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Induced Epidermal Cell Turnover in the Seawater-Adapted Guppy, *Poecilia reticulata*

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The turnover of epidermal cells after seawater adaptation of the freshwater fish was studied in the guppy (*Poecilia reticulata*) by means of proliferating cell nucleus antigen (PCNA) immunocytochemistry and transmission electron microscopy. The number of PCNA-immunoreactive cells in the epidermis of the seawater-adapted guppies, which becomes thinner than that in the freshwater, generally increases four times as much. Degeneration of filament-containing cells by necrosis or apoptosis occurs mainly in epidermal cells. Apoptotic filament-containing cells seem to be shed into the water in the environment instead of phagocytosis by adjacent macrophages. The apoptotic chloride cell has a highly condensed cytoplasm and the lumen of tubular system is distended. The apoptotic mucous cell, which has an electron-dense cytoplasm, is characterized by the presence of a large multivesicular body of different electron densities. Macrophages contain many electron-dense lysosomal bodies and large vesicles filled with cellular debris. It is concluded that mitosis and apoptosis of epidermal cells are greatly stimulated when fish are adapting to seawater. This result reflects an increase in epidermal cell turnover by change of environmental salinity.

Teleost skin has a multilayered epithelium consisting mainly of filament containing cells, mucous cells and chloride cells, which may serve various functions (Whitear, 1970; Schwerdtfeger, 1979; Kim et al., 1988). The densely adhering filament containing cells in the epidermis and the dermal layers of the skin reduce skin damage caused by mechanical influences, while the mucous layer on the external surface of the epidermis protects the fish against pathogenic organisms and may also reduce water resistance in fast-swimming fish. In addition, the skin mucous may facilitate ion uptake due to its ion-binding capacities, while the epidermal layer may form a selective permeability barrier for water and ions (Rosen and Cornford, 1971; Iger et al., 1988; Moon, 1995a). The important roles of the chloride cells on osmoregulation have been demonstrated from more recent physiological, biochemical and ultrastructural studies (Foskett and Scheffey, 1982; Whitear, 1986; Kim et al., 1993).

The aquatic environment is subject to chemical and physical changes adverse to life. Changes in habitat may induce stress in fishes and aquatic animals. Structural studies concerning the effects of environmental stressors on epidermal tissues in direct contact with the aquatic environment have been reported from aquatic animals (Leino and McCormick, 1984; Laurent et al., 1985; Avella et al., 1987; Wendelaar Bonga et al., 1990; Kim et al., 1993). Marked changes have been described for the epithelia of the intestine, gills and skin in those animals. In the case of the gills, the branchial epithelium has been extensively studied in different fish from waters with differing osmolarity and ionic composition or waters containing various kinds of pollutants. Many kinds of stressors were known to affect the branchial structures (McDonald, 1983; Wendelaar Bonga and van der Meij, 1989). Consequently, the gill epithelium has a high rate of cell renewal, with a half-life that in juvenile salmonids varies from 16 days in fresh water to 6 days in seawater (Mackinnon and Enesco, 1980; Chretien and Pisam, 1986; Zenker et al., 1987).

In contrast, the skin surface has received little attention, although it is a metabolically active tissue that plays an important role as a protective barrier between the water and the aquatic animal. It responds quickly to external stimuli such as pollutants or changes in the ionic content of the water. Some changes have been described for the skin tissue in fish exposed to stressors (Wendelaar Bonga and van der Meij, 1989; Iger and Wendelaar Bonga, 1994; Moon, 1995b).

In recent years, it has become well established that there are two types of cell death: necrosis and apoptosis (Wyllie, 1981). These are two different phenomena, although they correspond to cell death by extrinsic or intrinsic factors (Wyllie et al., 1980). Necrosis is characterized by the structural swelling of cells and compartments within a cell, followed by cellular disrup-

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tion. Apoptotic cells show a characteristic sequence of cellular shrinkage and increasing osmophilia of the cellular components, resulting in the transformation of each cell into one or more compact apoptotic bodies phagocytosed eventually by adjacent epithelial cells or macrophages (Wyllie, 1981). With respect to cell death, only a few approaches for describing an epidermal cell with necrotic or apoptotic ultrastructural characteristics in the skin of fish were mentioned in fish acclimated to seawater (Wendelaar Bonga and Meis, 1981; Iger and Wendelaar Bonga, 1994; Moon, 1995b).

