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The wheat germ agglutinin binding sites and development of the mitochondria-rich cells in gills of tilapia (*Oreochromis mossambicus*)

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Abstract

By labelling with gold-colloid-conjugated wheat germ agglutinin (WGA), the WGA-binding sites were identified in the apical region of certain mitochondria-rich (MR) cells in gills of freshwater-adapted tilapia (*Oreochromis mossambicus*). Bromo-deoxyuridine (BrdU) injection was used to discern the differentiation of the WGA binding sites during development of MR cells. Double labeling of gill sections of fish 2, 4, 6, and 8 days after a single BrdU injection showed that the BrdU labeled MR cells 2 and 4 days after injection were mostly WGA negative, whereas 6 and 8 days after injection, WGA and BrdU-labeled MR cell increased gradually in number. Furthermore, the ratio of the WGA-positive to total MR-cells was higher in gills of tilapia adapted to low calcium ([Ca] = 0.015 mm) freshwater than in hard freshwater ([Ca] = 1.898 mm). The results indicate that WGA binding site may be a marker expressed late during differentiation of MR cells, and physiologically functional in assisting uptake of calcium when environmental calcium is low. According to their WGA binding and also characteristics their likely function in calcium absorption, the WGA positive MR cells shown in this study are considered to be similar to the β cells described by Pisam et al. (1995).

Introduction

The mitochondria-rich (MR) cell (so called chloride cell) is specialized for ionic regulation in gill epithelia of fish (Hwang and Hirano 1985; Hwang et al. 1989; Goss et al. 1993; Zadunaisky 1996). Several studies have reported on cell renewal and proliferation of the MR cells in response to certain treatments, such as salinity change and cortisol injection (Conte and Lin 1967; MacKinnon and Enesco 1980; Chretien and Pisam 1986; Laurent et al. 1994). These studies showed that the MR cells are terminally differentiated cells and derived from 'undifferentiated' epithelial cells. The generation and differentiation of MR cells can be accelerated or slowed down in response to various hormones, e.g., cortisol, growth hormone and prolactin, or for adaptation to various milieus with varying ion composition.

In recent years, morphological differences in MR cells have been reported, particularly in fish adapted to freshwater (Avella et al. 1987; Laurent and Perry 1990; Pisam et al. 1995; Lee et al., 1996a, b). However, whether these different types of MR cells are differentiated independently from the 'undifferentiated' epithelial cells, or whether the morphological changes reflect different differentiation stages of the MR cells, remains to be determined.

Because morphological characters are often ambiguous and not easy to evaluate, a cellular marker for discriminating different types of MR cells may be useful for studies of the differentiation of the MR cells. Two types of MR cells, α and β, were characterized by transmission electron microscopy (Pisam et al. 1995). The β cells differ from α cells by the existence of electron-dense bodies in the apical region of the cell. Using the chromic acid-silver methenamine technique for staining carbohydrates, Pisam et al. (1995) demonstrated that abundant carbohydrates are accumulated at the apical region of the β cells. Based on this fact, we reasoned that lectins would be an excellent marker for

discriminating various types of MR cells, since lectins have been used extensively as biochemical and histochemical tools for identifying carbohydrate residues distributed on the cell surface (Roth 1983; Li et al. 1995; Van der Heijden et al. 1997) and also for investigating the differentiation of cells (Arenas et al. 1996).

Tritiated thymidine or bromo-deoxyuridine (BrdU) are commonly employed for tracing the differentiation of cells in renewing tissue (MacKinnon and Enesco 1980; Chretien and Pisam 1986; de Fazio et al. 1987; Laurent et al. 1994; Tamatani et al. 1995; Tsai 1996). BrdU is more convenient than tritiated thymidine in several respects (Laurent et al. 1994). Since it is incorporated into proliferating cells during the DNA synthetic phase and retained in the nuclei thereafter. Accordingly, cell differentiation can be traced by a time-course examination on the BrdU-labeled cells.

This study, therefore, was aimed at revealing the lectin binding during differentiation of the MR cells. We expected that the lectin binding might reflect the differentiation of MR cells and thus shed light on the relationship between morphological characteristics and differentiation of the MR cells.

Materials and methods

Fish

Mature adult tilapia (*Oreochromis mossambicus*) from the Tainan Branch of the Taiwan Fisheries Research Institute were reared in circulating freshwater at 27 °C and under a photoperiod of 12–14 h lighting. Juveniles of 30∼40 mm in fork length were used in the following experiments.

