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Quantitation OF ARGS aggrecan fragments in synovial fluid, serum and urine from osteoarthritis patients

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SUMMARY

Objective: To characterise ARGS neoepitope concentrations in various matrices from patients with knee osteoarthritis (OA) and assess performance of an immunoassay to facilitate clinical development of therapeutics affecting the A disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5) pathway.

Design: Matched sera, urine, and synovial fluid (SF) (surgical subjects only) were collected from healthy subjects, subjects with knee OA (non-surgical OA), and OA subjects undergoing total knee replacement (OA-TKR; $n = 20$ per group). Diurnal and inter-day variation was evaluated in the non-surgical OA group over 3 separate visits. Serum and urine samples were collected on two visits for the OA-TKR group with SF taken only at the time of surgery. ARGS neoepitope was quantitated using an optimized immunoassay.

Results: Serum ARGS neoepitope concentrations were elevated in OA-TKR subjects compared to non-surgical OA subjects ($P = 0.005$) and healthy subjects ($P = 0.0002$). Creatinine corrected urinary ARGS neoepitope concentrations were more variable, but were also elevated in the OA-TKR subjects compared to healthy subjects ($P = 0.008$). No significant diurnal effect or inter-day variance was observed in serum or urine. Serum ARGS neoepitope concentrations correlated with age ($P = 0.0252$) but not with total number of joints with OA involvement. SF ARGS neoepitope concentrations correlated with Western Ontario and MacMaster OA Index (WOMAC) stiffness score ($P = 0.04$) whereas a weaker, non-significant trend towards positive correlation with combined WOMAC score and the number of concurrent joints was observed.

Conclusions: This study utilized a sensitive and robust assay to evaluate ARGS neoepitope concentrations in various matrices in OA patients and healthy volunteers. ARGS neoepitope appears promising as a prognostic/stratification marker to facilitate patient selection and as an early pharmacodynamic marker for OA therapeutic trials.

Introduction

Characterizing the pathological processes involved in osteoarthritic cartilage degradation has been a significant focus of research over the past few decades with the ultimate goals of identifying key factors involved and development of novel osteoarthritis (OA) treatment strategies. Through the use of knockout mouse strains it has been widely reported that A disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5) is an instrumental protease responsible for driving cartilage loss in preclinical models of arthritis[1–3]. Further studies assessing the ability of fully selective, high affinity ADAMTS-4 and ADAMTS-5 antibodies to neutralise recombinant and native aggrecanase activity and modulate disease related endpoints in *in vivo* and *ex-vivo* models has confirmed ADAMTS-5 is a major protease involved in human OA[4]. To monitor the pharmacodynamic effect of a humanised anti-ADAMTS-5 monoclonal antibody (GSK2394002) as a potential disease modifying agent for OA, an assay that can quantify concentrations of the ARGS neoepitope, a product of ADAMTS-5 enzymatic degradation

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of aggrecan, was further developed[5,6]. Improvements have been made over previous assays[7–11] to reduce matrix effects and increase sensitivity by inclusion of additional blocking steps and ARGS neoepitope matrix depletion for standard curve dilutions. In addition the dynamic range has been increased over previous enzyme linked immunosorbent assay (ELISA) assays.

In this observational study, we evaluated ARGS neoepitope in patients with knee OA to support development of GSK2394002 and other drugs, which may affect cartilage degradation. Particular focus was to characterize the ARGS neoepitope assay performance and relative concentrations of ARGS neoepitope in blood, synovial fluid (SF) and urine of patients with knee OA managed conservatively (confirmed by X-ray but not scheduled or anticipated to require joint replacement in the coming year), or with end-stage knee OA undergoing TKR. Secondary objectives were to investigate diurnal, between- and within-subject variability of ARGS neoepitope. In addition correlations between serum and SF and to other demographic or clinical factors were assessed. An additional set of samples, obtained from age- and sex-matched healthy volunteers, served as a control group for comparative purposes.

Data from this study are intended to inform the biomarker strategy and study design of disease modifying treatments for OA. In addition, measurement of the ARGS neoepitope could serve as a prognostic or stratification marker to identify patient subsets more likely to respond to and benefit from treatment[12].

Materials and methods

Subject samples

Human biological samples were obtained with written informed consent from individuals in accordance with International Conference on Harmonisation Good Laboratory Practice (ICH GCP) under a protocol approved by the UK National Research Ethics Committee (Essex) (Registry No. 10/H0301/61, GSK ADM114261).

