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Nuclear and cytoplasmic lectin receptor sites in rat Py1a osteoblasts

M.G. Sabbieti[1], L. Marchetti[1], M.H. Hurley[2] and G. Menghi[1]

[1]Department of Comparative Morphological and Biochemical Sciences, University of Camerino, Camerino, Italy and
[2]Division of Endocrinology and Metabolism, University of Connecticut Health Center, Farmington, USA

Summary. The intracellular distribution of lectin receptor sites was studied in the rat Py1a osteoblasts using immunofluorescence at the confocal microscopy level. This immortalized cell line was found to represent a satisfactory model to study the occurrence and distribution of sugar moieties. Our data showed distinct affinity patterns of lectins recognizing different terminal or internal sugar residues. For some lectins, the binding patterns appeared to be cell cycle-independent, whereas for PNA the cell cycle greatly influenced the nuclear binding. By combining lectin affinity with sialidase degradation and alcoholic saponification the sialic acid acceptor sugars and derivatives were also visualized. In particular, glycoconjugates with sialic acids linked to ß-galactose, and mainly C_4 acetylated, were located in the cytoplasm, while glycoconjugates characterized by sialic acids linked to α-N-acetylgalactosamine, and devoid of acetyl groups at C_4, were almost exclusively found in the nucleus. The comparison of lectin affinities, with and without prior glycosidase digestions, allowed us to gain further insight into the chemical composition of glycoconjugates that act as the lectin receptor sites that appeared to belong to O- and N-linked glycoconjugates. The use of additional enzymatic treatments were useful to better establish the localization of nuclear receptor sites and results were compared with previous studies about endogenous and exogenous lectins in an attempt to reconcile the association of lectins and sugars within the nucleus and their possible involvement in modulation of cell proliferation and/or response to chemical signals. The above digestions also provided information about the cytoplasmic binding patterns.

Key words: Lectin histochemistry, Enzymatic degradations, CLSM, Py1a Osteoblasts, Rat

Offprint requests to: Prof. Giovanna Menghi, Dipartimento di Scienze Morfologiche e Biochimiche Comparate, Università di Camerino, I-62032 Camerino (MC), Italy. Fax: (737) 402708. e-mail: menghi@camserv.unicam.it

Introduction

The development of *in vitro* conditions which are permissive for the expression of the osteoblast phenotype, offers the possibility to examine various aspects of bone differentiation and growth (Lian and Stein, 1992; Aubin et al., 1993; Aubin and Turksen, 1996). While organ or tissue culture systems can sustain various osteogenic processes including bone deposition and resorption, cell cultures are best suited for investigating the cell differentiation and the earliest events of matrix elaboration.

Bone sialoproteins are synthesized very early during *in vitro* formation of bone-like tissue and constitute the major non-collagenous bone glycoproteins incorporated into the matrix (Kasugai et al., 1991a,b, 1992). The series of events clearly shows parallels with osteogenic processes occurring *in vivo* (McKee and Nanci, 1996). In this regard, culture systems are useful for examining the sequence of bone matrix component synthesis and transformed osteogenic cells have also been used in an attempt to obtain more homogeneous cell populations (Moseley and Suva, 1986).

Although there have been numerous investigations on osteoblast glycoconjugates (Takagi et al., 1983; Chopra et al., 1991; Chopra and Anastassiades, 1992; Kelm et al., 1992, 1994; Mintz et al., 1994; Ritchie et al., 1994), there are relatively few analyses concerned with *in vitro* visualization and characterization of individual glycosidic residues and specific carbohydrate sequences by lectin histochemistry. Lectins have been previously used to study the cytochemical properties of osteoblast cell membrane domains (Watson et al., 1989), to clarify the process of endochondral ossification (Nakamura and Ozawa, 1996), to induce the calvarial bone formation *in vivo* (Izbicka et al., 1997), and to investigate the osteoblast differentiation (Suzuki et al., 1993; Dunlop and Hall, 1995), odontogenesis (Zhang et al., 1994) as well as the tissue response to titanium implants (Nanci et al., 1994).

We have previously used the Py1a rat osteoblasts to study the regulation of FGF-2 in response to prostaglandin stimulation (Sabbieti et al., 1999a) and the

behavior of PNA lectin as marker of the Py1a cell cycle (Sabbieti et al., 1999b). In this study, we used the immortalized rat osteoblastic clonal cell line Py1a as model system to study the occurrence and distribution of nuclear and cytoplasmic lectin receptor sites and related glycocomponents, by combining a panel of fluorochrome-conjugated lectins with enzymatic digestions. The analysis of the affinity distributional patterns was performed by confocal laser scanning microscopy (CLSM) allowing the spatial localization of lectin receptor sites.

Materials and methods

Materials

F-12 medium and fetal bovine serum were obtained from Gibco; penicillin, streptomycin, lectins, DNase, RNase, collagenase, and exoglycosidases were purchased from Sigma; tissue culture dishes were obtained from Costar.

Cell cultures

Immortalized rat osteoblastic Py1a cells were plated in 6-well culture dishes (Corning), containing previously cleaned and sterilized coverslips, at a density of 3500 cells/cm^2 and were grown to about 70% confluence in F-12 medium containing 5% nonheat inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (50 mg/ml).

