Fucose in α(1–6)-linkage regulates proliferation and histogenesis in reaggregated retinal spheroids of the chick embryo

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We have used the lectin from Aleuria aurantia (AAL) which is highly specific for α(1–6)-linked fucose, to examine its effect on chicken retinogenesis in a reaggregation culture system. When dispersed cells of the embryonic chick retina are reaggregated to form histotypic retinospheroids, AAL elicits strong inhibition of spheroid growth. The action of AAL is specific, since its effect is dose-dependent, saturable, and inhibited by an excess of fucose. Fucosidase treatment entirely abolishes reaggregation. In contrast, Anguilla anguilla agglutinin (AAA) binding to fucose in α(1–2)-linkage does not show any effects. Incubation with CAB4—a specific monoclonal antibody for fucose in α(1–6)-linkage—reduces spheroid size and shape. AAL does not much affect primary aggregation, but rather subsequent processes of cell proliferation and histogenesis. In particular, AAL inhibits uptake of bromo-desoxyuridine (BrdU), most efficiently so during days in vitro 2 (div2) and div3. As a consequence, the histological differentiation is entirely disturbed, as evidenced by vimentin immunostaining; particularly, rosettes are not forming and the radial glia scaffold is disorganized. We conclude that glycoproteins exhibiting fucose in α(1–6)-linkage may play major roles in early processes of retinal tissue formation.

Key words: Aleuria aurantia lectin (AAL)/Anguilla anguilla agglutinin (AAA)/glycoproteins/proliferation/retinospheroids/rosettes

Introduction

Glycans are involved in biologically very diverse phenomena (Varki, 1993). In particular, the establishment of complex neural networks during brain development relies extensively on cell–cell specifications mediated through specific glycoepitopes, as indicated by their highly regulated expression during development (Blanks and Johnson, 1983; Liu et al., 1983; Whiteley and Scott, 1990; Lis and Sharon, 1991; Flaris et al., 1995; Song and Zipser, 1995). The list of glycoepitopes with significant biological functions is continuously increasing. The HKN-1 epitope with its sulfated glucuronic acid glycan structure is involved in neuritic differentiation (Keilhauer et al., 1985; Riopelle et al., 1986; Poltorak et al., 1987; Griffith et al., 1992). As other examples, α(2–8)-linked polysialic acid (PSA) is an essential functional constituent of the embryonic form of NCAM (Rutishauser, 1996); and peanut agglutinin (PNA) that recognizes Gal-β(1–3)-GalNAc, binds to areas avoided by neurite growth (Liu et al., 1988; Layer, 1990; Layer and Alber, 1990; Oakley and Tosney, 1991; Alber et al., 1994). Although belonging to the more rare terminal sugars, fucose may be of particular significance, as is evidenced by its major role in fertilization mechanisms (Johnston et al., 1998).

Lectins are tools to characterize glycoepitopes, since each lectin binds preferentially to distinct mono-, di-, or oligosaccharides (Kobata and Endo, 1992). Amongst the lectins which specifically recognize fucose are the Aleuria aurantia lectin (AAL) and the Anguilla anguilla agglutinin (AAA). AAL has been isolated from the fruiting body of the common orange peel mushroom (Kochibe and Furukawa, 1980; Debray and Montreuil, 1989; Fucumori et al., 1990); it has been used to isolate fucosyl-rich proteins (Hall and Karlsson, 1985; Ohlson and Karlsson, 1983; Ohlson et al., 1985). AAL is composed of two identical nonglycosylated 36 kDa protein subunits, each possessing one sugar binding site. Besides weaker interactions with various determinants (e.g., Fuc-α(1–2)-Gal; or the Lewis X-antigen Gal-β(1–4)-Fuc-α(1–3)-GlcNAc), AAL has its highest affinity towards fucosyl residues that are linked α(1–6) to the proximal GlcNAc residue of N-glycans (Yamashita et al., 1985). In contrast, AAA recognizes fucose in α(1–2)-linkage. As another tool to distinguish both types of fucose linkages, the antibodies CAB2 or CAB4 are highly specific for α(1–6)-linked fucosyl residues (Crandall and Newell, 1989; Srikrishna et al., 1997).

