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Lectins influence chondrogenesis and osteogenesis in limb bud mesenchymal cells

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Abstract The role of cell surface glycoproteins in cell behavior can be characterized by their interactions with plant lectins. This study was designed to identify the effects of lectins on chondrogenesis and osteogenesis in limb bud mesenchymal cells *in vitro*. Limb bud mesenchymal cells from mouse embryos were cultured in high-density micromass culture. Wheat germ agglutinin (WGA), concanavalin A (ConA), peanut agglutinin (PNA), Dolichos biflorus agglutinin (DBA) and Ricinus communis agglutinin (RCA) were added separately to the culture media. Cells were cultured for 5 or 9 days, and cell viability was assayed by neutral red on day 5. The micromasses were stained with alcian blue, alizarin red S and Von Kossa stains, and alkaline phosphatase assays were also done. Dolichos biflorus agglutinin induced an increase in chondrogenesis, calcium precipitation and proteoglycan production. ConA and PNA did not affect chondrocyte differentiation but induced chondrocytes to produce more proteoglycan. Wheat germ agglutinin reduced chondrification and ossification but induced mesenchymal cells to store lipid droplets. Ricinus communis agglutinin 1 was toxic and significantly reduced cell survival. In conclusion, DBA was the most effective inducer of ossification and chondrification. Wheat germ agglutinin induced adipogenesis instead. These assays showed that *lectins* play important roles in limb bud development.

Keywords Lectin · Mesenchymal cells · Ossification · Chondrification · Adipogenesis

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Introduction

The carbohydrate part of glycoproteins has a pivotal role in protein folding, oligomerization, sorting, and transport [1]. Evidence is accumulating that intracellular animal lectins play important roles in quality control and glycoprotein sorting along the secretory pathway. Some of the amino acid sequences of these lectins are similar to those of plant lectins. For instance, a mammalian lectin counterpart of wheat germ agglutinin (WGA) is potentially important in modifying cell signaling pathways [2]. Wheat germ agglutinin was also found to stimulate insulin receptors in the absence of insulin [3].

Both chondrogenesis and osteogenesis events are orchestrated by numerous signaling pathways [4]. Many of these pathways are mediated by surface glycoconjugates. The distribution patterns of cell surface glycoconjugates are highly dynamic and related with structural modifications [5]. The uptake of several lectins such as concanavalin A (ConA), WGA and Ricinus communis agglutinin (RCA) by undifferentiated mesenchymal cells has been reported [6], and chondrocytes were also found to internalize membrane-bound ConA [7] and WGA [8].

In the late blastema stage in the mouse embryo, peanut agglutinin (PNA) binding sites appear on the cell surface. Lectin binding sites disappear from the cell surface as

mesenchymal development progresses; however, galactose and galactose derivatives appear [9]. These modifications in lectin binding patterns may be important in signaling pathways that form during normal limb bud development.

It has been suggested that carbohydrates and their corresponding receptors (endogenous lectins) decode biological information [10]. Glycoconjugates that react with endogenous lectins can induce chondrogenesis and osteogenesis. For instance, the local administration of ConA increased demineralization [11]. The osteogenic cell population in mouse bone marrow cultures was found to bind WGA and synthesize bone-specific proteins such as alkaline phosphatase, type I collagen and osteocalcin, and to form mineralized nodules [12]. Peanut agglutinin-binding cells with chondrogenic potential were found in the chick embryo calvarium [13]. These lines of evidence all support a functional role of glycoconjugate-binding lectins in morphogenesis.

In the light of these considerations, we hypothesized that adding lectins to the culture medium would modify cell signaling pathways induced by the interaction of lectins (as ligands) with sugar-bearing glycoconjugates. Therefore, the aims of this study were to investigate the effect of glycoconjugates (induced by exposure to lectin) on mesenchymal cell differentiation toward osteocytes and chondrocytes in an *in-vitro* mouse limb bud model.

Material and methods

Cell culture

All experiments were performed in accordance with our center's guidelines for the ethical handling of animals. Fifteen pregnant mice (gestational age 12.5 days) were killed by cervical dislocation and the embryos were removed from the uterus through an abdominal incision. Limb buds (forelimbs and hindlimbs) were microdissected with forceps and scissors. Limbs were collected and exposed to dispase (Gibco, UK) for 1.5 h at 37°C to separate the ectoderm from the mesoderm. Then mesodermal cells were dissociated with 1% trypsin (Sigma) for 20 min at 37°C. Trypsin was neutralized by adding culture media containing fetal calf serum (FCS). A suspension of single cells was obtained by pipetteing. Cell viability was assessed by trypan blue. High-density micromass cultures were prepared from isolated mesenchymal cells. The isolated limb mesenchyme was dissociated into a suspension of 2.5×10^7 cells/mL in DMEM/F12 (containing 10% FCS, 1% glutamine and antibiotics), and then 20-μL drops of the cell suspension were spotted onto each well of a 24-well plate. The culture media, containing 10 μg/mL of each lectin, was added to each well for 5 or 9 days. Culture media were changed every 2 days [14].

