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Lectin cytochemistry on developing rat submandibular gland primary cultures

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Summary. Lectin cytochemistry was performed in vitro on primary cultures from the rat submandibular gland. For this purpose, prepubertal rats (17, 27, 33 days old) of both sexes were used. Several types of medium supplements were tested and it was found that cells survived until 15 days in presence of all medium supplements and extracellular matrix gel. The binding patterns of all FITC/TRITC-labeled lectins, with and without prior sialidase digestion and deacetylation, were analyzed in a confocal laser scanning microscope. In particular, the occurrence of C_4 acetylated sialic acid linked to ß-galactose at day 27 and the presence of fucose residues at day 33 indicated that lectin probes applied to cultured cells give results similar to those obtained in intact tissues and can be used as markers of growth and differentiation.

Key words: Lectins, CLSM, Primary cultures, Submandibular gland, Developing rat

Introduction

Lectins are sugar-binding proteins or glycoproteins of non-immune origin, which recognize saccharides with appropriate complementary sequences (Liener et al., 1986); accordingly, the affinity of different lectins to specific sugars make them useful as histochemical probes also *in vitro* (Sabbieti et al., 2000).

Over the past few years the authors have experimented lectin histochemistry to characterize at light, confocal and electron microscopy level, surface and cytoplasmic glycoconjugates of mammal salivary glands (Menghi et al., 1989, 1992, 1997, 1999; Menghi and Materazzi, 1994; Accili et al., 1996).

In particular, in rat developing submandibular glands, the use of lectins, combined with enzymatic digestion, pointed out marked variability, showing differential reactivity of secretory elements not only at different stages, but also within each stage testifying to progressive differences in carbohydrate content and arrangement (Accili et al., 1999, 2001) and according to Keryer et al. (1973), who have biochemically demonstrated that carbohydrate components are the parameters which vary most with age and sex, whereas peptic components only slightly vary.

In the present research, we investigated primary cultures obtained from prepubertal rat submandibular glands by means of specific and appropriately selected lectins, conjugated to fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) fluorochromes also in combination with enzymatic degradation.

The goal of the present study was to plan appropriate growth protocols for the rat submandibular gland primary cultures in order to investigate the capability of cultured cells to synthesize fuco- and sialo-glycoconjugates.

The occurrence and spatial distribution of lectin reactive sites were investigated and analyzed in a Confocal Laser Scanning Microscope (CLSM). Before lectin staining, appropriate growth protocols were planned; in particular, medium supplements and extracellular matrix were tested in order to study short- and long- term effects on cell secretory activity.

Materials and methods

Rat submandibular gland primary cultures

Prepubertal Wistar white rats (17, 27, 33 days old) of both sexes were used. At day 17 distinction between sexes was not performed because sexual dimorphism is just beginning at day 27. At day 33 submandibular glands from males and females were separately cultured. Animals were purchased from Morini (San Polo d'Enza, RE, Italy) and sacrificed according to the recommendation of the Italian Ethical Committee and under the supervision of authorized investigators. Submandibular glands were promptly excised and used for primary culture preparation.

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After Hank's Balanced Salt Solution rinsing (HBSS: 5.3 mM KCl, 0.4 mM KH_2PO_4, 120 mM NaCl, 4.2 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 5.7 mM D-glucose, 15 mM HEPES, 0.5 mM $MgCl_2x6H_2O$, 0.8 mM $MgSO_4x7H_2O$, 1.12 mM $CaCl_2$ anhydrous), pH 7.4, glands were cut and cells were enzymatically dispersed with Collagenase type I A (Sigma) and Hyaluronidase IV-S (Sigma) on vortex for 30 min. Comparison with *in situ* experiments indicated that glycoconjugate profile was not appreciably modified by hyaluronidase digestion. Cellular suspension was centrifuged at 600 rpm and rinsed three times in HBSS solution. Cells were then dispersed on nylon filter, and the eluate was collected, centrifuged and placed in DMEM/F12 (1:1 mix) (Gibco BRL) containing penicillin and streptomycin (Sigma). Some samples were grown in the presence of 2.5% fetal calf serum (Gibco BRL), while other ones were grown in absence of it. Finally, cells were plated on previously cleaned and sterilized glass coverslips contained in six-well culture dishes (Costar) at a density of 10,000-12,000/cm^2. Some glass coverslips were preventively treated with extracellular matrix (ECM) gel (Sigma). ECM gel is composed primarily of laminin, collagen type IV, heparan sulfate proteoglycan and entactin. All cultures were maintained at 37 °C and 5% CO_2.

In some experiments 20 nM sodium selenite (Gibco BRL), 5 μg/ml transferrin (Gibco BRL), 1.1 mM hydrocortisone (Sigma), 50 mg/ml insulin (Sigma), 2 nM T3 (Sigma) or 80 ng/ml epidermal growth factor (Sigma) were added to primary cultures maintained in serum-free conditions. To test the effect of each single medium supplement on cell growth and differentiation, parallel cultures were carried out by alternatively removing only one medium supplement.