In order to investigate a role for fucose-containing glycoproteins in proper development of the retina, we have probed the effect of AAL on an in vitro model of the reaggregating retina of the chick embryo. Fully dispersed cells of the 6-day-old chick embryo develop into histotypic spheres, after their reaggregation on a rotating culture system (Layer and Willbold, 1994). After primary reaggregation, the cells proliferate and, within a period of a week, highly organized areas that are homologous to all retinal layers can be characterized by appropriate markers. In particular, future photoreceptors are predominantly found within inner rosettes (equivalent to an outer nuclear layer, ONL), while ganglion cells are distributed...
within a neuritic matrix resembling an inner plexiform layer (IPL). We here report that incubation in the presence of AAL leads to a drastic change of development of retinospheroids. The concentration-dependent effects are specific for this lectin. Primarily, growth of spheroids due to diminished cell proliferation during the first days of culture is affected, but not so much primary reaggregation. The effects of AAL on differentiation and histogenesis of the spheroids are documented by changes of the spheroid size, of rosette formation, and particularly, of the radial glial marker vimentin (Willbold et al., 1997). We conclude that glycoproteins with α(1–6)-linked fucose are involved in the cell regulation of retinogenesis.

Results

**AAL inhibits growth of retinospheroids, particularly during the early period of reaggregation**

Rosetted spheroids are spherical reaggregates that regenerate in rotation culture of dissociated neuroepithelial cells from E6 chick eye central retinal tissue. The first cellular event is the aggregation of single cells into so-called primary rosettes, followed by proliferative and differentiative processes (Layer and Willbold, 1994; Layer et al., 1998). During that time, the size of aggregates and their state of histological differentiation increase (see below). The final appearance after 10 days of culture of untreated rosetted spheroids is depicted in Figure 1. The drastic difference in spheroid size becomes immediately evident by comparing a nontreated (Figure 1, left) with an AAL-treated sample after 10 days in vitro (Figure 1, right). Spheroid diameters in AAL-treated samples are less than ½ at div3, to increase to about ⅔ by day 6 and day 10 of culture. Temporally, the volume increase of control spheroids is most pronounced between div1 and div3 (see Figure 3, black bars). While staying always viable, AAL-treated spheroids (5 µg/ml) grew much slower than control spheroids and thus presented a much smaller aggregate size throughout the experiment (Figure 3, striped bars). Noticeably, within the first day, the difference in size is only minor indicating that primary aggregation of cells is not significantly disturbed by AAL. The difference in spheroid size becomes most pronounced at div3. The fact that only few rosettes are discernible by div5 (data not shown) indicates the low degree of tissue differentiation. These findings are further detailed by applying AAL for only a 1 day period, either at culture day div0, or div1, div2, or div3, to then document their growth after a total period of 5 days (Figure 2). The inhibition of growth in all these cases—although always detectable—is less pronounced than compared with a permanent application of AAL. Remarkably, rosettes are not
AAL acts specifically on fucose: fucose coincubated together with AAL counteracts the growth inhibition of spheroids by the lectin. Average diameter of spheroids is shown over a culture period of 5 days. Black (controls), striped (plus 5 µg/ml AAL), dotted (5 µg/ml AAL plus 20 mM fucose); 250 spheroids in each series were measured. Data are given as means ± SD and tested for statistical significance by Student’s t-test (p < 0.01).

Fig. 3. AAL acts specifically on fucose: fucose coincubated together with AAL counteracts the growth inhibition of spheroids by the lectin. Average diameter of spheroids is shown over a culture period of 5 days. Black (controls), striped (plus 5 µg/ml AAL), dotted (5 µg/ml AAL plus 20 mM fucose); 250 spheroids in each series were measured. Data are given as means ± SD and tested for statistical significance by Student’s t-test (p < 0.01).

detected, if AAL incubation occurred at div0 or div1, while a later application of AAL allowed formation of rosettes (arrows in Figure 2, lower right). This indicates, that histogenesis is severely disturbed, if AAL acts on the cells during the first 2 days of reaggregation. Thereby, the effect of AAL treatment on spheroid growth depends on the concentration of the applied lectin (Table I). Up to 1 µg/ml AAL, the decrease of spheroid diameter is constant from approximately 220 µm to 130 µm, while higher concentrations (from 2.5 to 10 µg/ml) of the lectin does not induce a further growth inhibition. Therefore, the effect of AAL is concentration-dependent and saturable.

