EXTENDED REPORT

Mitochondrial mutagenesis correlates with the local inflammatory environment in arthritis

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ABSTRACT

Background To examine the association between mitochondrial mutagenesis and the proinflammatory microenvironment in patients with inflammatory arthritis. Methods Fifty patients with inflammatory arthritis underwent arthroscopy and synovial tissue biopsies, synovial fluid and clinical assessment were obtained. Fifteen patients pre/post-TNF therapy were also recruited. Normal synovial biopsies were obtained from 10 subjects undergoing interventional arthroscopy. Macroscopic synovitis/vascularity was measured by visual analogue scale. Cell-specific markers CD3 (T cells) and CD68 (macrophages) were quantified by immunohistology. TNFα, IL-6, IFNγ and IL-1β were measured in synovial fluids by MSD multiplex assays. Synovial tissue mitochondrial mutagenesis was quantified using a mitochondrial random mutation capture assay (RMCA). The direct effect of TNFα on oxidative stress and mitochondrial function was assessed in primary cultures of rheumatoid arthritis synovial fibroblast cells (RMCA). Mitochondrial mutagenesis, reactive oxygen species (ROS), mitochondrial membrane potential (MMP) and mitochondrial mass (MM) were quantified using the RMCA and specific cell fluorescent probes.

Results A significant increase in mtDNA mutation frequency was demonstrated in inflamed synovial tissue compared with control (p<0.05), an effect that was independent of age. mtDNA mutations positively correlated with macroscopic synovitis (r=0.52, p<0.016), vascularity (r=0.54, p<0.01) and with synovial fluid cytokine levels of TNFα (r=0.74, p<0.024) and IFNγ (r=0.72, p<0.039). mtDNA mutation frequency post-TNF therapy was significantly lower in patients with a DAS<3.2 (p<0.05) and associated with clinical and microscopic measures of disease (p<0.05). In vitro TNFα significantly induced mtDNA mutations, ROS, MM and MMP in RASFcs (all p<0.05).

Conclusion High mitochondrial mutations are strongly associated with synovial inflammation showing a direct link between mitochondrial mutations and key proinflammatory pathways.

INTRODUCTION

Rheumatoid arthritis (RA) and psoriatic arthritis (PsA) are two of the most common forms of inflammatory arthritis characterised by synovial inflammation, pain and progressive damage. 1 The mechanisms involved in synovial inflammation and invasion are not fully understood; however, an early event in inflammation is angiogenesis, which facilitates the persistent infiltration of immune cells into the joint resulting in destruction of adjacent articular cartilage and bone. 2 Oxidative stress arises from an imbalance between reactive oxygen species (ROS) production and the capability of opposing antioxidant forces. Mitochondria provide cellular energy through oxidative phosphorylation in the mitochondrial electron transport chain with resultant ATP production and ROS generation as a by-product. 3 Antioxidant defence systems generally mop up leaked ROS production; however, under inflammatory and pathological conditions, ROS can exceed the capacity of antioxidant defence systems and damage occurs to lipids, proteins and DNA. DNA adducts such as 8-oxo-7,8-dihydro-2′-deoxyguanosine and lipid peroxidation-inducing agents such as 4-hydroxy-2-nonenal are highly elevated in synovial fluid and tissue of RA and PsA patients and correlate with angiogenic growth factor expression. 2, 4 Oxidative damage can induce cyclooxygenase-2, angiogenesis, MMP9/13 expression, can regulate antipapoptotic pathways and can alter nuclear factor kappa B (NF-κB) signalling, key processes involved in the pathogenesis of inflammatory arthritis. 5–8 Furthermore antioxidant treatments can improve disease progression in animal models of inflammatory arthritis. 9, 10

Mitochondrial DNA (mtDNA) lacks the intrinsic repair mechanisms of nuclear DNA and is thought to be more sensitive to increased oxidative damage and thus more vulnerable to high mutation rates. 11 Mutation of the mitochondrial genome in genes encoding proteins for subunits of mitochondrial respiratory chain complexes IV, ribosomal RNA and transfer RNA have been associated with neurodegenerative diseases and cancer. 11–13 Inherited mitochondrial disease is considered a distinct entity predisposing individuals to heritable conditions such as chronic progressive external ophthalmoplegia, lebers hereditary optic neuropathy and mitochondrial myopathy, encephalopathy, lactic acidosis and stroke syndrome. 14 Furthermore, type-1 tumour necrosis factor receptor (TNFR1) mutant cells show evidence of altered mitochondrial function paralleled by increased oxidative capacity and mitochondrial ROS generation, blockade of which resulted in reduced proinflammatory cytokine expression. This suggests a potential role for mitochondrial ROS in the regulation of cytokine pathways in inflammatory diseases. 15

