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Enhanced Chloride Cell Turnover in the Gills of Chum Salmon Fry in Seawater

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ABSTRACT—Cellular differentiation and renewal in the gill chloride cells were examined in freshwater (FW)- and seawater (SW)-adapted chum salmon (*Oncorhynchus keta*) fry and in fry during SW adaptation using 5-bromo-2'-deoxyuridine (BrdU) as a marker for newly-differentiated cells. Chloride cells and BrdU-labeled nuclei were immunocytochemically detected by using antisera specific for Na^+,K^+-ATPase and BrdU, respectively. Although the number of chloride cells located at the base of the lamellae and in the interlamellar region (filament chloride cells) was constant in FW, SW and SW-transferred groups, chloride cells located in the lamellar epithelium (lamellar chloride cells) were fewer in SW than in FW, and decreased during SW adaptation. Newly-differentiated cells with BrdU-immunoreactive nuclei were detected mainly in the filaments, and rarely observed in the lamellae. The turnover rates of filament chloride cells for FW, SW and SW-transferred fish during the first 24 hr were 8%, 21% and 28%, respectively. These results indicate that chloride cells in the filament are replaced continuously by newly-differentiated cells in both FW and SW, and that the turnover was about 3 times greater in SW than in FW. More frequent turnover of filament chloride cells in SW suggests a specific role, presumably in salt excretion.

INTRODUCTION

Chloride cells are involved importantly in the osmoregulatory processes of teleostean branchial epithelium (Evans, 1993). The cells are characterized by a rich population of mitochondria and by an extensive membranous tubular system continuous with the basolateral membrane (Karnaky *et al.*, 1976; Philpott, 1980; Pisam and Rambourg, 1991). Sodium-potassium adenosinetriphosphatase (Na^+,K^+-ATPase), a key enzyme of ion transport generating ionic and electrical gradients, is located in the basolateral membrane of chloride cells (McCormick, 1995). Chloride cells have been shown to be involved in salt secretion in seawater (SW) and possibly in ion uptake in fresh water (FW) (Foskett and Scheffey, 1982; Zadunaisky, 1984; Avella and Bornancin, 1990; Marshall, 1995).

Among Pacific salmon (genus *Oncorhynchus*), chum (*O. keta*) and pink (*O. gorbuscha*) salmon spawn close to the sea. The fry go down to the sea in early life stages (Salo, 1995). They acquire hypoosmoregulatory ability during alevin stages (Weisbart, 1968; Iwata *et al.*, 1982; Hasegawa *et al.*, 1987; Clarke and Hirano, 1995). We have recently demonstrated enhanced gill Na^+, K^+-ATPase activity and activation of gill chloride cells in chum salmon fry prior to SW entry (Uchida *et al.*, 1996). In FW chum salmon fry, chloride cells were observed in both filament and lamellar epithelia. The chloride cells showed marked morphological changes following SW transfer; filament chloride cells became activated, whereas lamellar chloride cells disappeared during SW adaptation. These observations suggest that the well-developed chloride cells in the filament may be responsible for salt secretion in SW, whereas those in the lamella may be the site of ion uptake in FW.

Using tritiated thymidine, Conte and Lin (1967) examined the kinetics of cellular morphogenesis in the gill epithelium in juvenile coho (*O. kisutch*) and chinook (*O. tshawytscha*) salmon during SW adaptation, demonstrating a greater turnover rate of nuclear DNA in SW-adapted fish than in FW-adapted fish. Chretien and Pisam (1986) also demonstrated that cell differentiation and renewal in the gill epithelium of 50% SW-adapted guppy were 3 times greater than in that of FW-adapted fish. However, most studies have been focused on the differentiation of gill epithelial cells as a whole, in which pavement (respiratory) cells predominate, often comprising more than 95% of gill epithelial cells, with chloride cells contributing only a small population (Goss *et al.*, 1995).

The aim of the present study is to explore the differentiation and renewal of gill chloride cells in FW- and SW-adapted chum salmon fry, and in fry during SW adaptation. To determine the degree of differentiation of gill chloride cells, antisera specific for Na^+, K^+-ATPase reacting with chloride cells (Ura *et al.*, 1996; Uchida *et al.*, 1996) and for 5-bromo-2'-deoxyuridine (BrdU) incorporated into nuclei during DNA synthesis (Gratzner,1982) were used as markers of newly-formed chloride cells.