AAL acts highly specifically on Fuc α(1–6)-linked N-glycans

The effects as induced by AAL on spheroid growth are highly specific. Growth inhibition of rosetted spheroids by AAL could be abolished by coincubation with 20 mM fucose. Figure 3 shows the average diameter of rosetted spheroids under control (black bars), AAL-treated (striped bars), and under AAL plus fucose-treated conditions (dotted bars) between div1 and div5. As morphologically documented in Figure 1, control spheroids increase their diameter from 70 to 230 µm between div1 and div5, while AAL-incubated aggregates less than doubled their size within the same time. When fucose (20 mM) was added together with 5 µg/ml AAA to the medium, the spheroids reached about 80–90% of the diameter of control aggregates. Thus, by competing off binding of AAL to spheroids, fucose is able to minimize the growth inhibition induced by AAL. When spheroids were incubated with 20 mM fucose only, they developed like control aggregates (data not shown). To further attest the essential role of fucose during spheroid formation, reaggregation was performed in the presence of 100 mU/ml fucosidase (Figure 4). Irrespective of whether AAL was added or not (cf. Figure 4, left and right), reaggregation of dispersed cells and spheroid growth were entirely abolished. Cells mostly remained dispersed, forming only irregular cell clumps, which stayed not viable longer than 3–4 days. The strong and additive effects of fucosidase plus AAL (cf. Figure 4, lower) indicate that not only AAL-sensitive fucose, but fucose in general exerts a most essential role in retinal reaggregation.

AAL acts specifically on α(1–6)-fucose linkages, as shown by two independent experiments. First, we incubated spheroids with another fucose-specific lectin (Anguilla anguilla aggumin; AAA; Figure 5). In contrast to AAL, which recognizes fucose in α(1–6)-linkage, AAA binds to fucose with a strong preference to α(1–2)-linkage. Figure 6 shows the average diameter of control spheroids (black bars), AAL incubated spheroids (striped bars; 5 µg/ml), and of aggregates treated with 5 µg/ml AAA (dotted bars) from div2 to div5. In contrast to the action of AAL, application of AAA showed no inhibition of spheroid growth. In a second experiment (Figure 6), spheroids were raised in the presence of CAB4, a monoclonal antibody highly specific for fucose in α(1–6)-linkage (Crandall and Newell, 1988). At the applied conditions, the shape and the size of spheroids is changed (Figure 6, right). In particular, cells appear more loosely packed in the antibody treated sample (cf. Figure 6, left, upper and lower). Thus, both experiments document that AAL binds to fucose in a sterically highly specific manner.

AAL inhibits BrdU-uptake during reaggregation

Since growth of aggregates is mainly due to cell proliferation during the early culture period (see Layer and Willbold, 1994), we investigated the effect of AAL on BrdU-uptake (Figure 7). Lectin-treated and control spheroids of different developmental stages (div1–div4) were supplemented with BrdU 16 h prior to fixation. BrdU-labeled cells were visualized by a BrdU-specific antibody on sections (Figure 7, right), from which the rate of proliferation could be determined quantitatively (Figure 7, left). At div1, in both types of spheroids we could find 35–40% proliferative cells. Then, in untreated spheroids the percentage of BrdU-uptake decreased continuously (by 30–50%/day). In contrast, AAL-incubated spheroids showed a much more rapid reduction of BrdU-incorporation from 40% to 5% between div1 and div2, further decreasing during the following days. Two BrdU-labeled sections show the significant difference in cell division at div4 (Figure 7, right). Evidently, AAL exerts an antiproliferative effect on spheroids which is most pronounced until div2. This result coincides with the data on growth

Table I. Decrease of spheroid diameter as a function of AAL concentration

<table>
<thead>
<tr>
<th>Conc. AAL (µg/ml)</th>
<th>Spheroid diameter (µm)</th>
<th>Diameter (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>220.0</td>
<td>100.0</td>
</tr>
<tr>
<td>0.01</td>
<td>175.3</td>
<td>79.7</td>
</tr>
<tr>
<td>0.1</td>
<td>169.6</td>
<td>77.1</td>
</tr>
<tr>
<td>1</td>
<td>119.2</td>
<td>54.2</td>
</tr>
<tr>
<td>2.5</td>
<td>72.4</td>
<td>32.9</td>
</tr>
<tr>
<td>5</td>
<td>80.7</td>
<td>36.7</td>
</tr>
<tr>
<td>10</td>
<td>66.9</td>
<td>30.4</td>
</tr>
</tbody>
</table>