In this study, we demonstrate a significant increase in mtDNA mutation frequency in the inflamed synovium compared with normal synovium. We demonstrate for the first time a significant relationship...
between mtDNA mutation frequency with macroscopic/microscopic measures of inflammation and with key proinflammatory mediators: TNFα and interferon γ (IFNγ). Finally, we demonstrate in vivo and in vitro that TNFα significantly alters mtDNA mutation frequency and mitochondrial function.

MATERIALS AND METHODS

Patient selection and arthroscopy
Fifty inflammatory arthritis patients with active disease (13 men, 37 women) were recruited prior to commencing biologic therapy (RA, n=39; PsA, n=11) fulfilling the criteria of the American College of Rheumatology16 and classification criteria for PsA,17 respectively, from the Rheumatology Clinic. Ten patients undergoing interventional arthroscopy for cruciate ligament tears were also recruited (eight men; two women; mean age 38 years (IQR 28–43)). All patients provided fully informed consent. The mean age of the inflammatory arthritis cohort was 52 years (IQR 41–60) and the clinical characteristics for these patients included tender joint count 13 (3–21), swollen joint count 11 (7–18), erythrocyte sedimentation rate 37 mm/h (27–65), C reactive protein 29 mg/l (9–57), disease activity score (DAS) 28 5.76 (3.9–6.32) and disease duration 19 months (5–57). Sixty-five per cent of patients were rheumatoid factor positive and 77% anticyclic citrullinated protein antibody positive. A subgroup of 15 patients (RA, n=8; PsA, n=7) were assessed at baseline and 3 months post-TNF inhibitor (TNFi) therapy; their median age was 52 years (IQR 55–49), 33% were rheumatoid factor and anticyclic citrullinated protein positive. Median baseline DAS28 was 4.76 (5.2–3.4). Seven patients achieved a low disease activity state (DAS28<3.2) after 8 months of treatment according to van Gestel et al.18

Arthroscopy, macroscopic assessment and synovial biopsy
Under local anaesthetic, arthroscopy of the inflamed knee was performed using a Wolf 2.7 mm needle arthroscope (Richard Wolf, Vernon Hills, Illinois, USA) as previously described.3 Macroscopic synovitis and vascularity were scored on a visual analogue scale (0–100 mm).19 Synovial tissue biopsies were obtained by 2 mm grasping forceps, under direct visualisation, embedded in mounting media for immunohistochemical analysis or snap frozen in liquid nitrogen for mitochondrial mutagenesis analyses. Paired synovial fluid was obtained and stored at −70°C for cytokine analysis. Control synovial biopsies were also obtained from 10 patients undergoing interventional arthroscopy for cruciate ligament tears, under direct visualisation, and with key proinflammatory cytokines (IFNγ, IL-6, IL-1β, TNFα) being measured using MSD technology.20

Mitochondrial random mutation capture assay (RMCA)
Levels of mitochondrial point mutations in snap frozen synovial biopsies were analysed in a blinded fashion using mitochondrial RMCA as described previously.20 Biopsies were homogenised (Precellys 24, Stretton Scientific Ltd, UK) in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20 mM EDTA, 0.5% sodium dodecyl sulphate buffer and digested with proteinase K (Sigma–Aldrich, St Louis, Missouri, USA), 0.5% H2O2 in phosphate buffered saline (pH 7.6). Slides were counterstained with haematoxylin and a negative control. Sections were incubated with primary mouse monoclonal anti-CD68 and anti-CD3 antibodies (DAKO, Glostrup, Denmark) and an appropriate isotype matched mouse monoclonal antibody as an appropriate control. Colour was developed in solution containing diaminobenzadine tetrahydrochloride (Sigma-Aldrich, St Louis, Missouri, USA), 0.1% H2O2 in phosphate buffered saline for 10 min and air-dried. A routine three-stage immunoperoxidase labelling technique incorporating avidin-biotin immunoperoxidase complex (DAKO, Glostrup, Denmark) was used. Sections were incubated with primary mouse monoclonal anti-CD68 and anti-CD3 antibodies (DAKO, Glostrup, Denmark) at room temperature for 1 h. Sections were also incubated with an appropriate isotype matched mouse monoclonal antibody as a negative control. Colour was developed in solution containing diaminobenzadine tetraydrochloride (Sigma–Aldrich, St Louis, Missouri, USA), 0.5% H2O2 in phosphate buffered saline (pH 7.6). Slides were counterstained with haematoxylin and mounted. Slides were assessed using a well-established semi-quantitative scoring method ranging from 0 to 4 (0=no staining, 1=<25%, 2=25–50%, 3=50–75%, 4=>75% staining).14 21 22 Sections were scored separately for lasing and sublining layers and results were expressed as the mean scores.14