Hypotonic milieus and acclimation conditions

For tracing differentiation of the MR cells, the fish were kept in local freshwater (FW). For determining ratio of MR cells with wheat-germ-agglutinin binding sites, the fish were adapted respectively to two hypotonic milieus with different calcium levels. Hard freshwater (HFW) was prepared by adding coral chips in FW (Lee et al. 1996b) and low calcium water (LCW) was prepared artificially by mixing $CaSO_4$, $MgSO_4$, NaCl, K_2HPO_4 and KH_2PO_4 to deionized water (double-deionized water, Milli-RO60, Millipore, USA). Water [Na^+], [K^+], [Ca^{2+}], and [Mg^{2+}] were determined by a Hitachi Z-8000 polaried zeeman atomic absorption spectrophotometer (Table 1).

Ultra-thin and semi-thin sections

Gills of tilapia juveniles were removed, fixed with phosphate buffered 4% paraformaldehyde plus 2% glutaldehyde, pH 7.4 for 8 h at 4 °C, dehydrated in alcohol and embedded in LR white resin (London Resin, U.K.). Polymerization of the resin was performed by incubation of the capsules under UV light at –20 °C for 24 h. Semi-thin (1 μm) and ultra-thin (80 nm) sections were prepared for cytochemical staining.

Lectin-gold labeling

Semi-thin and ultra-thin sections of gill filaments were attached to nickel grids or to slides coated with 0.5% gelatin, respectively. The sections were then blocked with 0.1% bovine serum albumin (BSA-c, Aurion, Netherlands), 0.1% Tween 20 in PBS for 30 min. After blocking, the sections were incubated respectively with wheat germ agglutinin (*Triticum vulgare*, WGA), concanvalin A (*Concanavalia ensiformis*, Con A) and *Ulex europaeus* agglutinin-I (UEA-I) for 30 min. All three lectins were 10 nm colloidal gold conjugated and were supplied by Sigma (U.S.A). They were 1:250 diluted as working solutions in 0.1% BSA-c, 0.1% Tween 20 in PBS. After reaction with lectin, the slides were washed three times with PBS containing 0.1% Tween-20, each for 5 min. Finally, silver-enhancement on the gold particles were performed by incubation for 18 min in aurion R-gent (Aurion, Netherlands) prepared as per the manufacturer's instructions. The sections were then placed in distilled water for 15 min, dried, and counter-stained with toluidine blue (semi-thin sections) or uranyl acetate and lead citrate (ultra-thin sections). Control sections of WGA were processed in parallel with treating excess WGA (10 μg ml^{-1}) for 15 min before the incubation with gold conjugated WGA. All the reactions were performed at room temperature. Photographs were taken using a transmission electron microscope (Hitachi, H-7000) or a light microscope (Olympus, BX60).

BrdU injection

BrdU is a thymidine analog that is incorporated by the proliferating cells during the DNA synthetic phase, and the BrdU is retained in the cell nuclei thereafter, as these cells proceed through G_2 and M phases, divide

Table 1. Elemental concentrations (mM) of three hypotonic milieus

	[Ca]	[Mg]	[Na]	[K]
FW	0.201 ± 0.009	0.129 ± 0.003	0.420 ± 0.067	0.027 ± 0.003
HFW	1.898 ± 0.186	0.312 ± 0.005	0.885 ± 0.063	0.312 ± 0.064
LCW	0.015 ± 0.02	0.160 ± 0.008	0.546 ± 0.013	0.326 ± 0.051

The number of determinations was 7. Values shown were mean ± SD.

to form two daughter cells, or even become differentiated (Laurent et al. 1994; Tamatani et al. 1995). Forty tilapia were used and each individual was injected once intraperitoneally with BrdU in a final dosage of 200 μg per g body weight. One hour later, 4 fish were killed, and 4 more were sampled every 2 days for the following 8 days after the injection. Several gill filaments were removed, and prepared for microscopy as described above.