Lectins

Wheat germ agglutinin, ConA, PNA, Dolichos biflorus (DBA) and RCA (all from Sigma) were used. These lectins bind, respectively, sialic acid, mannose [15], galactose/*N*-acetylgalactosamine (Gal/GalNac) [16], *N*-acetylgalactosamine (GalNac) [17] and D-galactose [18]. WGA was also binds *N*-acetyl glucosamine (GlcNAc) [19]. The cell culture media were supplemented with 10 μg/mL of each lectin.

Viability assay

On day 5 of culture, the culture medium was discarded and micromasses were washed with phosphate-buffered saline (PBS). One milliliter of 0.05% neutral red (wt/vol) in PBS was added to each well and kept at 37°C for 2 h. Cells were fixed with calcium formol for 1 min and washed with PBS. One milliliter of acid alcohol (0.5% glacial acetic acid [vol/vol] in 50% ethanol) was added to each well and incubated for 2 additional h. Acid alcohol was collected and optical density was assessed spectrophotometrically at a wavelength 550 λ (Shimadzu UV-120-01, Japan). The absorbance measured with this method was directly proportional to the surviving cells in each micromass culture [20].

Chondrification assay

On day 9 of culture, proteoglycan synthesis was evaluated by alcian blue staining. The micromasses were washed and fixed with Kahl's fixative. Samples were stained with 1% alcian blue, pH 2, overnight. Alcian blue stained foci that were evidence of chondrogenesis, indicating maturation and the presence of functional chondrocytes. The number and diameter of chondrification nodules were counted in each micromass island with a dissecting microscope, and the number and area of the nodules were used as an index of cellular differentiation [20].

Matrix mineralization

Calcium deposition was evaluated by staining cell with alizarin red S and silver nitrate. Mineralized matrix was evaluated with Von Kossa staining. Micromasses were fixed with formalin for 2 h and stained with a 2.5% silver nitrate (Sigma) solution in the absence of light at room temperature for 30 min. Cells were washed and allowed to air dry [21]. Micromasses were also stained with alizarin red S. Cells were washed and fixed in 70% ethanol, stained with 5% alizarin red S in PBS overnight, and then washed with PBS.

Alkaline phosphatase activity is involved in the initiation of inorganic phosphate generation for matrix mineralization

