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Lectin Receptor Sites During Postnatal Osteogenesis in Guinea Pigs

Receptory lektyn w postnatalnej osteogenezie świnek morskich

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Abstract

Background. Osteoporosis and its complications have become widespread, affecting large portions of the world's population. More advanced information is needed on these pathologies to expand the possibilities for pathogenetic therapy.

Objectives. Lectin histochemistry methods offer new insights into the structure of tissue carbohydrates and their rearrangement under physiological and pathological conditions. The aim of the present investigation was to use a set of lectins with different carbohydrate affinities to study postnatal remodelling of cartilage and bone in relation to the age and sex of experimental animals.

Material and Methods. A panel of five conventional lectins – Con A, PNA, RCA, WGA, SNA, supplemented with an original fucose-specific lectin from *Laburnum anagyroides* bark (LABA) – was used to investigate the femoral bones of female and male guinea pigs aged 3 months, 1 year and 3 years. Tissue samples were fixed in 4% formaline, decalcified in 7% HNO₃ and embedded in paraplast. The sections were subjected to a routine lectin-peroxidase-diaminobenzidine visualization technique.

Results. A pronounced labeling of growth plate chondromucoid was found with Con A, PNA and SNA. Articular cartilage showed much fainter labeling with these same lectins. Capsules of isogenic groups of chondrocytes expressed a strong affinity for SNA and WGA, encompassing a predominance of Neu5Ac/2-6Gal, DGlcnAc and NeuNAc determinants. Osseomucoid showed faint reactivity with all the lectins used, while SNA distinctly marked the line of ossification. Glycoconjugates within lacunas and osseous canaliculi, as well as cytoplasmic glycoconjugates of bone and cartilage cellular elements, expressed strong labeling with RCA and SNA. Osteoclasts reacted selectively with PNA. A comparison of lectin labeling between the experimental groups indicated that the tissue reactivity of males exceeded that of females, and that aging caused a decrease in the lectin reactivity of both genders.

Conclusions. The data extend current knowledge regarding the selective lectin labeling of osseous tissue constituents, and demonstrate the applicability of lectin histochemistry methods in osteoporosis studies (*Adv Clin Exp Med* 2012, 21, 1, 19–26).

Key words: bone, cartilage, postnatal osteogenesis, sex-related dimorphism, lectin histochemistry.

Streszczenie

Wprowadzenie. Wzrost osteoporozy w aspekcie wiekowym stał się przyczyną przeprowadzenia bardziej dokładnych badań patogenezy tej choroby wraz z rozszerzeniem zakresu metod badania tkanki kostnej.

Cel pracy. Przeprowadzenie eksperymentalnych badań ekspresji receptorów lektyn o różnej specyficy węglowodanowej w komponentach strukturalnych tkanki kostnej świnek morskich w aspekcie wiekowym, biorąc pod uwagę dymorfizm płciowy.

Materiał i metody. Zestaw, który stanowi 5 standardowych lektyn (Con A, PNA, RCA, WGA, SNA), uzupełniony oryginalnym fukozo-specyficznym lektynowym preparatem kory *Laburnum anagyroides* (LABA), został wykorzystany podczas przeprowadzenia badań ekspresji glikokoniuatów podczas postnatalnego remodelowania chrząstki i kości, uwzględniając płeć i wiek zwierząt doświadczalnych. Kości udowe świnek morskich, płci męskiej i żeńskiej, w wieku 3 miesięcy, 1 i 3 lat, odpowiednio zostały utrwalone w 4% neutralnej formalinie, odwapnione w 7% HNO₃, a również zatopione w paraplastie. Skrawki zostały potraktowane koniuatami lektyn-peroksydazy chrzanowej, a wizualizacja receptorów została wykonana w systemie DAB – H₂O₂.

