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ORIGINAL ARTICLE

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An in vitro model for abnormal skeletal development in the lysosomal storage diseases

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Abstract Lysosomal storage diseases such as G_{M1}-gangliosidosis are associated with skeletal abnormalities. Radiological and histological studies, both in human and corresponding animal models, indicate retarded bone formation. Since cartilage maturation leads to bone formation, we developed an in vitro system to study and compare the biological features of cartilage from dogs affected with G_{M1}-gangliosidosis with age-matched controls. Costochondral chondrocytes were grown in monolayer and in agarose culture. Both affected and control cells dedifferentiated in monolayer; however, in agarose culture they re-expressed the chondrocytic phenotype. Cells from affected dogs were enlarged and contained numerous large vacuoles when compared with control cells. This morphology was similar to that seen in vivo. In addition, the affected cells appeared to have a reduction in mitosis and alcian blue staining proteoglycans. Cultures from affected animals contained fewer cells positive for alkaline phosphatase activity. Both affected and control cells expressed collagen types I and II and were positive for the lectin *Ricinus communis* agglutinin-I. However, the staining of the control culture for type II collagen was more prominent than in the affected cells. These findings suggest that culture of chondrocytes in agarose may be a useful method for studying the biology of cartilage which leads to skeletal abnormalities in lysosomal storage diseases.

Key words G_{M1}-gangliosidosis · Chondrocytes · Tissue culture · Bone retardation

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Introduction

G_{M1}-gangliosidosis, which is characterized by deficient activity of lysosomal β-galactosidase, is one example of lysosomal storage diseases in which there is a storage of glycolipids, glycosaminoglycans and oligosaccharides [22], and is associated with skeletal abnormalities [19, 20, 24]. Radiological and histological studies both in human and corresponding animal models of G_{M1}-gangliosidosis indicate retarded bone formation [4]. In a previous study, we described the clinical, morphological, histochemical, and biochemical features (including bone dysplasias) in two canine models for G_{M1}-gangliosidosis, English Springer Spaniel and Portuguese Water Dog [3].

In G_{M1}- and G_{M2}-gangliosidosis, mucopolysaccharidosis, and glycoprotein storage diseases the chondrocytes are vacuolated and contain partially degraded compounds [1]. However, the mechanism(s) which lead to retarded bone formation and skeletal dysplasia in some of these disorders are unknown.

The purpose of this study was to select an in vitro system for growing chondrocytes from individuals with lysosomal storage disorders, in which the cells retain their phenotype. In this study we compared the morphology and histochemical properties of control and β-galactosidase deficient canine chondrocytes and their extracellular matrix grown in monolayer and in agarose culture.

Materials and methods

Tissue culture

Canine costochondral cartilage was obtained from two 8-month-old PWD affected littermates with G_{M1}-gangliosidosis and an age-matched control. Primary cultures were established as previously described using human costochondral cartilage [7]. The cultures were fed Dublecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 10% fetal bovine serum, 1% L-glutamine, and 0.1% penicillin-streptomycin twice weekly. At confluence the cells were further passaged and cultured either in agarose or in monolayer.

