Experimental and Molecular Pathology 101 (2016) 214–220

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Interleukin-1α induces focal degradation of biglycan and tissue degeneration in an in-vitro ovine meniscal model

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ARTICLE INFO

Article history:
Received 26 February 2016
and in revised form 5 September 2016
Accepted 7 September 2016
Available online 09 September 2016

Keywords:
Meniscus
MMPs
IL-1
ADAMTS
In-vitro model of meniscal
Degeneration
OA

ABSTRACT

We have developed an ovine meniscal explant model where the focal degradative events leading to characteristic fragmentation patterns of biglycan in human OA of the knee and hip, and evident in animal models of knee OA and IVD degeneration are reproduced in culture. Lateral and medial menisci were dissected into outer, mid and inner zones and established in explant culture ± IL-1 (10 ng/ml). The biglycan species present in conditioned media samples and in GuHCl extracts of tissues were examined by Western blotting using two C-terminal antibodies PR-85 and EF-Bgn. Clear differences were evident in the biglycan species in each meniscal tissue zone with the medial outer meniscus having lower biglycan levels and major fragments of 20, 28, 33 and 36, 39 kDa. Similar fragmentation was detected in articular cartilage samples, 42-45 kDa core protein species were also detected. Biglycan fragmentation was not as extensive in the IL-1 stimulated meniscal cultures with 36, 39, 42 and 45 kDa biglycan species evident. Thus the medial meniscus outer zone displayed the highest levels of biglycan processing in this model and correlated with a major zone of meniscal remodelling in OA in man. Significantly, enzymatic digests of meniscal tissues with MMP-13, ADAMTS-4 and ADAMTS-5 have also generated similar biglycan species in-vitro. Zymography confirmed that the medial outer zone was the region of maximal MMP activity. This model represents a convenient system to recapitulate matrix remodelling events driven by IL-1 in pathological cartilages and in animal models of joint degeneration.

1. Introduction

Biglycan is a widely distributed class I small leucine-rich repeat proteoglycan (SLRP) [1,2]. It is found in skeletal tissues [3] such as bone [4,5], cartilage [6,7], intervertebral disc [8,9] and tendon [10] as well as non-skeletal tissues such as lung [11–13], brain [14,15], heart [16], liver [17,18] and kidney [13,19,20]. Biglycan is also a vascular proteoglycan [21,22]. The biglycan core protein (Mr 37,983) [23] contains a characteristic cluster of N-terminal Cys residues that form two disulphide bonds, a central 10-12 leucine-rich repeat region and two glycosaminoglycan (GAG) chains, either chondroitin sulphate (CS) or dermatan sulphate (DS) attached at amino acids 5 and 10 in human biglycan [7]. Non-glycanated forms of biglycan have also been described in articular cartilage [24] and intervertebral disc [25] and their abundance increases with ageing. De-glycanated biglycan has been shown to bind bone morphogenetic protein (BMP)-2 and stimulate new bone formation by osteoblasts in a rat mandible model [26].

The SLRPs have diverse functions in musculoskeletal tissues as modulators of tissue organization, cellular proliferation, matrix adhesion, and response to growth factors and cytokines [6]. The SLRPs are involved in many aspects of mammalian biology both in health and disease and are now recognised as key signalling molecules with an extensive repertoire of molecular interactions with growth factors and a number of receptors which regulate cell growth, morphogenesis and immunity [1,2]. Biglycan in particular, has roles to play in the initiation of the inflammatory response during tissue stress [2,27]. It acts as an endogenous ligand analogous to the pathogen associated molecular patterns (PAMPs) for the innate immunity receptors; toll-like receptor-2 and −4 (TLR2 and TLR4) expressed by macrophages and can stimulate the expression of the inflammatory mediators TNF-1α and macrophage inflammatory molecule-2 (MIP2) [2,27]. Biglycan can also bind to bone morphogenetic protein/transforming growth factor-β (BMP/TGF-β) and modulate their activities influencing fibrosis [28,29] and skeletal

Abbreviations: MI, MO, LI, LO, the inner and outer zones of the medial and lateral menisci; AC, articular cartilage; IL-1, interleukin-1; IVD, intervertebral disc; GuHCl, guanidine hydrochloride; ADAMTS, a disintegrin adamalysin protease with thrombospondin type 1 motifs; MMP, matrix metalloproteinase; SLRPs, small leucine rich proteoglycans.

