Journal of Animal and Veterinary Advances 7 (4): 493-501, 2008
ISSN: 1680-5593
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Localization of Cathepsin B Enzymatic Activity in Equine Articular Cartilage, Skin Fibroblasts and Phenotypically Modulated and Dyschondroplastic Chondrocytes

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Abstract: The enzymatic activity of the cysteine proteinase cathepsin B (EC 3.4.22.1) was localized in equine normal unfixed cartilage, phenotypically modulated chondrocytes and horse skin fibroblasts, dyschondroplastic chondrocytes and cartilage by immunofluorescence. The final precipitation of the fluorescent reaction of cathepsin B enzymatic activity was monitored during the enzymatic reaction. An intense fluorescence was observed using N-carbobenzoxyl-L-alanyl-L-arginyl-L-arginine-4-methoxy-β-naphthyl-amide as specific substrate for cathepsin B and 5-nitrosalicylaldehyde as coupling reagent. The unspecific fluorescence was eliminated by the use of the specific inhibitor for cathepsin B, E-64. Continuous monitoring demonstrated the presence of fluorescent granules after a defined time, with the formation of crystals as the final reaction product. This method shows cathepsin B activity in different types of tissues and cells and can be used as kinetic analysis of the enzymatic activity. The intense enzymatic activity observed in equine chondrocytes and equine skin fibroblasts suggests that cysteine proteinase is not only involved in the degradation of collagen by fibroblasts, but it also plays an important role in the intracellular digestion of collagen within chondrocytes.

Key words: Horse, cathepsin B, enzymatic activity, skin fibroblasts, chondrocytes, dyschondroplasia

INTRODUCTION

Developmental Orthopaedic Diseases (DOD) is a common term used to describe a variety of problems that range from angular deformity to dyschondroplasia resulting in skeleton weakness, lameness and poor athletic performance of the horse. The specific aetiopathogenesis of DOD is unknown, but it is usually associated with an alteration in the developing cartilage affecting the endochondral ossification. Molecular events such as endocrine dysfunction (Glade and Belling, 1986), as well as alterations in the molecules of the cartilage extracellular matrix (Ekman *et al*., 1990; Chen *et al*., 1993; Henson *et al*., 1997), vasculars (Kincaid *et al*., 1985; Carlson *et al*., 1986; Woodard *et al*., 1987), in the process of maturation and differentiation of chondrocytes (Farnum *et al*., 1984) and in the function of the enzymes involved in the transition of the cartilage matrix in osseous matrix (Gal *et al*., 1985; Ekman and Ridderstrale, 1992; Farquharson *et al*., 1992; Hernández-Vidal *et al*., 2002) have been suggested amongst as the pathological causes of this process.

Changes in the articular cartilage due to a biomechanically weak extracellular matrix induce microfractures in the metaphyseal and subchondral bone. Since, chondrocytes are responsible for maintaining the

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integrity of the extracellular matrix, alterations in the cartilage can be the result of an imbalance in the production by the chondrocytes of the degradative enzymes. Chondrocytes synthesize and release several enzymes capable of degrading the collagen and the proteoglycans such as metalloproteinases (Sires *et al.*, 1995), cysteine proteinases and adenosine triphosphatases (ATPases) (Sasaki and Ueno-Matsuda, 1993).

Chondrocytes can be stimulated by cytokines to produce cartilage degradative enzymes (Jasin and Dingle, 1981). *In vitro* studies suggest that cathepsin B plays an important role in the degradation of the cartilage proteoglycans in the presence of interleukin 1 (IL-1) (Buttle *et al.,* 1992, 1993; Buttle and Saklatvala, 1992). These results agree with those of Baici and Lang (1990), where the intracellular storage and cathepsin B biosynthesis were stimulated by interleukin IL-1β.

In normal conditions, cathepsin B is storaged intracellularly in low quantities by the chondrocytes. It has been observed, in cellular monolayer cultures, that the biosynthesis and release of this enzyme increases by phenotypic modulation (Baici *et al.*, 1988). It is well known that the cellular phenotype changes during subculture series (Fröhlich *et al.*, 1995).