Lectin binding

Cells grown for different days were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer saline solution (PBS), pH 7.4, at room temperature for 20 min. After three rinsings in PBS, cells were incubated in 0.3% Triton-X100 and 1% bovine serum albumin (BSA) in 0.1 M PBS, pH 7.4, at room temperature for 30 min. After rinsing, primary cultures were subjected to single or double labeling for 90 min at room temperature with *Arachis hypogaea* (PNA, recognizing ß-Galactose), *Dolichos biflorus* (DBA, recognizing α-N-Acetyl-D-Galactosamine), *Lotus Tetragonolobus* (LTA, recognizing α-L-Fucose), *Triticum vulgaris* (WGA, recognizing N-Acetyl-D-Glucosamine >> sialic acid) and *Canavalia ensiformis* (Con A, recognizing α-D-Mannose > α-D-Glucose) lectins conjugated to FITC or TRITC and diluted in 0.1 M PBS, pH 7.4, containing 0.3% Triton-X100 and 1% BSA, respectively as previously detailed (Sabbieti et al., 2000). Then, cells were rinsed several times in PBS and coverslips were mounted on slides with PBS/glycerol (1:1).

Controls were performed by incubating cells with lectin solutions added with their specific competing sugars at concentrations ranging from 0.2-0.4 M (Menghi et al., 1989).

Sialidase digestion

Before labeling as described above, some cells were incubated at 37 °C for 60 min with sialidase (neuraminidase, Type V, from Clostridium perfringens) at a concentration of 0.86 U/ml in 0.1 M sodium acetate buffer, pH 5.5, containing 10 mM $CaCl_2$(Bolognani Fantin et al., 1989).

In order to verify the specificity and efficiency of the enzymatic degradation, control cells were incubated in the enzyme-free buffer solution under the conditions above.

Deacetylation technique

Deacetylation was carried out by pretreating cells with 0.5% KOH in 70% ethanol for 30 min at room temperature (Sabbieti et al., 2000).

Analysis by CLSM and image acquisition

The binding patterns were analyzed in an Ar/Kr Bio-Rad MRC-600 Confocal Laser Scanning Microscope (Bio-Rad, Hertfordshire, UK) attached to a Nikon Diaphot-TMD-EF inverted microscope equipped with a Plan Apo, oil-immersion, objective (x 60, NA=1.4). The instrument was routinely calibrated as previously detailed (Sabbieti et al., 2003). Image acquisition was performed by a PIC format file and then printed with an Epson Stylus photo 890 on Epson glossy photo paper.

Results

Primary cultures were prepared at different post-natal developmental stages. Day 27 represents a crucial step since granular tubule differentiation is just beginning (Gresik, 1994); in addition, day 27 was selected to study the secretory function of cultured cells since we had previously demonstrated that only at this stage acinar cells synthesize, in intact tissue, C_4 acetylated sialic acids (Accili et al., 2001). Day 33 was selected to identify convoluted granular tubule cells; however, differences between sexes were never found.

In order to maintain primary cultures in physiological conditions, the first step was devoted to testing low concentrations of serum since high concentrations of serum in the culture medium could cause an increase in mitosis and a return to a dedifferentiated state in several kinds of cultures.

To evaluate the influence of each medium supplement (MS), namely sodium selenite, transferrin, hydrocortisone, insulin, thyroid hormone T3, and epidermal growth factor (EGF), parallel cultures were prepared by omitting each time a specific MS. A panel