MSD multi-array technology
The four-spot MSD human proinflammatory cytokine ultra-sensitive kit, containing cytokines TNFα, IL-6, IFNγ and IL-1β (Meso Scale Discovery, Maryland, USA; Cat# K15009C-1) was used for the analysis of paired synovial fluid samples. Quantification was assessed using the Sector Imager 2400 (Meso Scale Delivery) instrument and software.

Primary RA and PsA synovial fibroblast culture
RA and PsA synovial biopsies were digested with 1 mg/ml collagenase type 1 (Worthington Biochemical, Freehold, New Jersey, USA) in RPMI medium (Gibco-BRL, Paisley, UK) for 4 h at 37°C in humidified air with 5% CO2. Dissociated cells were grown to confluence in RPMI 1640, 10% FCS (Gibco-BRL), penicillin (100 units/ml; Biosciences), streptomycin (100 units/ml; Biosciences) and fungizone (0.25 μg/ml; Biosciences) before passage. Cells were used between passages 4 and 8. K4IM cells, an immortalised normal human synoviocyte cell line (kind gift, Dr Evelyn Murphy), were cultured as above and used between passages 35 and 38. RA synovial...
fibroblast cells (RASFCs), PsA synovial fibroblast cells (PsASFCs) and K4IM were seeded onto 96-well plates and into T25 flasks and incubated with TNFα (10 ng/ml) for 24 h. mtDNA mutations, ROS, mitochondrial membrane potential (MMP) and mitochondrial mass (MM) were assessed as follows:

**In vitro mitochondrial dysfunction**

To determine the frequency of mtDNA point mutations, RASFC pellets were obtained following TNFα stimulation, were digested, purified mtDNA was extracted and RMCA was performed as outlined above.

To measure the level of ROS, following TNFα stimulation, cells were washed twice with a buffer (130 mM NaCl, 5 mM KCl, 1 mM Na2HPO4, 1 mM CaCl2, 1 mM MgCl2 and 25 mM Hepes, pH 7.4). Cells were loaded with 5 μM 2,7-dichlorofluorescein diacetate (DCFH-DA) (Invitrogen) for 40 min at 37°C. DCFH-DA is a non-fluorescent molecule which diffuses into the cells where it is deacetylated and rapidly oxidised to highly fluorescent 2,7-dichlorofluorescein in the presence of the generated ROS. DCF emits a fluorescent signal of the product which is linearly related to the intracellular hydrogen peroxide concentration. To measure MMP, following TNFα stimulation, cells were washed and loaded with 5 μM rhodamine-123 (Sigma) for 40 min at 37°C. Rhodamine-123 is taken up selectively by mitochondria which is dependent on MMP. Following 40 min incubation, ROS and MMP probes were removed, cells were washed and analysed using the Spectra Max Gemini System. DCFH-DA and rhodamine-123 were excited at 485 nm, and fluorescence emission at 538 nm was recorded. To measure MM, following TNFα stimulation, cells were incubated with Green-Fluorescent MitoTracker dye (Invitrogen). After
incubation for 45 min, cells were visualised using a fluorescence microscope. The probe has absorption and emission peaks at 490 and 516 nm, respectively, and the fluorescence intensity is proportional to the MM. Mean fluorescence values from four wells for each condition were obtained. Mitochondrial dysfunction assays were normalised to cell number.

**Statistical analysis**

SPSS15 system for Windows was used for statistical analysis. Non-parametric Wilcoxon signed rank, Spearman correlations with Bonferroni corrections and Mann–Whitney U test were used for analysis. p<0.05 was determined as statistically significant.