Based on *in vitro* cellular cultures, there is evidence that hypertrophic chondrocytes contribute to the endochondral ossification process through osteogenic differentiation (Descalzi Cancedda *et al.*, 1992; Gentili *et al.*, 1993; Galotto *et al.*, 1994). The phenotypic change from chondrocytes to osteoblasts is therefore an important point to study in biological research and can serve as a paradigm for other phenotypic changes in cellular differentiation.

The cysteinic proteinase cathepsin B belongs to a group of degradative enzymes associated with the destruction of the extracellular matrix in arthritis (Van Noorden *et al.*, 1988; Buttle *et al.*, 1993; Buttle *et al.*, 1995) and proteoglycans in cartilage (Morrison *et al.*, 1973). Increased levels of cathepsin B have been reported in cartilage with Osteoarthritis (OA) in humans (Bayliss and Ali, 1978; Martel-Pelletier *et al.*, 1990; Baici *et al.*, 1995a), in rheumatoid synovium (Mort *et al.*, 1984; Codorean *et al.*, 1981; Trabandt *et al.*, 1991) and in synovial fluid (Codorean *et al.*, 1981; Lenarcic *et al.*, 1988; Gabrijelcic *et al.*, 1990). It is also known that cathepsin B has the capacity to degrade extracellular components of the cartilage *in vitro* in several species (Sires *et al.*, 1995; Hernández-Vidal *et al.*, 1998; Goto *et al.*, 1993; Söderström *et al.*, 1999). It has been demonstrated that this enzyme degrades subunits of proteoglycans (aggrecan) (Nguyen *et al.*, 1990) and it has been implicated in bone degradation process (Goto *et al.*, 1994; Everts *et al.*, 1999). Cathepsin B also has a degradative action on the non-calcifying cartilage during the endochondral ossification process, since, it has been demonstrated that osteoclasts, primordial cells in this process, release high levels of this enzyme (Sasaki and Ueno-Matsuda, 1993; Goto *et al.*, 1993; Hernández-Vidal *et al.*, 2002).

Previous studies have demonstrated that specific inhibitors of this nzyme such as trans-epoxysucciny-L-leucylamido-4-guanidino-butane (E-64) inhibit osteoclastic bone reabsorption *in vitro* (Everts *et al.*, 1998) as well as *in vivo* (Delaisse *et al.*, 1984) establishing its important role in bone reabsorption (Goto *et al.,* 1994; Everts *et al.*, 1998, 1999). Experiments *in vitro* showed that the specific cathepsin B inhibitor CA-07 Me has a marked inhibitory effect in bone reabsorption (Hill *et al.*, 1994; Everts *et al.*, 1998).

Cathepsin B has been localized in the osteoclasts of different species (Goto *et al.*, 1994; Söderström *et al.*, 1999; Blair *et al.*, 2000) including the horse Hernández-Vidal *et al.*, 2002; Gray *et al.*, 2002) and its activity has been shown by the use of the specific substrate Z-Arg-Arg-MNA (Schutzen *et al.*, 1995; Everts *et al.*, 1999).

In the present study, the localization of the fluorescent final reaction of the activity of the cathepsin B was monitored to investigate its differential expression in equine articular cartilage, skin fibroblasts and phenotypically modulated and dyschondroplastic chondrocytes.

MATERIALS AND METHODS

Articular cartilage: Full depth samples of articular cartilage were obtained from the lateral crest of the femoral trochlea of 25 normal horses and horses with dyschondroplasia aged 1-5 years old. The samples were collected during the first 3 to 6 h. post-mortem, embedded in OCT and immediately frozen in liquid nitrogen (-186°C) for histochemical studies and immunolocalization of the enzymatic activity of cathepsin B. The frozen samples were cut perpendicular to the articular surface, (8 μm thick) at -30°C with a cryogenic microtome. The samples included the 3 articular cartilage zones (superficial, mid and deep zone).