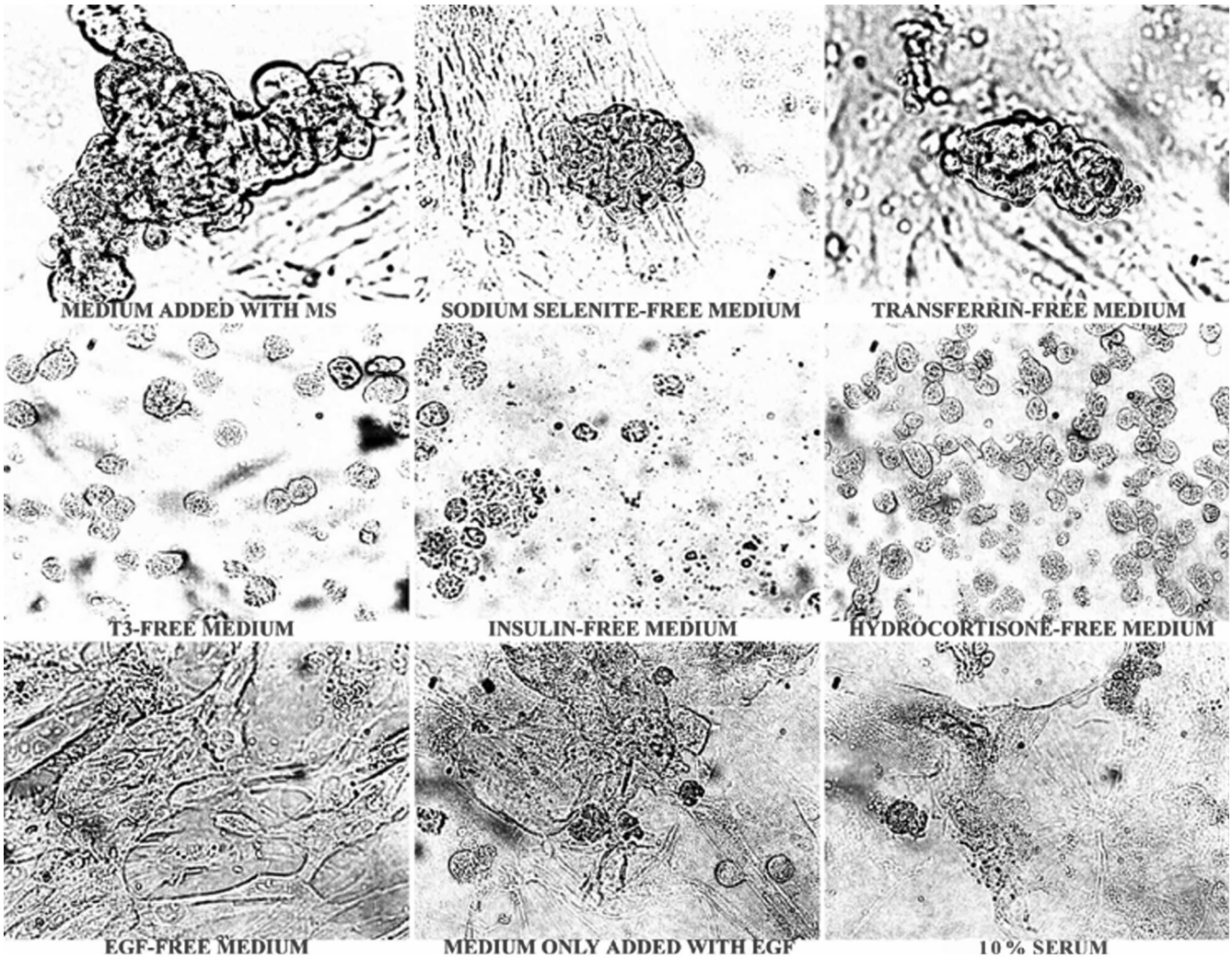

Fig. 1. Light microscopy micrographs of 8-day-old rat submandibular gland primary cultures grown in the presence of ECM. Clusters are evident in the presence of all MSs as well as in sodium selenite- and transferrin-free medium. T3-, insulin- and hydrocortisone-free medium fail to produce cell aggregation but do not influence the cell morphology as occurred with EGF-free medium, only EGF added medium and 10 % serum. Objective x 40

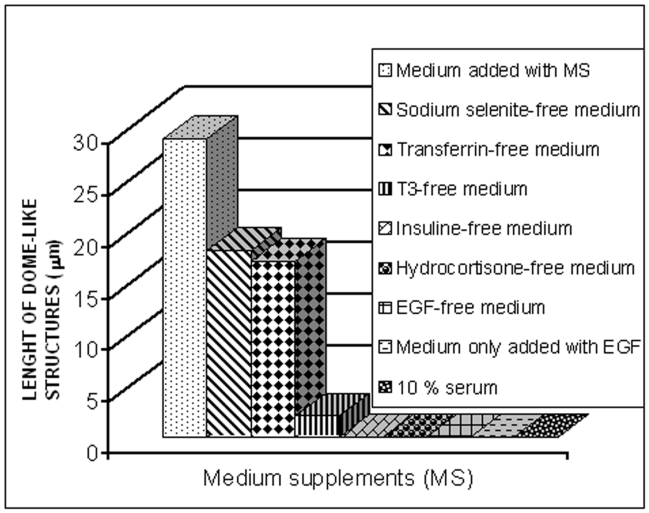

Fig. 2. Effects of MSs and serum on the length of dome-like structures in 8-day-old cultures grown in the presence of ECM. The analysis is performed by CLSM.

of unfixed and unstained cells, at 8 days of culture in the presence of ECM, is shown in Fig. 1. A cluster-like organization of cells was observed in presence of all MSs. The lack of each MS showed appreciable effects after 7 days of culture. In fact, after this period, T3-, insulin- and hydrocortisone-deprived cultures showed a marked reduction in cell aggregation. Cells grown in an EGF-free medium, in a medium with only added EGF or in a medium containing 10% serum alone, lost their original aspect and showed a fibroblast-like shape.