Wyniki. Badania ujawniły znaczną ekspresję receptorów lektyny Con A, PNA oraz SNA w składzie chondromukoidu nasadowych płytek wzrostu, w porównaniu ze słabszym wiązaniem tych lektyn z chrząstką powierzchni stawowej. Otoczki izogenicznych grup chondrocytów wykazywały dużą przynależność do lektyny SNA i WGA, co świadczy

o zwiększonej ekspresji w ich składzie Neu5Ac/2-6Gal, DG1cNAc i NeuNAc determinantę. Oseomukoid wykazał słabą reaktywność ze wszystkimi wykorzystanymi lektynami, podczas gdy lektyna SNA konturowała linie kostnienia. Glikokoniugaty zatok i kanalików kostnych, jak również cytoplazmatyczne glikokoniugaty elementów komórkowych chrząstki oraz kości, demonstrowały znaczną przynależność do lektyny RCA i SNA. Osteoklasty sporadycznie reagowały z lektyną PNA. Porównywanie wiązania lektyn między grupami zwierząt doświadczalnych ujawniło, że reaktywność komponentów tkankowych samców była wyższa niż u samic, biorąc pod uwagę te same struktury. W starszych grupach wiekowych reaktywność tkanek u obu płci była ograniczona.

Wnioski. Otrzymane wyniki pozwalają poszerzyć wiadomości o możliwościach wykorzystania lektyn jako selektywnych markerów komórek oraz ich subpopulacji, jak również można je wykorzystać jako podstawę badań dotyczących patologicznych zmian węglowodanów w tkance kostnej (*Adv Clin Exp Med* 2012, 21, 1, 19–26).

Słowa kluczowe: kość, chrząstka, postnatalna osteogeneza, płciowo uwarunkowany dymorfizm, histochemia lektyn.

Current knowledge about chondrogenesis and osteogenesis during normal development as well as under different physiological and pathological conditions is far from complete. The frequency of skeletal disorders, particularly osteoporosis, has been constantly increasing during past decades [1, 2], and various new approaches are being used for their investigation and treatment. Among these are lectin histochemistry methods for detecting the carbohydrate determinants of glycosaminoglycans, proteoglycans, glycoproteins and glycolipids [3–5, 8].

Lectin labeling *in situ* and *in vitro* has provided new insights into the early stages of osteogenesis and revealed the important role of glycoconjugates in the mechanisms of growth and metabolism in cartilage and bone [6, 7]. However, the current authors found no references in the available literature to the late stages of postnatal development, or to sex-related differences in glycoconjugate expression during endochondral ossification. The goal of the present investigation was to study the redistribution of lectin receptor sites in the chondroosseous junction at subsequent stages of postnatal development, particularly in relation to the sex of the experimental animals, and to compare these changes with parathormone levels and bone mineralization profiles.

Material and Methods

Male and female guinea pigs aged 3 months, 1 year and 3 years were divided into six experimental groups. Plasma parathormone levels were determined using an immunoradiological test system (BioSource hPTH-IRMA, Belgium). Serum levels of calcium, magnesium and phosphorus were detected with a standard kit (LaChema, Czech Republic). The calcium and magnesium content in bone tissue were detected using atomic absorption spectrometry on an AAC-115 spectrometer (LOMO, St Petersburg, Russia), following the standard protocol at wavelengths 422.7 nm for Ca and 285.2 nm for Mg. Phosphorus content was determined

using an SF-46 spectrophotometer (LOMO, St. Petersburg, Russia) at 360–370 nm.

For the lectin histochemistry, femoral bones were removed after administering an ether anesthesia overdose and decapitating the animals. Tissue samples were fixed in 4% neutral formaline for 24 hours; decalcified in several changes of 7% HNO₃, using a preparatory needle to assess the degree of decalcification; incubated for 48 hours in a 5% solution of aluminum–potassium–phosphate salts; washed for 24 hours in tap water and embedded in paraplast. Sections 7 µm thick were labeled with lectin-peroxidase conjugates with subsequent visualization in 3,3'-diaminobenzidine tetrahydrochloride-H₂O₂ as described in an earlier article [8].