Spheroid diameter was determined at div2. Note that growth inhibition is saturable and reaches a maximum at 2.5 µg/ml AAL. Statistics as in Figure 3.
inhibition by AAL, supporting the notion that the increase of spheroid size is due to cell proliferation. Moreover, the AAL effect on proliferation could be greatly reduced by fucose. As an example, Table II presents the relation between BrdU-uptake and spheroid diameter at div3 for control spheroids, AAL-treated spheroids (5 µg/ml) and spheroids incubated with 5 µg/ml AAL plus 20 mM fucose. Thereby, lectin-treated spheroids presented only 50% BrdU-positive cells and 41% of the diameter of the control, whereas in the presence of fucose only a slight reduction of both parameters occurred.

**AAL inhibits differentiation and histogenesis in retinospheroids**

To follow differentiative events in AAL-treated spheroids, we compared the histotypical architecture of 6-day-old untreated and AAL-treated spheroids by immunostaining their Müller glia system with a vimentin-specific antibody (Figure 8). In control aggregates, the radial glial scaffold is highly organized. In Figure 8 (upper and middle), large rosettes and several IPL-like areas are nicely outlined. Radial glial processes originate from an outer limiting membrane (OLM) at the internal face of the rosette to extend radially through an INL-like nuclear area, to finally concentrate within IPL-like areas. In contrast, AAL-treated spheroids showed a low degree of tissue organization (Figure 8, lower). While a rough distinction in nuclear areas and fiber-rich areas was indicated, a clear laminar formation is missing. In particular, rosettes and individual radial processes could not be resolved. This shows that AAL interfered strongly with the histotypical formation of spheroids.

**Discussion**

The specific sugar composition of glycoproteins is responsible for a number of molecular and cellular functions, including intercellular and intracellular target recognition (Lasky, 1992; Song and Zipser, 1995). We have used a well-established retinal reaggregation system of the chick embryo to investigate the effect of the fucose-specific lectin AAL on retinogenesis under...
Fucose regulates proliferation and histogenesis in chick embryo retina rotation culture conditions. For many questions, this system is superior to monolayer cultures, since it allows to create three-dimensionally organized spheres that resemble a normal retina in many respects. As has been shown by many markers, spheroids are highly homologous to a normal embryonic retina. In particular, constituents of all retinal layers are reestablished (Layer and Willbold, 1994).

Effect of AAL is highly specific

We have focused on fucose as the target sugar, since it is well-known to be developmentally significant (for references, see Introduction). Evidently, this holds also true for retinal reaggregation, since both AAL or/and fucosidase treatment strongly inhibited spheroid growth. Fucosidase, affecting all terminal fucose residues, elicited a much more dramatic response than AAL on reaggregating retinal cells, almost entirely abolishing spheroid formation. The additive effect of fucosidase plus AAL treatment was remarkable, since it indicates that fucosidase has a pleiotropic effect on cellular reaggregation, while AAL may affect distinctively early cell cluster formation (possibly primary rosette organization, see below).

A number of experiments showed that AAL acts highly specifically. First, the growth inhibitory effect of AAL is dose-dependent and saturable, whereby the necessary concentration of the lectin is very low (1 µg/ml). This action can be blocked by fucose, albeit only at high sugar concentration. A better inhibitory effect should be achieved by a (di-)saccharide containing fucose in α(1–6)-linkage, reflecting specifically the stereochemical constitution of the respective receptor glycoconjugate. That the type of sugar linkage is relevant, was demonstrated independently by two experiments. With the first, we showed that AAA—another fucose-specific lectin—does not show similar effects as AAL. Moreover, incubation in the presence of the monoclonal antibody CAB4 (Crandall and Newell, 1988; Srikrishna et al., 1997, 1998) reduced the size of spheroids and changed the packaging of cells. The effects induced in the presence of AAL and fucose, the rate of dividing cells is 81%, not far off the control value (cf. Figure 3).