MATERIALS AND METHODS

Fish

Fertilized eggs of chum salmon (*Oncorhynchus keta*), obtained

from the Otsuchi Salmon Hatchery (Iwate, Japan) in December 1994, were transported to the Ocean Research Institute, University of Tokyo. They were reared in a stock tank (200-*l*) with recirculating FW at 10°C, at which temperature hatching occurred after 7 weeks. After completion of yolk absorption, fry were fed commercial trout diets (Nissin Siryo, Tokyo) of suitable particle sizes at approximately 1.5% body weight per day.

BrdU administration

In early July, about 200 fry weighing 4-7 g were anesthetized with 2-phenoxyethanol (0.05%) and injected intraperitoneally with BrdU (Sigma, St. Louis, MO) at 100 μg/g body weight in saline. After the injection, the fry (n=100) were transferred either to FW (FW group) or directly to SW (SW-transferred group). Another group of intact fry (n=100) in FW were transferred directly to a SW tank (200-*l*) at the same time. After acclimation to SW for 2 weeks, they were injected with BrdU in the same manner and kept in SW (SW group).

Plasma Na^+ levels and gill Na^+,K^+-ATPase activity

To evaluate the adaptability to SW, plasma Na^+ levels and gill Na^+,K^+-ATPase activity were measured. The fish were sampled on days 0, 1, 2, 3, 4, 5 and 7 after BrdU injection for the determination of plasma Na^+ levels. Blood was collected from the caudal vessels with glass capillaries. Plasma was obtained by centrifugation at 10,000 rpm for 5 min and stored at -80°C until analyses. Plasma Na^+ concentrations were measured by atomic absorption spectrophotometry (Hitachi 180-50, Tokyo). For the measurement of gill Na^+,K^+-ATPase activity, gills were collected on days 0 and 7. Gill Na^+,K^+-ATPase activity was measured as described in Uchida *et al.* (1996).

Immunocytochemical detection of chloride cells and BrdU-incorporated nuclei

For the examination of chloride cell differentiation, chloride cells and BrdU-incorporated nuclei were detected immunocytochemically using antisera specific for Na^+,K^+-ATPase (Ura *et al.*, 1996) and anti-BrdU monoclonal antibody (Sigma, St. Louis, MO), respectively. For the immunocytochemical study, gills were sampled on days 1, 2, 3, 4, 5 and 7 after BrdU injection. The fish were anesthetized with 2-phenoxyethanol (0.05%), and whole gills were dissected out and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 hr at 4°C. After fixation, the second gill was removed, dehydrated in ethanol, and embedded in paraplast. Serial sections (4 μm) were cut on the plane parallel to the gill arch. Pairs of adjacent sections were mounted on separate slides: one section was mounted on a gelatin-coated slide for Na^+,K^+-ATPase and the other section on a Biobond (British BioCell International)-coated slide for BrdU. Both sections were stained by avidin-biotin-peroxidase complex (ABC) method (Hsu *et al.*, 1981), using commercial reagents (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA). Chloride cells were detected with anti-Na^+,K^+-ATPase antiserum (Uchida *et al.*, 1996). The sections were counterstained with Mayer's hematoxylin to visualize the nuclei. BrdU-incorporated nuclei were detected as described by Oinuma *et al.* (1992) with some modifications. Briefly, deparaffined sections were incubated sequentially with: 1) 0.6% H_2O_2 for 30 min, 2) 0.5% trypsin in phosphate-buffered saline (PBS, pH 7.4) at 37°C for 15 min, 3) 0.01% Tween 20 in PBS for 5 min, 4) 2N HCl at 37°C for 1 hr, 5) 0.05 M borate buffer (pH 8.5) for 5 min, 6) 2% normal goat serum for 30 min, 7) anti-BrdU monoclonal antibody at a dilution of 1 : 4000 overnight at 4°C, 8) biotinylated anti-mouse IgG for 30 min, 9) ABC for 1 hr, and 10) 0.02% 3,3'-diaminobenzidine tetrahydrochloride containing 0.005% H_2O_2 for 10 min. The sections were photographed with a microscope (Nikon, Tokyo) equipped with a Nomarski differential interference contrast device.

