months. Samples collected from 17 OA patients were used as a disease control. These patients had an average age of 67 years (ranging from 40-92), and average disease duration of 39 months. Blood from 27 NHDs were collected from volunteers with an average age of 47 years (from 21-80).

All RA patients satisfied the American College of Rheumatology (ACR) criteria for diagnosis of rheumatoid arthritis, and had clinically active disease as defined by serological and clinical parameters. All were IgM rheumatoid factor positive. The patients were taking non-steroidal anti-inflammatory drugs (NSAIDs) plus weekly Methotrexate. The osteoarthritis patients had clinically active disease as defined by diagnostic parameters, and absence of serological abnormalities. The patients were either on no medication or taking analgesics and/or NSAIDS (non-steroidal anti-inflammatory drugs). All samples were collected with full ethical approval from each site and included patients’ medical consent. Disease groups were not age or sex matched.

Separation of PBMCs and total RNA extraction: Histopaque-1077 (Sigma, UK) was used to separate PBMCs from heparinized whole blood. Total RNA was extracted using TRI Reagent (Sigma, UK). The crude extracts were subjected to RNase-free RQ-DNase I digestion at 37 C for 30 minutes. The total RNA concentrations were measured photometrically using a GeneQuant...
spectrophotometer. The extracts were analysed for possible structural damage and genomic DNA contamination by horizontal submarine gel electrophoresis using 1% agarose loening gel, which contained ethidium bromide (0.5 mg/l). Electrophoresis was performed in 1 x loening buffer (35.9 mM Tris-base, 34 mM Sodium dihydrogen orthophosphate, 1 mM EDTA in HPLC water, pH 7.7). The gels were then visualised under a BioVision™ gel imager (Biogene, UK).

**Multiplex RT-PCR:** One microgram of total RNA was converted into cDNA using the Reverse Transcriptase (RT) System kit with random hexamer primers and the avian myeloblastosis virus (AMV) RT enzyme (Promega, UK). RT cycles consisted of 10 min at room temperature, 1 hour at 42 °C, 5 min at 99 °C, and were then stored at 4 °C until used in the PCR reaction. PCR was performed on a TouchDown thermal cycler (Hybaid, UK) using primers for HERV-K10 reference sequence M14123 (sense: 5’GCAAGTAGCCTATCAACTGC-3’, (nt 1729 to 1751) antisense: 5’GCAGCCCTATTTCTTCCGGACC-3’, (nt 2241 to 2261). Intron spanning primers for histidyl tRNA synthetase (HtRNAS) were (sense: 5’-CTTCAGGGAGAGCGTCTGC-3’ antisense: 5’-CCTCCAGGTCTAGATAAGC-3’). PCR cycling conditions were as follows: 94°C for 3 min; 30 cycles of 1 min at 94°C, 1 min at 59°C, and 2 min at 72°C, with a final extension step of 10 min at 72°C. The PCR mix contained the two sets of primers at final concentrations of 0.5 µM (for HERV-K10) and 1 µM (for HtRNAS), 0.2 mM dNTP mix, 1.25 U Taq DNA Polymerase (Promega, UK), and 3 mM MgCl2 in total volume of 50 µl. Controls (omitting RNA templates and RT) were also included in each experiment to detect any traces of genomic DNA contamination. The PCR products were analysed by loading aliquots of products onto a 2% agarose-Tris Borate-EDTA (TBE) gel containing ethidium bromide, which then underwent electrophoresis. The bands on the gel were visualised under UV trans-illuminator (Biogene, UK). By comparing the size of amplified products with a molecular weight standard (pGEM DNA marker, Promega, UK), it was possible to confirm the size and identity of PCR products. PCR bands were semi-quantitatively analysed by measuring their pixel densities using Scion image™ Internet software. A testicular carcinoma cell line known to harbour HERV-K10 (Tera-1) was used as a positive control for HERV-K10 PCR development, optimisation and testing.

**Southern blot:** A specific 5'-biotin labelled HERV-K10 gag probe (5’-AAGGAGATAGCTGGCATGC-3’; position 1931-1950; 20 nt.), selected from HERV-K10 sequence published by Ono et al. [15] (manufactured by Oswel, UK) was designed to confirm the specificity of the HERV-K10 PCR product. RT-PCR products were run on agarose-TBE gel electrophoresis. The DNA bands were denatured by soaking the gel in 1M NaCl/0.5 M NaOH and neutralized by 0.5 M Tris/pH 7.5, 1.5 M NaCl and transferred onto a porablot nylon membrane (Amersham pharmacia, UK) overnight. The labelled probe was added to the hybridisation solution (5 % Denhardt’s solution, 0.5 % SDS, 5 x SSC) and UV-cross-linked membrane for overnight hybridisation at 65 °C. Probe concentration during the hybridisation was 20ng/ml (300ng of probe in 15ml of hybridisation solution). The bands on the nylon membrane were detected using enhanced chemiluminescent (ECL) detection system (Amersham pharmacia, UK).