Table II. Comparison between BrdU uptake and spheroid diameter

<table>
<thead>
<tr>
<th>BrdU-positive cells (%)</th>
<th>Diameter of spheroids (%)</th>
<th>Experiment</th>
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<tbody>
<tr>
<td>100</td>
<td>100</td>
<td>Control</td>
</tr>
<tr>
<td>50</td>
<td>41</td>
<td>+ 5 µg/ml AAL</td>
</tr>
<tr>
<td>96</td>
<td>81</td>
<td>+ 5 µg/ml AAL + 20 mM fucose</td>
</tr>
</tbody>
</table>

Note that in the presence of AAL and fucose, the rate of dividing cells is 81%, not far off the control value (cf. Figure 3). Statistics as in Figure 3.
by CAB4 were only weak. It is possible that the antibody did not optimally reach the relevant cells within the growing spheroids, or, alternatively, the antibody did not fully interfere with the particular fucose functioning. Nevertheless, both experiments taken together establish that AAL acts on spheroid formation specifically via fucose in \(\alpha(1\rightarrow6)\)-linkage. Thus, our findings have revealed that the distinct steric arrangement of terminal fucose in \(\alpha(1\rightarrow6)\)-linkage plays a decisive role in retinogenesis.

Fig. 8. Histogenesis of AAL-treated spheroids is disturbed, as revealed by anti-vimentin immunostaining of cryosections from spheroids raised for 6 days in absence (control, upper and middle) and presence of 10 \(\mu\)g/ml AAL (lower). The high power picture shows a large rosette and several IPL-like areas in control (middle), establishing the organized radial glia scaffold, while in the AAL-treated sample histogenesis is highly disturbed (below). Scale bar, 100 \(\mu\)m.

An early distinct AAL action during histogenesis of retinospheroids?

For understanding why AAL acts during a specific early time window, it is necessary to consider the early processes of spheroid formation. After the embryonic retinæ have been dissociated, the dispersed cells rapidly reaggregate in vitro (Sheffield and Moscona, 1970; Steinberg, 1970). A decisive step is a „sorting out” of particular cells organizing into primary rosettes. These are mostly mitotic, representing the precursors for the different retinal cell types (Layer et al., 1990). Rosettes grow bigger to produce more mitotic cells. New-born daughter cells are organized in radial cell columns, held in space by Müller glia cells (Layer and Willbold, 1994; Layer et al., 1997; Rothermel et al., 1997). Their organization was clearly affected by AAL, as shown by vimentin immunostaining. Rosettes are detectable by various means, the simplest of them being a mere microscopic inspection, as has been applied here. Since rosettes represent the core of retinal histogenesis in spheroids (Fujisawa, 1973; Sheffield and Moscona, 1970; Layer et al., 1990; Layer and Willbold, 1994, for review), their missing indicates that histogenesis in a respective spheroid sample is disturbed.

The BrdU studies have revealed that cell proliferation is strongly inhibited in the presence of AAL. By applying AAL during specific periods of spheroid development, we could discern that during the first day cell division is not much affected; rather, inhibition of cell division becomes effective only after the first day of incubation. This is revealed both by the BrdU-quantification and by morphological inspection of spheroids. Thus, cell numbers after primary reaggregation are still normal; in other words, the production of cells that constitute primary rosettes may not be affected by AAL. The effect of AAL becomes detectable only when a second generation of cells is born and begins to differentiate, including photoreceptors, ganglion, amacrine, horizontal, bipolars, and Müller cells. An inhibitory effect of AAL on differentiating cells was indeed revealed by following histotypical differentiation and formation of primary rosettes. Alternatively, AAL could specifically inhibit cell proliferation of particular subtypes of retinal cells. However, this latter possibility is less likely, since a much lower number of rosettes was detected in AAL-treated spheroids.