Isolation and culture of chondrocytes: Normal and dyschondroplastic cartilage of the lateral trochlear ridge

of the distal femur were aseptically obtained 3-6 h post-mortem from animals between 1 and 5 years old. Chondrocytes were isolated from the cartilage by enzymatic digestion as described by Davies *et al.* (1991). Articular chondrocytes were plated as primary cultures at a density of 2×10^5 cells and placed on 8-well chamber slides for incubation (Nunc. Inc, USA) (with Dulbecco's-modified Eagle's medium (DMEM) (Gibco, UK) containing 10% (v v^{-1}) of heat-inactivated Foetal Calf Serum (FCS), 200 UI mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin and 2.5 μg mL^{-1} of fungizone at 37°C, 95% air and 5% CO_2).

Cell monolayer cultures were fixed in 4% paraformaldehyde for 30 min. then rinsed in PBS for 10 min. The cells were then permeabilized with 0.1% Triton X-100 (v v^{-1}) for 5 min to facilitate intracellular access of the antibodies. Triton X-100 has a reactive effect on any inactive protease in cartilage (Ali *et al.*, 1967).

Chondrocytes propagation: Chondrocytes for phenotype modulation were cultured at a high density of 4×10^7 cells cm^2 in 25 or 75 cm^2 cell culture flasks with 10 or 30 mL of DMEM culture medium. The culture medium was replaced every 3 days. Cells were subcultured after being detached by trypsinization (0.25% of trypsin (EC 3.4.21.4), 0.537 mM of EDTA in 100 mL of buffered saline solution) and washed 3 times by centrifugation at 2500 rpm (Mistral MSE, 2000) for 5 min.

Isolation and propagation of horse skin fibroblasts: Fibroblasts were isolated from shaved horse skin under aseptic conditions. The skin was soaked in 70% ethanol (v v^{-1}) during 5 min and cut in large sections on a sterile Petri dish with PBS. Skin sections were washed 3 times with sterile PBS and incubated in 20 mL of trypsin-EDTA for 2 h at 37°C in an orbital shaker at low speed. After letting stand for 15 min., the supernatant containing the fibroblasts was collected and the trypsin removed by centrifugation at 2500 rpm for 5 min. The cells were plated at a density of 2×10^5 on 8-well chamber slides with DMEM containing 10% (v v^{-1}) of heat-inactivated Foetal Calf Serum (FCS), 200 UI mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin, 2.5 μg mL^{-1} of fungizone, 5μL mL^{-1} of 1% (w v^{-1}) stock ascorbic acid solution and 10 μL mL^{-1} of a 5% (w v^{-1}) stock L-glutamine solution at 37°C, 95% air and 5% CO^2. The cells were incubated for 3-4 weeks to allow fibroblast growth.

One day before their use, cells were incubated in a FCS-free cell culture medium, supplemented with antibiotics. On the day of the experiment, cells were coated in Ca^{2+}-and Mg^{2+}-free Hanks buffered salt solution.

***In situ* detection of cathepsin B activity:** Detection of cathepsin B enzymatic activity in unfixed normal cartilage, modulated chondrocytes, skin fibroblasts, dyschondroplastic chondrocytes and cartilage was performed essentially according the method of Van Noorden and Vogels (1987) and Van Noorden *et al.* (1987).

N-carbobenzoxyl-L-alanyl-L-arginyl-L-arginine-4-methoxy-β-naphthyl-amide (Z-Ala-Arg-Arg-4MβNA) was used at a concentration of 0.01% as specific substrate for cathepsin B, 1mM 2-hydroxy-5-nitrobenzaldehyde (5-nitrosalicylaldehyde) as coupling reagent and E-64 (10 μM) as specific inhibitor of cathepsin B.

The articular cartilage sections were rinsed in 100 mM phosphate buffered saline, pH 6.0, for 2-5 min and dried during 10 min at 25°C. Later, they were coated with 50 μL of enzymatic incubation medium. The incubation medium for the detection of cathepsin B enzymatic activity consists of 1.3 mM of EDTA, 1 mM of (Dithiothreitol (DDT), 2.65 mM of L-cysteine, 1mM of 2-hydroxy-5-nitrobenzaldehyde and 1mg mL of Z-Ala-Arg-Arg-4MβNA in 100 mM of phosphate buffered solution (pH 6.0).