Double stains of BrdU and WGA

One μm thick cross sections of gill filaments were cut and attached to the slides coated with 0.5% gelatin. The sections were incubated with 2N HCl for 30 min to denature the DNA and make the incorporated BrdU accessible to the BrdU antibody (Van de Kant et al. 1988), then washed thoroughly in distilled water and blocked in 0.1% BSA-c (Aurion, Netherlands) in PBS for 30 min. After blocking, the sections were treated with WGA conjugated 10 nm colloidal gold (Sigma U.S.A., 1:250 diluted) for 30 min, anti-BrdU monoclonal antibody (Boehringer Mannheim, diluted 1:20) for 1 h and goat anti-mouse IgG conjugated 10 nm colloidal gold (Sigma U.S.A., diluted 1:100) for 1 h. These reagents were diluted in PBS containing 0.05% Tween-20 and 10% normal goat serum (Jackson Immuno-research Laboratories, Inc. USA). Between each step, the slides were washed three times with PBS containing 0.1% Tween-20, each for 5 min. Finally, the slides were silver-enhanced for 18 min by aurion R-gent as described above, stopped in distilled water for 15 min, dried, and counter-stained with Toluidine blue. All reactions were performed at room temperature. Photographs were taken with a light microscope (Olympus, BX60).

Cell counting and labeling index

Background in the gill sections that were processed with silver enhancement was low. In the double labeling of BrdU and WGA, only the nuclei with five or more silver grains were identified as BrdU-labeled cells, MR cells with five or more silver grains in the apical region were considered as WGA positive cells. The MR cells are stained lightly and pinkly blue and the other small epithelial cells purplish blue by the toluidine blue staining; in addition, the MR cells are usually much larger than the epithelial cells. Using these two morphological criteria, the MR cells hence were discriminated from the epithelial cells. The labeling index used in this study represents the number of the BrdU labeled nuclei per cross section of gill filament. A whole cross section of gill filament is shown in Figure 1a. One hundred cross sections of gill filament, and all the MR cells in the gill epithelia, were identified and counted in each of five fish to determine the labeling index.

Percent of WGA-labeled MR cells in gills of tilapia adapted to hard freshwater and low calcium water

Two groups of 5 fish were kept in either hard freshwater or low calcium water for 4 days. The gill filaments of the fish were then taken and processed for WGA staining; 100 MR cells were identified and counted in each individual.

Statistics

Numbers of WGA- or BrdU-labeled cells in the sample fish were analyzed by t-test or by a one-way ANOVA (Tukey's pair-wise method).

Results

Lectins histochemistry in gill epithelia of tilapia

A whole cross section of gill filament is shown in Figure 1a. The observation and counting of the labeling of WGA and BrdU on the MR cells were restricted to the overlay epithelium of the gill filament. Among