AAL downregulates cell division most likely either by affecting signal transduction pathways, or/and by changing the cellular microenvironment. The effect of AAL must be achieved by binding to a specific AAL receptor. A number of cell adhesion molecules have been shown to be involved in retinal histogenesis, including cadherins (Takeichi, 1987), members of the IgG superfamily (Rutishauser, 1996) and the HT7 antigen (Fadool and Linser, 1992). In particular, NCAM appears a good candidate for AAL action, since a Fuc-\(\alpha(1\rightarrow6)\)-GlcNac residue on NCAM is essential to allow synthesis of
polysialic acid (PSA; Kojima et al., 1996). Thus, AAL by binding to NCAM would inhibit the sialyl transferase to attach to NCAM, and therefore inhibit PSA formation. A similar requirement of the AAL binding site on the P0 MAG has been shown to interact with NCAM, and therefore inhibit PSA formation. A similar binding to NCAM would inhibit the sialyl transferase to attach polysialic acid (PSA; Kojima et al., 1996). Thus, AAL by binding to NCAM would inhibit the sialyl transferase to attach to NCAM, and therefore inhibit PSA formation. A similar requirement of the AAL binding site on the P0 MAG has been established for the HNK-1 epitope (Burger et al., 1992). As the next step of analysis, we shall characterize the receptor for AAL.

In summary, this study has demonstrated that fucosyl plays an essential role in retinal reaggregation. AAL appears to affect a distinct early step of spheroid formation, most likely rosette formation. This, in consequence, affects proliferation to then disturb the entire spatiotemporal order of spheroid formation. In vivo studies are underway to show how AAL can interfere with normal retinogenesis. Since rosette formation represents a severe complication in a number of ophthalmic disorders (Layer et al., 1998), AAL might become a valuable tool not only to investigate retinogenesis but it also may have biomedical impact. To this end, the primary action of AAL, in particular the characterization of the AAL receptor(s) has to be further analyzed.

Materials and methods

Tissue preparation

Eyes of embryonic day 6 (E6) White Leghorn chicken were isolated, washed with F12 medium, and dissected free of connective tissue. The eyes were cut around the ora serrata, the lens and the vitreous body were removed, and the central part of the retina was isolated and collected in F12 medium on ice. For details, see Willbold and Layer (1994).

Retinospheroid production by rotary culture

The isolated tissue was incubated in 1 mg/ml trypsin (Cooper Biomedical/Technicon, Bad Vilbel, Germany) in F12 medium for 2 min at room temperature, washed in F12 medium and gently dissociated into single cells with a fire-polished Pasteur pipette in the presence of 0.05 mg/ml DNase I (Worthington, NJ, USA). After three washes in F12 medium, the retinal cells were resuspended in aggregation medium (10% fetal calf serum, 2% chicken serum, 1% glutamine, 0.1% penicillin/streptomycin, 0.02 mg/ml gentamicin in Dulbecco’s Modified Eagle medium (DMEM)); 2 ml suspension of retinal cells were reaggregated in 35 mm plastic dishes (eq. to two eyes per dish; Greiner, Nürtingen, Germany). After three washes in F12 medium, the retinal cells were resuspended in aggregation medium (10% fetal calf serum, 2% chicken serum, 1% glutamine, 0.1% penicillin/streptomycin, 0.02 mg/ml gentamicin in Dulbecco’s Modified Eagle medium (DMEM)); 2 ml suspension of retinal cells were reaggregated in 35 mm plastic dishes (eq. to two eyes per dish; Greiner, Nürtingen, Germany) by rotation on a gyroratory shaker (self made, 70 r.p.m., 37°C, 96% air, 4% CO2). The medium was changed every 3 days. Viability of spheroids was established by staining with 100 µl diacetyl-fluorescein solution (6 µl/ml of a 5 mg/ml DAF solution in acetone). Green fluorescence of spheroids indicates their viable state.

Lectin, fucosidase and monoclonal antibody CAB4 incubations

Aleuria aurantia agglutinin (AAL; Boehringer, Mannheim, Germany) was used at a concentration of 5 µg/ml; Anguilla anguilla agglutinin (AAA; Sigma, Deisenhofen, Germany) was used at concentrations of 5 and 10 µg/ml; α-L-fucosidase (EC 3.2.1.51, 10 units/mg protein, Sigma) was used at a concentration of 100 mU/ml. Final concentration of the antibody was 1:10 dilution of the original supernatant (generous gift of Drs. H. Freeze, J. Newell, and G. Srikrishna, La Jolla, CA). Along with the change of media, the lectins, α-L-fucosidase and CAB4-antibodies were added freshly to the aggregation medium. If not otherwise stated, lectins, α-L-fucosidase, and antibodies were present throughout the entire culture period. Inhibition of AAL was carried out by adding 20 mM α-L-fucoside to the culture medium. Along with each experiment, controls were carried out which were treated with the salt solutions in which the lectins, α-L-fucosidase and mAb CAB4 were dissolved. None of these controls showed an effect (data not shown).