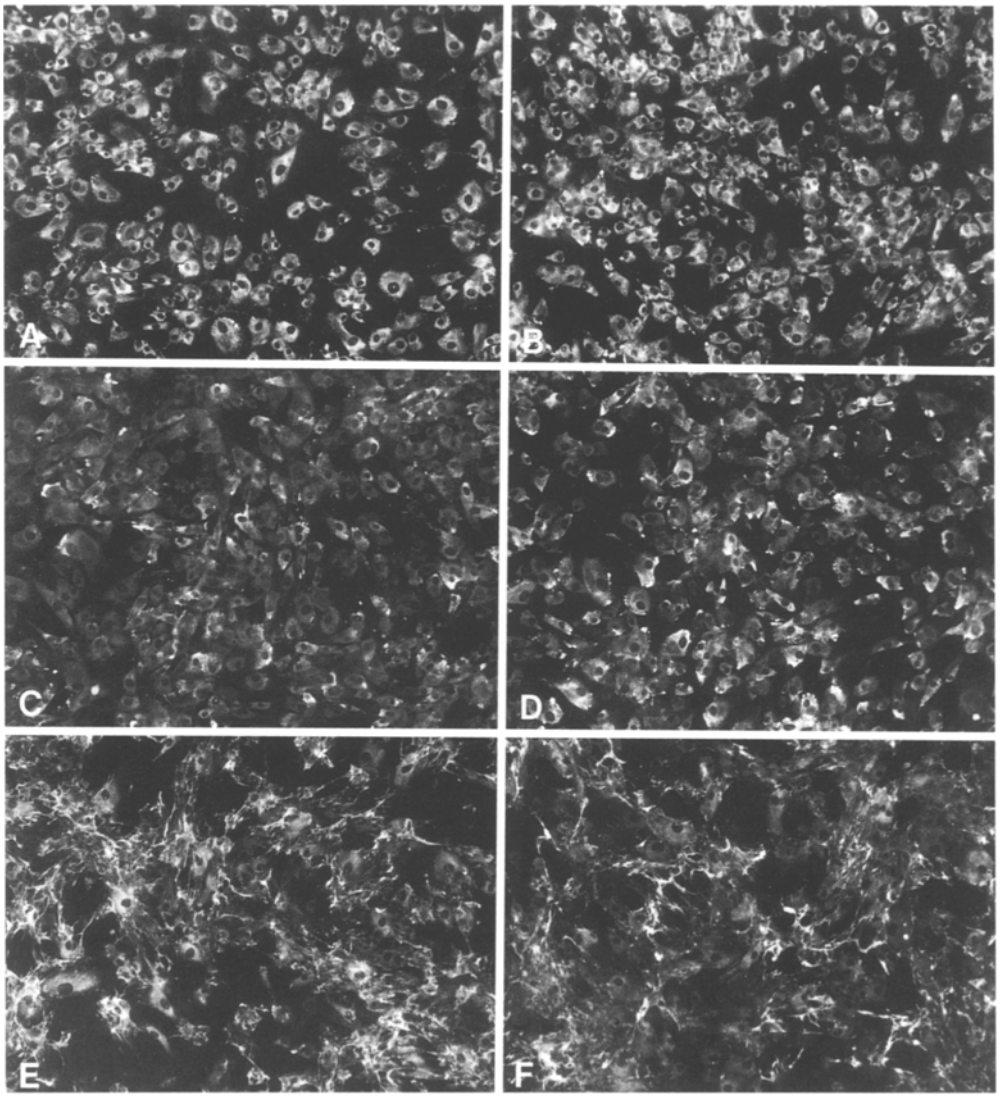

Fig. 1A–F Photomicrograph of the immunofluorescent detection for type I procollagen, type III collagen, and fibronectin in monolayer cultures of control and G_{M1}-gangliosidosis affected canine chondrocytes. Nearly every cell in the control (**A**) and affected (**B**) cultures was positive for type I procollagen. Many cells in control (**C**) and affected (**D**) cultures were also positive for type III collagen. The fibrillar staining pattern of fibronectin is readily apparent in both control (**E**) and affected (**F**) monolayer cultures. **A–F**, ×100

Agarose cultures were established as previously described using human chondrocytes [7]. Briefly, the cells were dissociated from monolayer, filtered, and resuspended at 5×10^5 cells in 1 ml of 0.5% low-temperature agarose in DMEM. A 10-µl drop of cell suspension was plated into 35-mm tissue culture dishes that were coated with 1% high-temperature agarose. Cultures were flooded with 2 ml DMEM and fed twice weekly with complete media change. Cultures were evaluated after 3 weeks. To compare the cells in agarose with cells grown in monolayer, cells suspended in DMEM were plated directly into 35-mm dishes (approximately 1.75×10^5 cells/dish) and grown to confluence.

Immunocytochemistry

Agarose and monolayer cultures were rinsed and fixed with methanol:acetone (1:1). The cultures were incubated overnight at 4°C in one of the following monoclonal antibodies [Developmental Studies Hybridoma Bank, University of Iowa, Iowa City: human type II collagen (CIICI, undiluted, [16]) or type I procollagen (Sp1.D8, undiluted, [15]). Some cultures were incubated in goat anti-type III collagen (1:20, Southern Biotechnology), fluorescein-conjugated rabbit anti-human fibronectin (1:20, Cappel, Organon Teknika Corporation), fluorescein-conjugated *Ricinus communis* agglutinin I (RCA-I, Vector Laboratories, 50 µg/ml) or fluorescein-conjugated RCA-I preincubated in 0.2 M lactose. Cultures incubated in phosphate buffered saline served as negative controls. The cultures were rinsed and incubated for 1 h at room temperature in fluorescein-conjugated rabbit anti-mouse IgG (1:40, Cappel, Organon Teknika Corporation) or in fluorescein-conjugated rabbit anti-goat IgG (1:10, Cappel, Organon Teknika Corporation). The cultures were photographed through an Olympus Vanox microscope (DPlanApo 20UVPL Olympus objective) equipped for fluorescence microscopy.