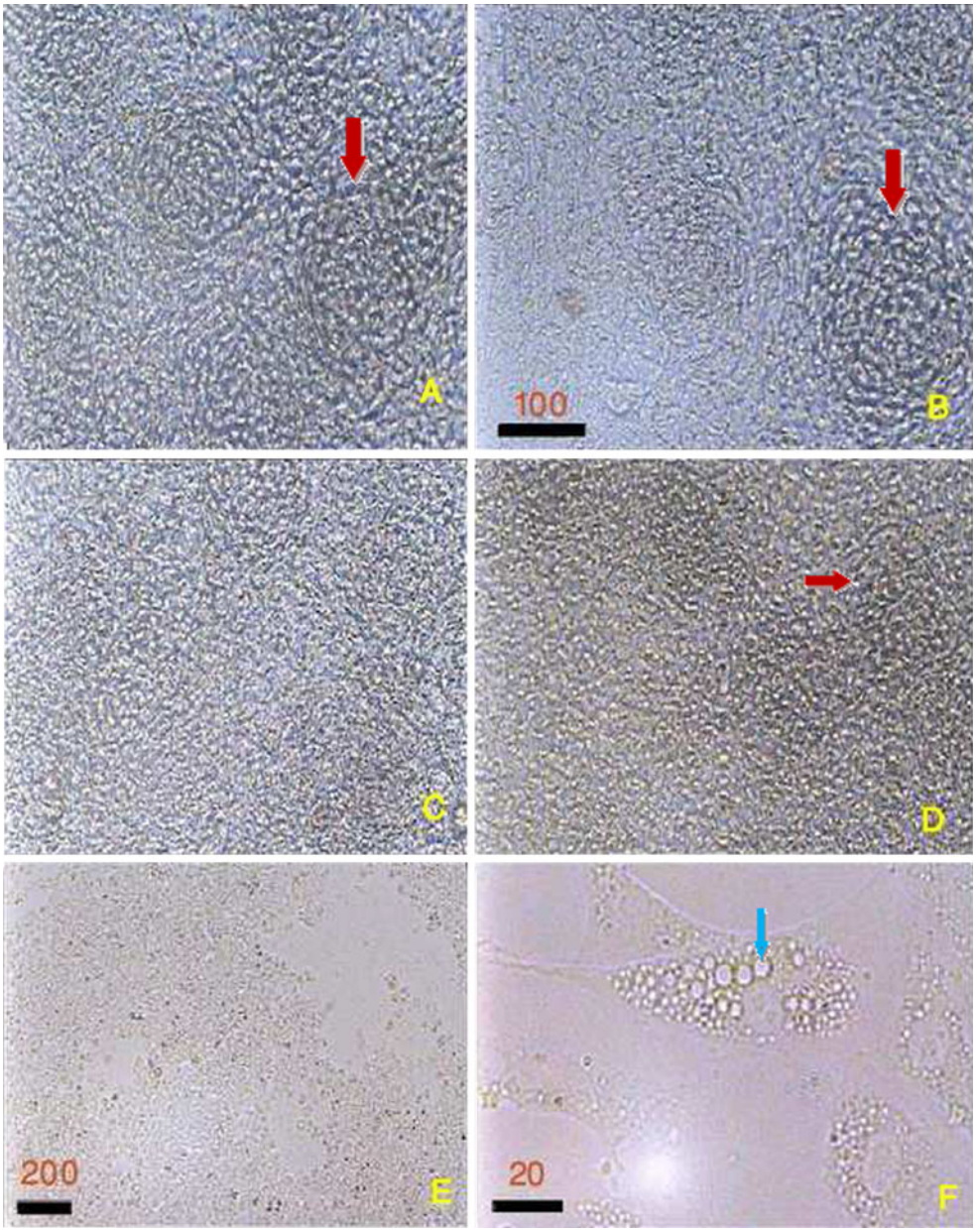

Fig. 1 Mesenchymal cells after 3 days of incubation with different lectins. Cells were treated with **a** ConA, **b** PNA, **c** DBA, **d** RCA1, and **e** WGA. *Red arrows* show nodules. The nodules are cell aggregates with extracellular matrix between them. Note lipid droplets in panel e (*blue arrow*)

[22, 23]. For alkaline phosphatase staining, cells were fixed with citric acid/formaldehyde/acetone fixative, washed in PBS, and stained with alkaline phosphatase solution (Sodium Nitrite Solution, FRV-Alkaline Solution and Naphthol AS-BI Alkaline Solution (Sigma)) in the dark at room temperature for 15 min.

Morphometrical analyses

Each sample section was analyzed with a video-microscopy system comprising a microscope (E-200, Nikon, Tokyo, Japan) linked to a video camera (SONY SSC Dc 18P, Minato, Tokyo, Japan,), a P4 PC computer, and an LG monitor (795 FT Plus, Seoul, GuroGu, South Korea). We

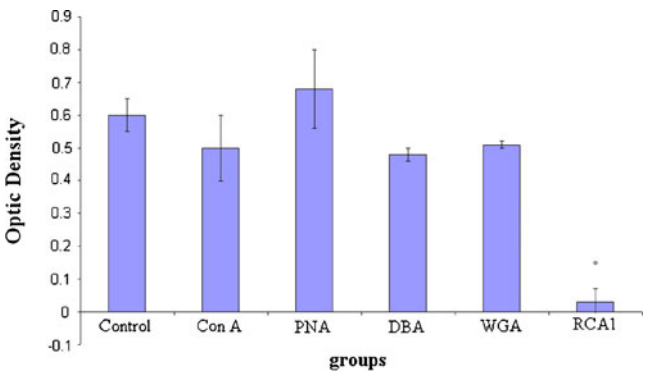

Fig. 2 Optic densities (OD) indicating cell viability according to neutral red staining (n=6). *Significantly different from control ($P \leq 0.05$)

used stereology software designed *ad hoc* at our lab. Stereological points were superimposed upon the images of tissue sections and viewed on the monitor. A transparent test system composed of 900 grid points (point lattice grid) were adjusted over the image of each culture and the number of nodules were calculated.

The diameter of each nodule was measured by a calibrated ruler using the image of a standard 0.01 mm