A routine set of five lectins (Con A, PNA, RCA, WGA, SNA) was supplemented with an original fucose-specific lectin from *Laburnum anagyroides* bark (LABA) [12] (Table 1). The lectins were purified and labeled with horseradish peroxidase at the Lectinotest Laboratory (Lviv, Ukraine; <http://www.meduniv.lviv.ua/depart/lectinotest.html>). In the control experiments, the lectins were omitted from the incubation medium. For general morphology, the sections were stained with hematoxylin and eosin. Morphological analysis was conducted with a Zeiss KM 470600-9901 microscope (Germany), and photographs were taken using an AVerMedia video system (Taiwan). The statistical analysis was performed using Microsoft Office Excel 2003 software (USA).

Results

An age-dependent elevation of parathormone levels was observed in males, compared to a decrease in the same parameter in females. In both sexes, aging caused an increase in Ca and P content in bone tissue. In blood serum these indices remained at approximately same levels in males, and decreased slightly in females (Table 2, Fig. 1).

Routine histological examinations revealed differences in ossification rates. In male guinea pigs

Table 1. Panel of lectins used, their sources and specificities**Tabela 1.** Lista wykorzystanych lektyn, ich źródła i specyfika

WGA	<i>Triticum vulgaris</i> (Wheat germ)	GlcNAc-[(β 1,4)-GlcNAc] ₁₋₂ > terminal [β -GlcNAc] _n >NeuAc
SNA	<i>Sambucus nigra</i> / Elder tree bark	NeuNAc α 2,6-Gal/GalNAc β 1
Con A	<i>Canavalia ensiformis</i> / Jack bean	α/β -Man > α -Glc > α -GlcNAc
RCA	<i>Ricinus communis</i> / Castor bean	Gal-(β 1,4)-GlcNAc > terminal β -Gal > terminal α -Gal
PNA	<i>Arachis hypogaea</i> / Peanut	β DGal $\rightarrow$ 3DGalNAcDGal
LABA	<i>Laburnum anagyroides</i> / Golden chain	α -LFuc

Table 2. Concentration of parathormone in blood, Ca, P, Mg in blood and bone of male and female guinea pigs during postnatal life**Tabela 2.** Stężenie parathormonu we krwi, Ca, P, Mg we krwi oraz w tkance kostnej samców i samic świnek morskich w postnatalnej osteogenezie

Groups, n = 5	Biochemical investigation (Badanie biochemiczne)						
	parathyroid hormone, pg/ml M ± m	mineral components in blood serum, mM/l			mineral components in bone, mg/g		
		total Ca level, M ± m	inorganic P, M ± m	total Mg level, M ± m	Ca, M ± m	P, M ± m	Mg, M ± m
	Males						
I	10.02 ± 0.21	2.32 ± 0.11	1.93 ± 0.09	0.96 ± 0.04	143.45 ± 7.31	82.45 ± 4.51	5.96 ± 0.17
II	11.54 ± 0.47*	2.48 ± 0.16	1.89 ± 0.06	0.83 ± 0.05	167.2 ± 12.16	84.56 ± 3.70	7.31 ± 0.21**
III	11.32 ± 0.54	2.43 ± 0.12	1.9 ± 0.11	0.95 ± 0.05	174.4 ± 9.25*	87.27 ± 7.14	6.87 ± 0.16*
Cr	–	0.9825	–0.9928	–	0.9403	0.75	–
	Females						
I	11.06 ± 0.34	2.56 ± 0.12	2.02 ± 0.04	1.12 ± 0.07	152.25 ± 8.31	92.65 ± 9.32	6.05 ± 0.18
II	10.81 ± 0.26	2.24 ± 0.17	2.04 ± 0.08	0.99 ± 0.03	167.8 ± 6.64	87.72 ± 5.82	7.16 ± 0.29*
III	9.94 ± 0.13*	2.16 ± 0.05*	1.95 ± 0.13	1.15 ± 0.02	175.05 ± 5.34	95.43 ± 7.91	6.30 ± 0.21
Cr	–	0.774	0.9269	–	–0.846	–0.659	–

Cr – correlation coefficient; * p < 0.05; ** p < 0.001; t – Student's coefficient.