Alcian blue detection of cartilage proteoglycans, both in agarose and monolayer cultures, was accomplished by simultaneous fixation and staining for 24 h in a mixture of 2.5% glutaraldehyde and 0.05% alcian blue [9]. Agarose and monolayer cultures were evaluated for alkaline phosphatase activity using the indoxyl-tetrazolium method [23]. Cultures were fixed in cold 100% ethanol and then incubated for 1 h at 37°C in a 5-bromo, 4-chloro, 3-indoxyl phosphate, toluidine salt (Sigma) and nitroblue tetrazolium (Sigma) substrate, pH 9.3. Agarose and monolayer cultures were also analyzed for lipids using Sudan black B [12] and for carbohydrates using the periodic acid-Schiff reaction (PAS) [18].

Transmission electron microscopy

Agarose cultures were fixed in a mixture of 1.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, with 0.15% ruthenium red. Cultures were rinsed and post-

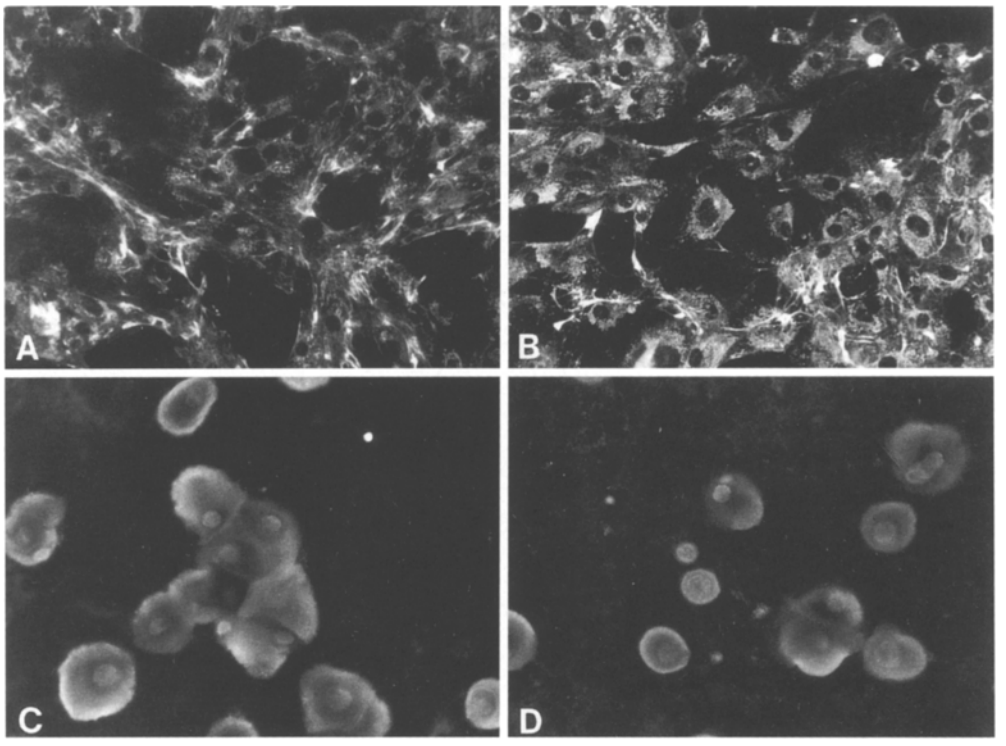

Fig. 2A–D Photomicrograph of the fluorescein-labeled lectin *Ricinus communis* agglutinin-I (RCA-I) pattern in control and affected canine chondrocytes cultured in monolayer and agarose. Monolayer culture of control (**A**) and affected (**B**) chondrocytes. Note the abundant label in affected cells when compared with control cells. After 3 weeks in agarose culture both the control (**C**) and affected (**D**) cells had a similar staining pattern. While every cell was positive, there was an overall decrease in the total number of cells in the affected cultures, giving the false impression that fewer cells are positive for RCA-I when compared with controls. **A–D**, ×200

fixed in buffered 2% OsO_4 and further processed for routine microscopy. Thin sections were viewed with a Siemens 102 transmission electron microscope.