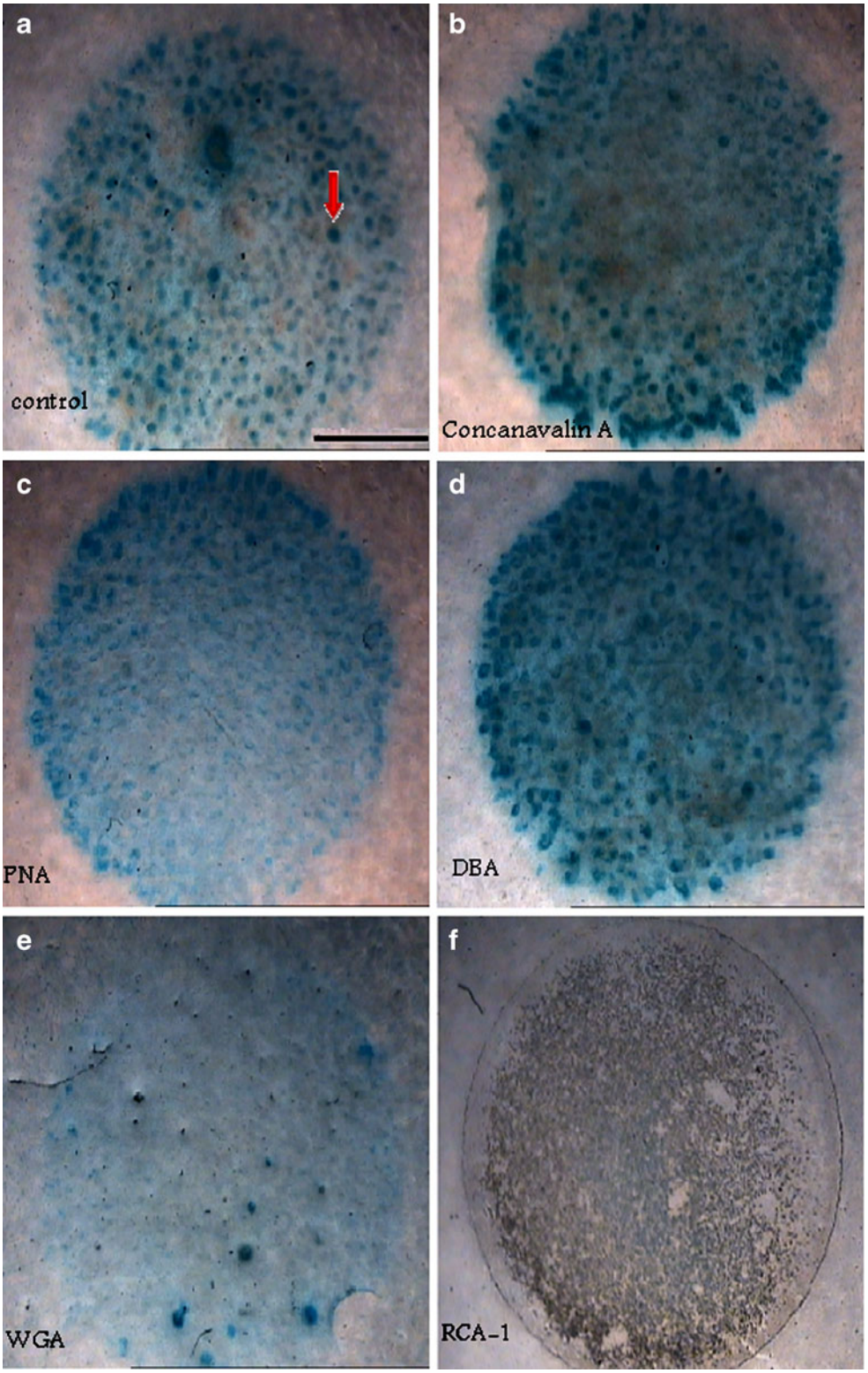

Fig. 3 Cartilaginous nodules (*arrow*) were stained with alcian blue. More nodules were formed after treatment with DBA; however, larger nodules were formed after exposure to DBA and ConA. Cells treated with RCA1 did not differentiate to chondrocytes. **a** Control culture, **b** ConA, **c** PNA, **d** DBA, **e** WGA, **f** RCA1

graticule (Zeiss; Germany) with similar magnifications for all images.

The intensity of the reaction was assessed with Java Image Analyses software (http://mac.softpedia.com/get/Graphics/ImageJ.shtml).

Statistical analyses

The results are presented as the mean±standard deviation. The data were analyzed by analysis of variance (ANOVA) and least significant difference (LSD) *post hoc* tests. A significance level of $P<0.05$ was accepted as statistically significant. All analyses were done with SPSS v. 11.5 software. The graphs were generated with Excel. All experiments were performed in triplicate.

Results

Condrified nodules were detected after 48 h of culture in all wells except those treated with RCA1. Some cells with lipid droplets in their cytoplasm were detected, especially where cell density was low (Fig. 1). These cells, with adipocyte-like morphological features, appeared after 3 days of culture.

Cell viability

Neutral red assays showed that lectins did not influence cell viability, except for RCA1. Most cells treated with RCA1 died ($P=0.000$), and we surmised that this agglutinin was toxic for mesenchymal cells (Fig. 2).

Chondrification

Morphometric studies of cells stained with alcian blue showed that the number of the nodules in DBA-treated wells was significantly increased ($P=0.02$). Chondrogenesis was not affected by either PNA or ConA. Cells treated with WGA formed only a few nodules in numbers significantly lower than in control cultures ($P=0.001$). Cells treated with RCA1 did not form any nodules at all (Fig. 3). Our measurements of the diameters of the nodules showed that ConA-, DBA- and WGA-treated cells formed larger nodules than the control cultures ($P=0.018$, $P=0.001$ and $P=0.001$, respectively).