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http://dx.doi.org/10.1016/j.yexmp.2016.09.001
0014-4800/

cell differentiation [1,2]. Biglycan is associated with collagen fibrils in connective tissues and predominantly with type VI collagen in the pericellular matrix. While biglycan influences collagen fibrillogenesis in-vitro, it does not interact with fibrillar collagens under all conditions; its major interactive arena lies in cell mediated regulatory processes in health and disease [30,31].

Direct evidence for the importance of the SLRPs in musculoskeletal tissues has been demonstrated using knockout mice. Although functional overlap between SLRP members is evident, a major phenotype of biglycan, decorin, fibromodulin and lumican single-knockout or double-knockout mice is age dependent tendon laxity, ectopic calcification and arthritis [32–36]. Biglycan is prominently expressed in the cartilaginous rudiments during spinal development[37], absence of biglycan accelerates degeneration of the IVD in mice [34]. Biglycan levels are elevated at sites of annular remodelling with fragmented biglycan core protein species prominent during IVD degeneration[38,39]. Fragmentation of biglycan has been observed with connective tissue remodelling in animal models of osteoarthritis (OA) and intervertebral disc degeneration[39,40]. Moreover, biglycan is extensively fragmented in pathological articular cartilage (AC) and menisci in OA and associated with focal areas of fibrillation in knee AC [41].

Our laboratory has recently developed an ovine meniscal model where we have evaluated the effect of the inflammatory cytokines interleukin-1α (IL-1α) and tumour necrosis factor-α (TNF-α) on the production of MMPs and ADAMTS-4 and 5 by the inner and outer regions of the medial and lateral menisci and compared this data with articular cartilage (AC) from the same knee joint. Focal differences were evident in the responsiveness of the meniscal regions to IL-1α and TNF-α with the outer meniscal regions more responsive to these inflammatory cytokines in terms of MMP expression than the inner meniscal regions [42]. The present study was undertaken to ascertain how similar the fragmentation patterns of biglycan in our model were to those observed in the cartilages of a number of pathological human joint tissues in animal models of joint degeneration [39–41] and in in-vitro digestions of biglycan with selected proteinases of relevance to degeneration of knee joint tissues [38,39,43]. Thus in the present study we determined if the regional biglycan core protein fragmentation patterns in an ovine meniscal explant model were similar to those observed in pathological human knee joint tissues, in animal models of joint pathology and in normal and degenerate ligament/tendon.

2. Materials and methods

The polyclonal antibody EF-Bgn was raised in rabbits by EZ Biolab (Westfield, IN, USA) using the biglycan C-terminal specific13-mer peptide TDRLAIQFGNYKK. The EF Bgn pAb was affinity purified from pooled immune serum from two rabbits using a (CGG) TDRLAIQFGNYKK peptide affinity column, CGG was added to the immunizing peptide sequence to facilitate linkage to a solid phase support for immunopurification purposes. Antibody specificity was confirmed by pre-absorption of the antibody preparations with the peptide TDRLAIQFGNYKK prior to immunoblotting. Comparative immunoblots with pAb EF Bgn were also undertaken with pAb PR-85 also raised to the same C-terminal sequence of biglycan. PR-85 has been used successfully in 3 earlier studies by our laboratory [39–41], this Ab was kindly supplied by Prof Peter Roughley, Shriners Hospital, Montreal, Quebec, Canada. All electrophoresis products, precast gels, blotting consumables, protein standards, application and running buffers were Novex products obtained from Invitrogen, Mt. Waverley, Vic, Australia. Chondroitinase ABC, were purchased from Sigma Aldrich, Sydney, Australia. Keratanase I and II were purchased from Seikegaku Corp, Tokyo. Japan. rh MMP13 was provided by Prof Gillian Murphy, University of Cambridge, UK. Recombinant human ADAMTS-4 and ADAMTS-5 were provided by Prof Hideaki Nagase, Kennedy Institute, Imperial College, London, UK.