Cr – współczynnik korelacji; * p < 0.05; ** p < 0.001; t – współczynnik Studenta.

aged 1 year there were epiphyseal growth plates in the knee and hip-joint cartilage, whereas in females of the same age group, growth plates had disappeared and the bones were completely ossified.

Intercellular spaces within the growth plates of the experimental animals expressed strong reactivity with Con A, PNA and WGA; moderate reactivity with SNA and RCA; and faint-to-negative reactivity with LABA (Figures 2A and 2C). In comparison to the growth plates, the articular cartilage expressed much fainter labeling with the lectins (Tables 3 and 4).

Chondrocytes from different locations were labeled with RCA and SNA, the lectins used to visualize different phases of the mitotic cycle (Fig. 2B).

Glycoconjugates in the perinuclear space of the developing chondrocytes expressed intense WGA affinity, whereas isogenic group capsules demonstrated strong reactivity with WGA and SNA (Figures 2B and 3A).

Osseomucoid demonstrated a rather faint affinity for all the lectins used except for SNA, which distinctly labeled the cement line (tidemark) (Fig. 3A). Islets of cartilage within the developing osseous tissue reacted to Con A and SNA; aging caused a decrease in the number of these islets.

Glycoconjugates within lacunas and osseous canaliculi, as well as cytoplasmic glycoconjugates of osteoblasts and osteocytes, reacted strongly to RCA and SNA (Figures 2D and 3B). Osteoclasts