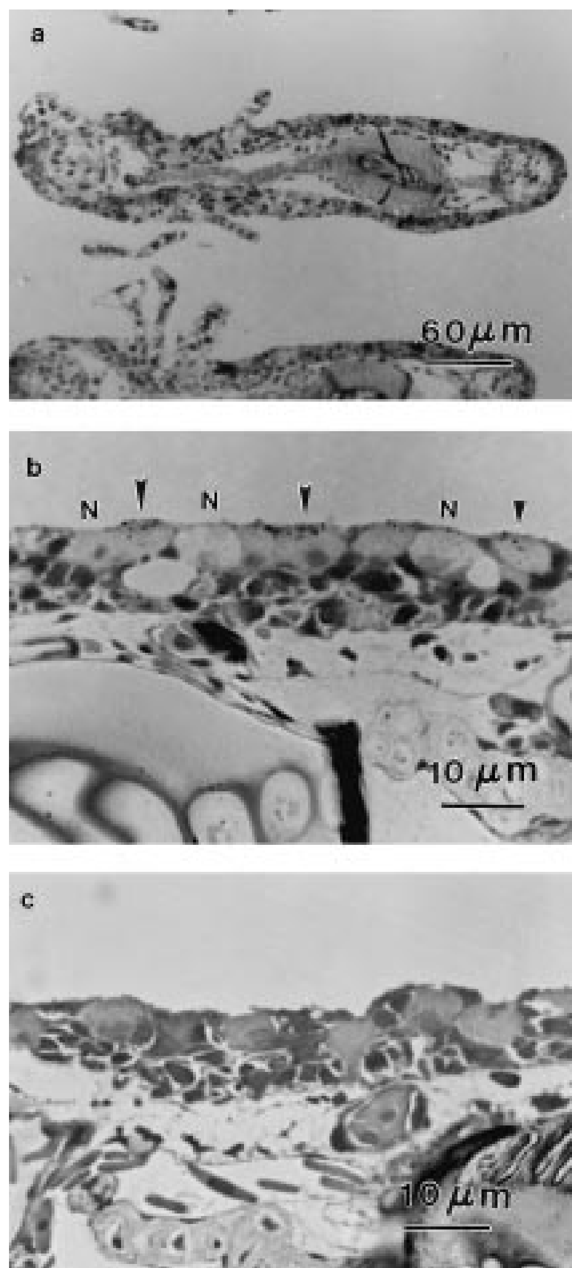

Figure 1. (a) A whole cross section of the gill filament, observation and counting of the labeling of WGA and BrdU on the MR cells were restricted to the overlay epithelium of the gill filament. (b) Localization of wheat germ agglutinin (WGA) binding sites in apical region of mitochondria-rich cells. The WGA positive MR cells, with silver grains more than four, are indicated by arrowheads. Note that not all MR cells are positively stained with WGA. N, WGA negative MR cells. (c) Negative control of the WGA labeling. This was conducted by treated the slide with excess WGA prior the labeling of gold conjugated WGA. No silver grains are seen in the tissue section.

the three lectins used in this study, wheat germ agglutinin (WGA) was the only one to label the MR cells of tilapia gills (Figure 1b). The reaction was confirmed in control sections treated with excess WGA prior the labeling of gold conjugated WGA (Figure 1c); tissues treated in this manner did not bind Con A and UEA-I (data not shown).

Localization of wheat-germ-agglutinin (WGA)-binding sites in apical structure of certain mitochondria-rich cells

The WGA-binding sites are localized by labeling with gold-colloid-conjugated WGA, both by light microscopy and transmission electron microscopy in the apical region of mitochondria-rich cells in gills of freshwater-adapted tilapia. It should be noted that not all MR cells are positively stained with WGA in their apical region. The other epithelial cells are all negatively stained. In electron micrographs (Figure 2a, b) the binding sites are distributed generally around vesicles that accumulated in apical region of the MR cells.

BrdU labeled cells in gill epithelia

The cells labeled with BrdU immediately after injection were all ‘undifferentiated’, no mature MR cells were labeled at that time (Figure 3a). Changes in labeling index are shown in Figure 4, and significant increase in number of the BrdU-labeled cells are seen particularly on days 1 and 7. At 1 h post-injection, 0.86 $\pm$ 0.32 cells per cross-section of gill filament were labeled. The labeled cells increase to 2.80 $\pm$ 0.45 cells at the 1st day, 3.06 $\pm$ 1.11 cells at the 2nd day. The increase was due to the first division of the labeled cells. The labeling index then slowly decline from the 2nd day to the 6th day. It was in the 7th day post-injection, another increase labeled cells (5.08 $\pm$ 1.33) was seen, which would indicate the BrdU-labeled cells divide again after another cell cycle.

Tracing MR cells by double labeling of WGA and BrdU

WGA and BrdU labeling by the silver enhancement method are clearly separated in the mitochondria-rich cells. WGA labeling is exclusively located in apical region, and BrdU labeling in the nuclei (Figure 3b, c). A few BrdU labeled MR cells, i.e., 0.13 cell per gill filament cross-section, appeared first in the 2 day samples, increasing gradually to 0.39 $\pm$ 0.07 on day 4,