Uptake of 5′-bromo-2′-deoxyuridine (BrdU) and growth assay

During the indicated periods, retinospheroids were treated with 50 mM BrdU for 16 h (final concentration; Boehringer, Mannheim, Germany). Subsequently, retinospheroids were washed twice with PBS and harvested by centrifugation. Incorporation of BrdU into cells was detected by immunostaining of cryosections from retinospheroids. For preparation of cryosections, see Histological procedures. The dried cryosections were incubated for 10 min with 4N HCl, followed by a wash with PBS (0.1% Triton X-100 in PBS) for 5 min. After neutralization with 0.1 M Na2B4O7, pH 8.5, the sections were washed twice in PBS and once more in PBS containing 5% BSA for 30 min. To detect incorporated BrdU, the sections were incubated with a monoclonal mouse anti-BrdU antibody (5.1 mg/ml; dilution 1:1000; Boehringer, Mannheim, Germany), and then with a Texas Red–conjugated anti-mouse secondary antibody (5 mg/ml; dilution 1:100; Dianova, Hamburg, Germany). BrdU–labeled cells (1500 cells counted each time) in spheroids from 3 independent experiments were counted. Quantification of the total number of cells was achieved by staining the cell nuclei with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI 24653; Merck, Darmstadt, Germany; final concentration 1 µg/ml). Proliferation rate was calculated as follows: % proliferation = BrdU-positive cells × 100/DAPI-positive cells. Growth inhibition due to lectin treatment was calculated as follows: % inhibition = ([% BrdU-positive cells (control) – % BrdU-positive cells (AAL-treated)] / % BrdU-positive cells (control)) × 100.

Histological procedures

For preparation of frozen sections, the retinospheroids were fixed in 4% formaldehyde for 30 min at room temperature. After fixation, the aggregates were washed three times in PBS for 15 min and then transferred into a solution of 25% sucrose in PBS; subsequently, they were sectioned at 10–16 µm on a cryostat (Microm, Walldorf, Germany).

Vimentin immunostaining of frozen sections

Frozen sections were soaked for 30 min in PBSAT (0.1% BSA, 0.5% Triton X-100 in PBS). For vimentin staining, they were incubated for 2 h in a 1:25 dilution of 50 µg/ml monoclonal antibodies directed against bovine vimentin (Boehringer, Mannheim, Germany) in PBS. The sections were washed three times in PBS, followed by an incubation with a Cy3-labeled rabbit-anti mouse IgG (Jackson Immuno Research) for 25 min at a 1:50 dilution in PBS. Finally, the sections were DAPI-stained as described above, washed in PBS, and mounted in Kaisers glycerol gelatin (Merck, Darmstadt, Germany).
Microscopy and photography

Stained sections were inspected and documented with a Zeiss Axioshot microscope equipped with fluorescence optics. Whole retinospheroids were examined with an Olympus IMT-2 or a Leitz Diavert inverted microscope with transmission illumination. Photomicrographs were taken using Kodak TMax 400 films.

Determination of spheroid size

Diameters of aggregates were measured on photomicrographs of whole spheroids. The determination of spheroid diameters was calculated as follows: spheroid diameter (cm) = measured spheroid diameter (cm)/objective magnification × microscope factor × proportion of photomicrograph to negative.

Acknowledgments

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Abbreviations

AAA, Anguilla anguilla agglutinin; AAL, Aleuria aurantia lectin; BrdU, 5'-bromo-2'-deoxyuridine; DAPI, 4',6-diamidino-2-phenylindol dihydrochloride; div, days in vitro; INL, inner nuclear layer; IPL, inner plexiform layer; mAb, monoclonal antibody; PSA, α(2→8)-connected polysialic acid.

References


S. Steleck et al.
Fucose regulates proliferation and histogenesis in chick embryo retina