For quantitative analysis, the following were counted on the pairs of adjacent sections along a 200-μm length of a gill filament: 1) chloride cells with a nucleus in the section (A), 2) cells with a BrdU-immunoreactive (ir) nucleus (B), and 3) chloride cells with a BrdU-ir nucleus (C). The percentage of BrdU-labeled chloride cells was calculated as C x 100 / A.

Statistics

All data are expressed as the mean ± S.E.M. Significance of differences was determined by Student *t*-test or Cochran-Cox test after variance analysis by F-test.

RESULTS

Plasma Na^+ levels and gill Na^+,K^+-ATPase activity

Plasma Na^+ levels of the fry kept in FW were constant throughout the experimental period for 7 days, except for a slight but significant decrease ($P<0.05$) on day 1 (Fig. 1). Following direct transfer from FW to SW, plasma Na^+ levels did not change until day 4, but significantly increased ($P<0.05$) on days 5 and 7 compared with the initial level. In the SW group, plasma Na^+ levals showed no significant changes, but were kept slightly higher than those in the FW group. There was no significant difference in gill Na^+,K^+-ATPase activity between days 0 and 7 after BrdU injection in each experimental group (Fig. 2). However, the activity was slightly but

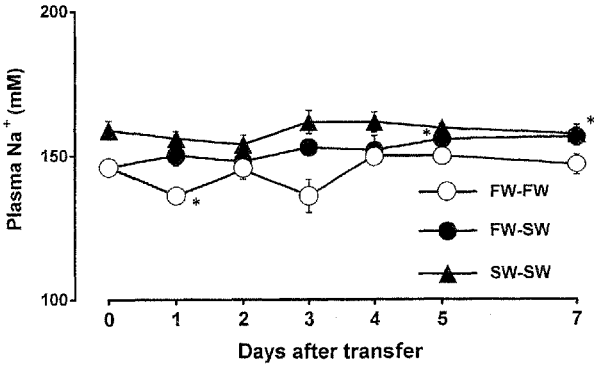

Fig. 1. Changes in plasma Na^+ levels in chum salmon fry kept in fresh water (FW-FW), transferred from fresh water to seawater (FW-SW) and acclimated to seawater for 2 weeks (SW-SW). Data are expressed as mean ± S.E.M. (n=10). *P<0.05, significantly different from the corresponding initial values (day 0).

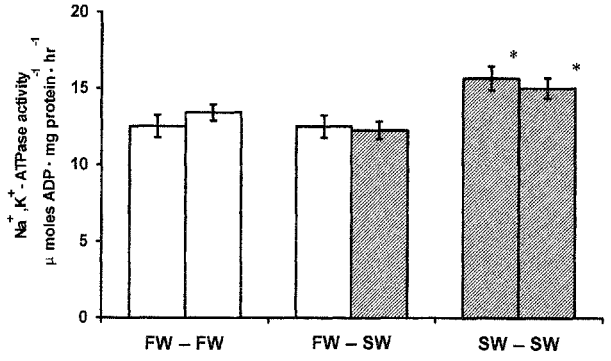

Fig. 2. Gill Na^+,K^+-ATPase activity before (left) and 7 days after (right) BrdU injection in chum salmon fry kept in fresh water (FW-FW), transferred from fresh water to seawater (FW-SW) and acclimated to seawater for 2 weeks (SW-SW). Data are expressed as mean ± S.E.M. (n=8). *P<0.05, significantly different from the initial value of FW group.

significantly higher ($P<0.05$) in the SW group than in FW and SW-transferred groups.

Immunocytochemical detection of newly-differentiated chloride cells

Na^+,K^+-ATPase-ir chloride cells were observed in both filament and lamellar epithelia of the gill (Fig. 3). The number of chloride cells located at the base of the lamellae and interlamellar regions (filament chloride cells) was constant in FW, SW and SW-transferred groups, ranging from 150 to 180 per mm gill filament. In contrast, chloride cells located in the lamellar epithelium (lamellar chloride cells) were fewer in the SW group than in the FW group, and decreased gradually in SW-transferred group.

Newly-differentiated cells with a BrdU-ir nucleus were detected mainly in the gill filament, and were rarely observed in the lamellar epithelia (Fig. 3). BrdU-ir cells in the lamella were 8%, 12% and 12% of total BrdU-labeled cells in the entire gills in FW, SW and SW-transferred groups, respectively, on day 7. Newly differentiated cells labeled with BrdU were frequently detectable in close vicinity to the central venous sinus in the filament on day 1 (Fig. 3). Thereafter, BrdU-ir nuclei spread out, especially at the basement of the lamellar epithelia.