The intensity of the reaction to alcian blue was also assessed by image analysis. More intense reactions indicated higher proteoglycan secretion by newly differentiated chondrocytes. The data showed ConA-, DBA- and WGA-treated cells secreted significantly more proteoglycan than cells in control cultures. In contrast, cells treated with WGA formed fewer nodules but they were larger and contained more proteoglycan. Table 1 summarizes the data for the number of the nodules, their diameters and the intensity of the alcian blue reaction in response to different lectins.

Ossification

Alizarin red S, silver nitrate and alkaline phosphatase assays showed evidence of ossification in almost all mesenchymal cell cultures treated with the various lectins. The only cells that did not form osteogenic nodules were those treated with RCA1. Morphometric studies showed that DBA-treated cells formed the highest number of ossified regions compared to control cultures and also other lectin-treated cells ($P=0.02$) (Fig. 4). Statistical analyses of the intensity of the silver nitrate reaction confirmed that calcium precipitation in DBA-treated cultures was greater than in other cultures ($P=0.02$) (Fig. 5). In addition, the diameter of the ossified areas was larger and staining for calcium was more intense than in the other cultures ($P=0.01$). In comparison, cells treated with WGA formed significantly fewer ossified nodules that were significantly smaller in diameter and less intensely stained with both alizarin red S and silver nitrate ($P=0.001$). Cells treated with ConA and PNA showed more intense staining for calcium, but the number and area of the ossified nodules were not significantly larger than in control cultures ($P<0.05$). These findings indicated that ConA and PNA induced osteogenic cells to precipitate more ossified matrix. However, as Fig. 5 illustrates, the reaction of the ossified nodules in cultures treated with ConA and PNA was more intense than in controls, although the difference was not significant. Table 2 summarizes the data for the number of the nodules, their diameters and the intensity of staining in cells exposed to different lectins.

The alkaline phosphatase reaction was more intense in control cultures compared to the other groups. This

Table 1 Number of nodules, their diameters and intensity of alcian blue staining ($n=3$)

Groups	Nodule diameter (mm)	Number of nodules	Staining intensity (micropixels)
Control	0.07±0.04	142.25 ±16.22	161.31±31.41
Concanavalin A	0.08±0.03[a]	145.25±21.97	185.69±23.63[a]
PNA	0.07±0.03	148.25 ±13.17	165.61±26.78
DBA	0.09±0.03[a]	241.75±36.92[a]	187.04±22.76[a]
WGA	0.09±0.04[a]	15.50±6.81[a]	189.00±23.33[a]

[a] Significantly different from control

Data for RCA1 are not show because this lectin was toxic and no nodules formed.

