Differential Immunohistochemical Expression Pattern of Galectin-3 in Normal and Osteoarthritic Human Articular Cartilage

Mahmoud Orazizadeh [1*], Donald M Salter [2]

[1]Department of Histology, Medical School, Ahwaz Jondishapour University of Medical Sciences (AJUMS), Ahwaz, Iran, [2]Department of Pathology, Medical School, Edinburgh University, Edinburgh, Scotland, UK

ABSTRACT

Background: Previous studies have shown that Galectin-3, a member of lectin family, is expressed in developing cartilage in mouse embryo and also in growth plate of long bones. **Objective:** In the present work, the expression pattern of Galectin-3 in normal and various grades of osteoarthritic (OA) human articular cartilage has been studied. **Methods:** Using immunohistochemistry and standard western blotting, the in vivo and in vitro expression pattern of Galectin-3 in normal and OA human articular cartilage were assessed. **Results:** Immunohistochemical studies showed a similar pattern of Galectin-3 expression in normal and mild OA but severe OA showed different strong expression in all zones of human articular cartilage. **Conclusion:** Increased expression pattern of Galectin-3 in advanced stages of OA may occur as a result of the imbalance of chondrocyte homeostasis that occurs in OA cartilage and provides a condition to modify normal chondrocyte to an OA chondroctye.

Keywords: Articular Cartilage, Galectin-3, Osteoarthritis

*Corresponding author: Dr. Mahmoud Orazizadeh, Department of Anatomical Sciences, Medical School, Ahwaz Jondishapoor University of Medical Sciences, Ahwaz, Iran. Tel: (+) 98 611 336 7562, Fax: (+) 98 611 336 1544, e-mail: m_orazizadeh@yahoo.com

INTRODUCTION

Galectin-3, a 30 kD non-glycosylated protein and a member of lectin family, is specific for β-galactosides, to which they bind in a Ca^{2+} independent manner (1). In addition to the characteristic carbohydrate recognition domain (CRD), Galectin-3 has a unique amino-terminal domain, which can be digested by collagenase and matrix metalloproteinases-2 and –9 (MMP-2 and MMP-9) in vitro (2,3) and is thought to be important for self-oligomerization, and hence for its functional multivalency (4).

Galectin-3 normally distributes in epithelia of many organs and various inflammatory cells, including macrophages, as well as dendritic cells (DC) and Kupffer cells (5). Galectin-3 is expressed in different cell compartments of eukaryotic cells, and appears to play specific functions at each of these locations. It has been previously shown that in the midgestation mouse embryo, the major site of Galectin-3 expression is the cartilage (6). Subcellular distribution of the protein in growth plate chondrocytes of fetal and neonatal mice is mainly cytoplasmic, with high amounts detectable in mature and early hypertrophic cells (7).

The previous works suggested an intracellular function for Galectin-3 in terminal differentiation of chondrocytes. Galectin-3 is recognized in the cytoplasm of proliferative, mature, and hypertrophic chondrocytes in the growth plate cartilage of developing long bones (8,9). They have also described several defects in chondrocyte differentiation in the long bones of Galectin-3 null mutant embryos, both at the cellular and at the molecular level with an acceleration of cell death in the absence of coordinated angiogenesis. The present study has focused on the expression pattern of Galectin-3 in normal and OA human articular cartilage. The results show the topographical variation in the expression pattern of Galectin-3 in normal and mild OA articular cartilage. The immunohistochemistry results from severe OA cartilage did not show any modification in strong expression of Galectin-3 in all zones of human articular cartilage.

MATERIALS AND METHODS

Tissue Sources and Handling. Human articular cartilage was obtained, with ethical approval and patients' consent, at operation from knee joint arthroplasty specimens and amputations for peripheral vascular in Royal Infirmary Hospital, Edinburgh, Scotland, UK during 1998-2003.

Assessment of Cartilage for Osteoarthritis (OA). The articular surface was assessed and graded macroscopically for the presence or absence of OA using the Collins/McElligott system (10) (Table 1). One set of full thickness pieces of normal and OA cartilage was fixed in 4% formalin for embedding in paraffin wax.