Experimental procedures

Fixation-permeabilization

The cultures were serum-deprived for 24 h in F-12 with bovine serum albumin (BSA) (1 mg/ml). Then cells were washed twice with 0.1M PBS (phosphate-buffered saline), pH 7.4, and fixed for 20 min at room temperature, with 4% paraformaldehyde diluted in PBS. After three washes with PBS, cells were permeabilized with 0.3% Triton X-100 for 20 min on ice and incubated with 0.5% BSA in PBS for 20 min at room temperature. For this study paraformaldehyde fixation was chosen in order to preserve the morphology of the cells and antigenicity of the lectin receptor sites; indeed, our preliminary experiments showed that, analogously to an embryonic human epithelial line (Bolognani Fantin et al., 1989), the above fixation was proper for lectin histochemistry.

Permeabilization-fixation

In order to verify the effects of other permeabilization methods, we also treated some serum-deprived cells with buffer A (0.5% Triton X-100, 300 mM sucrose, 3 mM $MgCl_2$, 100 mM NaCl, 10 mM 1,4-piperazinediethanesulfonic acid), pH 7.4, for 10 min at 4 °C. Cells were then fixed for 20 min at room temperature with 4% paraformaldehyde diluted in PBS. After three washes with PBS, cells were incubated with 0.5% BSA in PBS for 20 min at room temperature.

Lectin binding

Cells treated as above, were stained with the fluorescein isothiocyanate (FITC)- and/or tetramethylrhodamine isothiocyanate (TRITC)-conjugated lectins listed in Table 1. All lectins were diluted in 0.1 M PBS, pH 7.4, and samples were incubated for 1 h at room temperature. Finally, cells were washed three times with PBS and mounted on slides in PBS/glycerol (1:1). Single as well as sequential and/or simultaneous labeling methods were performed.

The nominal specificity of lectins was tested by incubating cells with the respective hapten sugars illustrated in Table 1 at concentrations ranging from 0.2 to 0.4 M; also unconjugated lectins were tested for control experiments line (Bolognani Fantin et al., 1989; Menghi et al., 1989).

Sialidase digestion

Before lectin labeling, cells were incubated with sialidase (neuraminidase, Type V, from *Clostridium perfringens*) at a concentration of 0.5 U/ml in sodium acetate buffer, pH 5.5, containing 10 mM $CaCl_2$ for 1-4 h at 37 °C. Control cells were incubated with the enzyme-free buffer to determine the effect of the buffer solution. The specificity of the enzymatic degradation was tested by immersing cells in sialidase solution and

Table 1. Lectin tested in Py1a cells.

LECTIN (acronym)	SOURCE	CONCENTRATION	SUGAR SPECIFICITY
PNA	*Arachis hypogaea*	0.05 mg/ml	Gal ß-(1-3)-D-Galactosamine
ECA	*Erythrina cristagalli*	0.05 mg/ml	Gal ß-(1-4)-D-Glucosamine
DBA	*Dolichos biflorus*	0.02 mg/ml	α-N-Acetyl-D-Galactosamine
UEA I	*Ulex europaeus*	0.05 mg/ml	α-L-Fucose
LTA	*Tetragonolobus purpureas*	0.05 mg/ml	α-L-Fucose
WGA	*Triticum vulgaris*	0.01 mg/ml	N-Acetyl-D-Glucosamine>>Sialic acid
Con A	*Canavalia ensiformis*	0.05 mg/ml	α-D-Mannose>α-D-Glucose
PSA	*Pisum sativum*	0.05 mg/ml	Fucosylated core region of bi- and tri-antennary N-linked oligosaccharides

then in different lectins having affinity for various sugar moieties (Menghi et al., 1989).

Deacetylation (saponification) technique

Deacetylation was carried out by treating cells with 0.5% KOH in 70% ethanol for 30 min at room temperature prior to sialidase digestion (Menghi et al., 1996).

DNase digestion

Cells were digested with DNase I (from bovine pancreas), 100 U/ml, for 20 min at room temperature and then reacted with lectins. The enzyme was diluted in the buffer A. Control cells were treated with the enzyme-free buffer under the same conditions.

RNase digestion

Cells were submitted to RNase A (from bovine pancreas) at a concentration of 100 mg/ml in buffer A for 60 min at room temperature. Then, the cultures were treated for lectin binding. Controls were performed by incubating cells with buffer A only.

$(NH_4)_2SO_4$ extraction

Cells were treated with 0.25 M $(NH_4)_2SO_4$ for 5 min at 4 °C and processed for lectin cytochemistry. Control stainings were carried out on untreated cells.

Collagenase digestion

Cells were incubated with PBS 0.1M, pH 7.4, containing 0.1 mg/ml collagenase (High Purity, Type VII) overnight at 37 °C. Cells were then washed three times with PBS and incubated with lectins. Control cultures were incubated with lectins without enzyme digestion.

ß-Galactosidase digestion

Some cells were immersed in a solution of ß-galactosidase (Grade VI, from *Escherichia coli*, 455 U/mg Prot.) in sodium citrate buffer 0.05M, pH 3.9, with added 25 mM EDTA and then reacted with lectins (Menghi et al., 1993).

Confocal laser scanning microscope (CLSM) analysis

Fluorescent lectin receptor sites were examined with a Nikon Diaphot-TMD-EF inverted microscope using a x60 oil immersion lens with numerical aperture 1.4 Plan Apo objective. The microscope was attached to the Bio-Rad MRC 600 confocal laser imaging system (Bio-Rad, Hertfordshire, UK) equipped with a krypton/argon laser. Black level, gain and laser intensity, Kalman averaging, excitation intensity, pinhole aperture, and Z-series analysis of cells were carried out as previously detailed (Menghi et al., 1997). Sections were examined and images were stored as PIC format file and then printed with Epson Stylus Photo 700 on Epson glossy photo paper.