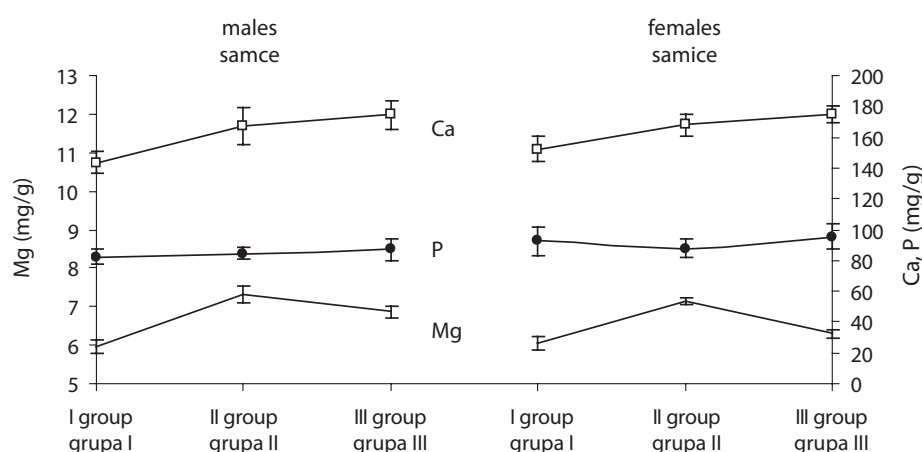


Fig. 1. Ca, P and Mg content in the bone tissue of male and female guinea pigs

Ryc. 1. Stężenie Ca, P, Mg w tkance kostnej samców i samic świnek morskich

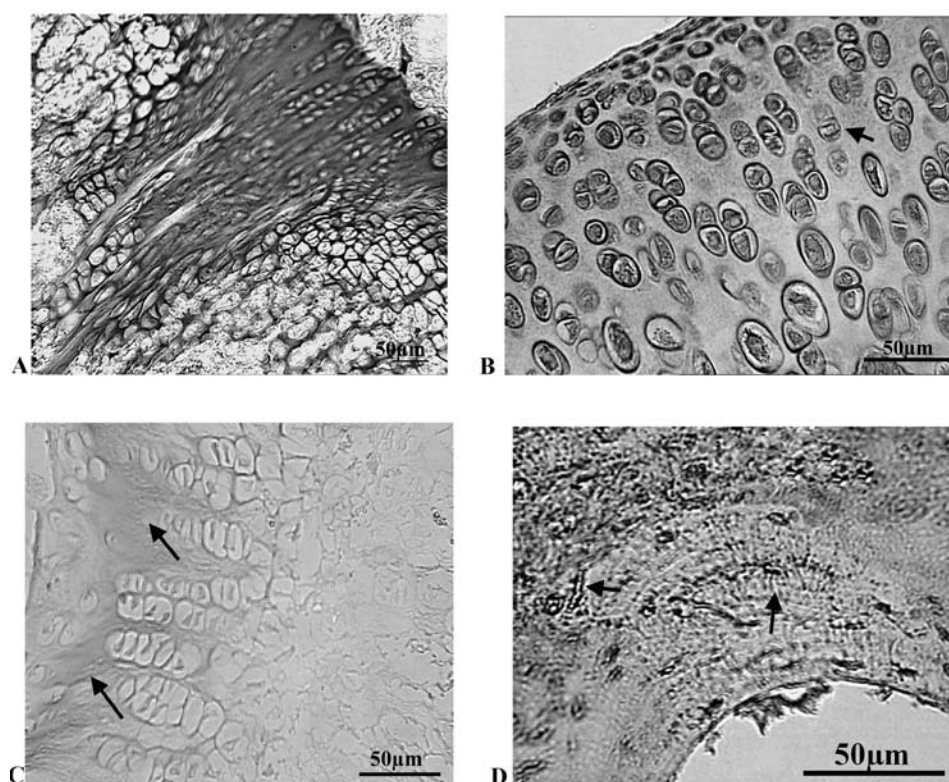


Fig. 2. Lectin labeling of the bone and cartilage of male guinea pigs aged 3 months. A) Expression of Con A receptor sites within the metaepiphyseal plate chondromucoid; B) Mitotic figures within isogenic cell groups of the articular cartilage revealed with SNA; C) Faint LABA reactivity of the metaepiphyseal growth plate; D) Expression of SNA receptor sites in bony lacunas and canaliculi during osteon formation

Ryc. 2. Ekspresja receptorów lektyn w tkance kostnej i chrzęstnej samców świnek morskich w wieku 3 miesięcy. A) ekspresja receptorów lektyny Con A w składzie chondromukoidu epifizarnej blaszki wzrostu; B) lektyna SNA, mitozę w komórkach chrząstki stawowej; C) słabe homogeniczne połączenie lektyny LABA w epifizarnej blaszce wzrostu; D) ekspresja lektyny SNA w systemie zatok i kanalików podczas formowania kości

were selectively labeled with PNA and SNA (Figures 3C and 3D).

A comparison among the groups of animals revealed that the lectin reactivity of the males' bone and cartilage exceeded that of the females, and that aging caused a reduction in lectin labeling in the tissues of both genders.

Discussion

A differential mode of parathormone secretion was detected, and reference levels of this hormone in male and female guinea pigs during their postnatal development were established. The main physiological action of parathormone is directed

Table 3. Lectin receptor sites in bone tissue of male guinea pigs during postnatal life**Tabela 3.** Receptory lektyn w tkance kostnej samców świnek morskich podczas postnatalnej ontogenezy