2.1. Tissues

Non-arthritic human knees were obtained from The International Institute of Advancement in Medicine (IIAM), Jessup, PA, USA; a division of the Musculoskeletal Foundation. Full-thickness cartilage was harvested from macroscopically normal tissue. Histological and immunohistological examination of full thickness osteochondral tissue sections confirmed the tissues were non-degenerate. Ovine stifle joints (6-12 month old) were obtained from a local abattoir.

2.2. Explant culture

Full-depth 5 mm^2 plugs of AC were harvested from the trochlear groove, and the lateral and medial menisci were removed aseptically from 6 to 12 month old sheep. The menisci were trimmed of marginal synovial tissue and ligamentous attachments at the anterior and posterior horns, and then radially dissected into outer, middle and inner regions. The middle zone was discarded to provide clear demarcation between the inner and outer meniscal regions. The inner and outer zones of the medial and lateral menisci (MI, MO, LI, LO, respectively) and AC explants were rinsed in phosphate buffered saline (PBS, pH 7.0) containing gentamycin (50 μg/ml) and placed in Dulbecco's Modified Eagles Medium (DMEM; Sigma, Castle Hill, NSW, Australia) buffered with sodium bicarbonate 3.7 g/L (Fronine, Riverstone, NSW, Australia) supplemented with 10% (v/v) Foetal Calf Serum (FCS; Trace Biosciences Pty. Ltd., Castle Hill, NSW, Australia), 2 mM L-glutamine (ICN Biochemicals Inc., Aurora, OH, USA), and 50 μg/ml Gentamicin (Pharmacia Pty. Ltd., Bentley, WA, Australia). The explants were equilibrated at 37 °C in an atmosphere of 90% humidity and 5% (v/v) CO_2 for 48 h, after which the tissues were washed (3 × 5 min) in serum-free-DMEM. The meniscal and AC explants were then cultured individually for 4 days in 24-well culture plates in 1 ml of serum free DMEM ± 10 ng/ml IL-1α (PeproTech Inc., Rocky Hill, NJ, USA).

2.3. Extraction of tissues

Ex-vivo and cultured explants were finely diced and extracted with 10 volumes of 4 M guanidine hydrochloride (GuHCl) extraction buffer (0.5 M sodium acetate pH 5.8 containing 10 mM EDTA, 20 mM benzamidine and 50 mM 6-aminohexanoic acid) using end-over end mixing for 48 h at 4 °C and the extracts separated from the tissue residues by centrifugation. The tissue residue was discarded.

2.4. Chondroitinase ABC and keratanase-I digestion of tissue extracts

Six replicate tissue extracts or media samples were pooled to provide a representative sample and precipitated overnight with 5 volumes absolute ethanol at 4 °C. The precipitated samples were recovered by centrifugation, resuspended in 0.1 M Tris acetate buffer (pH 6.5) and digested overnight at 37 °C with Chondroitinase ABC (0.1 U) and Keratanase I (0.05 U) (Seikagaku Corp, Tokyo, Japan). The samples were re-precipitated with 5 volumes ethanol; the precipitate collected by centrifugation and residual ethanol removed using a centrifugal vacuum concentrator (Speed vac).

2.5. Lithium dodecyl sulphate PAGE

Aliquots of the chondroitinase ABC, keratanase-I digested samples from 1 mg tissue (0.1 ml) were mixed with lithium dodecyl sulphate PAGE application buffer (35 μl) and 500 mmol/l dithiothreitol (15 μl). The samples were then heated at 70 °C for 30 min, cooled, and 25 μl aliquots containing the equivalent of the extract from 1 mg wet weight tissue or of culture media were electrophoresed under reducing conditions on 10% NuPAGE Bis-Tris gels at 200 V constant voltage for 50 min using NuPAGE MOPS (3- [N-morpholino]- propanesulfonic acid) sodium dodecyl sulphate running buffer. The gels were electroblotted to