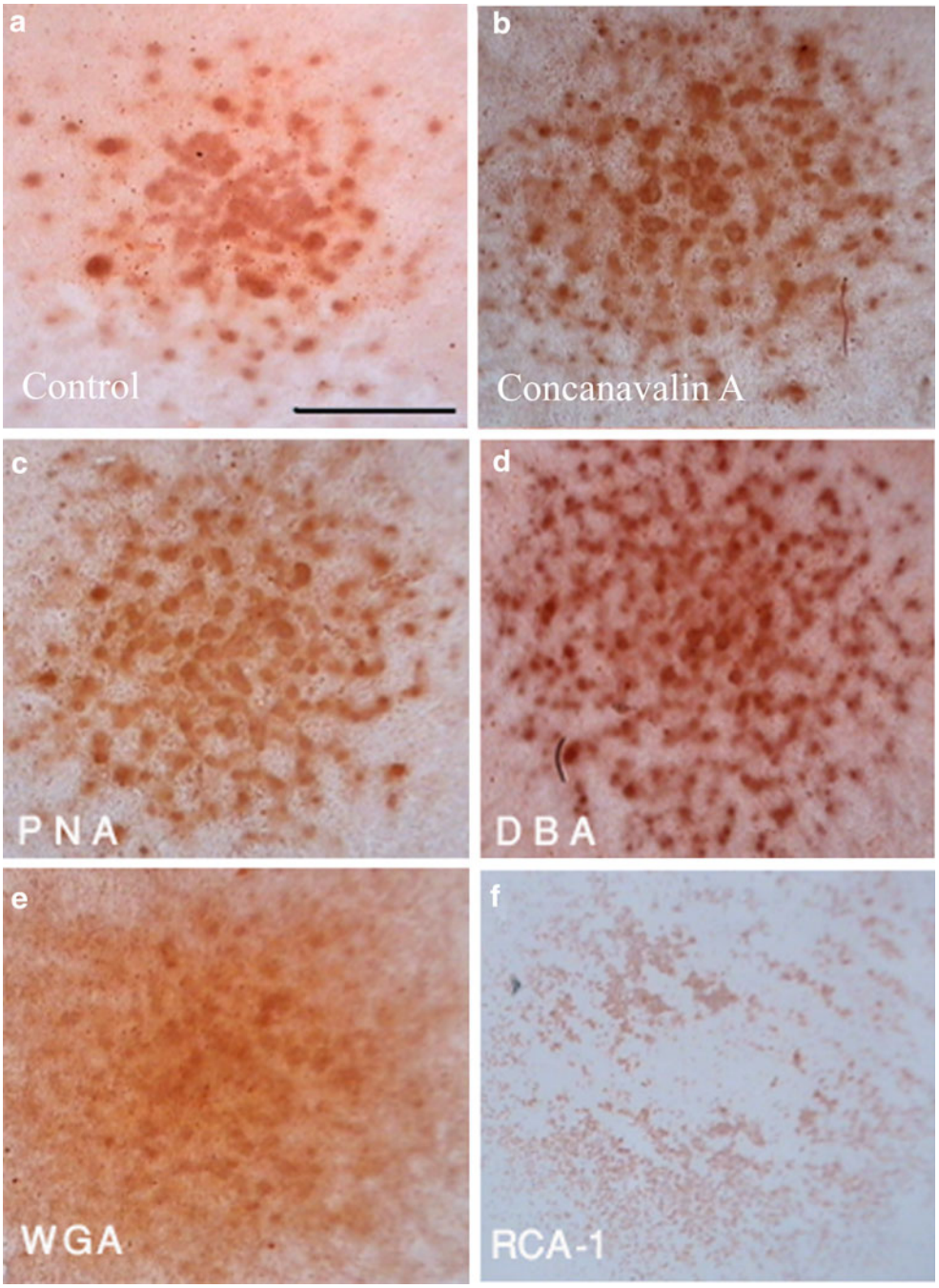

Fig. 4 Cells stained with alizarin red S. Cells exposed to DBA produced the most nodules with the largest diameters and greatest calcium content. PNA and ConA had no effect on the number of nodules but led to increased calcium precipitation by differentiated osteocytes. WGA led to the formation of fewer nodules. **a** Control culture, **b** ConA, **c** PNA, **d** DBA, **e** WGA, **f** RCA1

indicates that the capacity of mesenchymal cells to differentiate into osteoblasts was diminished by ConA and PNA (Fig. 6). Cells treated with WGA and RCA1 did not show alkaline phosphatase activity.

Discussion

Mesenchymal cells exposed to different lectins showed changes in morphology and glycosylation behavior. ConA induced the fibroblasts to form spherical cells with features characteristic of condrocytes, which were able to produce proteoglycans [7]. Some lectins induced an increase in the adhesion capacity of mesenchymal stem cells, chondrocytes and osteoblasts among other types of cell [24]. In contrast, some endogenous lectins inhibited osteoclastic differentiation and the ability of osteoblasts to mineralize the matrix [25].

Lectins, including PNA, are mitogenic and can induce cell proliferation [26]. On the other hand, some lectins such as that of *Rana catesbeiana* inhibit cell proliferation [27]. Our viability tests with neutral red showed that the number