Group (Grupa)	Elements of cartilage and bone (Tkanka chrzęstna i kostna)	Lectins (Lektyny)					
		WGA	LABA	Con A	PNA	RCA	SNA
I	Distant zone AC	++	+	++	++	+	+
	Chondromucoid AC	+	-	+	++	+	+
	Cells AC	+	-	±	±	+	++
	Epiphyseal plate						
	- ZUC	+/-	-	+	±	+	±
	- ZCC	++	+/-	+++	+++	++	+++
	- ZBC	++	+	+++	++	++	+++
	- ZC	++	±/-	++	++	+	++
	Periosteum	+	+	+	+	+	++
	Osseomucoid	±	-	±	±	+	±
	Osteocytes	±	-	±	±	+	++
	Osteoblasts	+	-	±	±	+	±
	Osteoclasts	-	-	-	++	-	-
	Endosteum	+	+	+	+	+	+
II	Distant zone AC	+	-	±	±	+	±
	Chondromucoid AC	±	-	±/+	±	±	±
	Cells AC	±	-	±	+	+	++
	Epiphyseal plate						
	- ZUC	±	-	+	-	±	±
	- ZCC	++	±	++/+	+	++	++
	- ZBC	+++/-	±	+++/-	+	++	++
	- ZC	+++/-	-	+	±	+	+
	Periosteum	+	+	+	+++	+	++
	Osseomucoid	±	-	±	±	±	±
	Osteocytes	±	-	±	±	+	++
	Osteoblasts	±	-	±	±	+	+
	Osteoclasts	-	-	-	-	-	++
	Endosteum	+	±	+	+	+	+
III	Distant zone AC	±	-	±	±	±	±
	Chondromucoid AC	±	-	±	±	±	±
	Cells AC	±	-	±	+	+	++
	Periosteum	+	±	+	++	++	+
	Osseomucoid	-	-	±	±	±	±
	Osteocytes	-	-	±	±	±	+
	Osteoblasts	-	-	±	±	±	+
	Osteoclasts	-	-	-	+	-	-
	Endosteum	+	-	±	+	+	+

AC – articular cartilage; ZUC – zone unaltered cartilage; ZCC – zone columnar cartilage; ZBC – zone bubbles cartilage; ZC – zone calcification. Reactivity: – unreactive, ± weak, + moderate, ++ strong, +++ intense or very strong.

AC – chrząstka stawowa; ZUC – strefa chrząstki rezerwowej; ZP – strefa proliferacji; ZMH – strefa dojrzewania i hipertrofii; ZC – strefa kalcynacji. Poziom zabarwienia: – niski, nieaktywny, ± słaby, + umiarkowany, ++ silny, +++ intensywny lub bardzo silny.

at stimulating the resorptive activity of osteoclasts, and at inhibiting the reabsorption of phosphate ions by nephrons. Both of these mechanisms cause an elevation of Ca levels in blood plasma [9].

In the current study, the data on plasma Ca and P levels in male and female guinea pigs correlate positively with parathormone levels, whereas the intensity of these ions' deposition in bone tissue is apparently influenced by other biologically active substances, i.e. estrogen and testosterone. The increased ossification rate of growth plates in

females in comparison with males is probably due to the stimulatory effect of somatotrophins and androgens on chondrocyte proliferation and the elongation of bones in males, and on the inhibitory effect of estrogens in females [9].

Large amounts of mannosyl and sialic acid residues were detected in the growth plates of all the investigated groups of animals. Cartilage ossification inhibited lectin reactivity and decreased the carbohydrate content of the mineralized bone. These results are in good agreement with the find-