The tissue sections as well as the isolated cells were incubated at room temperature during the fluorescent microscopy stage for up to 150 min, in this way, the product reaction bound to 5-nitrosalicylaldehyde could be continually monitored. The specificity of the enzymatic reaction was confirmed during the incubation period in the presence of inhibitor E-64 or in the absence of the specific substrate.

The slides were examined and photographed during the incubation period using a Nikon Diaphot microscope fitted with epifluorescent illumination and photographed during the time-course (5, 25, 45, 60, 80 and 150 min) on Kodak Ektachrome P1600 × film.

RESULTS

Cathepsin B activity was detected by the fluorescence method using Z-Ala-Arg-Arg-4MβNA as specific substrate for cathepsin B (McDonald and Ellis, 1975; Van Noorden *et al.*, 1987; Baici *et al.*, 1988; Shuja and Murnane, 1996) and 5-nitrosalicylaldehyde (Dolbeare and Vanderlaan, 1979; Van Noorden *et al.*, 1987) as coupling reagent providing a fluorescent end-product. Specificity of the enzymatic reaction was confirmed by use of the specific cathepsin B inhibitor E-64 (Barrett *et al.*, 1982; Baici and Lang, 1990; Kawada *et al.*, 1995; Aisa *et al.*, 1996).

Enzymatic activity in normal and dyschondroplastic samples: There was no enzymatic activity in any of the normal cartilage samples. These findings are in agreement with those of Baici *et al*. (1995b). Neither was enzymatic activity was localized in pathological samples fixed in paraformaldehyde at 4%. Graf and Sträuli (1983) have demonstrated that fixation with formaldehyde (4%) abolished immunoreactivity of cathepsin B in rabbit skin fibroblasts. Nevertheless, in freshly isolated dyschondroplastic chondrocytes, high levels of enzymatic activity were observed. These cells incubated with enzyme activity medium, showed both lysosomal and extracellular activity to cathepsin B. These results were comparable with those seen in the immunolocalization studies in dyschondroplastic growth cartilage Hernández-Vidal *et al*. (1998).

Dyschondroplastic cells showed a specific strong fluorescent stain, which progressively increased during the incubation period. After 25 min of incubation with the medium for enzymatic activity, intense star-like fluorescent particles were observed (Fig. 1). Based on these observations we can infer that in freshly isolated dyschondroplastic chondrocytes a proportional correlation exists between the presence of the enzyme and its activity.

Enzyme activity in phenotypically modulated chondrocytes: Cathepsin B activity was studied in phenotypically modulated chondrocytes by serial subculturing, which showed a gradual increase in the expression of this enzyme. In these chondrocytes there were particles with pronounced yellow fluorescence in the lysosomes of the cells (Fig. 2a). Nevertheless, there was also extracellular activity. It was also noticed that the intensity of the fluorescent particles increased during the incubation for cathepsin B activity and eventually star-like crystals were observed (Fig. 2b). These findings are in agreement with Van Noorden *et al*. (1987), where a recrystallization of the final reaction product was reported.

The lysosomal activity of the phenotypically modulated cells was comparable with that observed in dyschondroplastic chondrocytes.

Monitoring of cathepsin B activity in horse skin fibroblasts and phenotypically modulated chondrocytes during incubation: Figure 3 (a, b, c) shows the monitoring of cathepsin B activity in horse skin fibroblasts. A weak fluorescent stain was observed over the horse skin fibroblasts during the first 5 min of incubation (Fig. 3a). Some small yellow fluorescent particles began to appear after 7 min; however, increased lysosomal fluorescent particles per cell were observed during the first 25 min. after the reaction was started (Fig. 3b). After 45 min, further increased numbers and intensity of yellow

