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Localization of cathepsins B and D in equine articular cartilage

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Summary

The proteoglycan-degrading enzymes cathepsin B and cathepsin D have been identified in normal horse articular cartilage and isolated chondrocytes using immunocytochemical methods. Both enzymes were located intracellularly. Low levels of the enzymes were detected in about 50% of chondrocytes maintained in primary monolayer culture. All samples of cartilage from the lateral trochlear ridge (age range 5 months-1 year) demonstrated the presence of cathepsins B and D, and particularly high levels of both enzymes were seen in the majority of chondrocytes in the hypertrophic region.

keywords: cartilage, cathepsin B, cathepsin D, horse, chondrocytes

Lokalisation der Kathepsine B und D im Gelenkknorpel von Pferden

Die Proteoglycan abbauenden Enzyme Kathepsin B und Kathepsin D wurden in normalem Pferdegelenkknorpel mit der immunocytochemischen Methode identifizert. Beide Enzyme waren intrazellular lokalisiert. Niedrige Konzentrationen der Enzyme wurden in etwa 50% der Chondrozyten, die in einer Einschichtprimärkultur gehalten wurden, nachgewiesen. Alle Knorpelproben von der lateralen Gelenkrolle des distalen Femur (Altersbereich fünf Monate bis ein Jahr) zeigten die Anwesenheit des Kathepsin B und Kathepsin D. Besonders hohe Konzentrationen von beiden Enzymen wurden in der Mehrzahl der Chondrozyten in der Region mit übermässigem Wachstum beobachtet.

Schlüsselwörter: Knorpel, Kathepsin B, Kathepsin D, Pferd, Chondrozyten

Introduction

Dyschondroplasia, a common developmental orthopaedic disease in horses is characterized by a failure of endochondral ossification and retention of cartilage. The cartilage lesions that develop, leading eventually to microfractures of metaphyseal and subchondral bone, are thought to be due to formation of a biomechanically inferior extracellular matrix (ECM) (*Hurtig* et al., 1993).
Since the chondrocyte is solely responsible for maintaining the integrity of the ECM, we propose that these cartilage defects might result from an imbalance in the production of matrix-degrading enzymes by these cells. Chondrocytes synthesize and secrete a range of degradative proteinases which are essential for the normal turnover of ECM. How-ever, under certain pathological conditions there is evidence that overproduction of these enzymes, due to cytokine stimulation, results in irreversible degradation of the cartilage (*Morris* and *Treadwell*, 1994; *Roughley* et al., 1993; *Koolwijk* et al., 1995). In the few reported studies on the role of degradative enzymes in equine joint disease interest has focussed exclusively on the metalloproteinases (*May* et al., 1992; *Hurtig* et al., 1993). There is currently no information available in the horse on the endogenous hydrolases, cathepsin B and cathepsin D. Both of these enzymes have been identified in human cartilage and are able to degrade aggrecan (*Roughley* et al., 1993; *Baici* et al., 1995a), one of the major components of the ECM. Although the metabolism of aggrecan (aggregated proteoglycan) has been well studied in normal equine articular cartilage (*MacDonald* et al., 1992; *Platt* and *Bayliss*, 1994; *Morris* and *Treadwell*, 1994) the involvement of cathepsins B and D has not received attention.
The aim of the present study therefore was to examine equine articular cartilage, using immunocytochemical methods, to detect the presence of cathepsins B and D.

Materials and methods

Cartilage samples

Articular cartilage was removed aseptically within 3h of euthanasia from macroscopically normal femorotibial joints of 8 horses aged 5 months–1 year. Full thickness cartilage samples were cut from the lateral trochlear ridge of the distal femur and were either snap-frozen in OCT embedding medium and stored at -20°C for immunocytochemistry, or were used immediately for isolation of chondrocytes.

Isolation and culture of chondrocytes

Chondrocytes were released from the cartilage by enzymic digestion as previously described (*Davies* et al., 1991) and plated as primary cultures in 8–well cell-culture slides at a density of $1x10^5$ cells/well in DMEM supplemented with 10% heat-inactivated foetal calf serum, 120g/ml benzyl penicillin and 200g/ml streptomycin. After 24–48h in culture the chondrocyte monolayers were fixed in 4% paraformaldehyde and permeabilized with Triton X-100 prior to immunocytochemistry.

