SPECIES SPECIFICITY OF NEOEPITOPE ANTIBODIES TO AGGRECANASE- AND MMP-GENERATED SEQUENCES IN THE INTERGLOBULAR DOMAIN OF AGGRECAN

+*Little, C B (A-Arthritis Research Campaign); *Flannery, C R; *Hughes, C E; **Otterness, I G; *Caterson, B
+*Cardiff University, Cardiff, Wales, UK. Connective Tissue Biology Laboratories, School of Biosciences, Museum Ave., Cardiff, CF10 3US, UK, (0)2920874593, Fax: (0)2920874594, LittleCB@Cardiff.ac.uk

Introduction

Proteolysis of the interglobular domain (IGD) of the aggrecan core protein with loss of the glycosaminoglycan (GAG) bearing portion of the molecule from articular cartiage is an early and persistent event in joint disease which renders the tissue suceptible to further mechanical disruption. Aggrecanases and the matrix metalloproteinases (MMPs) have been implicated in the metabolism of aggrecan, however the relationship between these two proteolytic activities in normal turnover and in early and late disease remains controversial. Monoclonal antibodies (MAbs) which specifically recognise the N- and C-terminal neoepitope sequences generated in human aggrecan by cleavage at ^{373}E-A^{374} and ^{341}N-F^{342} in the IGD by aggrecanases and MMPs, respectively provide powerful tools not only to investigate the pathophysiology of cartilage degeneration but also as potential early diagnostic markers of joint disease(1). However, the amino acid sequences surrounding the two cleavage sites differs between species (Table 1), and this could affect the utility of these MAbs in animal models used to investigate the processes involved in cartilage destruction. In the present study we have determined the reactivity of these MAbs in a variety of species using Western blotting and surface plasmon resonance.

Materials and Methods

Isolation and digestion of aggrecan by aggrecanase or MMPs. Proteoglycans were extracted from human, bovine, equine, porcine, rat, mouse, guinea pig and chicken articular cartilage and subjected to cesium chloride density gradient centrifugation. Equal portions of D1 proteoglycan (measured as GAG) from each species were digested with purified porcine articular cartilage aggrecanase or recombinant human MMP-3.

Western blot analyses of aggrecanase- or MMP-generated aggrecan catabolites. The digested proteoglycans were deglycosylated and analyzed by SDS-PAGE and Western blotting as described (2) using MAbs BC-3 and BC-13 (recognizing the aggrecanase-generated sequences ^{374}ARGSV... and …ITEGE^{373}, respectively) or BC-14 and BC-4 (recognizing the MMP-generated sequences ^{341}FFGVG... and …DIPEN^{342}, respectively). Some digests were analysed using MAbs 2-B-6 recognising chondroitin-4-sulphate stubs on aggrecan, MAb AF-28 (provided by Dr. AJ Fosang) an antibody to the FFGVG.. neoepitope or affinity purified polyclonal antisera recognising the neoepitope sequence DIPEN… (provided by Dr. JS Mort).

Analysis of binding affinity. Synthetic peptides corresponding to the N-terminal neoepitope sequences resulting from aggrecanase cleavage of human, bovine, ovine, equine, porcine, canine, rat, mouse, guinea pig and hamster aggrecan were synthesised. These peptides were conjugated to biosensor chips and the IC_{50} for inhibition of BC-3 binding to the peptides measured using a BIAcore 2000 as previously described (3).

Table 1: Amino acid sequences surrounding the MMP and aggrecanase cleavage sites in the IGD of aggrecan in a variety of species. The amino acid differences from the human sequence are shown in bold typeface.

Species	MMP cleavage site	Aggrecanase cleavage site
Human	..FVDIPEN-FFGVGGE..	..RNITEGE-ARGSVIL..
Porcine	..FVDIPEN-FFGVGGE..	..RNITEGE-ARG**T**VIL..
Equine	..FVDIPEN-FF**A**V**S**GE..	..**Q**NITEGE-ARG**N**VIL..
Bovine	..FVDIPE**S**-FFGVGGE..	..RNITEGE-ARGSVIL..
Rat	..FVDIPEN-FFGVGGE..	..RNITEGE-ARG**N**VIL..
Guinea Pig	..FVDIPEN-FF**S**VGGE..	..RNITEGE-ARG**N**VI**H**..
Chicken	..F**EALVPG-LFTDEVG**..	..RN**V**TE**E**E-ARGS**IAT**..
Murine	..FVDIPEN-FFGVGGE..	..RN**V**TEGE-A**L**GSVIL..
Hamster	..F**I**DIPEN-FFGVGGE..	..**Q**N**T**TEGE-**V**RG**NE**IL..