Results

Monolayer culture

In monolayer both affected and control chondrocytes had dedifferentiated and were generally polygonal in shape. They had lost their chondrogenic phenotype and expressed type I procollagen and type III collagen and fibronectin (Fig. 1). Type II collagen and alcian blue staining proteoglycans were not detected (data not shown).

While both control and affected chondrocytes dedifferentiated in monolayer, there were several notable differences. Transmission electron microscopy revealed that the affected cells possessed abundant vacuoles containing empty spaces and lamellated membrane structures similar to those present in affected cells cultured in agarose (data not shown). Histochemical analysis revealed that both control and affected cells in monolayer expressed alkaline phosphatase activity. However, control cultures contained more positive cells than cultures of affected cells (data not shown).

Additional analyses revealed that neither cell type was positive with Sudan black. Both cell types were positive when incubated in RCA-I (Fig. 2). However, numerous large perinuclear vesicles were positive for RCA-I in affected cells, while fewer smaller vesicles were apparent in control cells. The RCA-I staining pattern was blocked by preincubation in lactose (data not shown).

Agarose culture

While both affected and control chondrocytes dedifferentiated in monolayer, they expressed their respective chondrogenic phenotypes in agarose culture. During culture in agarose the cells maintained a round morphology. However, there were notable differences in morphology between the affected and control chondrocytes in agarose. The affected cells were larger than control cells and contained numerous, large vacuoles (i.e. secondary lysosomes) which had empty spaces and lamellated membrane structures (Fig. 3). These large, vacuolated cells were viable as determined with the trypan blue exclusion assay. At 1 week in agarose culture an alcian blue staining matrix was visible in cultures of both affected and control chondrocytes. This matrix accumulated throughout the 3-week culture period. However, more single cells, not associated with an alcian blue staining matrix, were seen in cultures of affected cells than in control cultures (Table 1). Moreover, in the affected cultures, the chondrogenic clusters were typically smaller and contained fewer cells. Immunocytochemistry revealed that cells in affected and control agarose cultures expressed both type I procollagen and type II collagen (Fig. 4). It is noteworthy that cells surrounded by an alcian blue halo were positive for both collagen types.

Histochemical analysis revealed that there were no notable differences between affected and control chondrocytes with regard to PAS staining distribution (Table

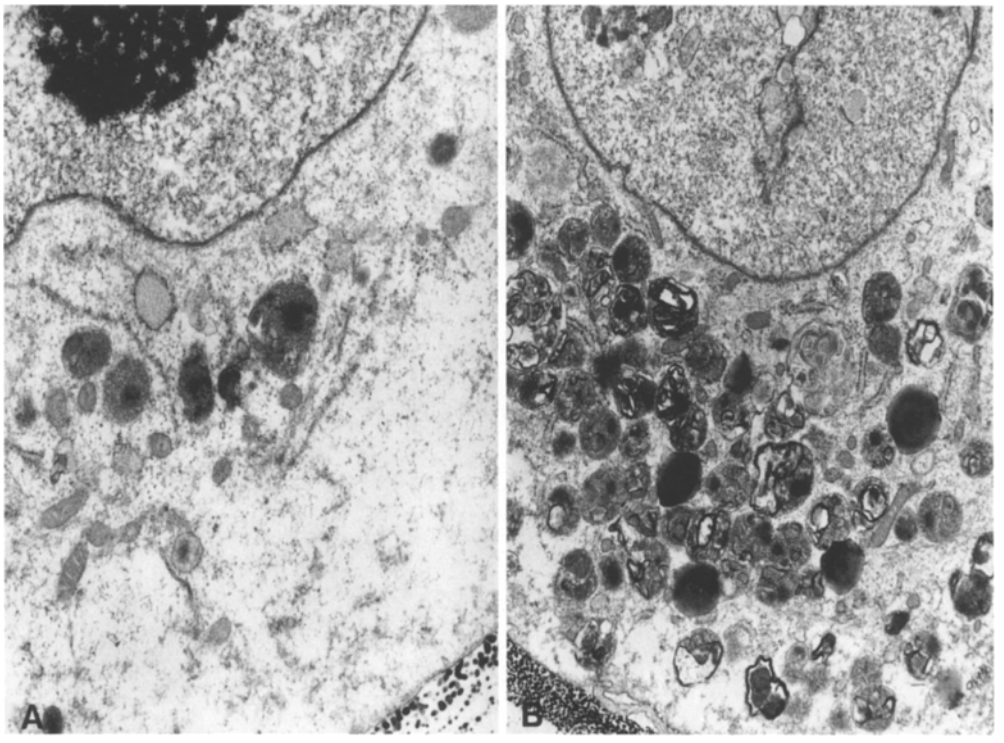

Fig. 3A,B Transmission electron micrograph representative of a control (**A**) and of a G_{M1}-gangliosidosis affected (**B**) canine chondrocyte in agarose culture. Note the abundance of secondary lysosomes in the affected cell. The lysosomes have empty spaces and lamellated membrane structures. **A**, **B** ×25,000