Results

Py1a rat osteoblasts showed distinct and selective binding patterns for all the lectins tested. Appreciable differences were not found between single and double labeling method. In addition, double stainings, simultaneously and/or sequentially performed, gave overlapping binding patterns both at the cytoplasm and nuclear level. Similarly, comparable results were obtained using lectins conjugated alternatively with FITC or TRITC. For each lectin, we described the results originating from the enzymatic and histochemical treatments which were significant to gain insight into the nature and composition of the receptor sites.

PNA lectin

In fixed-permeabilized cells, the most evident labeling was observed in the nucleus during mitosis (Fig. 1b, c); binding sites were also present during interphase.

Permeabilization prior to fixation slightly decreased the signal at the nuclear level which preferentially appeared as diffuse spots.

Sialidase digestion

After sialic acid removal, few PNA affinity sites were seen in the perinuclear zone suggesting scarcity of terminal sialic acid linked to ß-galactose (Fig. 2b,c).

KOH/Sialidase digestion

Pretreatment with potassium hydroxide, to remove the acetyl groups of sialic acid, showed a strong positivity, supporting a significant amount of neuraminidase-resistant C_4 acetylated sialic acids linked to ß-galactose, occurring throughout the cell cytoplasm with increased staining in the perinuclear region (Fig. 3b,c).

$(NH4)_2SO_4$ extraction

Permeabilized-fixed cells treated with 0.25M $(NH4)_2SO_4$, in order to extract non-histone nuclear proteins and to maintain chromatin, nuclear matrix and associated RNAs, showed decreased PNA labeling within nuclei of dividing cells (Fig. 4a).

DNase digestion

DNase digestion in permeabilized-fixed cells did not qualitatively affect the labeling (Fig. 4b).

RNase digestion

RNase digestion slightly modified the nuclear PNA binding patterns. For instance, nuclei showed both diffuse peripheral labeling and darker regions in which bright spots were present (Fig. 4c).

DBA lectin

Binding sites showed cytoplasmic location (Fig. 1a,c) which was particularly intense in non dividing cells.

Permeabilization with buffer A prior fixation caused the cytoplasmic DBA reactive sites to disappear and to be internalized into the nucleus (Fig. 5b).

Sialidase digestion

The cleavage of sialic acid prior to DBA labeling induced the appearance of new binding sites primarily in the nucleus except for unreactive dark holes, in contrast the cytoplasmic affinity appeared unmodified (Fig. 2a,c).

The acetate buffer did not modify DBA labeling (Fig. 5a).

KOH/Sialidase digestion

The alcoholic saponification with potassium hydroxide did not increase the sialidase-induced DBA staining suggesting a lack of neuraminidase-resistant sialic acid both at the cytoplasm and nuclear level (Fig. 3a, c).

$(NH4)_2SO_4$ extraction

Cells treated with 0.25M $(NH4)_2SO_4$ and stained with sialidase/DBA showed a labeling similar to that of permeabilized-fixed cells except for the presence of bright spots in the nucleus.

ß-Galactosidase digestion

The removal of terminal ß-galactose residues did not influence DBA affinity indicating that the terminal sequence ß-galactose-N-acetylgalactosamine is not present within glycoconjugates of Py1a cells.

ECA lectin

Numerous binding sites were located at the level of the cytoplasm and were particularly evident in a perinuclear, triangle-shaped, region.

There was a complete superimposition of ECA reactive sites with PSA, WGA and Con A at the triangle-shaped zone supporting the occurrence at this level of N-linked oligosaccharides.

Sialidase digestion

The cleavage of sialic acid before ECA binding did not affect the native affinity sites indicating the absence of the terminal dimer sialic acid-ß-galactose linked to N-acetylglucosamine.

UEA I lectin

UEA I lectin showed reactive sites in the cytoplasm and, in particular, at triangle-shaped zone; variable reactivity was also found at the nuclear level (Fig. 6a).

Nuclear signaling was marked in permeabilized-fixed cells which could be interpreted as mobilization rather than unmasking of accessible residues; indeed, concurrent with the increased nuclear affinity, the cytoplasmic binding disappeared.

$(NH_4)_2SO_4$ extraction

This treatment slightly modified UEA I labeling.

RNase digestion

The enzyme degradation, slightly decreased the nuclear labeling.