Antisera

A polyclonal sheep anti-human cathepsin D (donated by Dr. *R. Hembry*, Strangeways Research Lab, Cambridge) and a polyclonal sheep anti-human cathepsin B (donated by Dr. *D. Buttle*, University of Sheffield Medical School) were used as the primary antibodies. These antisera were specific for the human enzymes and cross-reactivity with the horse enzymes had been confirmed by Western blotting (*Hernandez-Vidal*, unpublished observations). The secondary antibody was either an FITC-conjugated rabbit anti-sheep Ig (DAKO) or a biotinylated rabbit anti-sheep Ig(Vector).

Immunocytochemistry

Sections of frozen cartilage were cryostat cut at a thickness of 5–8 µm and fixed in 4% paraformaldehyde. Cartilage sections and chondrocyte monolayers were treated with blocking buffer (10%FCS, 0.5% casein, Tris-buffer pH 7.4) followed by incubation for 1 h with sheep anti-cathepsin B or sheep anti-cathepsin D (dil 1:50). As controls these primary antisera were replaced with PBS or normal sheep serum (1:50). After thorough washing and a further blocking step the sections and cell monolayers were incubated for 1h either with FITC-conjugated rabbit anti-sheep Ig (1:200) or with biotinylated rabbit anti-sheep Ig(1:200) followed by 30min incubation with 1:100 HRP/streptavidin complex (Amersham) and colour development with diaminobenzidine.
For immunofluorescence, slides were mounted in Citifluor (UKC, Canterbury) and viewed with a Nikon Diaphot micro-scope fitted with epifluorescent illumination.
Peroxidase-stained slides were mounted in Apathy's medium (BDH) and viewed under Nomarski optics.