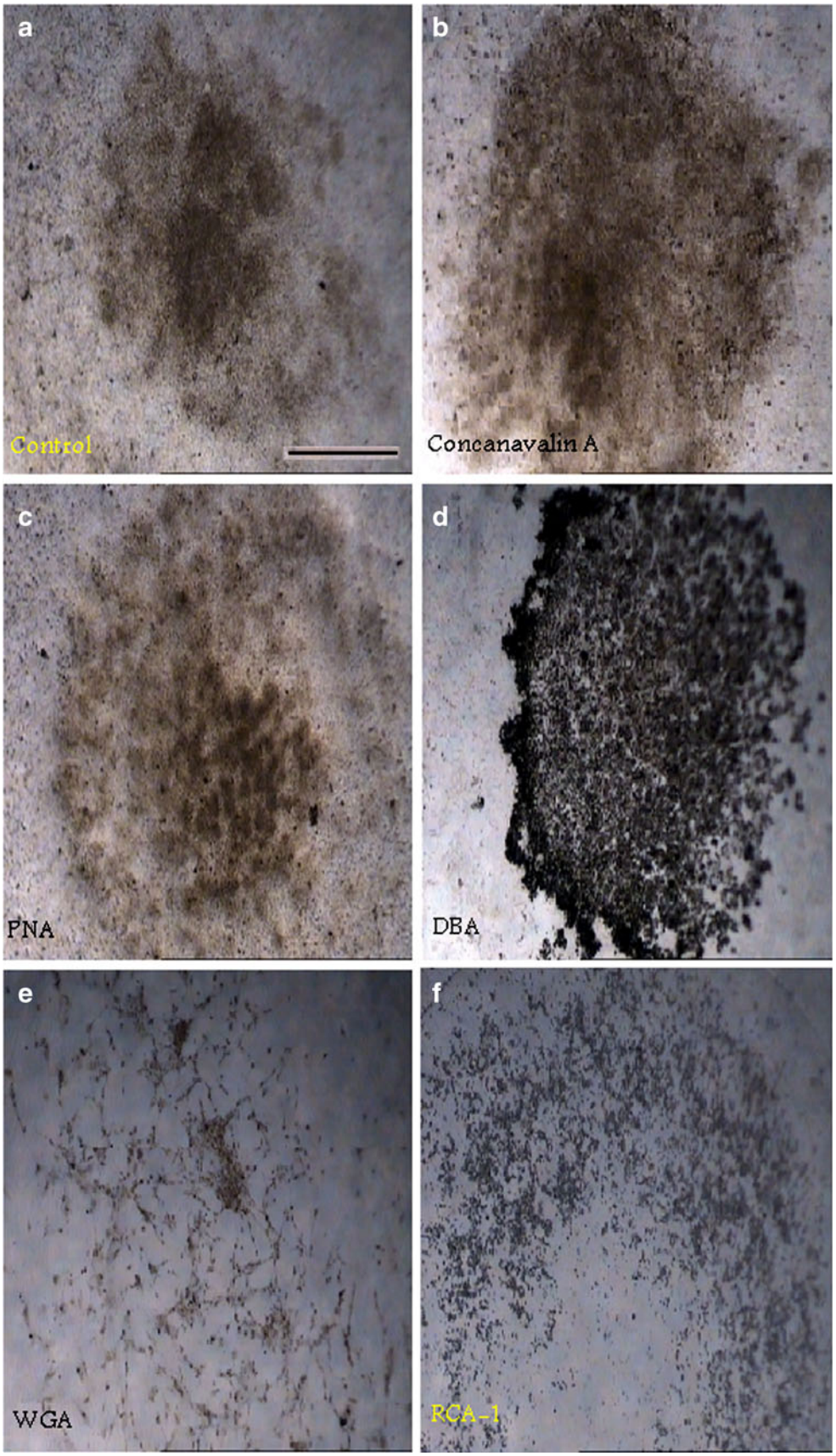

Fig. 5 Cells stained with silver nitrate. Calcium precipitation was greatest in cells exposed to DBA. **a** Control culture, **b** ConA, **c** PNA, **d** DBA, **e** WGA, **f** RCA1

Table 2 Number of nodules, their diameters and intensity of alizarin red S and silver nitrate staining (n=3)

Groups	Nodule diameter (mm)	Number of nodules	Staining intensity for alizarin red S (micropixels)	Staining intensity for silver nitrate (micropixels)
Control	0.09±0.03	165.75±37.16	119.24±16.57	152.37±4.83
Concanavalin A	0.08±0.03	180.75±10.69	129.24±14.82[a]	155.64±9.65
PNA	0.09±0.45	179.00±27.90	127.50±19.37[a]	143.78±4.44
DBA	0.15±0.81[a]	216.75±36.60[a]	165.02±30.25[a]	181.78±6.60[a]
WGA	0.07±0.51	47.00±10.13[a]	118.39±23.46	0.00±0.00[a]

[a] Significantly different from control ($P \leq 0.05$).

Data for RCA1 are not show because this lectin was toxic and no nodules formed.

of viable cells in cultures exposed to PNA was greater than in control cultures and cells exposed to other lectins. This may be attributable to the mitogenic properties of PNA.

Because the number of cartilage nodules in cells incubated in ConA and PNA did not differ significantly, we surmmised that ConA did not influence chondrogenesis or chondrocyte differentiation. However, ConA and PNA exposure increased the size of the nodules and proteoglycan concentration. Earlier research found that ConA induced resting chondrocytes to convert to the hypertrophic phase [28] and produce proteoglycan [7]. Our data showed that PNA had the same effects in our mesenchymal cell cultures. Our alkaline phosphatase assays confirmed that ConA and PNA reduced osteoblast differentiation compared to control cultures. This is consistent with the previous finding that ConA was not able to increase alkaline phosphatase activity [29]. However, our data showed that these lectins increased calcium production. In this connection, it has been suggested that ConA may be useful as a novel model of endochondral bone formation [28]. It has been also reported that ConA induced bone formation in bone marrow-derived mesenchymal stem cells [30].