**RESULTS**

**Mutation of mtDNA in the synovium of active RA and PsA patients**

A significant increase in mtDNA mutations was demonstrated in RA and PsA synovial tissue (mean 4.8×10⁻⁴) compared with normal synovium (7.3×10⁻⁵) (p<0.013). When mtDNA mutations were analysed separately for RA and PsA synovium, significantly higher mutation frequency was demonstrated for both RA and PsA compared with normal synovium (p=0.017 and p=0.041) (figure 1A). No significant difference in mutation frequency was observed between RA and PsA (p=0.673) (figure 1A). The frequency of mtDNA mutations did not correlate with age in normal synovium (r=0.006, p=0.98) or in age-matched RA and PsA synovium (r=0.236, p=0.511) demonstrating that the increase in mtDNA mutation frequency was independent of age. The spectrum of mutations identified demonstrated substitution of purines for pyrimidines in 49% of mutations and substitution of pyrimidines for purines in 51% of mutations (figure 1B).

**Table 1**

<table>
<thead>
<tr>
<th>Frequency of point mutation</th>
<th>r Value</th>
<th>p Value</th>
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</thead>
<tbody>
<tr>
<td>Synovial fluid cytokine levels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>0.74*</td>
<td>0.024</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.464</td>
<td>ns</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.645</td>
<td>ns</td>
</tr>
<tr>
<td>IFNγ</td>
<td>0.718*</td>
<td>0.039</td>
</tr>
</tbody>
</table>

*p<0.05 statistically significantly.

IL, interleukin; IFN, interferon; ns, not significant; r, Spearman’s correlation co-efficient; TNF, tumour necrosis factor.

**Mutations in mtDNA correlate with macroscopic measures of inflammation**

Representative images of macroscopic synovitis and vascularity from a patient with low mtDNA mutations (figure 2A,D) versus high mtDNA mutations (figure 2B,E) are shown. Significant correlation was demonstrated between mtDNA mutation frequency and macroscopic synovitis (r=0.52, p=0.016) and vascularity (r=0.55, p=0.01) suggesting that mtDNA mutation frequency is associated with the degree of inflammation in the joint (figure 2C,F).

**Mutations in mtDNA correlate with mediators of inflammation**

Synovial tissue mtDNA mutation frequency significantly correlated with synovial fluid expression of TNFα (r=0.74, p=0.024) and IFNγ (r=0.718, p=0.039) demonstrating a strong relationship between mitochondrial instability and proinflammatory pathways (table 1, see online supplementary figure 1). Associations...
In this study, we examine mitochondrial mutagenesis in patients with inflammatory arthritis and assess its relationship to inflammatory mechanisms in vivo and in vitro. We demonstrate that mitochondrial random mutations are significantly increased in inflammatory arthritis compared with normal synovial tissue. mtDNA mutations significantly correlate with level of inflammation at the macroscopic level. In particular, we demonstrate a significant association between mtDNA mutation frequency and macroscopic/microscopic scores of inflammation and pro-inflammatory cytokines: TNFα, INFγ. Following TNFi biologic therapy, we demonstrate a significant decrease in synovial mtDNA mutations. Finally in vitro, we show that TNFα stimulation drives mitochondrial dysfunction in primary RASF and PsASF along with mtDNA mutations.

In this study, we used a newly validated mitochondrial RMCA which relies on single-molecule amplification to detect rare mutations among millions of wild-type bases and analyses mitochondrial mutagenesis at single base pair resolution and detects one mutation among 10 million wild-type bases. Many mitochondrial point mutations associated with mitochondrial dysfunction have been characterised. By assaying the random mutation frequency of a phenotypically neutral locus, 12S RNA, we estimate the likelihood at which any specific mitochondrial mutation and associated dysfunction, might occur. By using a TaqI site that does not span a protein coding region of the mitochondrial genome, we are able to extrapolate the extent of mutagenesis throughout the mitochondrial genome (ie, mutagenesis unaffected by phenotypic selection).

