IDENTIFICATION OF CHONDROCYTE HYALURONIDASE ISOZYMES AND REGULATION OF THEIR EXPRESSION BY PRO-INFLAMMATORY CYTOKINES

*+Flannery, CR, Little, CB, Hughes, CE and Caterson, B
*+Connective Tissue Biology Laboratories, Cardiff University, UK Tel. +1222 874593; FAX +1222 874594; Email: FlanneryCR@CF.AC.UK

Introduction

The load-bearing capacity of articular cartilage is facilitated by the presence of high concentrations of hydrophilic sulfated-glycosaminoglycans (S-GAGs) which are attached to the core protein of aggrecan. Maintenance of high S-GAG concentrations is achieved via the binding of the N-terminal G1 domain of aggrecan monomers to hyaluronan (HA), resulting in the formation of large multimolecular aggregates. Studies conducted using model cartilage explant culture systems have demonstrated that both HA and aggrecan are actively metabolized. Independent reports have demonstrated that under steady state conditions, aggrecan and HA are catabolized in a coordinated fashion (1, 2). In these studies, aggrecan lost from the extracellular matrix was released into culture media, while HA appeared to be internalized and presumably degraded by the chondrocytes (a process consistent with CD44-mediated endocytosis; Ref 3). Conversely, under conditions of elevated matrix catabolism (e.g. treatment with IL-1) aggrecan fragments and HA are detected in culture medium (4), thereby implicating the potential for the presence of extracellular hyaluronidase(s), an enzyme activity which has not previously been detected in cartilage. In order to identify putative chondrocyte hyaluronidases, we adopted a gene homology cloning approach and performed RT-PCR reactions on chondrocyte mRNA using degenerate primers based on amino acid sequences which are identical in two vertebrate (human) hyaluronidases, the plasma hyaluronidase Hyal-1 (5) and the sperm-associated hyaluronidase PH-20 (6). Three forms of chondrocyte hyaluronidase were identified, and the expression of mRNAs for these “isozymes” in freshly isolated and cultured human articular cartilage and chondrocytes was performed using primers specific for each gene product.

Methods

Articular cartilage was obtained from the knee joints of 3 patients undergoing arthroplasty for osteoarthritis. Bovine and porcine cartilage was obtained from the metacarpophalangeal joints of joints of 1-2 week old calves and 3-6 month old pigs. Portions of intact cartilage were taken for direct RNA extraction or maintained as explant cultures in DMEM prior to RNA extraction. In addition, chondrocytes liberated by pronase/collagenase digestion were grown as monolayer cultures in DMEM prior to RNA extraction. Individual explant or monolayer cultures were treated for 3 days with DMEM ± 10 ng/ml IL-1 beta, 100 ng/ml TNF-alpha or 1 μM all-trans retinoic acid. Total RNA was extracted from monolayer cultures by direct addition of Tri-Reagent (MRC, Cincinnati, OH, USA). For fresh tissue and explant cultures, samples were snap-frozen in liquid nitrogen and pulverized prior to addition of Tri-Reagent and RNA isolation. Homology cloning RT-PCR was performed using primers based on cDNA sequences encoding amino acid sequences AVDIWÉ, ALYP51 and FPDCCYN (identical for Hyal-1 and PH-20). Gene-specific “hot-start” RT-PCR was performed using cDNA sequences specific to three hyaluronidase “isozymes” identified by homology cloning reaction. A microtitre plate assay for hyaluronidase activity was developed based on the method of Frost and Stern (7).

Results and Discussion

Homology cloning RT-PCR of human chondrocyte monolayer RNA resulted in the generation of six distinct PCR products (~180bp to ~400bp). Each PCR product was isolated and sequenced. A homology BLAST search of the GenBank database revealed that three of the sequences obtained had significant homology to human cDNAs with the following accession numbers: U96078 (plasma hyaluronidase Hyal-1; Ref. 5), C03056 (candidate tumor suppressor LUCA-1; unpublished), AJ000099 (lysosomal hyaluronidase Hyal-2; unpublished), U90577 (candidate tumor suppressor LUCA-2; unpublished), AF040710 (LUCA-3; unpublished) and AC024255 (cosmid clone LUCA-13; unpublished). The sequences for LUCA-1 and -2 are identical to those for plasma Hyal-1 and lysosomal Hyal-2, respectively, and are contained within the cosmid clone LUCA-13 (29,341bp from chromosome 3p21.3) with LUCA-2 occurring 5’ to LUCA-1. LUCA-3, a third hyaluronidase gene, is also located in the 3p21.3 region. Sequence alignment of lysosomal Hyal-2/LUCA-2 and LUCA-3 with plasma Hyal-1/LUCA-1 revealed ~26% amino acid identity, with highest homology occurring around peptide sequences used for primer design.

Gene-specific RT-PCR was then performed using human chondrocyte RNA with primers selective for Hyal-1/LUCA-1, Hyal-2/LUCA-2 and Hyal-3/LUCA-3. Hyal-1 and Hyal-3 mRNAs were present in all monolayer cultures (not shown) and in extracts of fresh cartilage (Fig. 1, lanes 1-3). For explant cultures (Fig. 1, lanes 4-7), Hyal-2 and, to a greater extent, Hyal-3 mRNA levels appeared to be upregulated by pro-inflammatory cytokines, while Hyal-1 mRNA levels remained constant.

No hyaluronidase activity was detected in assays of porcine and bovine culture media and membrane extracts at neutral pH (pH 7.5), although when samples were adjusted to acid pH (pH 4.5), hyaluronidase activity was detectable in culture media. Collectively, these data indicate that chondrocytes may express Hyal-1 constitutively and that this isozyme could function as a housekeeping enzyme. The potential upregulation of Hyal-3 and/or Hyal-2 expression by IL-1 and TNF suggest that these isozymes may be responsible for the extracellular hyaluronidase activity observed in culture media, although the acid pH optima observed is consistent with intracellular hyaluronidase activity following internalization of HA. In this regard, extracellular hyaluronidases may utilise mannose-6-phosphate receptor pathways for their uptake in other cells from synovial joint tissues.

References


This work was funded by the Arthritis Research Campaign.