DETECTION OF MEMBRANE ASSOCIATED AGGRECANASE AND MATRIX METALLOPROTEASE ACTIVITIES IN MONOLAYER CULTURES OF BOVINE CHONDROCYTES.

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Introduction. Catabolism of aggrecan core protein, an early and important event in the pathogenesis of joint diseases such as osteoarthritis, occurs via cleavage of two peptide bonds (Asn^{341}-Phe^{342} and Glu^{373}-Ala^{374}) located in the interglobular (IGD) of the molecule. Cleavage of the Glu^{373}-Ala^{374} bond is believed to be the primary catabolic event in aggrecan degradation, however the identity of the enzyme responsible (termed 'aggrecanase') remains unknown. The Asn^{341}-Phe^{342} bond is susceptible to cleavage by many of the known matrix metalloproteinases (MMPs). Elegant studies conducted some 20 years ago (1) demonstrated that the catabolism of exogenous aggrecan applied to organ cultures of IL-1 and retinoic acid (RA) stimulated cartilage was dependent upon contact with the tissue, thereby suggesting a pericellular location for the proteolytic activity rather than cleavage by soluble enzymes. Whilst both soluble and membrane associated MMP activity has been demonstrated in chondrocytes (2-4), to date, direct evidence only exists for the presence of soluble aggrecanase (2). In the present study we have examined membrane preparations from cultured chondrocytes for the presence of IGD degrading aggrecanase and MMP activities. In addition, the expression by cultured chondrocytes of several of the transmembrane disintegrin metalloproteinases (ADAMs) were examined to determine whether their expression correlated with the aggrecanase and MMP activities detected on chondrocyte membranes.

Methods. Chondrocytes were isolated from 2 week old bovine metacarpophalangeal articular cartilage by sequential pronase and collagenase digestion. Isolated cells were grown in monolayer at high density ($6x10^6$ cells/60mm plate) in serum free DMEM for 120 hours. Cells were treated for the entire culture period ± RA (10^{-6}M) or IL-1α (10ng/ml). At the termination of culture, aggrecanase and MMP activities in the conditioned medium were evaluated using a recombinant IGD substrate ($rAgg1_{mut}$) and western blotting with monoclonal antibodies BC-3 (recognizing the aggrecanase generated N-terminal neoepitope ARGSV...) and BC-14 (recognizing the MMP-generated N-terminal neoepitope FFGVG...) as previously described (2). In addition the endogenous proteoglycan fragments in an aliquot of conditioned medium from all cultures were collected by ethanol precipitation, deglycosylated with chondroitinase ABC, keratanase and keratanaseII and separated by 4-12% gradient SDS-PAGE. Separated proteoglycan fragments were transferred to nitrocellulose and western blotting performed using BC-3 and BC-14 to detect aggrecanase and MMP generated aggrecan fragments. The cell layers were washed with PBS and cell membrane samples prepared by homogenization and ultracentrifugation as described previously (3). Aggrecanase and MMP activity in the cell membrane preparations were evaluated using $rAgg1_{mut}$ as for conditioned medium. Total RNA was isolated from duplicate cultures and amplified by RT-PCR using primers specific for ADAM 9 and ADAM10 mRNAs.

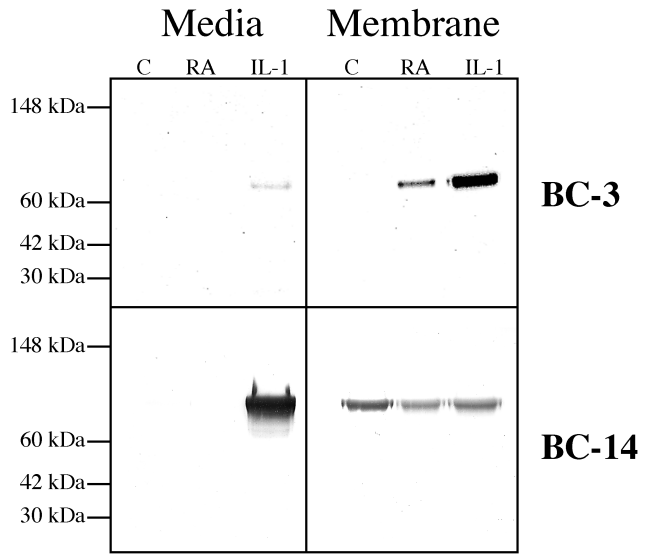
Results. In control culture medium there was no evidence for aggrecanase (BC-3 positive) or MMP (BC-14 positive) generated endogenous aggrecan degradation products. Aggrecanase generated proteoglycan fragments (150-75 kDa) were detected in conditioned medium from RA and IL-1 stimulated cultures. However, MMP degraded aggrecan fragments (BC-14 positive) were not detected in the medium RA or IL-1 stimulated cultures.

No IGD degrading aggrecanase activity (detected as BC-3 reactive $rAgg1_{mut}$ catabolites) was detected in conditioned medium from control or RA stimulated monolayer cultures (Fig 1). In response to IL-1 stimulation however, aggrecanase activity was detected in conditioned medium at very low levels (Fig 1). Similarly, IGD degrading MMP activity was only detected in conditioned medium from IL-1 stimulated chondrocytes (Fig 1).

In membrane preparations from both RA and IL-1 stimulated but not control chondrocytes, aggrecanase activity was readily detectable (Fig 1). IL-1 stimulated chondrocyte membranes preparations appeared to have greater aggrecanase activity than RA stimulated cells. In contrast with the results for soluble MMP activity, membrane associated MMP activity was detected in all (control, RA and IL-1) cultures (Fig 1).

Messenger RNA's for both ADAMs 9 and 10 were detected in control, RA and IL-1 chondrocyte cultures. There appeared to be no difference in the expression of the two ADAMs in control compared with RA or IL-1 stimulated cultures.

Figure 1 BC-3 and BC-14 Western blot of $rAgg-1_{mut}$ catabolites generated by conditioned media or membrane preparations from bovine monolayer chondrocytes cultures without (C) and with RA and IL-1.



Discussion. This study has clearly demonstrated the presence of aggrecanase activity in crude plasma membrane preparations of cultured bovine chondrocytes stimulated with RA or IL-1. The presence of aggrecanase generated proteoglycan fragments in the culture medium correlated with the detection of membrane associated but not soluble aggrecanase activity as the latter was not detected in RA medium. The reason why little aggrecanase activity was detected in the medium in the present study, whilst it has been readily detected in previously published work (2) may relate to the use of monolayer versus three-dimensional agarose culture methods. The expression of ADAMs 9 and 10 did not correlate with the presence of aggrecanase generated aggrecan catabolites in the culture media, nor with the detection of membrane associated aggrecanase activity. Whether the soluble aggrecanase activity found in IL-1 conditioned medium is derived from the membrane associated form or represents a distinct enzyme activity remains to be determined. It has recently been reported that MT-MMP-1 is able to cleave the IGD of aggrecan at both the aggrecanase and MMP sites and thus may be responsible for the membrane associated aggrecanase activity detected in the present study (5). The failure to detect BC-14 positive aggrecan fragments in the face of MMP activity in medium (IL-1) and membrane preparations (C, RA and IL-1) may indicate cleavage by these enzymes at a different site within the aggrecan core protein (5).

References. (1) Dingle, JT and Dingle TT, Biochem J 1980;190:431-438. (2) Hughes CE, et al. Trans Orthop Res Soc 1998;23:82. (3) Cowell S. et al. Biochem J 1998;331:453-458. (4) Büttner FH et al. Arthritis Rheum 1997;40:704-709. (5) Büttner et al, Biochem J 1998;333:159-165.

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