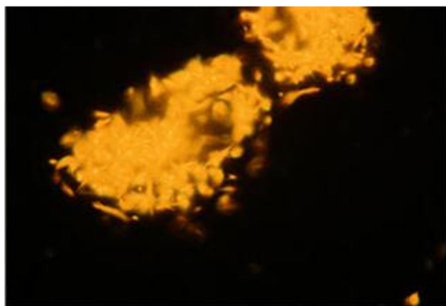

Fig. 1: Cathepsin B activity in dyschondroplastic chondrocytes (40X). Monolayer of unfixed articular chondrocytes, incubated with enzyme activity medium containing Z-Ala-Arg-Arg-4MβNA and 5-nitrosalicylaldehyde. Activity after 25 min of incubation

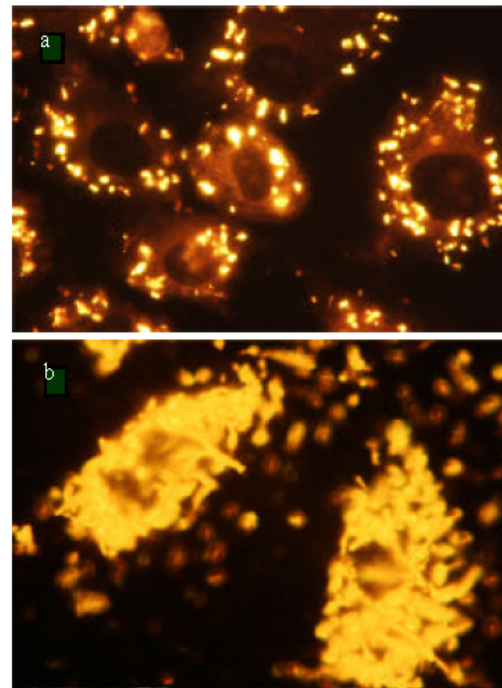

Fig. 2: Cathepsin B activity in chondrocytes phenotypically modulated (100X). Monolayer of unfixed articular chondrocytes, incubated with enzyme activity medium containing Z-Ala-Arg-Arg-4MβNA and 5-nitrosalicylaldehyde. a) Activity after 25 min of incubation. b)Activity after 45 min of incubation. the incubation

Fig. 3: Monitoring of the cathepsin B activity in horse skin fibroblasts during incubation (100X). Monolayer of unfixed fibroblasts, incubated with enzyme activity medium containing Z-Ala-Arg-Arg-4MβNA and 5-nitrosalicylaldehyde. a) Activity after 5 min of incubation. b) Activity after 25 min of incubation. c) Activity after 45 min of incubation

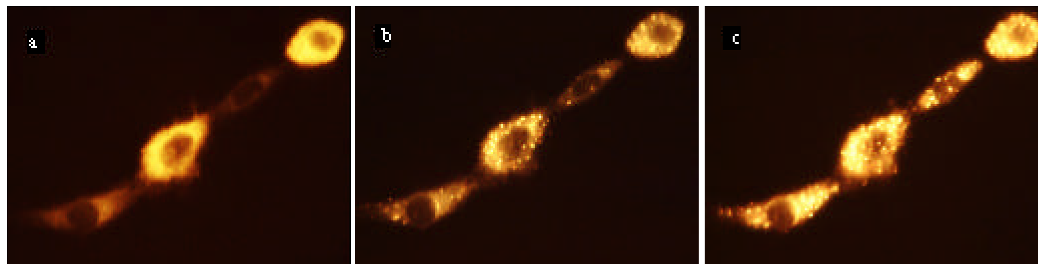

Fig. 4: Monitoring of cathepsin B activity in phenotypically modulated chondrocytes during incubation (40X). Monolayer of unfixed phenotypically modulated articular chondrocytes incubated with enzyme activity medium containing Z-Ala-Arg-Arg-4MβNA and 5-nitrosalicy-laldehyde. a) Activity after 5 min of incubation. b)Activity after 25 min of incubation. c) Activity after 45 min of incubation

fluorescent particles were observed in the lysosomes of the cell (Fig. 3c). One of the observed characteristics in period between 7 and 50 min was the increment of the fluorescent intensity of the enzymatic particles. The controls were incubated with enzymatic activity medium for 60 min in the absence of specific cathepsin B substrate (Z-Ala-Arg-Arg-4MβNA) or in the presence of the specific inhibitor (E-64) and did not show any fluorescent activity.