Confocal analysis allowed was to better establish the

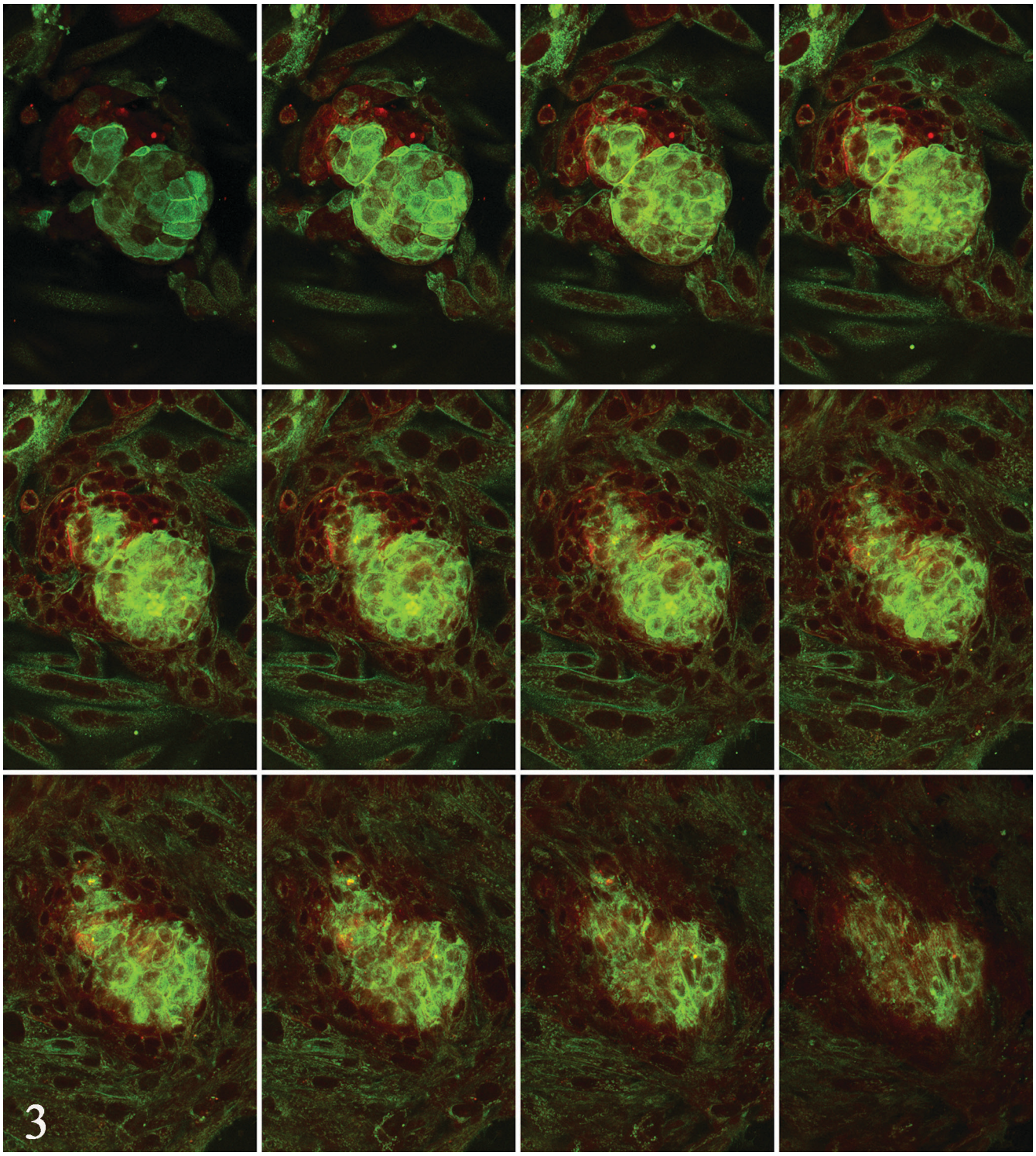

Fig. 3. CLSM. 33 post-natal day male rat. Cells grown in the presence of ECM. 8-day-old primary cultures grown in the presence of all MSs. Number 12 optical sections. Double DBA-FITC (green pseudocolor) and LTA-TRITC (red) staining. Merged images obtained from the apex (top left) to the base (bottom right) of a dome-like structure. x 60