Table 1 Histochemical analyses of affected and unaffected 3 week agarose cultures[a] including both cell clusters and single cells. The relative amount of staining detected in affected versus unaffected agarose cultures is indicated by a plus sign (+). Both alcian blue and alkaline phosphatase label were markedly reduced in the affected cultures when compared to controls.

	Portuguese Water Dog (affected)	Control (unaffected)
Alcian blue	+	++
Sudan Black	+	+
Periodic acid-Schiff	+	+
Alkaline phosphatase	+	++

[a] Entire culture analyzed

1). PAS was localized intracellularly and did not extend into the alcian blue staining halo. Although Sudan black staining lipid droplets were not detectable in monolayer, they were visible in both affected and control cells cultured in agarose (Table 1). Cells with and without an alcian blue staining halo were positive for PAS and Sudan black. In contrast to similarities in PAS and alcian blue staining, there were differences in alkaline phosphatase activity between affected and control cultures. Control cultures contained more cells positive for alkaline phosphatase activity when compared with parallel affected cultures (Table 1). Lastly, both affected and control cells were positive for RCA-I (Fig. 2). The staining distribution was similar to that of the alcian blue halo which surrounds the cells. This RCA-I-positive label was blocked by preincubation in lactose (data not shown).

Discussion

To characterize further our understanding of the mechanisms that contribute to skeletal lesions in lysosomal storage diseases, we compared chondrocytes from control and G_{M1}-gangliosidosis affected canine cartilage in vitro. Canine cartilage from the costochondral junction was cultured in the monolayer/agarose scheme, which has been used for several species including humans [7, 10]. Both the control and affected chondrocytes were analyzed in monolayer and in agarose culture. In monolayer, the canine chondrocytes dedifferentiated and expressed type I procollagen and III collagen and fibronectin. Type II collagen was not detected; nor was there any alcian blue staining material present. Numerous studies have indicated that chondrocytes from other species (i.e. humans, rabbit) also dedifferentiate in monolayer [7, 10].

In agarose culture, the morphology of the affected canine chondrocytes was similar but not identical to that seen in vivo for G_{M1}-gangliosidosis [2–4]. In vivo the lysosomes of affected chondrocytes contained mostly fine fibrils and few membrane fragments. In vitro the lysosomes contained mostly lamellated membrane structures and empty spaces; the latter is indicative of stored mucopolysaccharides or oligosaccharides [11, 14]. This difference in storage material between affected chondrocytes in vitro and in vivo may be attributed in part to the differences in the microenvironment and the rate of membrane versus extracellular matrix recycling. The affected chondrocytes were enlarged when compared with the control cells and there was an increase in vacuoles (i.e. lysosomes) when compared with the control cells which