Immunohistochemistry (IHC). To assess the in vivo expression pattern of Galectin-3, paraffin sections of normal and OA cartilage were deparaffinized and rehydrated. To block endogenous peroxidase activity sections were incubated with 0.3% hydrogen peroxide (H_2O_2) in methanol for 15 minutes. The metal rack containing dewaxed, rehydrated sections was placed over the top of the 50x10 mm magnetic bead in 2-litre glass beaker of the Jenway hot plate stirrer. It was filled with 1 litre 1mM EDTA buffer solution containing 0.1% Tween 20 (Invitrogen Life technologies). The pH was adjusted to 8.00 by adding 1 M NaOH and the heater was set to 65°C for overnight incubation.

The next day, the immunostaining method was applied using DAKO ChemMate ^{TM} Detection Kit, Peroxidase/DAB, and Rabbit/Mouse conjugated secondary antibody with the DAKO automatic immunostaining on the TechMate^{TM} instruments. Immunohistochemical assessment was performed by using a mouse anti-Galectin-3 mAb (9C4) at 1:400.

Table 1. The following histological/histochemical system was used to assess the immunohistochemical staining of normal and OA sections

	1. Classification of healthy and diseased joints
A. Normal	Smooth intact cartilage (Collins grade 0)
B. Mild OA	Roughening and cartilage loss (Collins grades I & II)
C. Severe OA	Areas of complete cartilage loss and bone exposure (Collins grades III& IV)
	2. Zonation of intact cartilage with no degeneration
a. Surface	Three cell thick layer
b. Superficial	Top 25%
c. Mid	Middle 50%
d. Deep	Bottom 25%
	3. Zonation of cartilage with degenerative changes
a. Assessment degenerative surface	Clones immunostaining
b. Superficial	Top 25%
c. Mid	Middle 50%
d. Deep	Bottom 25%
	4. Scoring scale
Criteria	Grade
A. No staining	1
B. More cells negative than positive	2
C. Equal number of positive and negative cells	3
D. More cells positive than negative	4
E. All cells positive	5

Negative controls were provided by omitting the primary antibody. Paraffin sections of normal human tonsil were used as positive controls. Positive staining was recognized as a dark brown colour associated with the cell membrane or cytoplasm. As chondrocytes were either strongly positive or negative no grading of the positivity was made. Areas that showed folding of the cartilage or exhibited non-specific background staining were not scored.

The positive immunoreactivity in different sections was graded in a range of 1-5. Galectin-3 staining for each zone was scored as: 1 = no staining; 2 = more cells negative than positive; 3 = equal number of positive and negative cells; 4 = more cells positive than negative; and 5 = all cells positive. Each zone of cartilage sections was evaluated and scored separately (Table 1). Cases studied for IHC assessment of human articular cartilage are shown in Table 2.

Table 2. Cases studied for IHC assessment of Galectin-3 expression in human articular cartilage

No. of cases (sex)	27 (13M/9F)*
Knee joint	9 (4M/2F)*
Hip joint	14 (6M/6F)*
Ankle joint	4 (3M/1F)
Age range (mean)	Knee: 57-78 (77) Hip: 67-92 (78) Ankle: 71-80 (76)

*More information were not recorded.
M= male, F= Female

Chondrocyte Culture. Normal and OA cartilage were kept separately. Chondrocytes were isolated by sequential enzyme digestion. Briefly, cells were seeded in Iscove's modified Dulbecco's medium (Gibco, UK) supplemented with 10 % fetal calf serum (Sigma, UK), 100 I.U./ml penicillin (Gibco, UK) and 100 μg/ml streptomycin (Gibco, UK) to a final density of $2x10^5$ cells /ml in 55 mm plastic petri dishes (Nunc, USA). Primary, non-confluent, 1-2 week cultures of chondrocytes were used in all experiments. Morphologically, the studied cells were typically flattened with a polygonal shape and did not show the fibroblastic appearance.

The day (16-20 hrs) before experiments, the culture medium containing serum was replaced by serum-free medium.