Our findings for DBA-treated wells showed that these cultures contained more cartilage nodules with larger diameters and more intense alcian blue staining (indicating more proteoglycan production) than in control cultures. This lectin also induced calcium precipitation. In the rat tibia epiphyseal plate, binding of DBA, which reacts with *N*-acetylgalactoseamine residues, was detected on the surface of perivascular cells in the erosion zone. Other cell types such as osteoblasts and chondrocytes did not react to DBA [31]. It has been suggested that DBA-positive cells could differentiate to osteoblasts. However, DBA-positive cells did not show alkaline phosphatase activity [31]. Zschäbitz and colleagues reported a general reduction in lectin reactivity during chondrogenesis [10]. Therefore, DBA interactions with cells may induce chondrification and ossification as DBA reactivity is lost.

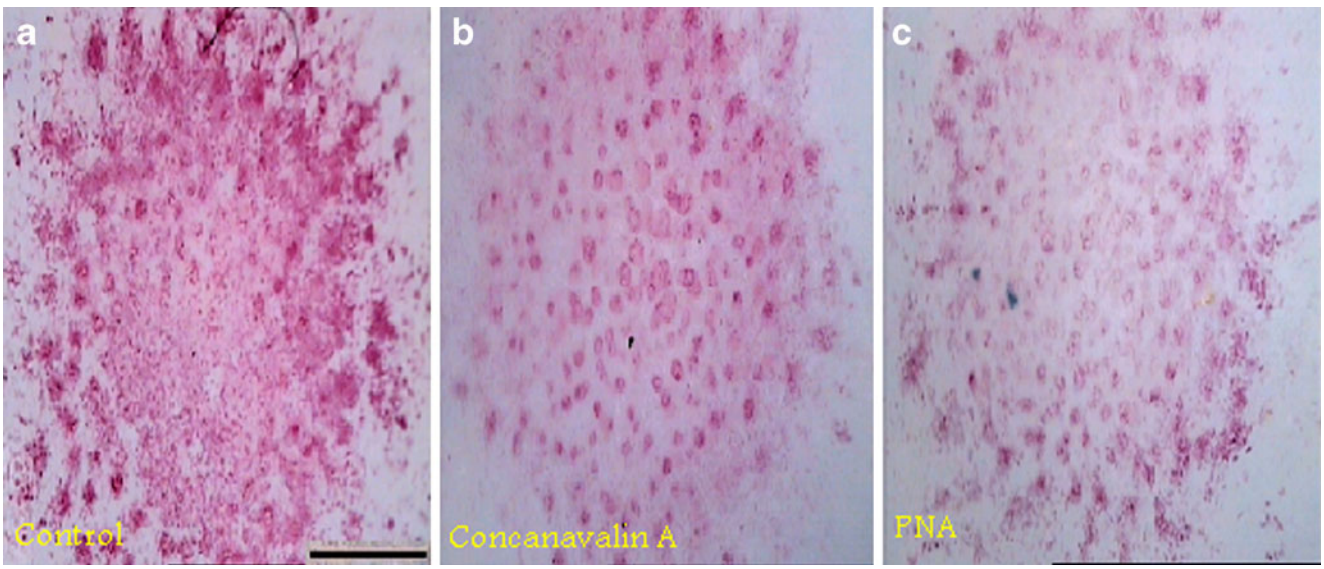

Fig. 6 Cell reactivity to alkaline phosphatase enzyme. Differentiation toward osteoctyes was most evident in mesenchymal cells exposed to PNA and ConA. **a** Control culture, **b** ConA, **c** PNA

In cells exposed to WGA, chondification and ossification decreased significantly in comparison to control cultures. However, WGA-treated cells were induced to store lipid droplets in their cytoplasm. It has been shown that low concentration of WGA (2.5–20 μg/mL) increased the binding affinity and sensitivity of insulin receptors in adipocytes [32]. The adipogenic properties of WGA may thus be due to insulin uptake. Chondrocytes also internalized membrane-bound WGA [8], and the osteogenic cell population in a mouse bone marrow culture also bound WGA and subsequently produced bone matrix [12]. In contrast, our data indicated that WGA reduced bone matrix formation.

In our model, RCA1 was toxic and reduced cell viability. Earlier work found that this lectin bound preferentially to the blastemata [33]. Recently, insulin-like growth factor-binding protein was found to bind to RCA1-agarose [34]. Present in serum, insulin-like growth factor is important for cell growth. RCA1 may bind to the growth factor-binding protein and thereby reduce the availability of this growth factor.

In conclusion, the lectins we tested had various impacts on mesenchymal differentiation in a cell culture model. The most effective inducer of chondrogenesis and osteogenesis was DBA. Matrix production by differentiated cells was favored by ConA and PNA, but these lectins did not affect the efficiency of cell differentiation. WGA induced the cells to store lipid droplets, and RCA1 was toxic. Our *in vitro* findings regarding the influence of lectins on mesenchymal cell differentiation merit further study to shed light on potential clinical applications.

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