Observations on paired adjacent sections stained with anti-Na^+,K^+-ATPase and anti-BrdU revealed that a large proportion of BrdU-labeled cells corresponded to Na^+,K^+-ATPase-ir chloride cells. Chloride cells with a BrdU-ir nucleus were 50%, 56% and 48% of total BrdU-labeled cells in the entire gills in FW, SW and SW-transferred groups, respectively, when examined on day 7.

In the filament, BrdU-labeled cells increased during the experimental period (Fig. 4). The percentage of BrdU-labeled chloride cells to total chloride cells in the filament in the FW group increased gradually and reached the maximal value of about 30% on day 5. In the SW group, the percentage of BrdU-labeled chloride cells was 3 times greater than that of the FW group on day 1, and reached a plateau level of about 30% on day 4. In the SW-transferred group, the percentage rapidly increased and attained a maximal level (about 30%) on day 1. The occurrence of BrdU-labeled chloride cells during the first 24 hr was 8%, 21% and 28% of the total chloride cells in the filament in FW, SW and SW-transferred groups, respectively.

In sharp contrast to frequent occurrence of BrdU-labeled chloride cells in the filament, lamellar chloride cells labeled with BrdU were rarely observed during the experimental periods in the three groups.

DISCUSSION

The present study clearly indicate that a proportion of chloride cells are consistently replaced by newly-differentiated cells; chloride cell turnover was much greater in SW than in FW, and greatest during SW adaptation.

Changes in plasma Na^+ levels indicate excellent hypoosmoregulatory ability of the chum salmon fry; plasma Na^+ levels were not increased greatly following direct transfer from FW to SW. Similar observations have been reported in chum salmon fry (Hasegawa *et al.*, 1987; Uchida *et al.*, 1996). Gill Na^+,K^+-ATPase activity of SW-adapted fish was slightly but significantly higher than that of FW-adapted fish, but there was no change 7 days after SW transfer. Uchida *et al.* (1996) have reported that gill Na^+,K^+-ATPase activity of the chum salmon fry had already been enhanced prior to SW entry and increased only by 40% 21 days after transfer from FW to SW. The well-developed hypoosmoregulatory ability of chum salmon fry also indicate that BrdU injection did not disturb their hydromineral balance.

Na^+,K^+-ATPase-ir cells coincide well with chloride cells (Uchida *et al.*, 1996; Ura *et al.*, 1996; Witters *et al.*, 1996). In accordance with our previous study (Uchida *et al.*, 1996), Na^+,K^+-ATPase-ir chloride cells were observed in both filament and lamellar epithelia in the chum salmon fry. The number of filament chloride cells was maintained at constant levels in the three experimental groups. On the other hand, lamellar chloride cells were fewer in SW-adapted fish and decreased in SW-transferred fish. These observations suggest possible significant roles for filament and lamellar chloride cells in SW and FW, respectively (Uchida *et al.*, 1996).

Recently, BrdU has been widely used to investigate cellular differentiation and renewal (Gratzner, 1982). BrdU is an analogue of thymidine, which is incorporated into nuclei during DNA synthesis and readily detectable by immunocytochemistry. In the present study, BrdU-ir nuclei were detected mainly in the filament and rarely in the lamella. BrdU-labeled nuclei in the filament were first observed near the central venous sinus and then spread to various regions. These observations are in accordance with Conte and Lin (1967) using ^3H-thymidine; undifferentiated stem cells were frequently observed in contact with the basement membrane and absent in the lamellae. Thus, pavement cells and chloride cells, two major cell types in the gill epithelia (Perry and Laurent, 1993), appear to develop from undifferentiated stem cells lying near the central venous sinus.

Although the total numbers of chloride cells were constant, BrdU-labeled chloride cells in the filament increased during the experimental period in all three groups. Chloride cells in the filament are thus replaced continuously; newly-differentiated cells are recruited and the equivalent number of cells degenerate. Furthermore, about half of the BrdU-ir cells in the filament corresponded to Na^+,K^+-ATPase-ir chloride cells on day 7 in the three groups. Chloride cells comprise only a small population (less than 5%) of gill epithelial cells, whereas pavement cells are the predominant cell type (Perry and Laurent, 1993; Goss *et al.*, 1995). Thus, the turnover of chloride cells appears much greater than that of the other cell types in the gill.