Protein Extraction and Western Blotting. The following methods for protein extraction and western blotting were used. In brief, cells at rest were washed with ice-cold PBS containing 100 μM Na_3VO_4 (Sigma, UK) and lysed in situ with ice-cold lysis buffer at 4°C for 15 min. Lysis buffer contained 1% Igepal (Sigma, UK), 100 μM Na_3VO_4, and protease inhibitor cock-tail tablet (Boehringer Mannheim, Germany). Supernatants were collected after centrifugation at 13,000 rpm for 15 min. Concentration of protein within lysates was determined using Fo-lin-Lowry assay method with Dynatech MR5000 (11).

Whole cell extracts were separated by a 15% SDS-PAGE method under reducing conditions. Following electrophoresis, whole cell lysates were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Immobilon-P, Sigma, UK). Membranes were blocked overnight at 4°C with 2% BSA in TBST (12.5 mM Tris/HCl, pH = 7.6, 137 mM NaCl, 0.1% Tween 20). After washing three times with TBST, blots were incubated for 1 h at room temperature with primary antibodies and then HRP labelled secondary antibodies. Membranes were rewashed extensively and the binding was detected using Enhanced Chemiluminescense Plus Western blotting detection system (Amersham, UK), according to the manufacturer's instructions.

Statistical Analysis. The mean, standard deviation (SD) and standard error of the mean (SEM) were determined in each experiment. For statistical comparisons, the Student's *t* test was applied.

RESULTS

Expression of Galectin-3 in Normal Human Articular Cartilage. Thirty five sections of normal articular cartilage were obtained from 9 males (age range = 64-92, mean = 76), 6 females (age range = 57-90, mean = 79) and 2 cases whose age and sex were not recorded.

Normal articular cartilage sections were isolated from tibia (3 males, 1 female), talus (2 males, 2 females), fibula (1 male) and calcaneous (1 female), normal ankles (3 males, 2 females, age range = 71-80, mean = 77), femoral heads (3 males, 4 females, age range = 72-90, mean = 82), femoral condyle (1 male, age 80) and tibia plateaux (3 males, 1 female, age range = 57-87, mean = 70).

Table 3. Immunoreactivity of Galectin-3 in cytoplasm of chondrocytes isolated from normal, mild and severe OA articular cartilage sections. Significant difference was observed between surface and the deep zone of normal and mild OA articular cartilage (P<0.001). In contrast, different zones of severe OA sections did not show significant difference in Galectin-3 expression (P > 0.05)

Sample	n	Surface	Superficial	Clones	Middle	Deep
Normal	35	2.8 ± 0.1	3.5 ± 0.1	---	3.7 ± 0.1	4.4 ± 0.1
Mild OA	11	2.7 ± 0.2	3.3 ± 0.2	---	3.4 ± 0.2	4.3 ± 0.1
Severe OA	6	N/A*	N/A	4	4.3 ± 0.2	4.8 ± 0.1

* N/A: Not Available

The pattern of expression in all sections showed significant difference between surface and the other zones ($P < 0.001$) (Table 3). The immunoreactivity (cytoplasmic expression of Galectin-3) increased progressively from surface towards the deep zone. The median scores and ranges for surface, superficial, mid and deep zones were 3 (2-4), 4 (2-5), 4 (2-5) and 4(3-5), respectively. Articular cartilage isolated from different joints showed a similar expression pattern (Fig. 1). The age and sex of donors had no noticeable effect on the pattern of Galectin-3 expression in different joints.