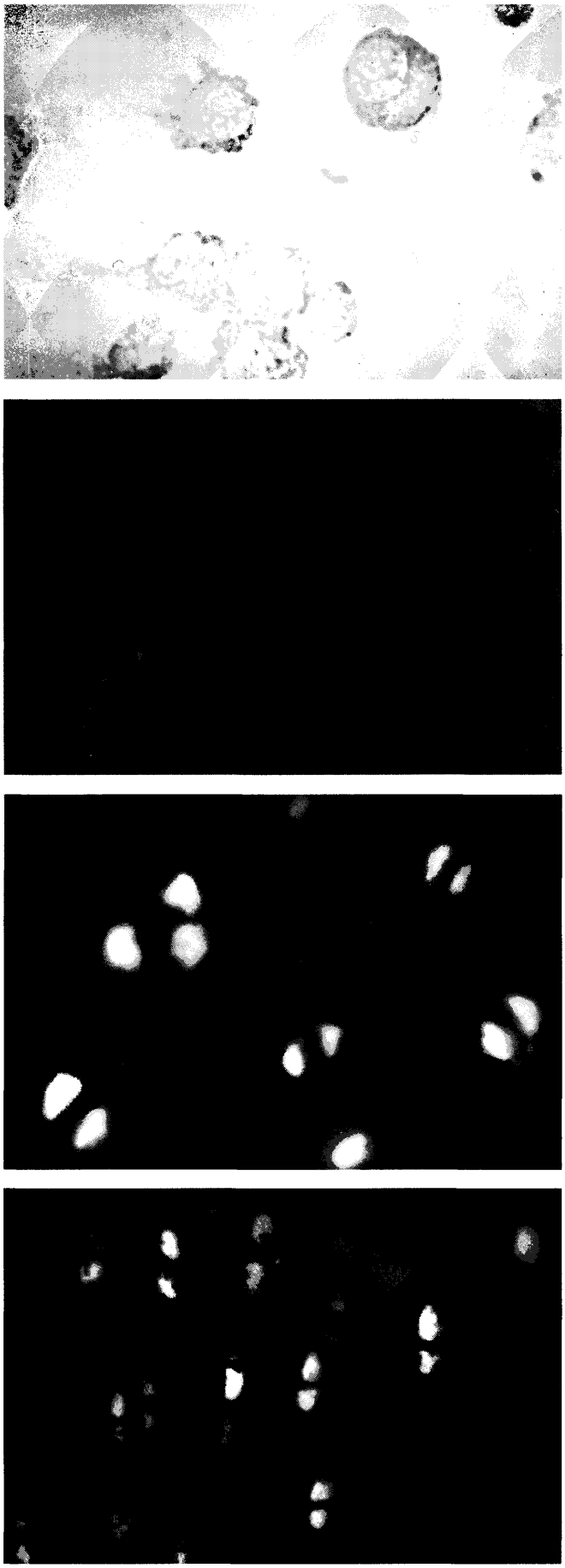

Figure 1A – 1D

Fig. 1: Immunolocalization of cathepsin B and cathepsin D in equine articular cartilage and isolated chondrocytes.

Lokalisation von Kathepsin B und Kathepsin D in Pferdegelenkknorpel und isolierten Chondrozyten

(A) Isolated chondrocytes treated with anti-cathepsin D and stained with biotin-streptavidin-peroxidase secondary antibody complex. (B-D) Frozen sections showing the hypertrophic zone of cartilage treated with (B) normal sheep serum (control); (C) anti-cathepsin B; (D) anti-cathepsin D. Sections were fluorescent stained with FITC-conjugated secondary antibody.

Results

Cathepsins B and D in isolated chondrocytes

Approximately 50% of chondrocytes which had been isolated from 8 samples of normal horse articular cartilage and maintained in monolayer culture for 24–48h showed positive immunoreactivity for cathepsin B and cathepsin D by both staining methods used.
Permeabilization of the cells during processing allowed entry of the antisera. Staining for both enzymes was intracellular with no evidence of membrane staining. Figure 1A shows chondrocytes staining for cathepsin D with the peroxidase method. Controls in which the primary antibody was replaced by PBS or normal sheep serum were negative (not shown).

Cathepsins B and D in cartilage

Frozen sections of cartilage showed little immunoreactivity of cathepsins B and D at the articular surface and in the mid zone. Strong intracellular staining for both enzymes was seen in the majority of chondrocytes in the hypertrophic region (Fig. 1C and D). Control sections were negative (Fig. 1B). There was little evidence of extracellular staining. Using both staining methods, the distribution of immunoreactivity for cathepsin B and D was similar in all samples within the age range studied so far (5 months – 1 year).

Discussion

In this paper we report, for the first time, the presence of the enzymes cathepsin B and cathepsin D in horse chondrocytes, and show that significant levels of these enzymes are found only in the hypertrophic region of articular cartilage. These findings in the horse are in agreement with the observations of *Ali* and *Bayliss* (1975) and *Baici* et al. (1995a, b) in humans, who found these enzymes at sites of matrix remodelling, particularly in osteoarthritis. Cathepsin B was considered perhaps to be the feature of phenotypically modulated chondrocytes (*Baici* et al., 1995b). Our study so far has been restricted to cartilage from a single anatomical site, the lateral trochlear ridge, from horses aged 5 months to 1 year. Since our results indicate that we might expect to see changes in the levels and distribution of these enzymes at sites of active remodelling i.e. in the growth plate in foetal and very young horses, studies to include this site in a wider age range are now in progress.
The presence of these PG-degrading enzymes in the hypertrophic region of the cartilage suggests that they may function in the normal process of matrix turnover and mineralization, and provokes speculation of their pathological involvement in dyschondroplasia. In this disease the reasons for the cartilage retention and failure of endochondral ossification remain unknown.
Based on the proposals that either production of an abnormal matrix (*Hurtig* et al., 1993) or defective chondrocyte differentiation (*Stromberg* and *Rejno*, 1978) are responsible, it would not be unreasonable to implicate cathepsins B and D. Proteoglycan is a mayor component of the ECM, its synthesis is controlled solely by the chondrocytes, and its presence is thought to inhibit calcification (*Sarathchandra* and *Ali*, 1994). It is therefore not difficult to imagine that disturbance of the overall PG turnover rate, intracellularly as well as extracellularly as a result of imbalance of cathepsin B or D production, may well be a contributory factor to the disease process.
The distribution of cathepsins B and D in pathological cartilage from lesions of dyschondroplasia are currently being investigated and will be compared with normal horse cartilage.

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