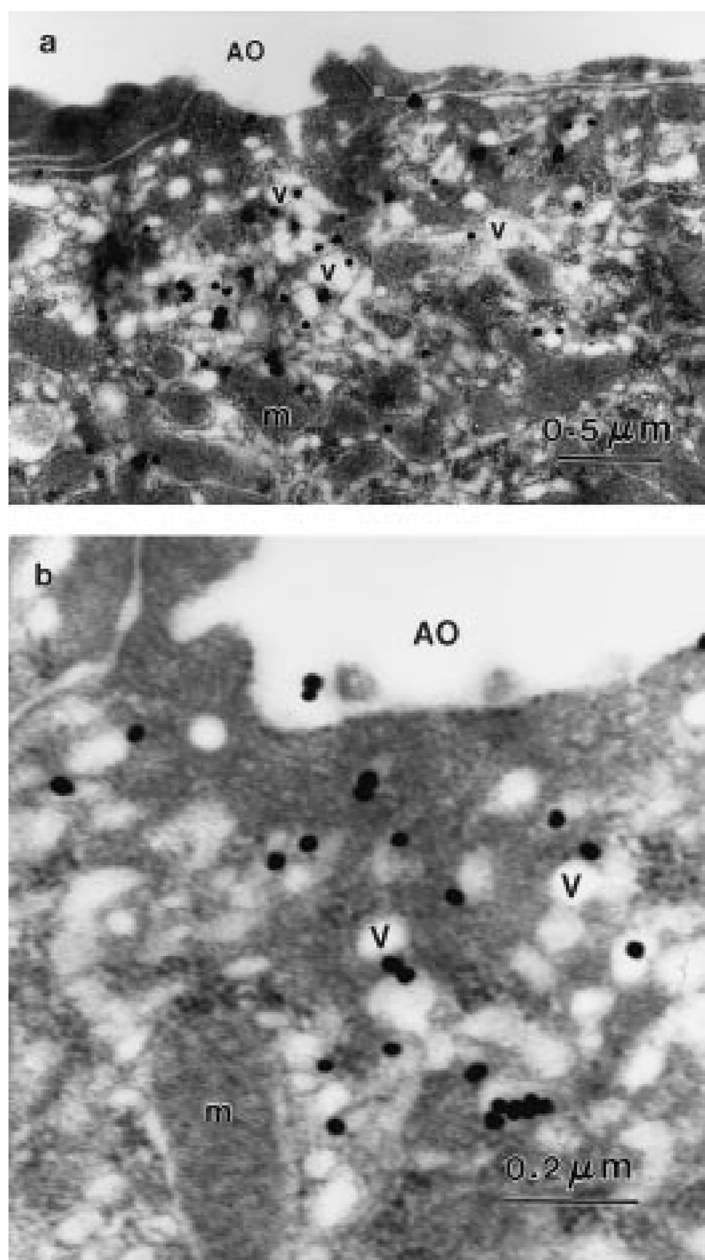

Figure 2. a. & b. WGA binding sites shown by transmission electron microscopy. A0, apical opening of the MR cell; V, vesicles in the apical region. m, mitochondria.

0.68 ± 0.05 on day 6, but had slowed down by day 8 (0.78 ± 0.12 cells) (Figure 5. solid line). Those BrdU-labeled MR cells were further subdivided into two fractions according to the WGA staining in their apical region. After 2 days, all the BrdU labeled MR cells were WGA negative (Figure 5, up-triangle-dashed-line). In samples taken on day 4, a few BrdU labeled MR cells were WGA positive (Figure 5, open-circle-dashed-line), however, most of the MR cells remained to be WGA-negative. In the day 6 sample, the number of WGA positive and BrdU-labeled MR cells was 0.24 ± 0.06, whereas the WGA-negative BrdU-labeled MR cells was 0.43 ± 0.03. A significant change was found in the day 8 sample in which WGA positive and BrdU-labeled MR cells increased significantly to 0.61 ± 0.06, whereas the WGA-negative, BrdU-labeled MR