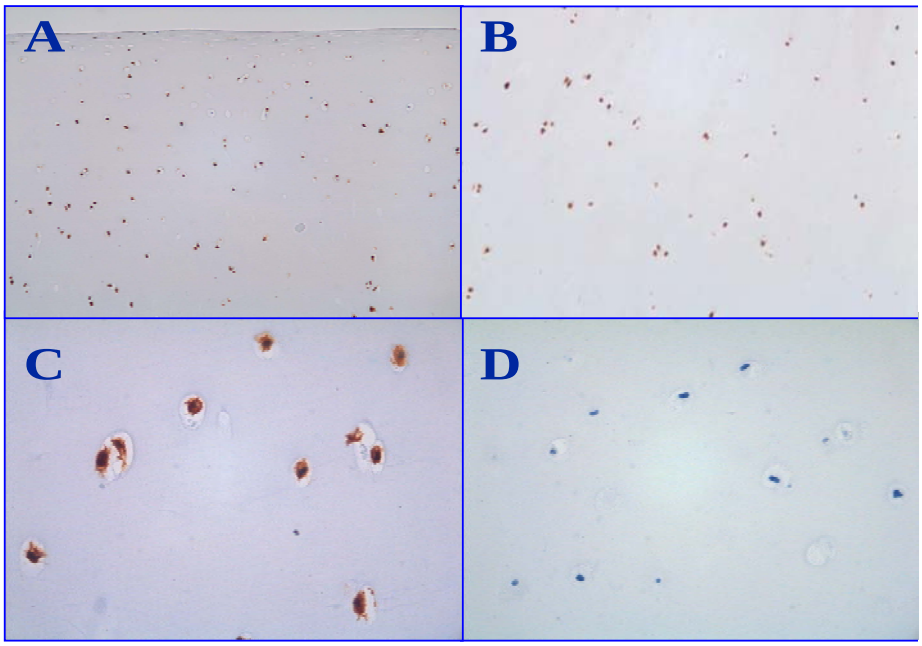

Figure 1. Histochemical localization of cytoplasmic expression of galectin-3 in normal human articular cartilage sections. Chondrocytes from normal articular cartilage sections showed positive Galectin-3 immunoreactivity in comparison to negative controls. The sections were immunostained by standard ABC method using mouse mAb anti-galectin-3 (9C4) (1:400) and DAKO detection kit. **A.** Femoral condyle, normal (FN) (male, age = 80), superficial-mid zone, x100. Galectin-3 immunoreactivity is increased from surface towards the deep zone. **B.** Tibial plateaux (male, age = 73), Mid-deep zone, x100. Chondrocytes in middle zone showed strong expression of Galectin-3. **C.** Tibia (ankle, male, age = 80), mid-zone, x400. High magnification of chondrocytes in mid-zone of Tibia, with strong expression of Galectin-3. **D.** Negative control, talus (ankle, female, age = 80), mid-zone, x100. No positive signal at all was observed in this section as negative control

Expression Pattern of Galectin-3 in Mild OA Human Articular Cartilage. To assess the expression pattern and determine precise immunolocalization of Galectin-3 in OA articular cartilage, 11 mild (2 grade I and 9 grade II), and 6 severe OA (5 grade III and 1 grade IV), isolated from femoral heads, and different anatomical regions of knee (femoral condyle, tibia plateaux and patella) were assessed by using immunohistochemical experiments. Samples of OA articular cartilage were obtained from 5 males (age range = 64-92, mean = 79), 9 females (age range = 57-90, mean = 79) and one case whose age and sex was not recorded.

Samples of mild OA articular cartilage were obtained from 2 males (age range = 64-92, mean = 78) and 5 females (age range 57-90, mean 78). These samples were isolated from femoral heads (4 males, 3 females, age range = 71-92, mean = 83), femoral condyle (1 male, age = 64), tibial plateaux (1 male, 1 female, age range = 57-64, mean = 60) and patella (1 male, age = 80). Articular cartilage from mild OA grades I and II exhibited significant difference be-

tween expression pattern of surface and the other zones ($P < 0.001$). The median scores and ranges for surface, superficial, mid and deep zones were 2 (2-4), 4 (2-4), 4 (2-4) and 4(3-5), respectively. The pattern of Galectin-3 expression in mild OA sections was similar to normal articular cartilage sections. In normal and mild OA cartilage sections, mid and deep zone chondrocytes in all samples showed stronger immunoreactivity in comparison to surface zone which fewer cells were positive.