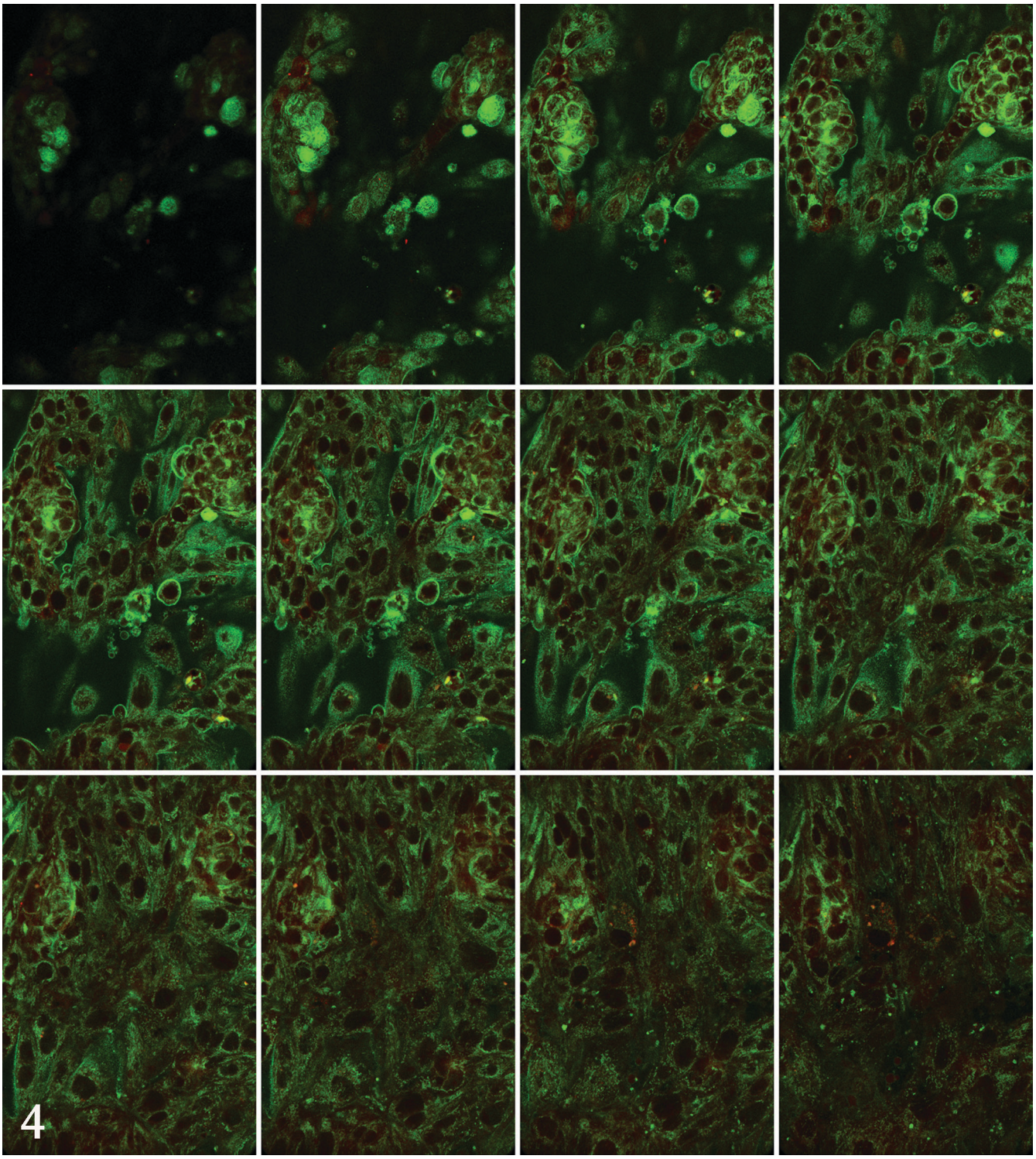

Fig. 4. CLSM. 33 post-natal day male rat. Cells grown in the presence of ECM. 8-day-old T3-deprived cells. Number 12 optical sections. Double DBA-FITC (green) and LTA-TRITC (red) staining. Merged images. Colocalized sites of α-N-Acetyl-D-Galactosamine and α-L-Fucose are not found. x 60

spatial organization of the cultured cells; 4-day-old cultures, grown in the presence of all MSs and ECM, aggregated in cobblestone-like structures. 8-day-old cells showed dome-like structures similar to those of a transfected epithelial cell line (Quissell et al., 1997). Moreover, CLSM allowed the height of dome-like structures on the z-axis to be analyzed (Fig. 2).

All lectins gave results comparable to those obtained in intact tissues (Accili et al., 1999, 2001). The optical sectioning of lectin binding patterns of 8-day-old cultures showed the spatial distribution of reactive sites. In this section we have only documented DBA as a marker of acinar cells and LTA as a marker of convoluted granular tubule cells. The presence of all MSs (Fig. 3) as well as the absence of sodium selenite and transferrin did not appreciably affect growth or DBA and LTA labeling in dome-like structures. Conversely, cell cultures grown without T_3 (Fig. 4) showed a decrease in dome-like structures and LTA binding. Similar results were also seen in insulin- and hydrocortisone-deprived cultures as well as in EGF-deprived cells or cells grown in the presence of EGF alone; indeed, both the dome-like structures and lectin labeling were considerably reduced.

In the present study we also investigated the cell growth and differentiation taking into consideration the influence of the time of culture and the addition of ECM.

Regarding the time of culture, we noted a cell survival until 15 days; after this period of time cells showed evident signals of degeneration when they were analyzed at transmission electron microscope level (preliminary unpublished results).