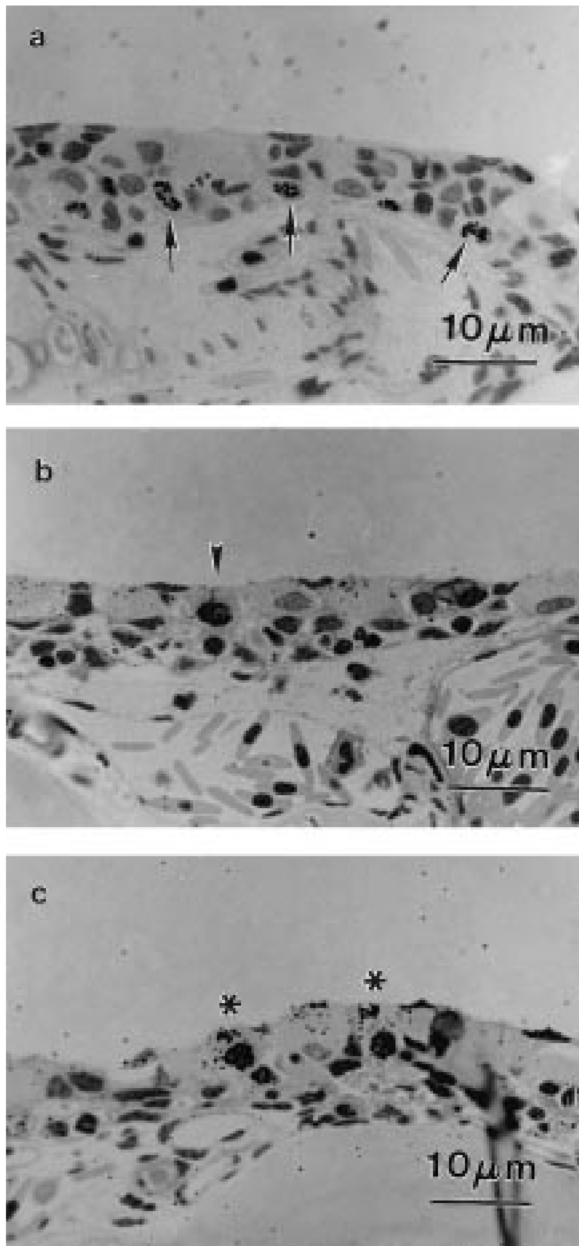

Figure 3. Double labeling of WGA and BrdU in tilapia gills. (a) Several BrdU-labeled cells (arrows) appear 1 hour after BrdU injection. They are all undifferentiated epithelial cells. No MR cell was labeled at this time. (b) A WGA (–) BrdU-labeled MR cell (arrowhead). This cell was found in sample of two-days after injection. (c) Two (WGA+) BrdU-labeled MR cells (asterisks), eight days after injection.

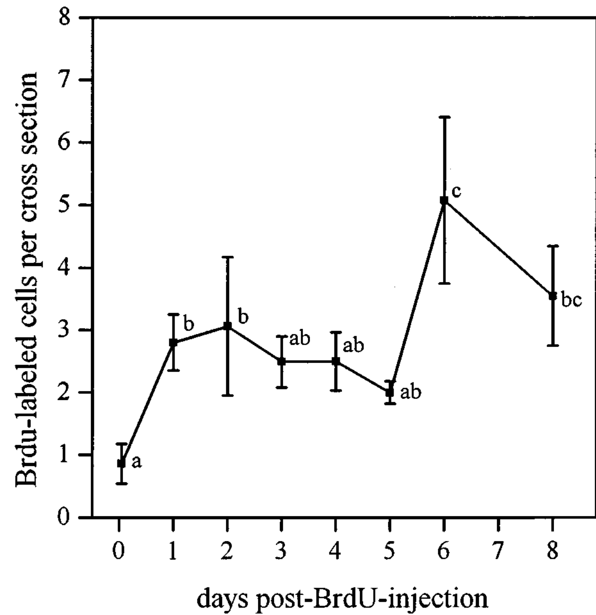

Figure 4. Ratio of BrdU-labeled cells per gill cross-section with time, after a single BrdU injection. The curve connects the mean values. Different letters indicate significant difference as analyzed by ANOVA.

cells decreased, also significantly, to 0.17 ± 0.09 cells per cross-section of gill filament.

Percent of WGA-labeled MR cells in gills of tilapia adapted to hard freshwater and low calcium water

The percent of WGA-labeled MR cells to total MR cells in gills of tilapia adapted to two media were significantly different ($p<0.05$). In gill of tilapia adapted to hard freshwater, 17.7 ± 7.2% of the MR cells were WGA positive. On the contrary, 48.2 ± 6.7% of MR cells were WGA positive in fish adapted to low calcium water.

Discussion

This study demonstrated the existence of sub-cellular structures in the apical region of the MR cells in tilapia gills which could be labeled by wheat germ agglutinin. The binding sites appear late during differentiation of the MR cells, and may participate in calcium uptake by tilapia gills.

The WGA positive MR cells are considered to be similar to the β cells described by Pisam et al. (1993, 1995) and Prunet et al. (1994), because WGA has binding affinity to carbohydrates, and the apical region