Expression Pattern of Galectin-3 in Severe OA Human Articular Cartilage. Samples of severe OA articular cartilage were obtained from 3 males (age range = 73-87, mean = 80) and 3 females (age range = 71-86, mean = 79). These samples were isolated from 4 femoral condyles, all male (age range = 73-80, mean = 80), tibial plateaux (both male, age range = 64-87, mean = 75). Chondrocytes in all zones of severe OA cartilage sections (n = 6) showed a similar strong expression of Galectin-3 (Table 3). Clones of degenerated surface zone showed stronger expression of Galectin-3 in comparison with the surface zone of normal and mild OA. The median scores and ranges for clones, superficial, mid and deep zones were 4 (4), 4 (4), 4 (4-5) and 4.5 (4-5), respectively. Galectin-3 expression pattern in various zones of severe OA sections was similar ($P > 0.05$). In severe OA, chondrocytes of clusters, showed strong immunoreactivity of Galectin-3, as did chondrocyte of various zones (Fig. 2).

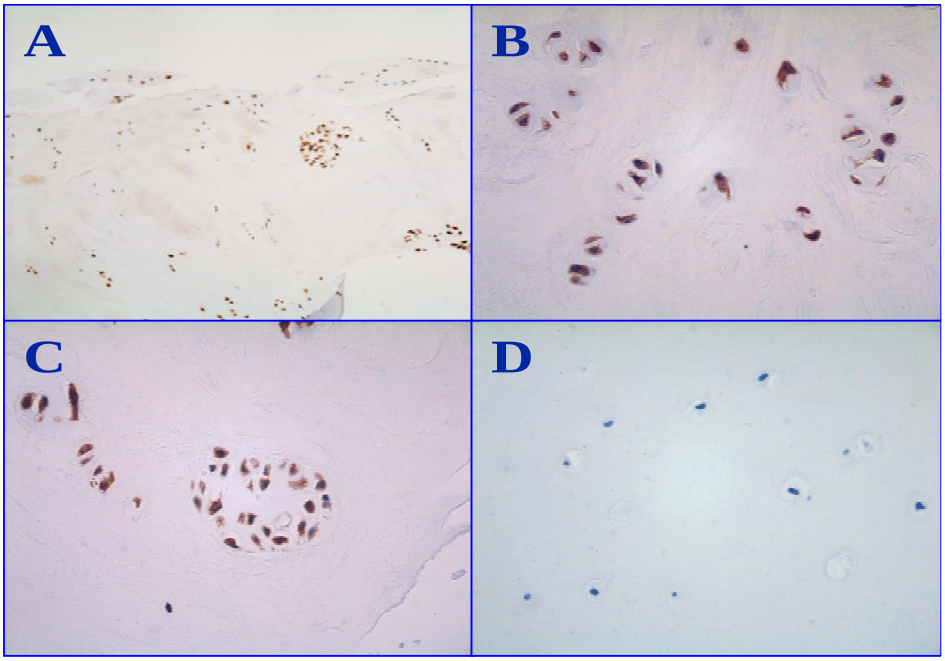

Figure2. Histochemical localization of cytoplasmic expression of galectin-3 in OA human articular cartilage sections. Chondrocytes from OA articular cartilage sections showed positive Galectin-3 immunoreactivity in comparison to negative controls. The sections were immunostained by standard method using mouse mAb anti-galectin-3 (9C4) (1:400) and DAKO detection kit. **A.** Tibial plateaux, OAIII, (male, age = 87), surface-deep zone, x100. All zones showed a similar strong expression of Galectin-3. **B.** Femoral condyle OA, FAIII, (male, 80), mid-zone, x200. Chondrocytes in middle zone showed strong cytoplasmic staining for galectin-3. **C.** Clone in tibial plateaux, OAIII, x400 (male, 87). Clones in severe OA sections showed strong expression of galectin-3. **D.** Negative control, talus (ankle, female, age = 80), mid-zone, x100. No positive signal at all was observed in this section as negative control

