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Characterization of nitrotyrosine as a biomarker for arthritis and joint injury

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SUMMARY

Objectives: To characterize the utility of nitrotyrosine (NT) as a biomarker for arthritis and joint injury.
Design: Synovial fluid, plasma, and urine from patients diagnosed with osteoarthritis (OA), rheumatoid arthritis (RA), anterior cruciate ligament (ACL) injury, meniscus injury and pseudogout, and knee-healthy volunteers were analyzed for concentrations of NT, nitrate and nitrite (NO_x), matrix metalloproteinase (MMP)-3, MMP-1, MMP-9, more than 40 chemokines and cytokines.
Results: In OA, plasma and synovial fluid NT were increased *versus* healthy volunteers. Synovial fluid to plasma NT ratios were elevated in OA patients. Synovial fluid from patients with ACL and meniscus injury and pseudogout had increased levels of NT ($P < 0.001$). In these samples, NT levels significantly correlated with ARGS-aggrecan neoepitope generated by aggrecanase cleavage of aggrecan ($P \leq 0.001$), cross-linked C-telopeptides of type II collagen ($P < 0.001$), MMP-1 ($P = 0.008$), and MMP-3 ($P \leq 0.001$). In RA, plasma NT decreased following 6 months of anti-tumor necrosis factor (TNF) treatment. For every 1.1% change in $\log_{10}$ NT, there was a 1.0% change in the $\log_{10}$ disease activity scores (DAS28-3 CRP). Both predicted and observed DAS28-3 CRP showed a robust linear relationship with NT. RA plasma NT positively correlated with CRP, MMP-3 and interferon γ-induced protein 10.
Conclusions: NT may serve as a useful biomarker for arthritis and joint injury. In RA, NT is highly correlated with several biomarkers and clinical correlates of disease activity and responds to anti-TNF therapy.

Introduction

Increased nitric oxide synthase (NOS) activity has been linked to joint injury and is associated with increased chondrocyte apoptosis, increased matrix metalloproteinase (MMP) activity and decreased extracellular matrix synthesis[1]. Osteoarthritis (OA) and rheumatoid arthritis (RA) show increased tissue staining for both inducible nitric oxide synthase (iNOS) and its downstream product, nitrotyrosine (NT)[1–6]. NT is a stable marker resulting from the generation of peroxynitrite, a powerful oxidant arising from the diffusion-limited reaction of nitric oxide (NO) with superoxide[1]. Although diet is a source of nitrate *in vivo* and should be considered when assessing this metabolite as a biomarker for NOS activity, numerous studies have used nitrate and nitrite (NO_x) as a measure of NO production in arthritis[7–12]. Studies on RA showed increased levels of plasma and synovial fluid nitrite[7–12], and synovial fluid NT[13] but have not demonstrated a therapeutic response of these biomarkers.

Recent work has shown increased nitrated type II collagen in serum from patients with OA and RA[14], although this measure is specific to type II collagen and would not include synovial and other joint tissue targets for peroxynitrite. A biomarker, such as NT that is derived from multiple nitratively modified joint proteins rather than solely from type II collagen, may better reflect the extent of joint pathology and provide a more robust response to therapeutic intervention. In the present investigation, we therefore determined NT and NO_x in plasma, urine and synovial fluid samples from patients with joint injury, OA, pseudogout and RA, and related these levels to arthritis biomarkers and clinical correlates of disease activity.

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Methods

Human samples

For all human samples, the protocol and informed consent documentations were reviewed and approved by Institutional Review Board(s) and/or Independent Ethics Committee(s) at each study center. Written informed consent was received from all eligible patients before procedures were initiated. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Samples (plasma, synovial fluid, and urine) were obtained from the following cohorts.

Cohort 1: human plasma samples

Subjects with knee OA were all female and obese [body mass index (BMI) $\geq$30] with symptomatic OA as evidenced by frequent knee symptoms during the course of the year. These included pain, aching or stiffness on most days of the month, along with the frequent use of medication. Subjects with Kellgren and Lawrence grades 2 or 3 of the signal knee (with either the same or less severe OA or no OA of the contralateral knee) were included in this OA cohort ($n = 83$). The control group ($n = 92$) had no evidence of knee OA in either knee (i.e., Kellgren and Lawrence grade 0 diagnosed by X-ray on anteroposterior view; infrequent knee pain, aching or stiffness the year prior to the study; or infrequent use of medication for treatment of knee pain).