Figure 4 (a, b, c), shows modulated chondrocytes during monitoring for cathepsin B activity. A weak non-specific fluorescence staining (Dolbeare an Vanderlaan, 1979) was noticed in the cytoplasm of the cells during the first 5 min of incubation (Fig. 4a). In unfixed phenotypically modulated chondrocytes, the yellow fluorescent reaction product gradually increased with incubation time as described for the horse skin fibroblasts (Fig. 4b and c).

DISCUSSION

In the present study, cathepsin B activity in equine isolated chondrocytes and skin fibroblasts, dyschondroplastic chondrocytes and cartilage by immunofluorescence is reported for the first time. In this study, it was demonstrated that freshly isolated chondrocytes from normal and dyschondroplastic equine cartilage showed an interesting distribution of cathepsin B enzymatic expression. Primary cultures of isolated normal chondrocytes presented cathepsin B in the lysosomes, but only in a low percentage of cultured cells. This result shows again the renowned heterogeneity of the articular cartilage cellular population and it is consistent with previous observations of the significant difference between the enzymatic levels of cathepsin B expressed by chondrocytes in different areas of the normal (Hernández-Vidal *et al*., 1996) and dyschondroplastic (Hernández-Vidal *et al*., 2002) equine articular cartilage.

The levels of lysosomal cathepsin B expression freshly isolated chondrocytes of normal horses were low; however, after some days in cellular monolayer, chondrocytes began to express intracellularly detectable quantities of cathepsin B, which was more evident after the phenotypical modulation by subculture. With the course of time of the cellular subculture, the reactivity of cathepsin B in the cell increased as well as the number of positive cells.

This study reports that the levels of cathepsin B activity increase in articular cartilage cells of horses with dyschondroplasia. The importance of high levels of cathepsin B in dyschondroplastic cartilage is unknown, but it is reasonable to suggest that the presence of cathepsin B in these cells has some pathological importance. It is known that in *in vitro* studies, cathepsin B has the capacity to degrade the majority of extracellular matrix components, including PG (Nguyen *et al*., 1990) and collagens type II, IX, X and XI (Maciewicz *et al*., 1991; Sires *et al*., 1995). Degradation of some elements of the extracellular matrix such as collagen type X can be due to abnormal activity of cathepsin B. This could cause a fault in the endochondral ossification process, even weakness of the growth plate, two of the characteristics that define dyschondroplasia.

Phenotypically modulated normal chondrocytes showed morphologic and biochemical characteristics similar to the ones observed in dyschondroplastic chondrocytes. Common features between dyschondroplastic and phenotypically modulated cells were larger sizes and increased expression and secretion of cathepsin B activity.

The cytochemical technique used to demonstrate the cathepsin B activity with 5-nitrosalicylaldehyde as coupling reagent is specific. The substrate selectivity is well known (Van Noorden *et al*., 1987) and the specific inhibitor (E-64) of the proteinase avoids the formation of star-like fluorescent end-product. This method offers the advantage of being a tool for the kinetic analysis of the enzymatic activity, since, it allows study of individual of cells during the incubation process and shows the relative quantities of cathepsin B activity in different cell types or in a heterogeneous population of a tissue.

The intense enzymatic activity observed in equine chondrocytes and equine skin fibroblasts suggests that the cysteine proteinase is not only involved in the degradation of the collagen by fibroblasts, but also plays an important role in the intracellular digestion of the collagen by chondrocytes.

These findings together with those of Baici *et al.* (1988), form a sound base upon which to rest the hypothesis that cathepsin B is a characteristic product of phenotypically modulated chondrocytes.

ACKNOWLEDGEMENT

We would like to thank Rossdale and Partners (Newmarket) and the Equine Fertility Unit (Newmarket) for the provision of post mortem material. We would also like to thank MSc. Alfredo Uzeta Navarrete from the Centro de Apoyo y Servicios Academicos de la Universidad Autonoma de Nuevo Leon for kindly printing the images used in this investigation. This work was funded by the CONACYT, Mexico.

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