The BrdU-labeled chloride cells exhibited different turnover rates among three experimental groups. In all the groups, however, the BrdU-labeled chloride cells were saturated at about 30%. This may be partly due to unavailability

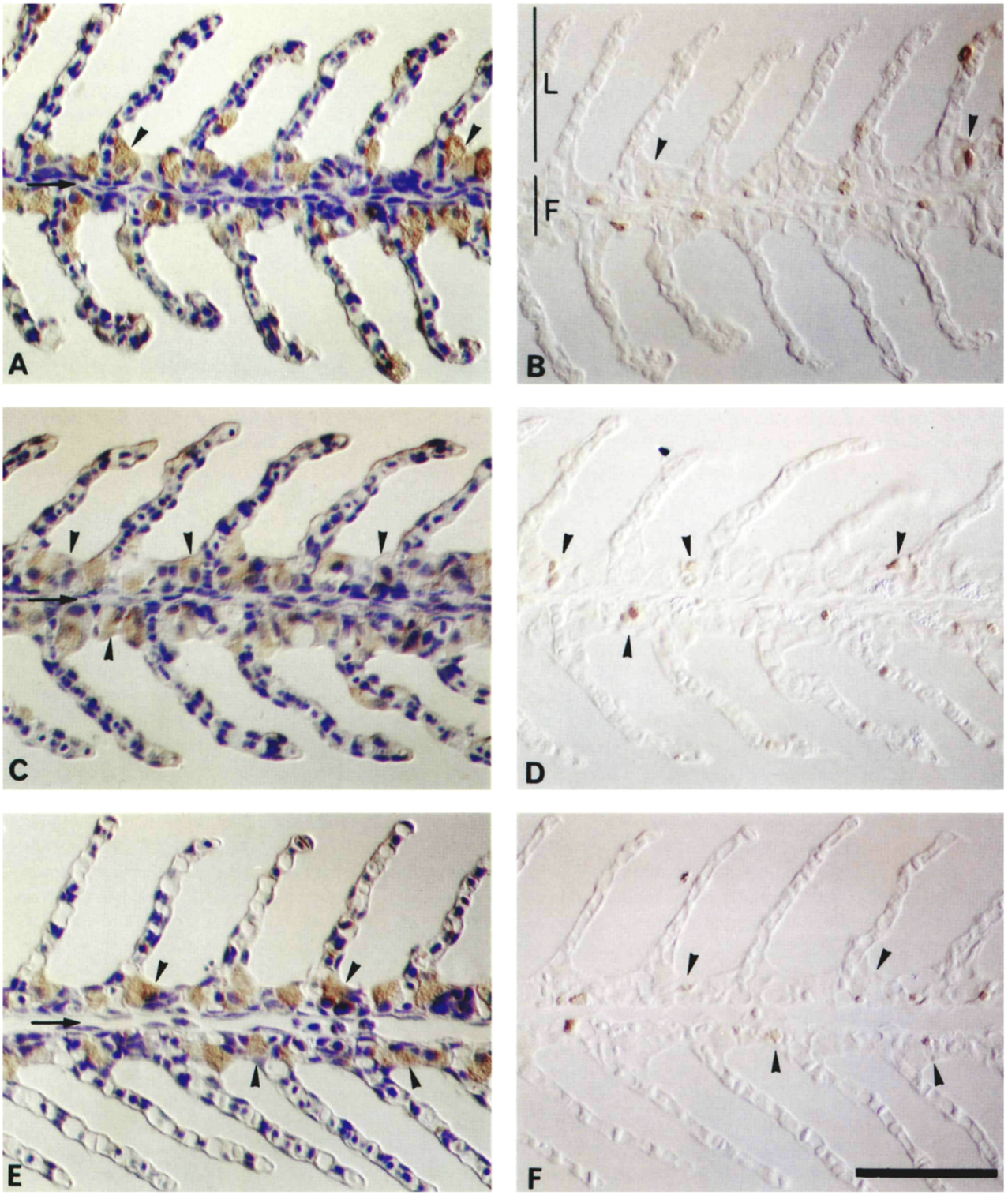

Fig. 3. Paired adjacent sections of gills on 1 day after BrdU injection, stained with anti-Na^+,K^+-ATPase serum (A, C, E) and anti-BrdU monoclonal antibody (B, D, F), in chum salmon fry kept in fresh water (A, B: FW-FW), transferred from fresh water to seawater (C, D: FW-SW) and acclimated to seawater for 2 weeks (E, F: SW-SW). Note that BrdU-labeled chloride cells (arrowheads) are detectable mainly in the filament (F), and rarely observed in the lamellae (L). Arrows; central venous sinus. Bar; 50 μm.