Up to three days we did not observe differences with or without ECM, while appreciable differences were

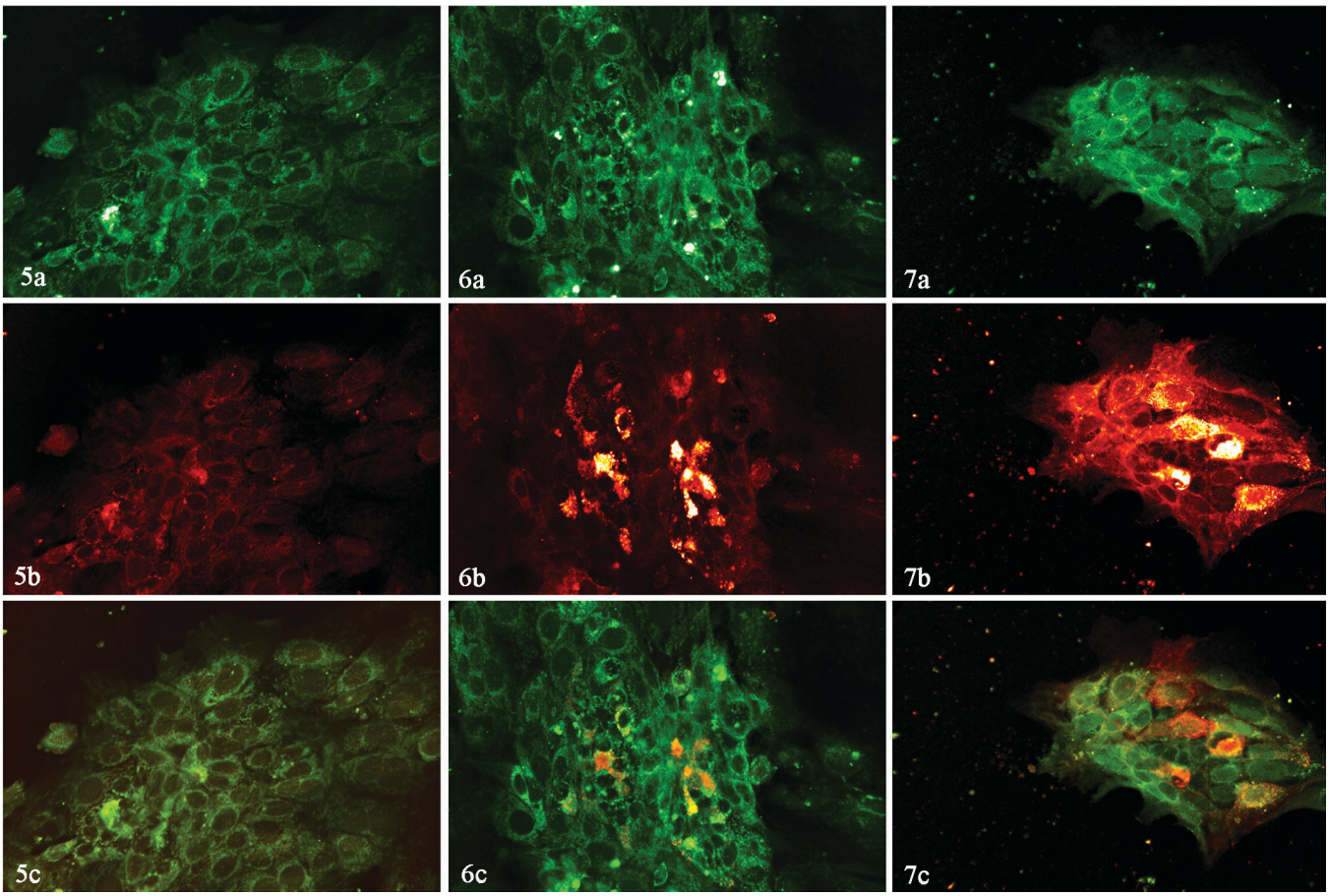

Fig. 5. CLSM. 27 post-natal day male rat. 1-day-old culture with ECM. Optical section. Double DBA-FITC **(a)**, PNA-TRITC **(b)** staining. Merge **(c)**. Note the prominent occurrence of α-N-Acetyl-D-Galactosamine. x 60

Fig. 6. CLSM. 27 post-natal day male rat. 1-day-old culture with ECM. Optical section. Sialidase/DBA **(a)**, sialidase/PNA **(b)** staining. Merge **(c).** Note the only presence of the terminal disaccharide sialic acid-ß-Galactose. x 60

Fig. 7. CLSM. 27 post-natal day male rat. 1-day-old culture with ECM. Optical section. KOH/sialidase/DBA **(a)**, KOH/sialidase/PNA **(b)** staining. Merge **(c)**. Note the appearance of the terminal disaccharide C_4 acetylated sialic acid-ß-Galactose. x 60

noted in the subsequent days. In particular, ECM-free cultures showed an evident alteration of cell aggregation and branching after 10 days.