PCNA was first described as a nuclear antigen in proliferating cells that react immunologically with sera from systemic lupus erythrematosus patients (Miyachi et al., 1978). PCNA has been identified as a DNA polymerase δ auxiliary factor (Bravo et al., 1987), a highly conserved 36-kDa nuclear protein directly involved in DNA synthesis (Mathews et al., 1984), and synthesized during all phases of the cell cycle except the resting (G_0) phase (Kurki et al., 1986). The antigen is stable and can be detected in cells of all tissues (Kurki et al., 1986; Foley et al., 1991).

PCNA methodology was developed for and has applied mostly to mammalian tissues (Foley et al., 1991). But it has been demonstrated in a few non-mammalian species including the fruitfly (Yamaguchi et al., 1991) and some fishes (Negishi et al., 1991; Negishi and Shinagawa, 1993; Alfei et al., 1994; Ortego et al., 1994). The latter studies demonstrated the successful application of mammalian-based PCNA technology to aquatic species.

Previous studies have demonstrated the effects of seawater adaptation on the gills and skin of fishes (Kim et al., 1993; Moon, 1995a; Kang et al., 1996). We have shown that seawater adaptation leads to a proliferation of chloride and mucous cells in the gills. We also observed ultrastructural changes in the epidermis of guppies adapted to seawater (Moon, 1995b). In the present study, we attempted not only to examine the ultrastructural characteristics of cell degeneration and death in the seawater-adapted guppy but also to compare cell renewal in the epidermis between the freshwater and seawater-adapted guppy using PCNA immunocytochemistry.

Materials and Methods

Experimental animals

Guppies (*Poecilia reticulata*) were obtained from a commercial aquarium and maintained in rearing facilities prepared in the laboratory, which were supplied daily with oxygen and Teramin (tropical fish food). The seawater 3.2% (salt concentration) transported from the adjacent ocean of Wallmeedo at Incheon, was kept at 4℃ and mixed with freshwater treated gradually with increasing salt concentration. The salt concentration was measured by a Salinity Refractometer (S-Mill).

For the experiment on environmental adaptation, groups of fish which were kept in the freshwater tank for one week, were made to adapt to seawater by gradually increasing the salt concentration until reaching a final concentration of 3.2% on day 7.

The skins were obtained by dissection of five seawater-adapted fishes. Normal skin was also dissected from five freshwater fishes.

PCNA immunocytochemistry

Skin tissues were fixed in 4% paraformaldehyde for 2 h, dehydrated, and embedded in wax. Wax sections were washed twice in 0.1 M phosphate-buffered saline (PBS) and pre-incubated at room temperature for 30 min in Triton X-100 and 1% BSA in 0.1 M PBS. Sections were rinsed in 0.5% BSA in 0.1 M PBS and incubated in a primary antibody solution (anti-PCNA, 1 : 100 dilution) in 0.1 M PBS containing 0.5% BSA and 0.5% sodium azide overnight at 4℃.

Tissues were washed twice in 0.1 M PBS, processed with the Vectastain ABC kit (Vector Laboratories) and developed with a diaminobenzidine substrate. Slices were rinsed in 0.1 M phosphate buffer (PB, pH 7.2), dehydrated, and coverslipped with Permount.

Electronmicroscopy

Tissues were prefixed with 2% paraformaldehyde - 2.5% glutaraldehyde solution in 0.1 M PB for 2 h, postfixed in 2% osmium tetroxide for 1 h, and dehydrated in a series of increasing solutions of ethanol. The dehydrated tissues were embedded in epon mixture and polymerized on the Polymerizer (Reichert-Jung) for 72 h at 60℃. The tissue block was ultrasectioned 70 nm thick and stained with uranyl acetate and lead citrate. The ultrathin specimens were examined with a transmission electron microscope (JEM-1200EX).

Statistical analysis

To compare the number of PCNA-immunoreactive cells between freshwater and seawater-adapted fish, we took the epidermis from both conditions. After making serial sections (each 8 μm in thickness), we counted the number of PCNA-immunoreactive cells per unit area on every fifth section under the light microscope (X400). Data were analyzed by student's t test for comparison.

Results

PCNA immunocytochemistry

The epidermis of the freshwater guppies was thicker than that of the seawater-adapted guppies because the epidermal cell shape become more flattened (Fig. 1A, B).