Western Blotting Analysis. To assess in vitro expression of Galectin-3 in human articular chondrocytes, analysis of the whole cell lysates obtained from normal and OA cultured human articular chondrocytes was performed. Normal samples were obtained from femoral condyles (both male, mean = 71), tibial plateaux (1 male, age not recorded), femoral heads (2 males and 1 female, age range = 70-83, mean = 78). OA samples were obtained from femoral condyle (1 male, age = 69, grade II), tibial plateaux (all female, mean = 76, 1 grade II, 2 grade III), femoral heads (1 male, 1 female, age range = 87-95, mean = 91, both grade II). Cultured primary chondrocytes at $2x10^5$ cells/ml concentration for a period of 10-15 days were lysed and their proteins extracted. Extracts from normal and OA cultured chondrocytes taken from different joints were analysed and probed with monoclonal anti-Galectin-3 (9C4) antibody at 1:500. A similar 30 kD band was expressed by normal and OA chondrocytes obtained from knee and femoral head, under reducing conditions (Fig. 3). The band expressed from normal and OA chondrocytes did not show identifiable modification. All extracts obtained from chondrocytes isolated from mild and severe OA, removed from donors of different age and sex, when separated under reducing conditions and probed with anti-Galectin-3 monoclonal (9C4) antibody, showed a single band at ~30 kD (Fig. 3). The intensity of Galectin-3 band in normal and OA extracts from different joints or donors with different age and sex under reducing conditions was similar.

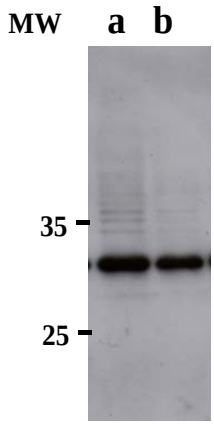

Figure 3. Recognition and comparison of galectin-3 expression in normal and OA human articular cultured chondrocytes. Equal amounts (40μg/lane) of total extracted proteins prepared from the cultured chondrocytes derived from normal (lane **a**, femoral condyle, male, age 69) and OAIII (lane **b**, tibial plateaux, female, age = 79) were run in a 15% SDS-PAGE under reducing conditions. The detection of galectin-3 was assessed by probing the blots with mouse mAb anti-gal-3 (9C4) at 1:500. The blot shown is representative of a series of experiments including three different donors (normal and OA). Molecular weights (MW) in kilo Daltons (kD) are indicated on the left

DISCUSSION

This study demonstrated that Galectin-3 is expressed by human articular chondrocytes. Galectin-3 was expressed in the cytoplasm of chondrocytes in both normal and OA human articular cartilage. There was topographical variation in the expression of Galectin-3 in normal and mild OA articular cartilage. In both normal and mild OA sections the immunoreactivity was increased progressively from surface towards the deep zone. Severe OA did not show any modification in strong expression of Galectin-3 in all zones of articular cartilage. The immunohistochemistry results from different cases were consistent. Biochemical analysis showed that Galectin-3 is expressed by cultured normal and OA articular chondrocytes obtained from different joints.

Human articular cartilage is a non-homogeneous tissue, with variation in matrix composition, chondrocyte morphology, and metabolism occurring with depth from the articular surface. Previous studies have demonstrated that cells isolated from different zones of articular cartilage retain their morphological and metabolic characteristics when maintained in homogeneous cell culture conditions such as monolayer (12) and agarose culture (13). Variation in the behavior of subpopulations of chondrocytes can be maintained in culture for as long as 3 weeks, demonstrating that intrinsic differences between subpopulations do exist (14).

The present study confirmed that Galectin-3 is expressed as a cytoplasmic protein in articular chondrocytes from different joints. The effect of Galectin-3 seems to be mediated via binding to Bcl-2, a proto-oncogen protein suppressor of apoptosis. Bcl-2 is present in chondrocytes of epiphyseal plate cartilage whereas it may be involved in the survival of early maturing chondrocytes. The spatio-temporal distribution of Bcl-2 and Galectin-3 in the plate cartilage is very similar (7-9).

It has been reported that the Galectin-3 null mutation in mouse has several effects on chondrocyte differentiation, most notably premature cell death of chondrocytes without concomitant vascular invasion in the metaphysis during endochondral bone formation (9). This suggested that there is intracellular regulation of chondrocyte apoptosis by molecules implicated in programmed cell death, like the proto-oncogene Bcl-2.

Other studies have demonstrated a role for Galectin-3 in chondrocyte differentiation and endochondral ossification (8,9). An increase in expression of Galectin-3 in advanced stages of OA may occur as a result of the imbalance in chondrocyte homeostasis that occurs in OA cartilage and provides a condition to modify normal chondrocyte to an OA chondrocyte.