LTA lectin

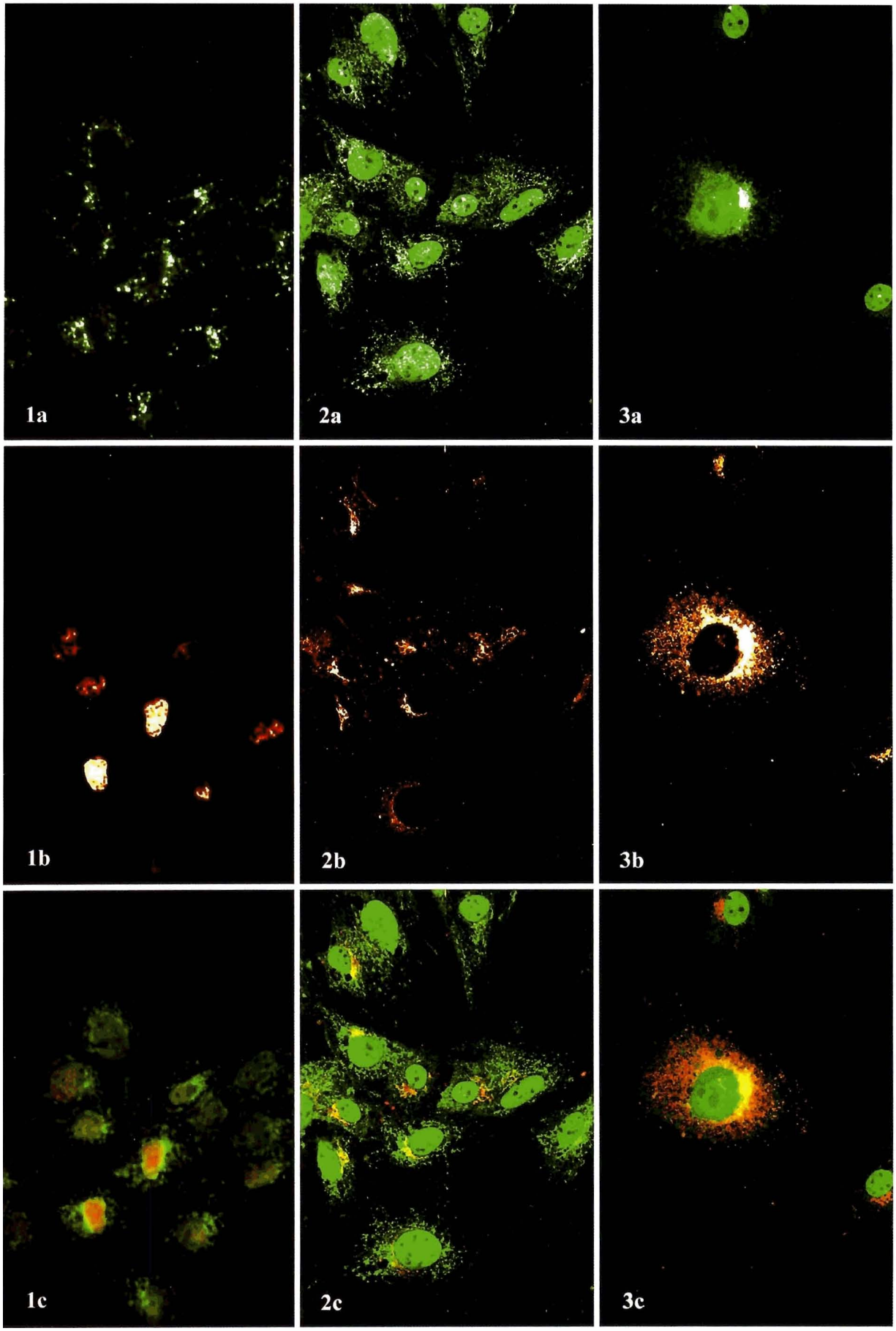
The labeling was primarily located in the cell cytoplasm although a few positive sites were noted in the nucleus of some cells.

All of the treatments tested gave results comparable to those obtained with UEA-I lectin.

Fig. 1. DBA-FITC and PNA-TRITC simultaneous double staining. A more or less evident DBA affinity was found in all cell cytoplasm with particular concentration around the nuclear envelope **(a)**. PNA labeling on the same cells indicated the presence of strongly positive to negative nuclei and always negative cytoplasm **(b)**. Merged image of DBA- and PNA-stained cells confirmed the exclusion of binding sites between the two lectins tested **(c)**. x 600

Fig. 2. Sialidase/DBA-FITC/PNA-TRITC simultaneous double staining. The cleavage of terminal sialic acid promoted the appearance of numerous DBA nuclear receptor sites except for black holes **(a)** and of few PNA perinuclear positive sites in non mitotic cells **(b)**. The superimposition of images supported further the sialidase- induced DBA and PNA reactive sites **(c)**. x 600

Fig. 3. KOH/sialidase/DBA-FITC/PNA-TRITC simultaneous double staining. The removal of acetylated groups did not affect the sialidase-induced DBA binding **(a)** while it strongly increased the sialidase-induced PNA binding, indicating that most sialic acids linked to the subterminal ß-galactose possess acetyl groups at C_4 that prevent the sialidase efficacy **(b)**. The merged image suggested that a codistribution of reactive sites is restricted to the perinuclear region **(c)**. x 750


1a
2a
3a
1b
2b
3b
1c
2c
3c

WGA lectin

Numerous reactive sites were concentrated in the paranuclear triangle-shaped zone. Some binding sites were also present at the nuclear level (Fig. 7a). Permeabilization before fixation strongly modified the binding patterns for WGA. For instance, there was modest labeling of the cytoplasm compared to fixed-permeabilized cells and the triangle-shaped zones were not found; conversely, the nuclear labeling was strongly increased (Fig. 7b).

$(NH_4)_2SO_4$ extraction

$(NH_4)_2SO_4$ extraction markedly decreased the nuclear binding sites that were restricted to the periphery near the nuclear envelope (Fig. 7c).

DNase digestion

DNase digestion after permeabilization-fixation showed spotted intense labeling inside the nuclei sometimes more evident around a less labeled core (Fig. 7d).

RNase digestion

WGA binding consequent to RNase digestion was decreased (Fig. 7e).

Collagenase digestion

No differences were found between collagenase-treated and untreated cells.

ß-Galactosidase digestion

The enzymatic treatment with ß-galactosidase did not produce affinity changes.

PSA lectin

PSA seemed to resemble the WGA labeling at the paranuclear triangle-shaped zone. Conversely, nuclei did not show binding (Fig. 8).

Con A lectin

Con A exhibited numerous binding sites which also appeared as intensely stained aggregates located outside the positive paranuclear triangle-shaped region. Nuclei failed to react (Fig. 9a).

There was codistribution of Con A and PSA binding patterns only in the paranuclear triangle-shaped region.