PCNA immunoreactivity appeared within the nucleus of the epidermal cells of both freshwater and seawater-

Fig. 1. Light and electron micrographs of epidermal cells of freshwater- and seawater-adapted guppies. PCNA-immunoreactive cells (arrowheads) are mainly located in the surface layer of the freshwater-guppy epidermis (A), but throughout the epidermis in the seawater-adapted guppy (B). The epidermis in the freshwater guppy is thicker than that of the seawater-adapted guppy. Electron micrograph of epidermal layers in the seawater-adapted guppy (C). The epidermis consists of filament containing cells (fc), chloride cells (cc), and mucous cells (mc). Electron micrographs of the filament containing cells showing various stages of necrosis and apoptosis (D, E, F, G). In F and G, note ruptured cell by necrosis (asterisk) and apoptotic cells with high electron density (star). bm, basement membrane; d, desmosome; is, intercellular space; l, lysosome; m, mitochondria; n, nucleus; pc, pigment cell; rer, rough endoplasmic reticulum. Scale bars=2 μm (C, D, E, F, G) and 10 μm (A, B).

adapted guppies. PCNA-immunoreactive cells of freshwater guppies were located usually in the surface layer of the epidermis, but those of seawater-adapted guppies were observed throughout the epidermis (Fig. 1A, B). The number of PCNA-immunoreactive cells in the skin of the seawater guppy increased four times