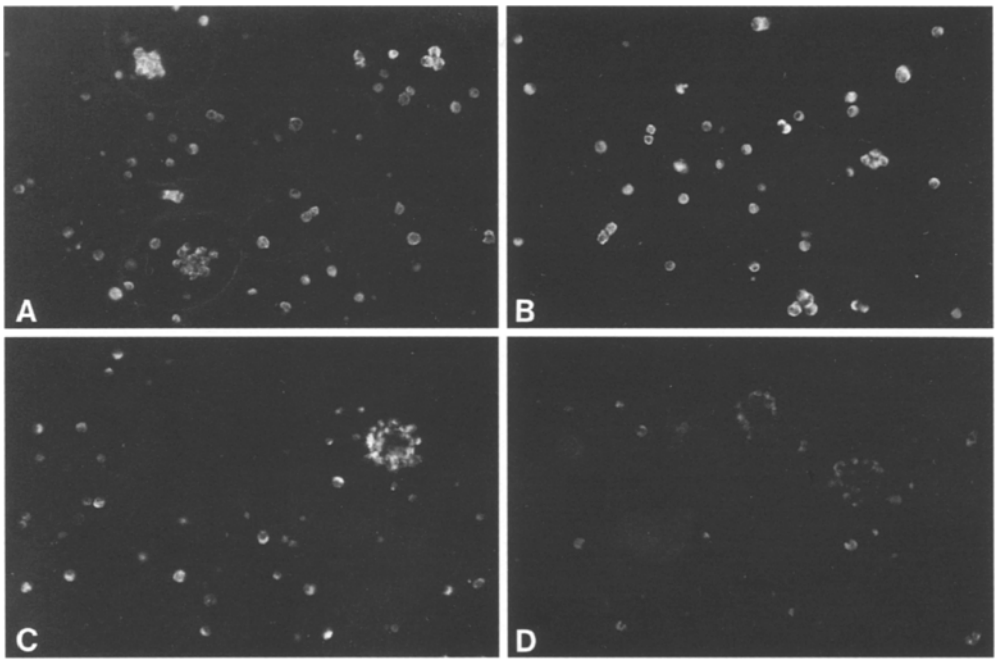

Fig. 4A–D Immunofluorescent detection of type I procollagen and type II collagen in control and affected chondrocytes cultured for 3 weeks in agarose. Type I procollagen was apparent in both control (**A**) and affected (**B**) cultures. Type II collagen label was found in both control (**C**) and affected (**D**) cultures. The label for type II collagen appeared stronger in the control than in the affected cultures. ×200

accumulated over time in culture. Trypan blue exclusion assay indicated that these cells were viable.

The agarose culture system is advantageous in that mitotic activity and matrix production can be analyzed independently [5–8]. The affected chondrocytes cultured in agarose contained chondrogenic clusters that were smaller when compared with control cultures. In addition, there were more single cells in the affected cultures than in controls. These findings suggest that mitosis is reduced in the affected culture. Moreover, when the cultures were stained with alcian blue there was a notable increase in the number of single cells without an alcian blue staining matrix. These data suggest that both mitosis and matrix production (proteoglycans) are reduced in the affected cultures when compared with the controls. Since the growth of cartilage depends on both mitosis and matrix production, a reduction in either component would compromise the size of the cartilage anlage and the growth plate. These data, therefore, would support the observation of retarded bone formation in G_{M1}-gangliosidosis.

Histochemical studies were conducted to analyze further the contents of the large vacuoles in the affected cells. Both PAS and Sudan black staining were present and there were no notable differences in staining distribution. Differences, however, were observed between the affected and control cultures when analyzed for alkaline phosphatase activity. Histochemistry revealed that cultures from affected dogs contained fewer cells with alkaline phosphatase activity when compared with controls in both monolayer and agarose cultures. Furthermore, there was no correlation between alkaline phosphatase activity and alcian blue staining matrices (enzymatic positive cells were not always associated with alcian blue staining cells) in agarose culture. It is noteworthy that in growth plate the zone of hypertrophic chondrocytes is rich in alkaline phosphatase, which is thought to be essential for cartilage septal calcification [17]. Therefore, a reduction in alkaline phosphatase may contribute to an overall reduction in endochondral ossification seen in the G_{M1}-gangliosidosis condition.

Immunocytochemistry revealed that both the affected and control cultures expressed type I procollagen and type II collagen in agarose. Both collagen types were associated with cells surrounded by alcian blue staining proteoglycans. However, there were some differences in staining intensity. It appeared that the control cultures stained more intensely for type II collagen when compared with affected cultures. The occurrence of both type I and type II collagen could indicate that the cells had not fully redifferentiated when cultured for 3 weeks in agarose. However, it has been shown under certain culture conditions that chondrocytes can simultaneously express both type I and type II collagen [5, 13, 21, 25].

Recently, we have shown, using lectin histochemistry, that chondrocytes in vivo, from both affected and control dogs, stained with RCA-I, which binds to β-galactosyl residues, but the staining intensity was greater in the affected dogs [4]. In the present study we observed more RCA-I staining in monolayer cultures of the affected cells than in controls. However, there were no notable differences between the affected and control cells cultured in agarose.

In conclusion, both affected and control canine chondrocytes dedifferentiated in monolayer and expressed their respective chondrogenic phenotypes in agarose. However, morphological and histochemical differences between the two cell types indicate that the affected cells are behaving similarly to that in vivo. Hence, this in vitro system may provide a means to analyze lysosomal stor-

age diseases through manipulation and biochemical dissection of the cells and their matrices. Indeed, culture of affected chondrocytes in agarose may be a useful method for further studies of the cellular and extracellular alterations and the mechanisms which lead to skeletal dysplasia in many lysosomal storage diseases.

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