Results and Discussion

None of the aggrecan preparations reacted with the neoepitope MAbs following incubation in digestion buffer alone (data not shown). Western blot analysis with MAbs BC-4 and BC-14 of samples that had been digested with MMP-3 is shown in Figure 1A and B, respectively. After MMP digestion a predominant BC-4 band of expected size (approx. 60kDa) was present in all species other than the bovine and chicken. The smaller mass BC-4 bands may be related to proteolysis within the disulfide-bonded loops of G1 and become apparent upon running the samples under reducing conditions (4). The lack of BC-4 reactivity in the bovine is consistent with the altered sequence in this species, however similar staining to that seen in other species was apparent when a polyclonal antiserum was used (data not shown). MMP site cleavage in the bovine was confirmed by the BC-14 blot where, as in most other species, several bands were readily apparent (Fig 1B). However, substitution of G^{344} with an S (guinea pig) abrogated BC-14 binding whereas substitution of G^{344} with an A (equine) did not. Neither the horse nor the guinea pig reacted with AF28 (data not shown). The complete lack of sequence similarity at the MMP site in the chicken resulted in no reactivity with BC-4 or BC-14 although digestion by MMPs was confirmed with the 2B6 immunoblot banding pattern (not shown).

Fig. 1. Western blot analysis of aggrecan metabolites generated by MMP catabolism of the IGD. A) MAb BC-4 recognizing ...DIPEN and B) MAb BC-4 recognizing FFGVG...

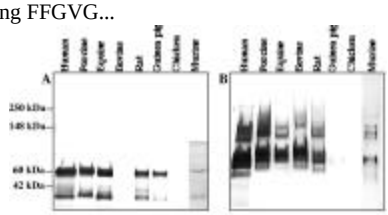
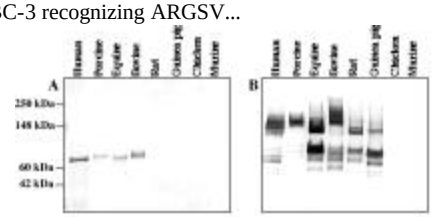


Fig. 2. Western blot analysis of aggrecan metabolites generated by Aggrecanase catabolism of the IGD. A) MAb BC-13 recognizing ...ITEGE and B) MAb BC-3 recognizing ARGSV...



Western blot analysis with BC-13 and BC-3 after aggrecanase digestion is shown in Figure 2A and B, respectively. Positive reactivity with BC-13 was demonstrated in human, porcine, equine, and bovine samples. However, in the rat and guinea pig, aggrecanase activity could be demonstrated by positive BC-3 immunoblotting. The reason for the lack of BC-13 reactivity in these two species is unclear given their sequence identity with the human but could relate to different N-linked glycosylation of the R**N**IT. The lack of BC-13 and BC-3 reactivity in the mouse and chicken could be related to sequence differences as digestion by aggrecanase was confirmed using 2B6 (not shown).

Quantitation of BC-3 reactivity was performed using surface plasmon resonance with synthetic peptides corresponding to the amino acid sequences of a number of species. The IC50's ($x10^{-8}$ M) for BC-3 binding to the different species were: human - 4.3, porcine – 6.7, equine – 4.3, bovine – 7.4, rat – 4.3, guinea pig – 3.7, Mouse - >1000, and Hamster - >1000. These results are consistent with those obtained by BC-3 Western blotting.

Conclusions

These studies have validated the predicted use of several neoepitope MAbs in different animal species utilized as models for aspects of the pathogenesis of arthritis in humans. Interestingly, some negative results were obtained with predicted immunopositive sequences which were homologous to those which were immunopositive in other species. These results suggest that additional post-translational or conformational changes must occur at some of the matrix proteinase cleavage sites in aggrecan.

Refs. (1) Caterson B et al (2000) Matrix Biol *in press.* (2) Little CB et al (1999) Biochem. J. 344, 61. (3) Otterness IG et al (1999) Matrix Biol 18,331. (4) Mort JS et al Biochem J (1985) 232, 805.

**Pfizer Inc., Groton, CN 06340.