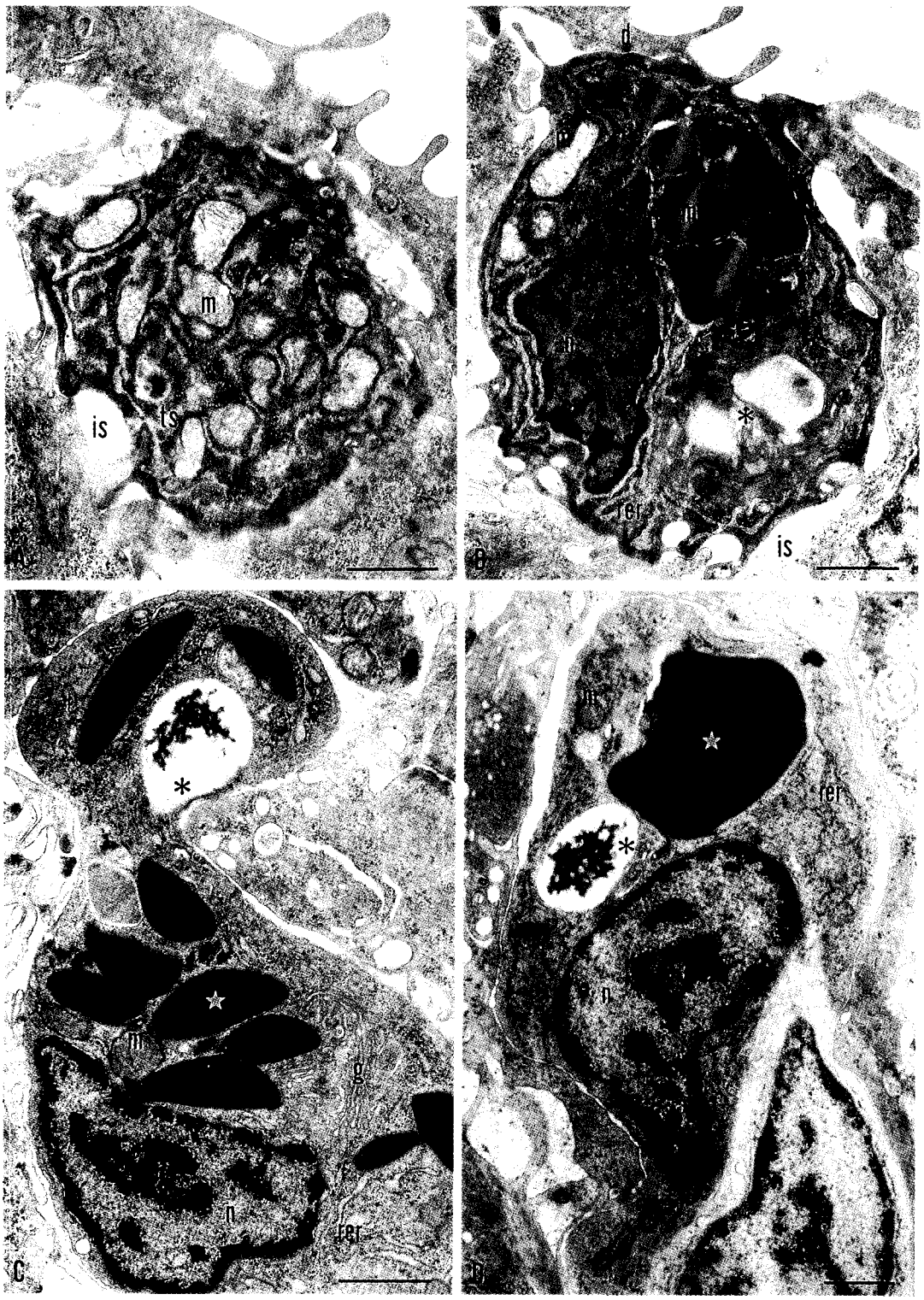

Fig. 2. Electron micrographs of the apoptotic chloride cell (A), mucous cell (B), and macrophages (C, D). The apoptotic chloride cells (A) are characterized by the occurrence of mitochondrial deformation and distended tubular system in the highly electron-dense cytoplasm. A large multivesicular body (asterisk) is present consisting of both many vesicles with various electron densities and membranous structures in the highly electron-dense cytoplasm of the apoptotic mucous cell (B). Macrophages contain many electron-dense lysosomal bodies (star) and large vesicles (asterisk) with fuzzy material and membranous structures which are represented as cellular debris (C, D). d, desmosome; g, Golgi complex; is, intercellular space; m, mitochondria; md, mucous droplet; n, nucleus; rer, rough endoplasmic reticulum; ts, tubular system. Scale bars=1 μm.

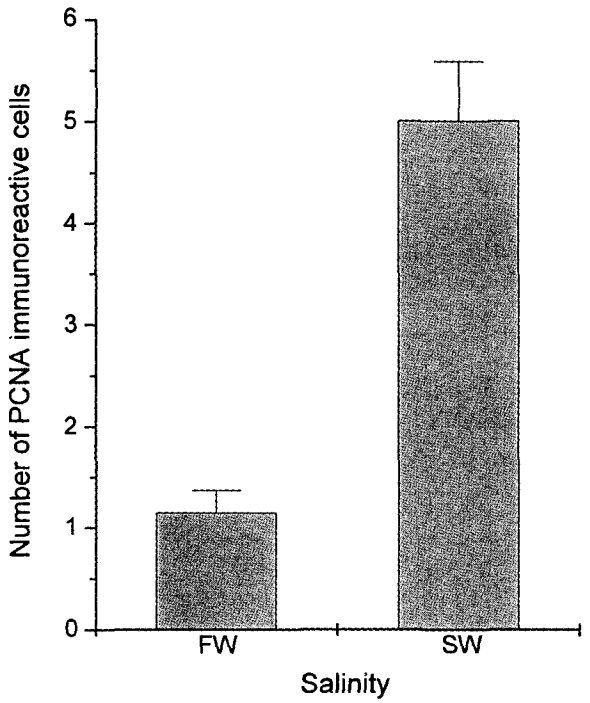

Fig. 3. The number of PCNA-immunoreactive cells per unit area in the freshwater (FW: 0%) and seawater-adapted guppies (SW: 3.2%). Statistical significance level for differences between seawater-adapted and freshwater guppies ($P<0.01$).

as much in comparison to that of the freshwater guppy ($P<0.01$, Fig. 3).

Ultrastructure

The epidermis of the seawater-adapted guppy consisted mainly of several layers of filament containing cells. Particularly, the flattened filament containing cells in the surface layer were characterized by the regular folds of the apical membrane or microridges. These cells also contained a web of microfilaments, and were connected to adjacent cells by desmosomes. Chloride cells and mucous cells were rarely found among filament containing cells (Fig. 1C).

Many filament containing cells in the epidermal surface layer showed a sign of cell degeneration. These cells in the early phase of necrosis were characterized by the cytoplasm with low electron density and their swelling. Microridges were swollen with low electron density (Fig. 1D). In the later stages of necrosis, the cytoplasmic organelles showed irregular distribution and the microridges were elongated to slender shape. The basal part of the cell showed low electron density while the upper part were seen as transparent (Fig. 1E). The junctional complexes were maintained only between lateral plasma membranes of the cells (Fig. 1E) and their cell boundary was eventually ruptured (Fig. 1F).

Some of the filament containing cells showed high electron density in the cytoplasm. It was interpreted that these cells were proceeding apoptosis. In cells with only a slight degree of cytoplasmic condensation, the lumen of the rough endoplasmic reticulum was distended (Fig. 1F). In other cells, these phenomena were more pronounced and, in addition, the indentations of the nuclear envelope and chromatin condensation were also observed. Multivesicular body with high electron density was observed in the condensed cytoplasm. There were extended intercellular spaces between the apoptotic cell and adjacent cells (Fig. 1G).