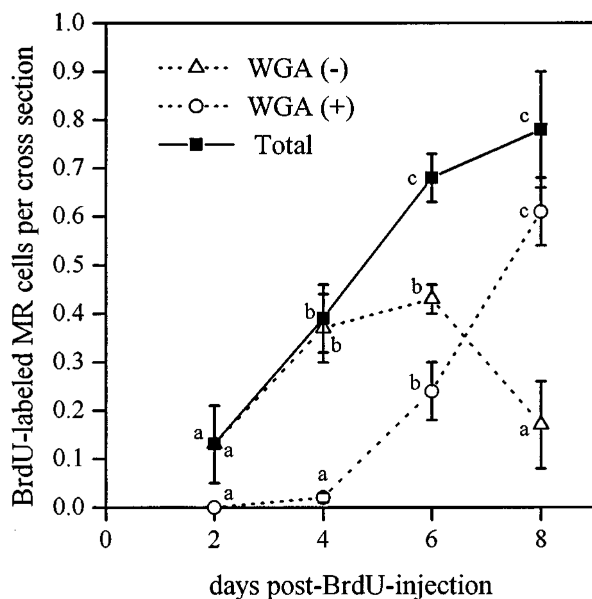

Figure 5. WGA-binding sites in the mitochondria-rich cells during differentiation. BrdU-labeled MR cells are mostly WGA (–) in the tissue two days after BrdU injection, while WGA (+) cells increase in number gradually in four-, six-, and eight-day's samples. The curve connects the mean values. Comparisons achieved by ANOVA were made among the four points in each line. Different letters indicate significant difference.

of the β cells was revealed by Pisam et al. (1995) to be rich in carbohydrates; also, the WGA labeling was limited to the apical region of certain MR cells. This hypothesis is further supported by the known link between MR cells and calcium uptake by tilapia gills. The MR cells in the gills have been demonstrated to be the main sites of calcium uptake (Ishihara and Mugiyu 1987; McCormick et al. 1992; Perry et al. 1992; Hwang et al. 1994). Pisam et al. (1995) proposed that the β cells are a specialized type of MR cells responsible for calcium uptake, since they can be induced by prolactin (Prunet et al. 1994), and calcium influx is increased by the same hormone (Flik et al. 1986). We propose that the WGA-binding sites in the MR cells of tilapia gills are associated with calcium absorption since the number of WGA positive MR cells was increased in gills of tilapia adapted to low calcium media; this would be consistent with the hypothesis that these cells are similar to the β cells.

Lee et al. (1996b), using scanning electron microscopy, distinguished three types of MR cells in freshwater-adapted tilapia. Based on the morphology of the apical opening, they were named wavy convex, shallow basin, and deep hole, respectively. Moreover, the prominence of each type of MR cell was found to depend on the ion composition of the water. They suggested a correlation between the shallow-basin MR-cells and calcium uptake since this type of MR cell is dominant in gills of tilapia adapted to comparatively low calcium medium. In reference to the present study, the WGA positive MR cells would be similar to the shallow-basin MR cells since they were both seen increased in number when the fish were kept in the low calcium freshwater.

Morphological changes have been observed and described during the life span of MR cells in tilapia gills (Wendelaar Bonga and van der Meij 1989). The WGA binding site found in this present study would further be viewed as a marker of the differentiation of MR cells. Moreover, that the WGA positive cells increase during differentiation of the MR cells, in turn, implies that different types of MR cells represent different differentiation stages of the cells. It is possible that the differentiation of the MR cells is programmed and changes of cellular structures will thus occur during the process. However, the process may be accelerated, or slowed down, in responding to certain changes in the environment, e.g., the WGA positive MR cells were induced when water calcium level is low, the α or β cells were increased in the gills of tilapia injected with growth hormone or prolactin, respectively (Pisam et al. 1993; Prunet et al. 1994). On the other hand, Laurent et al. (1994) also demonstrated that differentiation, but not proliferation, of the chloride cells in gills of salmon can be induced by cortisol injection or by transferring the fish into ion-poor water.

Con-A did not bind to the glutaldehyde- and paraformaldehyde-fixed plastic sections of tilapia gills in the present study. This differs from the work of Li et al. (1995) and Van der Heijden et al. (1997) in which the Con-A were applied successfully for labeling the apical opening of the MR cells in tilapia gills by incubating the tilapia larvae vitally with this reagent. It is possible that fixation of the gills will be critical for the Con-A binding to the MR cells. Furthermore, the WGA binding to the MR cells was different from the Con-A binding. Firstly, the WGA binding sites were localized in the area beneath the apical membrane of the MR cells, but the Con-A was found to be vitally stained in all the apical surface of the MR cells. Secondly, the labeling of Con-A was seen in the surface of most MR cells, but the WGA binding was seen only in certain MR cells. Therefore, it is more likely that these two lectins bind to different sub-cellular components of the MR cells.

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