Also Con A and UEA-I showed codistribution of receptor sites (Fig. 6b,c) indicating that the terminal fucose residues, located in the paranuclear triangle-shaped region, belong to carbohydrate chains bearing mannose in the core region as occurs in many fucoglycoconjugates.

Collagenase digestion

After collagenase digestion, decreased labeling was observed. In particular, the peripheral intensely stained aggregates disappeared leaving "empty" black vesicles (Fig. 9b).

ß-Galactosidase digestion

The removal of terminal ß-galactose induced a few Con A-positive sites in the nucleus (Fig. 9c).

Controls

All control stainings for lectin histochemistry gave negative results lending further to support the nominal specificity of the tested lectins.

Controls for the exoglycosidase digestions confirmed the specificity and efficacy of the treatments as well as the lack of effects consequent to the enzyme-free buffer.

Also controls for all the other enzymatic degradations allowed the minimal influence of the enzyme-free buffer to be ascertained.

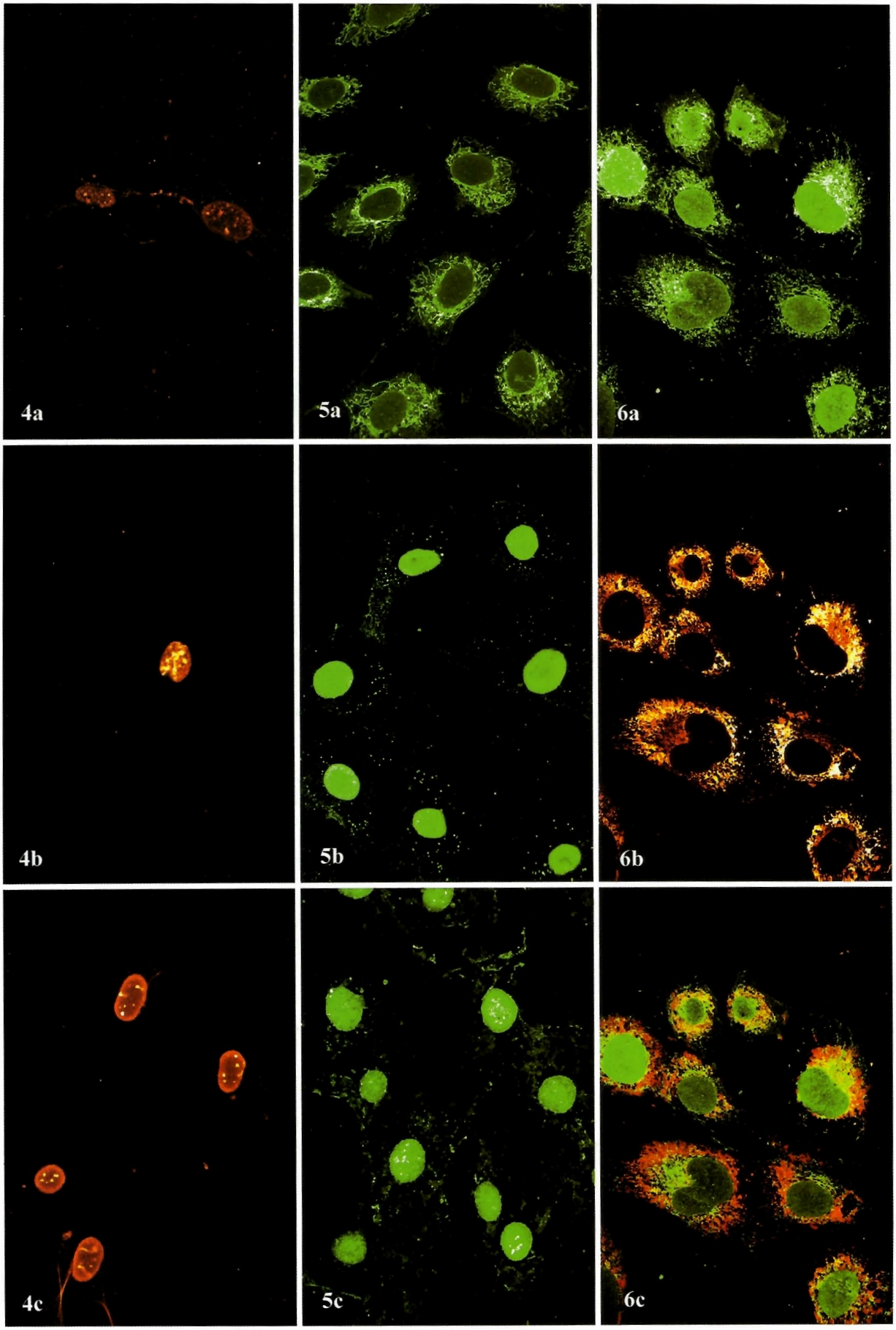
Discussion

The labeling of the rat osteoblasts Py1a was characteristic for each lectin and did not exhibit, except for PNA lectin, obvious differences between proliferating and quiescent cells. PNA labeling, restricted to the nucleus, was particularly evident in dividing cells, during G1 and S phases as well as mitotic phases as previously detailed (Sabbieti et al., 1999b). Taking into account that terminal non-reducing ß-galactose residues were never found in the cytoplasm at any stage of the cell cycle, we propose that during the cell division there is no mobilization of the ß-galactose-1-3-α-N-acetylgalactosamine disaccharide from the

Fig. 4a-c. PNA-TRITC staining after: -extraction with $(NH_4)_2SO_4$ that caused a decrease in the nuclear binding of dividing cells **(a)**, -DNase digestion that did not affect binding **(b)**, -RNase that promoted a characteristic redistribution of nuclear PNA affinity **(c)**. x 600