Some chloride cells and mucous cells also represented the apoptotic figures (Fig. 2A, B). Normal chloride cells were characterized by many mitochondria and specially developed tubular systems. Apoptotic chloride cells showed a very condensed cytoplasm. The lumen of the tubular system was also expanded. The intercellular spaces around the chloride cell may enlarge, probably as a result of cellular shrinkage (Fig. 2A).

The mucous cell, located in the upper epidermal layer, showed apoptotic characteristics with high electron density of the cytoplasm. The condensed chromatin was scattered within the nucleus. Electron-dense mucous droplets and the multivesicular body, which contains various types of vesicles and membranous structures, were found. There were wide intercellular spaces between the mucous cell and other neighboring cells because of cellular shrinkage and loss of cellular junctional complexes (Fig. 2B). Macrophages contained many electron-dense lysosomal bodies and large vesicles with fuzzy material and membranous structures represented by cellular debris (Fig. 2C, D).

Discussion

The disturbance of water and ion balance is a main environmental factor that influences the structures of the epidermis along with cell differentiation and cell degeneration (Schwerdtfeger, 1979; Wendelaar Bonga and Meis, 1981; Iger and Wendelaar Bonga, 1994; Moon, 1995b). It may provide further evidence that the environmental changes cause cells to age rapidly. Thus, we attempted to compare the turnover of epidermal cells from freshwater and seawater-adapted guppy, using PCNA immunocytochemistry. This study might be the first trial to use PCNA immunocytochemistry for identifying the epidermal cell turnover following seawater-adaptation of the freshwater fish. PCNA-immunoreactive cells in seawater-adapted guppy epidermis were located throughout the epidermal layer, while those cells in the freshwater guppy were observed mainly in the surface layer. The PCNA-immunoreactive cells increased four times in number in the seawater-adapted guppy. Quantitative analysis provided evidence that the mitotic ratio of epidermal cells significantly increases during seawater adaptation.

Apoptosis, the most common type of physiologically controlled cell degeneration and death, has been described for many cell types in higher vertebrates

(Wyllie, 1981; Bursch et al., 1985). In fish, it has been reported in cells producing hatching enzymes in pike embryos (Schoots et al., 1983) and in endocrine cells of the Stannius bodies in tilapia (Wendelaar Bonga and Pang, 1986). Dense, apoptotic bodies have also been reported in various tissues of fish exposed to mercury and copper (Daoust et al., 1984; Wester and Canton, 1992). The progressive densification of the cells, resulting in the formation of large globular structures, was interpreted as a sign of apoptotic degeneration of the chloride cells in the gills of *Oreochromis mossambicus* (Wendelaar Bonga and van der Meij, 1989). In the gills of the killifish adapted to seawater, various stages of apoptosis were also found to occur in chloride cells (Kang et al., 1996). Whereas cytoplasm and nuclei show shrinkage, the lumen of the tubular system distended. The progressive condensation of these cells leads to the formation of globules with characteristic apoptotic bodies which were eventually engulfed by macrophage-like cells. We also identified apoptotic chloride cells in the epidermis of the seawater-adapted guppy.

In respect to the filament-containing cells located in the epidermal surface layer, we observed extended intercellular space between the filament-containing cells, which showed specific high electron density, and adjacent cells. It was suggested that they were apparently shed into water in the environment, while other apoptotic bodies were phagocytosed by macrophages or adjacent cells (Wyllie et al., 1980). This type of cell degeneration has also been described in the pavement cells of the branchial epithelium (Wendelaar Bonga and Meis, 1981).

It has been shown that the secretion of mucous is greatly stimulated in seawater-adapted fishes (Solanki and Benjamin, 1982; Moon, 1995a), as well as in fish exposed to pollutant or acid (Benedetti et al., 1989; Iger and Wendelaar Bonga, 1994). The number of mucous cells were low in the epidermis and gills of seawater-adapted fish (Solanki and Benjamin, 1982; Moon, 1995a). Even though newly differentiating mucous cells occurred at the skin surface when exposed to lower pH or high salinity (Iger and Wendelaar Bonga, 1994; Moon, 1995a), there were no observations related to the degeneration of mucous cells. In this study, some mucous cells exhibiting signs of apoptosis were found in the epidermis of the seawater-adapted guppy.

In conclusion, we found a higher mitotic ratio of the epidermal cells following adaptation of the freshwater guppy to seawater, together with the frequent appearance of many apoptotic cells in the seawater-adapted epidermis. It is suggested that environmental changes may affect the turnover of epidermal cells.

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