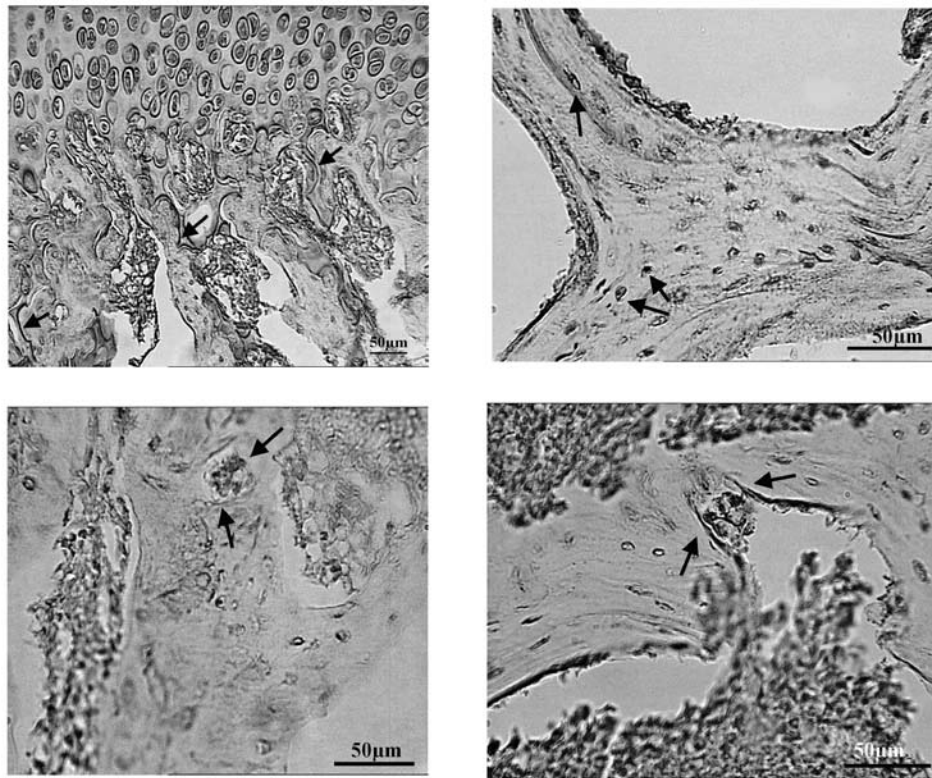


Fig. 3. A) Expression of SNA receptor sites in the chondro-osseous junction of a male guinea pig 3 months of age; B) RCA labeling of osteocytes within bony lacunae in a male guinea pig 3 years of age; C) PNA receptor sites within the cytoplasm of an osteoclast in Howship's lacuna in a female guinea pig 3 years of age; D) SNA binding to osteoclasts in a male guinea pig aged 1 year

Ryc. 3 A) Ekspresja receptorów lektyny SNA wzdłuż linii cementacji u samca świnki morskiej w wieku 3 miesięcy; B) receptory lektyny RCA w osteocytach w składzie zatok kostnych u samca w wieku 3 lat; C) receptory lektyny PNA w cytoplazmie osteoklasty, która znajduje się w zatoce Gauszypa u samicy w wieku 3 lat; D) ekspesja receptorów lektyny SNA w osteoklastach u samca w wieku 1 roku

ings of other researchers [10, 11], in spite of the use of different species, different fixatives and different lectin labeling protocols.

The strong Con A reactivity of developing cartilage and bone may be due partly to high mannosyl residue content in type II collagen molecules in the cartilage, and partly to the presence (reported by other researchers) of D-mannopyranosyl, D-glucopyranosyl and D-fructopyranosyl residues on the surface of osteoprogenitor cells [7, 12] or elevated expression of α -D-mannose-rich glycoproteins by apoptotic cells [13]. In this context mannosoglycans apparently play an important role during cartilage growth and development. This notion is confirmed by the phenomenon of the stimulation of osteogenesis by subcutaneous injection of Con A [6]. It is also noteworthy that mannosoglycans (Con A receptor sites) are expressed in the early stages of chondrohistogenesis [14]; this reactivity decreases in the ossification centers [5, 15].

Sex-related differences in lectin binding were also observed: Con A, PNA and SNA labeling in the growth plate of males exceeded that of females, especially when comparing 1-year-old animals. The ex-

pression of Con A receptor sites in the growth plates was higher than in the articular cartilage. Similar enhancement of Con A reactivity was described by Zschabitz et al. [12] in developing rat cartilage.

The large amounts of WGA and SNA receptor sites detected in the isogenic group capsules is in agreement with Cormack's report of high glycoconjugate content in these elements [16], and with the observations of Farnum and Wilsman, who described strong WGA binding to the intercellular septa in growth plates and pericellular glycoconjugates around chondrocytes. The low content of fucosoglycans in cartilage and bone noted in the current study is in agreement with the observations of Zschabitz et al. [12].