Subjects who had knee OA that was not end-stage ($n = 20$) were recruited from the GSK clinical unit (Cambridge, UK). Confirmation of knee OA was based on X-ray reports from General Practitioners or hospital opinion letters where possible or MR scan findings were accepted if accompanied by a specialist opinion indicating a diagnosis of knee OA. If none of these were available then a confirmatory X-ray was performed. Biological samples were collected on three visits in this cohort. On visit 1, subjects provided blood and urine samples at 5 timepoints between the hours of 0800 and 1,800 to assess diurnal variation. Single morning donation of blood and urine was obtained on visits 2 and 3. Approximately 1–2 weeks separated each study visit.

OA subjects undergoing total knee replacement (TKR) ($n = 20$) were recruited from the Robert Jones and Agnes Hunt Orthopaedic Hospital (Oswestry, UK). Biological samples were provided on two visits corresponding to the subjects' scheduled pre-surgical visit (visit 1) that typically took place between 3 and 6 weeks prior to surgery, and on the day of TKR (visit 2). On visit 1, matched blood and urine were collected. On visit 2, matched blood, urine and SF were collected. OA history was obtained from each OA patient at screening along with a subject completed WOMAC questionnaire[13] (Table II). Matched serum and urine were obtained via commercial sources (Tissue Solutions, Glasgow, UK; Cambridge Bioscience, Cambridge, UK) from 20 age- and sex-matched healthy individuals. This sample set served as a non-OA reference group.

Materials

Human recombinant ADAMTS-5 was generated by GlaxoSmithKline[14]. Recombinant human aggrecan (G1-IGD-G2 domains) was purchased from R&D systems (Minneapolis, USA). The mouse monoclonal anti human aggrecan antibody (binding to globular domains 1 and 2 (G1 and G2) of human aggrecan was purchased from Invitrogen (Carlsbad, USA). The monoclonal antibody, OA-1, which recognizes an N-terminal neoepitope '^{374}ARGS' (ARGS) following aggrecanase cleavage of the interglobular domain (IGD) of aggrecan, was developed at GSK[7] Standard bind Mesoscale-discovery (MSD) 96-well microtitre plates, blocker A, cytokine assay diluents, antibody diluents, 4× read buffer T with surfactant, sulfo-TAG normal human serum (NHS) ester and the Sector Imager 6000 with Discovery Workbench software were obtained from Meso Scale Discovery (MSD, Gaithersburg, USA).

Table I
Subject demographics

	Subject number	Age (years) (mean ± S.E.M.)	Gender (female:male)	Visits	Samples
Healthy controls*	20	64.1 ± 1.3	12:8	1	Serum, urine
OA	20	66.5 ± 1.6	12:8	1†	Serum, urine
				2, 3	Serum, urine
OA-TKR	20	68.7 ± 2.5	12:8	1	Serum, urine
				2	Serum, urine, SF

S.E.M.: Standard error of the mean.
* Subjects without signs or symptoms of OA or previous diagnosis of OA.
† Samples collected at five timepoints during the day. All other samples collected at 9:00 am.

Generation of ARGS neoepitope standard

A complete ADAMTS-5 digest of recombinant human aggrecan IGD (R&D Systems, Minneapolis, USA) was prepared and used as a standard for quantification of samples with the ARGS neoepitope assay. 50 μL of 1 mg/mL aggrecan was digested with 2.25 μL of 1.2 μM ADAMTS-5 in 10 mM hydroxyethyl piperazineethanesulfonic acid (HEPES), 1 mM $CaCl_2$, 150 mM $NaCl_2$, 0.05% NP-40, 1 μM $ZnCl_2$ at pH 7.4 for 4 days at 37°C. Complete digestion was confirmed by protein fragmentation Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting according to published methods[7]. In the absence of aggrecanase treatment a single ~92 kD band is observed, while 58 kD NITEGE and diffuse ~52 kD ARGS neoepitope specific immunoreactive bands arise following ADAMTS-5 mediated digestion (Supplementary Fig. 1).

Table II
Summary of OA history

OA history*	OA ($n = 20$)	OA-TKR ($n = 20$)
Knees affected by OA (% subjects)		
Both	75	70
Left	5	–
Right	20	30
OA in joints other than the affected knee(s) (%)	60	60
Average number of OA joints†	2.8	3.5
Concurrent hand OA (% subjects)	50	30
Concurrent foot OA (% subjects)	15	20
Concurrent hip OA (% subjects)	10	30
Concurrent spine OA (% subjects)	5	20
Concurrent shoulder OA (% subjects)	5	25
Knee injury within 30 days*	0	0
Disease duration (mean years ± S.E.M.)	9.0 ± 1.9	8.2 ± 1.2
Combined WOMAC (visual analogue 100 mm scale, mean ± S.E.M.)	26.1 ± 1.9	55.2 ± 4.45

* Based on subjects' self report.
† OA joints are counted using all the effected joints including the affected knee(s), 1 for single OA knee and 2 for both OA knees.