**DNA sequencing:** Authenticity of the HERV-K10 PCR products were further characterized by DNA sequencing and any resulting sequence data compared to the published HERV-K10 reference sequence [15].

**Statistical evaluation:** Statistical analysis was performed on the ratio of pixel intensities between bands for HERV-K10 and HtRNAS mRNA expression. Wilcoxon (Mann-Whitney) rank-sum test and two-tailed unpaired Student’s t-test were applied to the results. P< 0.05 was considered significant.
Results:

**Multiplex RT-PCR: Detection and semi-quantification of HERV-K10 expression:** In order to detect and relatively quantify HERV-K10 mRNA expression in RA patients, total RNA was initially extracted from PBMCs. The extracts were validated for possible structural damage and genomic DNA contamination by horizontal submarine agarose gel electrophoresis (fig.1). Using two sets of primers specific for HERV-K10 gag2 and HtRNAS genes, amplicons of 533bp and 319bp, respectively, were amplified from healthy and diseased samples (fig.2).

PCR results of the PBMCs showed a significant increase in HERV-K10 expression when compared to expression of the Housekeeping gene - Histidyl tRNA synthetase in RA patients. 68% (27/40) of PBMCS samples taken from RA patients showed significantly elevated HERV-K10 expression levels (p=0.02) when compared with disease controls (fig.3). Just 2% (2/40) of RA patients showed levels comparable to the housekeeper gene i.e. level of baseline cellular expression. 17% (3/17) of disease controls (OA) showed elevated HERV-K10 expression and other controls groups (NHD) had comparable levels (18.5% or 5/27). There was no significant difference in levels of HERV-K10 expression between OA and NHD (p=0.34). Levels of the HtRNAS were consistent in all samples tested.

**Southern blot and DNA sequencing:** A 5'-biotin labelled probe specific for HERV-K10 gag confirmed the specificity of the PCR and identity of the PCR products (fig. 4). The authenticity of the HERV-K10 PCR product was further characterized by DNA sequencing. Sequence analysis showed that PCR product had 96% homology with HERV-K10 [15] using blastn2 programme from NCBI (http://www.ncbi.nlm.gov/blast/bl2seq).

Discussion:

Rheumatoid arthritis is a systemic disease, producing inflammation in diarthroidal joints, as well as affecting lungs, heart, nervous system and skin. It affects about 0.5-1.0 % of the adult population and is found worldwide [16]. Of the myriad of agents, environmental and genetic, implicated in RA disease mechanisms and pathology, considerable evidence indicates that viruses may be important environmental factors in triggering the disease. Viruses associated with arthritis-like symptoms include Rubella, Hepatitis, Alphaviruses and Parvovirus B19 [17].

A number of viruses, including EBV [18], Parvovirus B19 [19] and CMV [20] have already been identified within the joint; although no clear link has been established definitively linking these pathogens with RA. One such study by Stahl and colleagues [21] detected the presence of multiple viral DNA in synovial tissue and fluid taken from patients in the early stages of idiopathic arthritis. Up to two thirds of patients tested were positive for at least one virus, with 20% of those patients positive for two or more.

One group of viruses in particular have attracted a large amount of interest over the past two decades with respect to RA. These are the retroviruses [10, 22]. Amongst the mounting evidence, implicating retroviruses in RA are the parallels that can be drawn between human and animal
retroviral infections. Animal retroviral pathogens such as Caprine Arthritis Encephalitis Virus (CAEV) and Maedi Visna Virus (MVV) cause a chronic arthritis in sheep and goats [23]. Both diseases show similarities to human RA in their pathology. Furthermore, the spontaneous development of RA-like disease in mice transgenic for the HTLV-1 \textit{tax} gene hints at retroviral involvement [24]. Also evident is the increased prevalence of rheumatic diseases in areas endemic for retroviruses e.g. HTLV in Japan and Southern Central America [25].

Seemayer \textit{et al.} [26] conducted an elaborate series of experiments involving the co-culture of RA patient synovial tissue samples with cell lines known to be permissive for retroviruses. This approach however failed to implicate any exogenous retroviral agents in RA, although the authors did not exclude any potential endogenous retroviral activity. Neidhart and colleagues also isolated and identified a retroviral transcript from RA synovial fluid cells corresponding to ORF2 of the L1-retrotransposon [27]. Additionally Human Retrovirus-5 (HRV-5) has been also implicated as playing a role in RA disease pathogenesis [28] although these conclusions were not supported by follow-up studies [29].

In the present study we showed that levels of HERV-K10 expression were significantly elevated in RA patient PBMC samples in comparison to both disease controls and NHD (p=0.02). There was no significant difference between OA and healthy individuals (p=0.34). This data was supported by previous evidence put forward by Nakagawa \textit{et al.} who also showed that HERV-K10 levels were elevated in RA patients as opposed to NHD [30]. Furthermore, our data confirms earlier work, which showed that NHDs have a baseline level of HERV expression in the peripheral blood [31; 32]. Consequently it is possible that differential expression of HERVs may be critical in disease states.

It was also shown that HERV-K10 expression in RA patients was high at the site of disease (n=17) with levels of expression almost twice as high as those seen in the peripheral blood (data not shown). These findings should be noted as preliminary, and require further verification with additional synovial samples.

In the context of the disease process, the precise role of HERV-K10 in RA remains unclear. It is plausible that the virus could be triggered by the ongoing immune response, or is indeed itself a trigger for RA [33]. Evidently cells within the microenvironment of the joint (e.g. B-cells) may harbour HERVs themselves [34] and therefore also have the potential to play a role in the disease pathology. It is also possible that external factors may contribute in its activation, leading to changes in HERV expression through a process of bystander activation. Many exogenous viruses such as EBV [35], HIV [36], and HSV [37] are known to interact, either directly or indirectly, with endogenous retroviruses. Infection with many of these viruses also leads to an activation of specific host cells (e.g. T-cells with reference to HTLV-I) during the course of infection thus furthering the potential for immune dysregulation. Numerous cytokines are also known to modulate viral expression [38]. Thus it is therefore possible that host cells infected and activated by exogenous or endogenous viruses could migrate into the synovium releasing pro-inflammatory cytokines such as IL-6 [39] and TNF-\(\alpha\) [40]. Such events are likely to activate HERVs within the joint, and may explain why levels of HERV activity were increased in the synovial fluid.

In this study, we have optimised a novel multiplex RT-PCR system for the simple detection and
semi-quantification of HERV-K10 gag gene against HtRNAs in RA patient versus controls. Overall there appears to be higher levels of K10 expression in peripheral blood and synovial fluid cells in RA patients. The possible contribution of HERV-K10 to RA is intriguing and merits further investigation in a larger cohort of patients. Furthermore, the advent of new technologies such as quantitative RT-PCR and micro-arrays, applied to this field of research, will undoubtedly help our understanding of the role of these HERVs in RA.

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Image Legends:

**Figure 1:** A good quality total RNA band pattern in healthy samples. No top band (DNA contamination) was seen. Two central bands (28S + 18S rRNA) were visible, with the upper 28S band twice as bright as the 18S fraction. Between 28S and 18S bands mRNA (weak smear) was visible. Degraded RNA bands were weak. Total RNA extractions were of high quality and suitable for RT-PCR.

**Figure 2:** Multiplex RT-PCR system using HERV-K10 gag and HtRNAs primers in (a) healthy individuals, (b) OA patients and (c) RA patients: lanes M, pGEM DNA Markers; lanes 1-5, individual samples tested in each group; lane 6, RT-PCR control (no RNA template).

**Figure 3:** The average ratio of pixel intensity of the PCR bands for HERV-K10 to HtRNAs in RA patients was significantly higher than patients with OA (p = 0.02) and healthy subjects (p = 0.01). There was no significant difference in mean ratio of pixel intensity of HERV-K10/HtRNAs mRNA expression between OA and healthy subjects (p value = 0.34).

**Figure 4:** Southern blot- using specific HERV-K10 gag biotin-labelled probe to verify the HERV-K10 PCR products. Lane 1-3, are PCR products from RA patients; lane 4 & 5 from OA patients; lane 6 dot blot of probe only (control).
References:


Figure 1

28s RNA
18s RNA
Degraded RNA

1  2  3
Figure 4

1  2  3  4  5

6
The potential role of human endogenous retrovirus K10 in pathogenesis of rheumatoid arthritis: a preliminary study


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