Fig. 5a-c. DBA-FITC staining of Py1a cells treated with sialidase-free acetate buffer that did not affect the cytoplasmic binding **(a)**. Buffer A caused a mobilization of DBA reactive sites to the nucleus **(b)**. $(NH_4)_2SO_4$ extraction further increased the DBA nuclear binding in form of white spots **(c)**. x 600

Fig. 6a-c. UEA I-FITC and Con A-TRITC simultaneous double staining. Note the distribution of UEA I- **(a)** and Con A-positive sites **(b)** that show co-localization restricted to a paranuclear triangle-shaped region and differential localization in the rest of the cytoplasm **(c)**. x 750


4a
5a
6a
4b
5b
6b
4c
5c
6c

cytoplasm to the nucleus. Condensation or exposition of terminal non-reducing ß-galactose moieties recognizable by PNA lectin is most likely due to the fact that removal of terminal ß-galactose did not promote DBA binding in the corresponding nuclear sites. Conversely, part of the PNA nuclear receptors were found to be characterized by the sequence ß-galactose-glucose as deduced by findings originating from Con A labeling after ß-galactosidase digestion. In particular, the PNA lectin receptors might be involved in modulation of DNA synthesis and mitotic events rather than in phenotypic differentiation due to the fact that in non-dividing cells the nuclear PNA binding is very weak or absent, probably because they are already bound to endogenous lectins as occurs in differentiated cells. It is of interest that galectin-3, a galactose/lactose-specific lectin has been found in association with RNP complexes in many animal cells and has been identified as a splicing factor (Dagher et al., 1995). Mouse 3T3 fibroblasts, fixed with paraformaldehyde and permeabilized with Triton X-100, yielded both nuclear and cytoplasmic staining with anti-galectin-3 (Hubert et al., 1995); in this case, the nuclear staining of galectin-3, was sensitive to RNase but not to DNase, implicating the association of the lectin with RNP structures. Also other authors have found that galectin-3 is present in the nucleus of mouse 3T3 fibroblasts in the form of ribonucleoprotein complexes (Wang et al., 1992). In particular, studies about the expression and regulation of galectin-3 in rat osteoblastic cells have demonstrated that galectin-3 mRNA and protein expression increases with time *in vitro* concomitant with other markers of osteogenesis, including expression of osteoblast-associated markers such as bone sialo-protein and osteocalcin (Aubin et al., 1996). In addition, owing to the potential ability of nuclear lectins to reversibly interact with nuclear glycoproteins as different as DNA polymerase alpha, transcriptional factors, and nuclear pore proteins (Hubert et al., 1989), nuclear lectins could be multifunctional regulators that can influence the binding between exogeneous lectins and related hapten sugars.

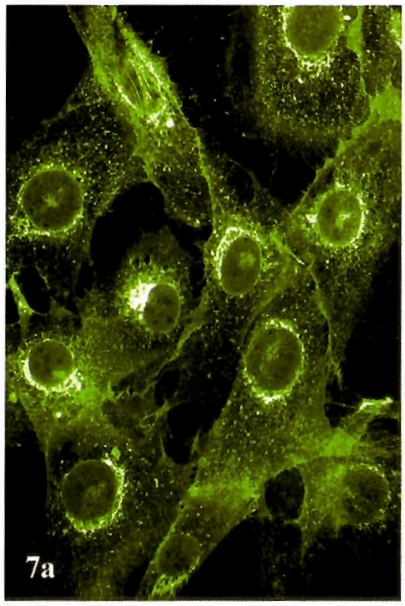
In addition to ß-galactose and sialic acid linked to N-acetylgalactosamine, we visualized at the level of the nucleus, receptor sites for LTA, UEA I, and WGA indicating the presence of fucose and N-acetyl-glucosamine residue. By comparing our data with previous ones about the nuclear lectin receptor sites, it should be noted that carbohydrate binding proteins have been detected in the nucleus of different cell types. In the nucleus of mammalian cells, a 43-kDa glycoprotein, concentrated over the dense chromatin periphery and interchromatin granule clusters, has been found to specifically bind WGA lectin, but not Con A nor UEA I, supporting the notion that p43 is a glycoprotein bearing an N-acetylglucosamine moiety (Soulard et al., 1991). Conversely, chromatin glycoproteins recognized by Con A have been isolated from pig, rabbit, and chicken tissues and appear to be tissue-specific rather than species-specific (Ferraro et al., 1989; Eufemi et al., 1991). Glycoproteins are mainly present in the group of proteins which are tightly bound to DNA; their tissue specificity, together with their capability to interact not only with DNA but possibly also with other nuclear components, suggested a role for these proteins in the mechanism of genome expression. Carbohydrate content of non-histone proteins from pig liver chromatin has been measured in different groups of chromatin fractions and did not seem to be related to the affinity of the proteins for DNA. Glycoproteins have been preferentially located in the nuclease-sensitive fractions of chromatin. A 59,000 dalton glycoprotein has been identified as a characteristic component of a chromatin fraction solubilized by DNase II and has been found to contain galactose, glucose, mannose, and xylose (Ferraro et al., 1988). Glycoproteins recognized by *Ulex europaeus* I have been identified in chromatin prepared from pig liver, heart and kidney nuclei and have been found among the proteins dissociated with urea and guanidine. In any case, these fucose-containing glycoproteins appear to have a marked tissue specificity, which suggests that they have specific regulatory roles in the processes occurring in the nuclei (Cervoni et al., 1990).