of BrdU because of its provision by a single injection. Similar saturation of BrdU-incorporated cells was reported in the fundic gland of *Xenopus laevis* (Oinuma *et al*., 1992). The BrdU-labeled chloride cells in the filament of SW fish rapidly increased, being three times greater than in FW fish on 1 day, and transfer from FW to SW resulted in the greatest increase

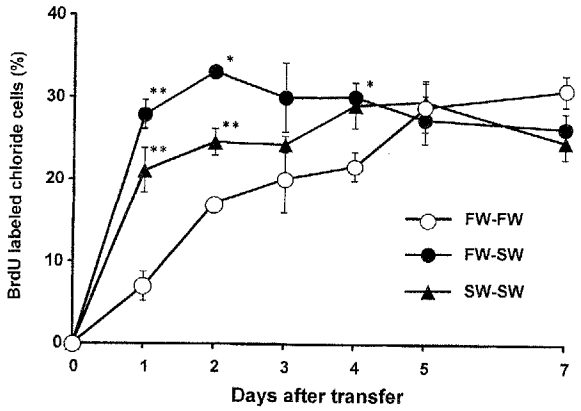

Fig. 4. Changes in percentage of BrdU-labeled chloride cells in the gill filament following BrdU injection in fry kept in fresh water (FW-FW), transferred from fresh water to seawater (FW-SW) and acclimated to seawater for 2 weeks (SW-SW). Data are expressed as mean ± S.E.M. (n=5). *P<0.05, **P<0.01, significantly different from the value of FW group.

among the three groups. Judging from their increased size and intensity of immunoreaction to Na^+,K^+-ATPase, filament chloride cells were markedly activated following transfer from FW to SW (see discussion in Uchida *et al.*, 1996). The activated chloride cells seem to be differentiated rapidly, probably within a few days after SW entry. The enhanced turnover of filament chloride cells in response to SW transfer adds support to the notion that filament chloride cells are responsible for salt secretion in SW.

BrdU-labeled cells were much fewer in the lamellar epithelia, being 8-12% of the total BrdU-ir cells 7 days after BrdU injection in all groups. Furthermore, BrdU-labeled chloride cells were rarely observed in the lamellar epithelium. Lamellar chloride cells in chum salmon fry gradually decrease after SW transfer, and almost disappear after 21 days (Uchida *et al.*, 1996). At the same time, degenerating chloride cells were frequently observed in the lamellar epithelia of SW-adapted fish by electron microscopy (Uchida *et al.*, 1996). Chloride cell differentiation in the gill lamellae seems less active than in the gill filaments.

Lamellar chloride cells have been suggested to play significant roles in hypoosmotic environments, presumably acting as sites for ion uptake (Laurent *et al.*, 1985; Avella *et al.*, 1987; Perry and Laurent, 1989). According to Laurent *et al.* (1994), no cell proliferation was seen in the lamellar epithelium of rainbow trout within 48 hr after transfer to ion-poor water. They also concluded that cell proliferation occurs primarily in the filament, and that migration from the filament is the main mechanism for the appearance of chloride cells on the lamellar epithelia. The filament epithelium is typically multi-layered, whereas the lamellar epithelium is normally composed of a double layer of cells separated by extracellular space. In chum salmon fry, lamellar chloride cells may also be derived from undifferentiated cells in the filament epithelia.

In conclusion, the filament chloride cells are replaced continuously by newly-differentiated cells both in FW and SW, whereas lamellar chloride cells are renewed much less frequently. The turnover of chloride cells in the filament was much greater in SW than in FW, and transfer from FW to SW resulted in the greatest turnover. Considering the excellent SW adaptability of chum salmon fry, filament chloride cells seem to be activated concomitant with accelerated cellular turnover during SW adaptation, and these cells may play a central role in salt secretion in SW.

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