An additional parameter examined in the present research was the maintenance of the secretory function. For this purpose, we analyzed the presence of sialoglycoconjugates, typical secretory products of intact rat submandibular glands (Menghi, 1984; Accili et al., 2001). 1-day-old cells grown in the presence of ECM showed the results indicated in Figs. 5-7. In particular, DBA and PNA were used in order to study the respective distribution of α-N-acetylgalactosamine and ß-galactose residues in terminal position (Fig. 5) or as acceptor sugars of sialic acid (Figs. 6, 7), whereas WGA and Con A were used to investigate internal sugars like N-acetylglucosamine and mannose. After sialidase digestion few cells showed a marked increase in only PNA labeling (Fig. 6). Also, preventive KOH deacetylation, performed to visualize C_4 acetylated sialic acids resistant to sialidase digestion, resulted in an increased number and intensity of PNA- positive cells (Fig. 7). At this time of culture positive sites for WGA and Con A were also found; in particular, WGA and Con A exhibited binding patterns similar to those of sialidase/PNA staining suggesting the synthesis of N-linked glycoproteins.

ECM-free cultures indicated that ECM produced no relevant effects during the early days of culture on cell morphology and function evaluated on the basis of the expression of different glycosidic products. Up to 3 days, cells grown without ECM showed both typical organization and lectin labeling. Also, after KOH/sialidase/PNA staining, C_4-positive sites were detected (Fig. 8). The effects consequent to the absence of ECM for longer periods are illustrated in Fig. 9 which evidences how ECM is important for the normal

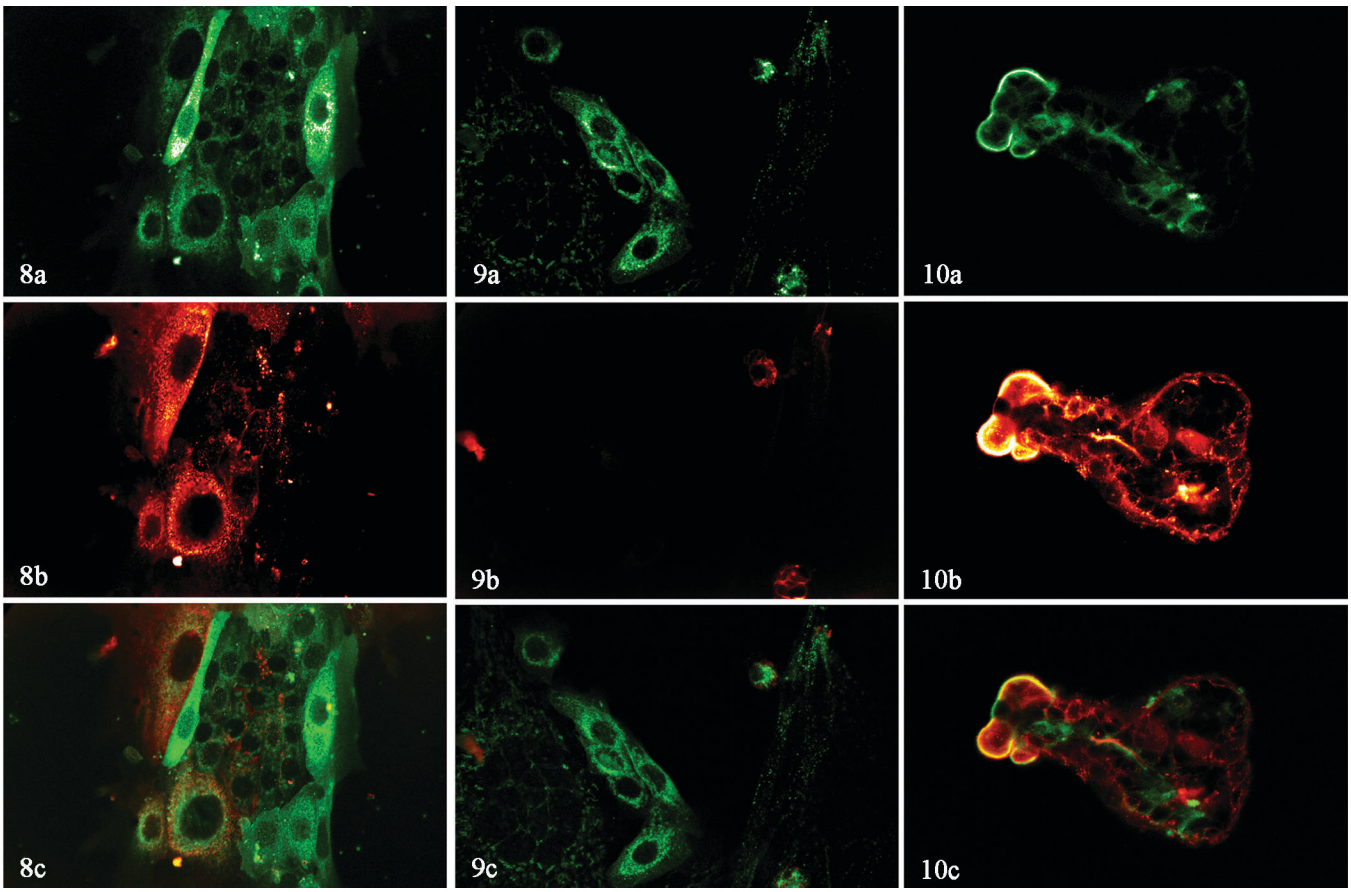

Fig. 8. CLSM. 27 post-natal day male rat. 3-day-old culture without ECM. Optical section. KOH/sialidase/DBA **(a)**, KOH/sialidase/PNA **(b)** staining. Merge **(c)**. Note the presence of exclusive binding patterns. x 60