In the cytoplasm of the rat osteoblasts Py1a, we found a complete overlapping of reactive sites between LTA and UEA-I lectins which have similar nominal specificity for terminal α-L-fucose with some differences in reacting with fucoglycoconjugates in accord with the link between fucose and its neighbor (Allen et al., 1977; Sugii et al., 1982). The colocalization of fucose-recognizing lectins and PSA, specific for the fucosylated core region of bi- and tri-antennary N-glycosidically linked oligosaccharides (Kornfeld et al., 1981), further attest to the occurrence on the triangle-

Fig. 7. WGA-FITC staining. Affinity sites are mainly present in the cytoplasm where they are concentrated in a paranuclear triangle-shaped zone **(a)**. Buffer A caused the confinement of reactive sites to the nucleus **(b)**. $(NH_4)_2SO_4$ extraction strongly decreased the nuclear binding induced by buffer A **(c)**. DNase digestion produced the exposition of nuclear positive sites above all near to the nuclear envelope **(d)**. RNase digestion decreased the nuclear binding except for white spots **(e)**. a, b, c, e, x 600; d, x 1200

Fig. 8. PSA-TRITC staining. Note the most reactive sites located on the triangle-shaped paranuclear zone and the lack of binding at nucleus level. x 600

Fig. 9. Con A-TRITC staining. Numerous positive sites are located in the cytoplasm with particular evidence in peripheral white vesicles **(a)**. Collagenase digestion caused the loss of the most reactive peripheral vesicles **(b)**. ß-Galactosidase did not affect the cytoplasmic binding patterns but induced some reactive sites in the nucleus **(c)**. a, b, x 1000; c, x 600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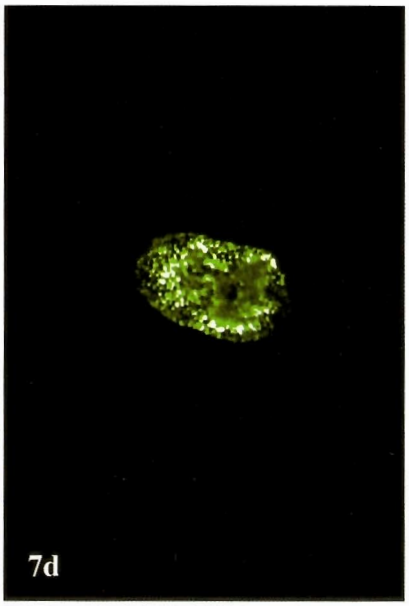
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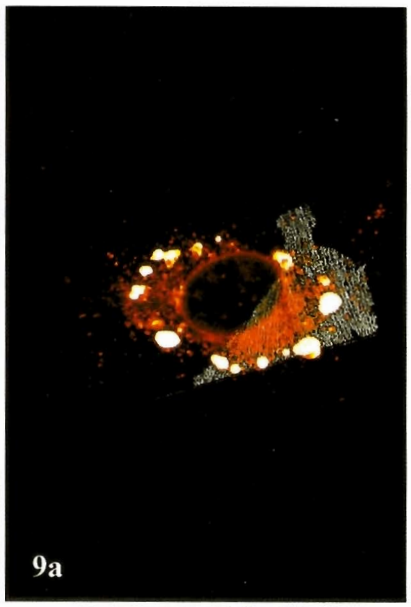
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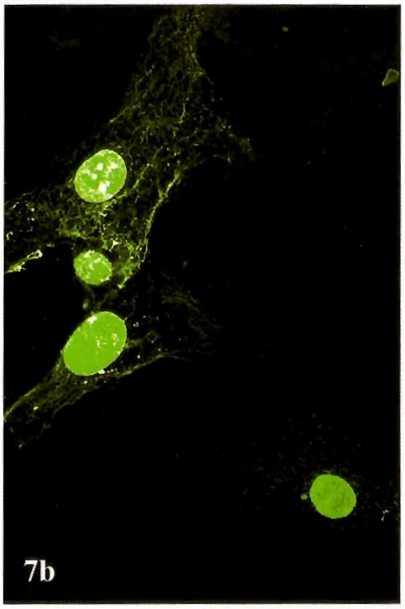
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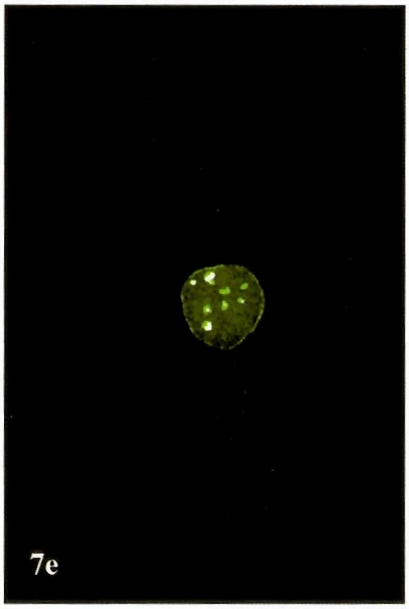
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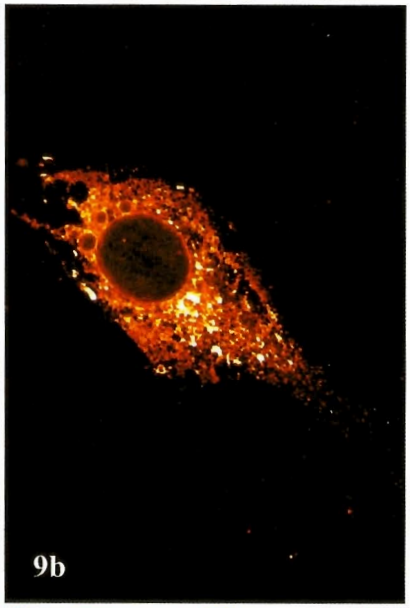
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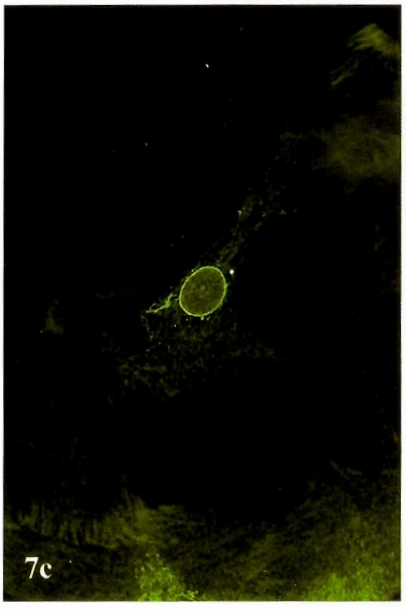
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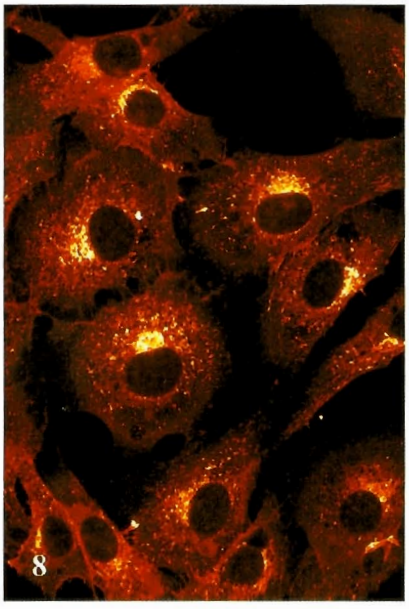
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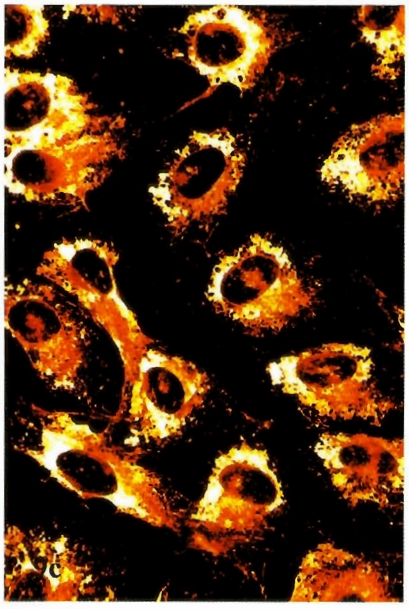
9c