In the current investigation, osteoclasts were intensely labeled by PNA and SNA, this observation is in agreement with other researchers' reports of a high content of DGal(β 1-3)DGlcNAc determinants (receptor sites for *Maclura pomifera* lectin) in these cells [10, 11], and with Howard and Batsakis's observations of PNA strong affinity for macrophages-histiocytes [17], which are closely related to osteoclasts.

Table 4. Lectin receptor sites in bone tissue of female guinea pigs during postnatal life**Tabela 4.** Receptory lektyn w tkance kostnej samic świnek morskich podczas postnatalnej ontogenezy

Group (Grupa)	Elements of cartilage and bone (Tkanka chrzęstna i kostna)	Lectins (Lektyny)					
		WGA	LABA	Con A	PNA	RCA	SNA
I	Distant zone AC	++	–	++	++	+	+
	Chondromucoid AC	++	±	+	++	+	+
	Cells AC	±	–	±	±	+	++
	Epiphyseal plate						
	– ZUC	+	–	+	±	±	±
	– ZCC	++	±	++	+++	++	+
	– ZBC	++	±	++	++/+	+	++
	– ZC	++	–	++	++/+	+	+
	Periosteum	+	+	+	+	+	++
	Osseomucoid	±	–	±	±	+	±
	Osteocytes	±	–	±	±	+	++
	Osteoblasts	±	–	±	±	+	±
	Osteoclasts	–	–	–	++	–	–
	Endosteum	+	+	+	+	+	+
II	Distant zone AC	+	–	±	±	+	±
	Chondromucoid AC	±/+	–	±	±	±	±
	Cells AC	±	–	±	+	+	++
	Periosteum	+	+	+	++	++	++
	Osseomucoid	±	–	±	±	±	±
	Osteocytes	–	–	±	±	±	++
	Osteoblasts	–	–	±	±	±	+
	Osteoclasts	–	–	–	–	–	++
	Endosteum	+	±	+	+	+	+
III	Distant zone AC	±	–	±	±	±	±
	Chondromucoid AC	±	–	±	±	±	±
	Cells AC	±	–	±	±	+	++
	Periosteum	+	±	+	++	++	+
	Osseomucoid	–	–	±	±	±	±/+
	Osteocytes	–	–	±	±	±	+
	Osteoblasts	–	–	±	±	±	+
	Osteoclasts	–	–	–	+	–	–
	Endosteum	+	±	±	+	+	+

AC – articular cartilage; ZUC – zone unaltered cartilage; ZCC – zone columnar cartilage; ZBC – zone bubbles cartilage; ZC – zone calcification. Reactivity: – unreactive, ± weak, + moderate, ++ strong, +++ intense or very strong.

AC – chrząstka stawowa; ZUC – strefa chrząstki rezerwowej; ZP – strefa proliferacji; ZMH – strefa dojrzewania i hipertrofii; ZC – strefa kalcynacji. Poziom zabarwienia: – niski, nieaktywny, ± słaby, + umiarkowany, ++ silny, +++ intensywny lub bardzo silny.

In the current study a decrease was observed in the expression of carbohydrate determinants during postnatal development of cartilage and bone. In guinea pigs both tissues demonstrated high content of Con A and SNA receptor sites (mannosoglycans and sialoglycans), with faint LABA reactivity (low fucosyl content). The findings regarding the lectin reactivity of guinea pig cartilage and bone are in agreement with the results of other researchers data on rat and human tissues,

in spite of the use of different fixatives, processing and staining protocols. Sex-related dimorphism included Con A, SNA and PNA reactivity. PNA and SNA showed selective labeling of osteoclasts, whereas RCA and SNA exposed selective labeling of osteoblasts and osteocytes. These data extend current knowledge on the applications of lectin for selective labeling of cells and their subpopulations, and for investigating pathologically changed osseous tissue, particularly in hyperparathyroidism.

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