Fig. 9. CLSM. 27 post-natal day male rat. 13-day-old culture without ECM. Optical section. Double DBA-FITC **(a)**, PNA-TRITC **(b)** staining. Merge **(c)**. x 60

Fig. 10. CLSM. 27 post-natal day male rat. 13-day-old culture with ECM. Sialidase/DBA **(a)**, sialidase/PNA **(b)** staining. Merge **(c)**. x 60

aggregation and the in vitro preservation of the secretory activity. After 10 days of culture (Fig. 10), in the presence of ECM, cells still showed a moderate synthesis of sialoglycoconjugates and the aggregate aspect was found to be very similar to that observed by Hieda and Nakanishi (1997).

Discussion

Primary cultures from rat submandibular gland could represent a valid tool to study the *in vitro* lectin cytochemistry. Distinctive binding patterns were detected with different lectins. These results can provide a basis for the comparison of carbohydrate changes that may occur during salivary gland differentiation.

Experimental procedures carried out by us (Sabbieti et al., 2003) and other authors (Quissell and Redman, 1979; Quissell et al., 1997, 1998) were integrated in order to get a protocol ready for the preparation of dispersed rat submandibular gland cells. The preservation of morphology and secretory products was monitored at light and confocal microscopy level.

On the basis of specific literature (Yang et al., 1982; Anderson, 1986, 1988; Redman et al., 1988; Mork et al., 1996), the influence of different medium supplements was firstly investigated. Moreover, using appropriate lectins (LTA, DBA, PNA, WGA, and Con A combined with sialidase digestion and deacetylation), single glycosidic residues, specific carbohydrate sequences and sialic acid derivatives were detected.

In particular, CLSM analysis was useful for following the effects of each medium supplement on cell aggregation and lectin binding. Indeed, insulin, T3 and hydrocortisone were determinant in survival, maintenance of differentiation and dome-like structure formation. The importance of insulin in stimulating healthy growing of cells from rodent submandibular glands was established by other authors (Yang et al., 1982; Anderson, 1988; Quissell et al., 1994). Several years ago, Scacchi et al. (1988) demonstrated the presence of insulin binding sites in rat submaxillary gland cells. Wigley and Franks (1976) also indicated that salivary cell proliferation is enhanced by insulin and hydrocortisone. More recently, Rocha et al. (2000) showed that insulin receptor is expressed in lacrimal and salivary glands. Dome-like structures are cell aggregates that have been observed in the rat submandibular cells also by Quissell et al. (1997).

Some authors (Wigley and Franks, 1976) selected protease activity as a distinctive product of granular tubule cells to monitor their secretory activity; in the present study, we used LTA lectin as a marker of α-L-fucose residues expressed by male and female convoluted granular tubule cells (Zhang et al., 1994; Menghi et al., 1997).

α-N-Acetyl-D-galactosamine, ß-galactose, and sialic acid were selected as markers of secretory products of acinar cells in which histochemical analyses evidenced the presence of neutral and acid glycoproteins (Quissell and Barzen, 1980; Accili et al., 2001). Biochemical data also demonstrated the presence of 30% galactose, 16% sialic acid and 30% N-acetylhexosamine in submandibular gland acinar cells of growing rats (Keryer et al., 1973).

By applying this method of dispersion and growth, rat submandibular gland cells survived well for at least 8 days and retained glycoconjugate expression at least until 15 days of culture. Previous data indicated 3-7 days of survival for rat acinar cells with retained phenotypical and functional features (Kanamura and Barka, 1975; Quissell et al., 1986; Redman et al., 1988). Although primary cultures could show physiological properties different from those of intact tissues (Hongpaisan et al., 1996), we obtained comparable results in cultured and in situ submandibular gland cells (Accili et al., 1999, 2001).

We also demonstrated that ECM plays an important role in preserving the secretion activity for long-term cultures. It has been shown that branching morphogenesis of mouse submandibular gland is dependent on cell-cell talking between and within epithelium and mesenchyme and such signaling is mediated, analogously to other branching organs, by hormones, growth factors and cytokines (Jaskoll and Melnick, 1999).

In addition to studying glycoconjugates in vitro, primary cultures could also be used for pharmacological and toxicological studies as well as for cellular and molecular biology. Also transduction mechanisms in response to effectors such as isoproterenol or pilocarpine (Bylund et al., 1982; Ryberg et al., 1987) could be investigated.

Finally, tissue engineering could represent a development of primary cultures entrapped on scaffolds; in particular, hydrogels may represent a valid structural support in maintaining phenotypic expression and metabolic function of immobilized cells.

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