shaped zone, probably the Golgi apparatus, of secretory glycoproteins typical of the bone matrix. For example, the phosphorylated glycoprotein osteopontin contains 16.6% carbohydrates (fucose, mannose, galactose, N-acetylgalactosamine, N-acetylglucosamine, N-acetyl-neuraminic acid), possibly including 1 N-linked oligosaccharide and 5-6 O-linked oligosaccharides; in particular, of the more than 30 residues of carbohydrate, about one-third are sialic acid linked to ß-galactose (Price et al., 1987; Butler, 1989). On the other hand, this terminal sequence was found in the cytoplasm of Py1a osteoblasts by using sialidase followed by PNA lectin. In addition, we found that part of the sialic acid residue is acetylated at carbon 4, as deduced by applying the KOH/sialidase/PNA technique.

Perinuclear and peripheral cytoplasmic Con A receptor sites appeared to be, by enzymatic digestions, structurally different. In particular, the peripheral cytoplasmic, vesicles-like, Con A receptor sites seem to belong to procollagenous units confined into deep cell membrane invaginations as deduced by the loss of Con A staining consequent to collagenase digestion. The remaining paranuclear cytoplasmic reactive sites support the occurrence of N-linked glycoconjugates whose presence was also confirmed by the concomitant distribution patterns of ECA, Con A, WGA, PSA, LTA, and UEA I. Indeed, in addition to osteopontin (Price et al., 1987), osteonectin also possesses a complex type oligosaccharide, as observed in the osteoblast-like cell lines SaOs-2 and MG-63, unlike the high mannose moiety found on bone matrix-derived osteonectin (Kelm et al., 1992). In the cytoplasm of Py1a cells we also found the presence of terminal α-N-acetylgalactosamine that has been previously found to be differentially expressed during odontogenesis. In this case, the authors suggested that growth hormone may regulate this component which may be a proteoglycan or a glycoprotein, essential for normal growth of the teeth (Zhang et al., 1994). In a clonal osteosarcoma cell line, UMR 106-06, competitive binding studies by lectins to compete for binding of EGF revealed that Con A, WGA, and to a lesser extent LCA (lentil lectin) could inhibit binding. It has also been found that treatment of cells with sialidase resulted in total loss of binding while α-glucosidase, ß-N-acetyl-glucosaminidase and α-mannosidase were without effect, indicating a specific interaction of EGF with terminal sialic acid residues of the EGF receptor (Moseley and Suva, 1986). Probably the removal of other carbohydrate residues that appeared not to modify EGF binding was ineffective due to their internal position.

In conclusion, the aim of the research was to develop a scientific basis for evaluating the influence of prostaglandins and endocrine disrupting chemicals on the glycosidic components of the Py1a osteoblasts. A preliminary report focused on the effects of $PGF_{2\alpha}$ has already been published (Sabbieti et al., 1998). Research is also in progress in order to evaluate the individual or synergic action of phthalate esters on FGF-2 and lectin receptor sites in Py1a cells.

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