We demonstrated increased mtDNA mutation frequency in the inflamed synovium compared with normal controls, levels of which correlate with clinical measures of disease activity and with the local inflamed synovial tissue microenvironment. Our data is supported by previous studies showing increased levels of DNA damage and lipid peroxidation in patients with inflammatory arthritis. Increased levels of lipid peroxidation and depolarised mitochondria in RA peripheral blood mononuclear cells have been shown to correlate with disease activity. Increased expression of 8-oxo-7,8-dihydro-2′-deoxyguanine and 4-hydroxy-2′-nonenal have been demonstrated in RA synovial tissue and serum, and correlate with disease activity and angiogenic factors. Increased clonal mtDNA mutation frequency in the MT-ND1 gene for mitochondrially encoded NADH-dehydrogenase-1 has been detected in RASF. Furthermore, potential mutation sites in the major histocompatibility complex epitope in RA patients, but not osteoarthritis, have been identified suggesting mtDNA may become antigenic and drive immune mediated responses. In mouse fibroblasts, TNFR1 mutant cells show evidence of altered mitochondrial function, resulting in increased oxidative capacity, mitochondrial ROS generation and proinflammatory cytokines. In addition, in vivo synovial tissue oxygen levels correlate with oxidative damage and mitochondrial dysfunction.

Mitochondrially derived ROS can induce TNFα cytotoxicity and may mediate the activation of transcriptional factor NF-kB which in turn can stimulate mitochondrial NADPH oxidase. TNFRI mutant cells exhibit altered mitochondrial function, increased production of IL-6, TNFα, IL-8 and phosphorylation of mitogen-activated protein kinase pathways. Altered mitochondrial membrane permeability and Bcl-X overexpression and K41M TNFα significantly induced MMP (p<0.05), ROS production (p<0.05) and mitochondrial mass (p<0.05) (see online supplementary figures 2, 3).
leads to resistance to TNF cytotoxicity. Combination of IL-1β and IFNγ can induce mitochondrial Bax translocation, cytochrome c release and caspase-3 cleavage. In addition, ROS can mediate IL-1-induced experimental arthritis, and cyclooxygenase-2 expression in RASFC. CD3+ T cells and CD68+ macrophages are potent producers of cytokines critical to synovial inflammation. In this study, reduction of synovial mtDNA mutation frequency and its association with measures of clinical disease activity and microscopic CD3+ cells following TNFi therapy, strengthens the hypothesis of a pathological link between mitochondrial dysfunction and pro-inflammatory pathways.

We have shown using RASFC that TNFα significantly increases mtDNA mutation frequency, coupled with increased ROS production, MMP and MM, thus recapitulating the in vivo responses to TNFi therapy. This is consistent with mice models showing interactions between oxidatively damaged mtDNA, increased NF-κB activity and TNFα production. TNFα increases mitochondrial ROS production and induces lipid-derived aldehyde formation. High mutations were demonstrated at baseline in TNFi non-responders suggesting TNF independent pathways may be involved in mtDNA mutations. We and others previously showed strong associations between mitochondrial dysfunction and synovial tissue oxidative stress, angiogenic growth factors and cytokines. Furthermore, hypoxia can increase mtDNA mutations in primary RA synoviocytes.

Direct functional consequences of mitochondrial random mutations in inflammation are unclear; however, they may have an important role in the regulation of the innate immune response. Zhou et al. have demonstrated that the NLRP3 inflammasome can sense mitochondrial dysfunction, altering the inflammatory response. Furthermore, mitochondrial dysfunction regulates IL-1 and IL-18 secretion from macrophages in response to lipopolysaccharide. Additionally in an experimental arthritis model, manipulation of mitochondrial dysfunction reduces severity of cartilage lesions and synovial inflammation.

Acknowledgement In summary, we have shown, for the first time that mtDNA mutations are significantly higher in the inflamed synovium compared with the control. We have shown significant associations between mtDNA mutations and macroscopic/microscopic measures of synovial tissue inflammation and with local production of pro-inflammatory cytokines. Furthermore, we demonstrate both in vitro and in vivo, that DNA mutagenesis and mitochondrial function are altered by TNFα regulation. These data further support the concept of complex interplay between inflammatory pathways, oxidative damage and oxygen metabolism in the pathogenesis of inflammatory arthritis.

Funding This study was funded by the Centocor Newman Fellowship, the Health Research Board of Ireland and EU FP6 AutoCure.

Competing interest None.

Patient consent Informed consent obtained.

Ethics approval This study was conducted in accordance with the Declaration of Helsinki and approved by the St Vincent’s University Hospital medical research and ethics committee.

Provenance and peer review Not commissioned; externally peer reviewed.

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*Ann Rheum Dis* published online November 25, 2011
doi: 10.1136/annrheumdis-2011-200245