Western blotting analysis did not show difference between normal and OA cartilage in Galectin-3 expression pattern. Despite the differential expression of Galectin-3 in normal and severe OA cartilage, no identifiable difference was found between normal and OA cultures chondrocytes. For a precise comparison of the immunohistochemistry results and western blotting analysis, further studies are needed to analyse Galectin-3 expression of numerous cultured chondrocytes from different grades of OA cartilage. In addition, it has been shown that in vivo and in vitro conditions affect the expression pattern of different molecules (15).

Further work is needed to determine which proteins interact with Galectin-3 in normal and OA articular cartilage, to control chondrocyte metabolism during development and in disease processes such as OA. Further studies are needed to elucidate the precise functional role of this molecule in the healthy and diseased chondrocyte metabolism and possibly in mechanotransduction pathway and progression of OA.

ACKNOWLEDGEMENT

This work has been supported by a scholarship from the Iranian Ministry of Health and Medical Education (MHME).

REFERENCES

1. Barondes SH, Cooper DN, Gitt MA, Leffler H. Galectins. Structure and function of a large family of animal lectins. J Biol Chem. 1994;269:20807-10.

2. Ochieng J, Fridman R, Nangia-Makker P, Kleiner DE, Liotta LA, Stetler-Stevenson WG et al. Galectin-3 is a novel substrate for human matrix metalloproteinases-2 and -9. Biochemistry. 1994;33:14109-14.

3. Ochieng J, Leite-Browning ML, Warfield P. Regulation of cellular adhesion to extracellular matrix proteins by Galectin-3. Biochem Biophys Res Commun. 1998;246:788-91.

4. Massa SM, Cooper DN, Leffler H, Barondes SH. L-29, an endogenous lectin, binds to glycoconjugate ligands with positive cooperativity. Biochemistry. 1993;32:260-7.
5. Flotte TJ, Springer TA, Thorbecke GJ. Dendritic cell and macrophage staining by monoclonal antibodies in tissue sections and epidermal sheets. Am J Pathol. 1983;111:112-24.
6. Fowlis D, Colnot C, Ripoche MA, Poirier F. Galectin-3 is expressed in the notochord, developing bones, and skin of the postimplantation mouse embryo. Dev Dyn. 1995;203:241-51.
7. Colnot C, Sidhu SS, Poirier F, Balmain N. Cellular and subcellular distribution of Galectin-3 in the epiphyseal cartilage and bone of fetal and neonatal mice. Cell Mol Biol (Noisy-le-grand). 1999;45:1191-202.
8. Colnot C, Fowlis D, Ripoche MA, Bouchaert I, Poirier F. Embryonic implantation in Galectin 1/Galectin 3 double mutant mice. Dev Dyn. 1998;211:306-13.
9. Colnot C, Sidhu SS, Balmain N, Poirier F. Uncoupling of chondrocyte death and vascular invasion in mouse Galectin 3 null mutant bones. Dev Biol. 2001;229:203-14.
10. Collins DH, McElligott TF. Sulphate (35SO4) uptake by chondrocytes in relation to histological changes in osteoarthritic human articular cartilage. Ann Rheum Dis. 1960 Dec;19:318-30.
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193:265-75.
12. Aydelotte MB, Greenhill RR, Kuettner KE. Differences between sub-populations of cultured bovine articular chondrocytes. II. Proteoglycan metabolism. Connect Tissue Res. 1988;18:223-34.
13. Lee DA, Bader DL. Compressive strains at physiological frequencies influence the metabolism of chondrocytes seeded in agarose. J Orthop Res. 1997;15:181-8.
14. Lee DA, Frean SP, Lees P, Bader DL. Dynamic mechanical compression influences nitric oxide production by articular chondrocytes seeded in agarose. Biochem Biophys Res Commun. 1998;251:580-5.
15. Salter DM, Godolphin JL, Gourlay MS. Chondrocyte heterogeneity: immunohistologically defined variation of integrin expression at different sites in human fetal knees. J Histochem Cytochem. 1995;43:447-57.