Cohort 2: matched human OA plasma and urine samples

Matched plasma and urine samples were obtained from male and female patients ($n = 20$) with painful knee OA, X-ray confirmed Kellgren and Lawrence grade 2 or 3, aged $\geq$40 years and a BMI $\leq$35. Any evidence of inflammatory arthritis resulted in exclusion from the study. Patients had no non-steroidal anti-inflammatory drug use for 7 days prior to sample collection. Control subjects ($n = 20$) were age- and gender-matched with the OA group and were confirmed by radiography to have no evidence of OA in the knees, hips or dominant hands.

Cohort 3: human synovial fluid samples (OA, joint injury, pseudogout, controls)

Because NT in plasma and urine could be derived from sources outside OA joints, we analyzed synovial fluid from OA and joint injury [anterior cruciate ligament (ACL) and/or meniscus rupture] patients who often develop OA following injury[15]. In addition, because increased synovial fluid levels of NT support a role for peroxynitrite-mediated joint tissue injury in OA and ACL injury, we assessed its level in pseudogout, an acute inflammatory condition involving joint damage. Briefly, human synovial fluid samples ($n = 382$) were aspirated without lavage from a cross-sectional convenience cohort with informed consent and approval of the Lund University research ethics committee. Diagnosis was made by arthroscopy, radiography, assessment of joint fluid and clinical examination. Diagnostic groups were knee-healthy references with no history of joint injury or joint pain ($n = 10$); pseudogout (pyrophosphate crystal arthritis, $n = 34$); joint injury sustained between less than 1 week and 20 years before sample acquisition (knee ACL rupture, with or without concomitant meniscus lesions, $n = 136$) or isolated knee meniscus injury ($n = 118$); and knee OA ($n = 84$). Patients with pseudogout had radiographic OA corresponding to Kellgren and Lawrence grade 1–3, whereas patients with OA had radiographic OA corresponding to grade 2 or greater. Patients with joint injury generally showed mild to moderate cartilage damage on arthroscopic examination, with a few additionally having radiographic signs of OA corresponding to Kellgren and Lawrence grade 1 or 2. The samples of this cohort were partly identical with those used in previous studies[16–19].

Cohort 4: matched human OA plasma and synovial fluid samples

To better understand the relationship between synovial fluid and plasma NT in OA, we obtained matched plasma and synovial fluid samples from male and female knee OA patients ($n = 40$; $n = 20$ per gender) just prior to knee replacement surgery from Clinomics BioSciences Inc. (Pittsfield, MA, USA). In addition, age- and gender-matched control samples ($n = 40$) were purchased from Clinomics BioSciences, Inc.

Cohort 5: RA plasma samples

Plasma was collected from RA patients ($n = 18$) before and after treatment with anti-tumor necrosis factor (TNF) biotherapeutics (etanercept, infliximab, or adalimumab) over a 6-month period. Control plasma samples ($n = 21$) from an age-matched cohort of healthy volunteers were obtained from PrecisionMed (San Diego, CA, USA).

Disease activity score (DAS) of 28 joints

The disease activity score of 28 joints using C-reactive protein (DAS28-3 CRP) was the outcome measure used as an indicator of RA disease activity and response to treatment. It is the basis for several other RA measurement tools and is widely used as an indicator of RA disease activity and response to treatment[20].

3-NT and NO_x assays

We used a newly described assay for total NT (protein-containing and protein-free) to measure NT levels in biological fluids (plasma, synovial fluid and urine)[21]. Briefly, quantification of NT from the human body fluid samples was performed using immunoaffinity two-dimensional (2D) liquid chromatography–tandem mass spectrometry (LC–MS/MS) using an HP 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) and a switching valve (Valco Instruments, Houston, TX, USA) plumbed in-line with a pump and interfaced to an API 4000 mass spectrometer (Applied Biosystems/MDS-Sciex, Toronto, Canada) operated in the negative ion electrospray and multiple reaction monitoring modes.

NO_x was measured using a fluorescent assay as previously described[22].

Immunoassays for MMPs, cytokines, chemokines, aggrecan fragments, aggrecan epitope 846 and cartilage oligomeric matrix protein (COMP)

MMP-1, MMP-3 and MMP-9 were measured in plasma from patients with RA using a multiplex sandwich-based enzyme-linked immunosorbent assay (ELISA) format (Meso Scale Discovery, Gaithersburg, MD, USA). Typically, a 5–10-fold dilution of biological fluid was analyzed. For measurement of cytokines and chemokines, approximately 25 μl of each sample was analyzed for 42 different human antigens as defined in the manufacturer's protocol (HCYTO-80K-42PMCX; Millipore, Billerica, MA, USA): epidermal growth factor (EGF), eotaxin, fibroblast growth factor (FGF-2), flt3 ligand (Ftl3L), fractalkine, granulocyte colony-stimulating factor (G-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF),