

Morphology and histochemistry of the skin of the Korean spined loach, *Iksookimia koreensis* (Cobitidae), in relation to respiration

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Received 1 March 2001; Accepted 10 April 2002

A b s t r a c t. The epidermis of the Korean spined loach *Iksookimia koreensis* was divided into three layers: an outermost layer, middle layer and stratum germinativum. And the dermis was composed of two layers: stratum laxum and compactum. The outermost layer consisted of polygonal cells or flattened cells, small amount mucous cells, and the middle layer consisted of two types of skin glands, a small mucous cell and a large club cell. The mucous cell was acid sulfomucins (some sialomucins), but the club cell did not show any histochemical reaction to mucosubstances. The stratum germinativum was composed of a cuboidal epithelial cell and a well-defined lymphatic space containing small lymphocytes. There was a large number of blood vessels just below the basement membrane. The stratum laxum of dermis has small scales and a definite area that was AB positive and PAS positive.

Key words: mucous cell, club cell, cutaneous respiration

Introduction

Fishes have an aquatic respiratory system that uses dissolved oxygen from the water through the gills. Otherwise, some fishes are capable of absorbing oxygen through the skin, although in many bony fishes the uptake might not be enough to satisfy the local cutaneous requirement. Gas metabolism through the skin in fishes plays a very important role in their respiration and can provides from 5 to 30 percent of their required oxygen (F e d e r & B u r g g r e n 1985, B o n d 1996). Many fishes with cutaneous respiration inhabit warm and stagnant reservoirs or environments, which undergo periodic drought, low activity or relatively low temperature. Many amphibious fishes such as *Monopterus*, *Boleophthalmus* and *Periophthalmus* depend on cutaneous absorption of oxygen for significant proportions of their respiratory requirements (L i e m 1967, M i t t a l & M u n s h i 1971, P a r k et al. 2000). Mud loaches (*Misgurnus*) and reedfish (*Calamoichthys*) are also cutaneous breathers (J a k u b o w s k i 1958, J o h a n s e n 1970, S a c c a & B u r g g r e n 1982, W h i t e a r 1986, P a r k & K i m 1999).

The Korean spined loach, *Iksookimia koreensis*, is a small, slender, benthic, freshwater cobitid fish in Korea, inhabits the pebbly substratum of shallow streams, which are sometimes subjected to drought. In aquaria, the fish frequently rise to the surface to obtain air and can live for extended periods without a constant supply of air. The present work was to study the structure and cytochemistry of the skin of *I. koreensis* belonging to the family Cobitidae, and to discuss the relationship between skin and respiration.

Material and Methods

Two males and ten females were collected in the non-breeding season from Chonju stream, a tributary of Mankyong River (Korea). They were ranged from 72.5mm to 95.8mm in standard length. The specimens were fixed in 10% neutral buffered formaldehyde. Skin fragments were taken from four regions; the top of the head, the dorsal, the lateral and the abdominal region.

These fragments were dehydrated through a standard ethanol series to 100%, cleared in xylene and then embedded in wax (Paraplast, Oxford). 5µm sections were deparaffinized and stained with Harris's hematoxylin, iron alum hematoxylin, counter-stained with eosin, and Masson trichrome stain (Gurr 1956) for general histology. For blood cells the giemsa method was used. Mucins of gland were demonstrated by alcian blue solution (AB) at pH 1.0 and 2.5 (Steedman 1950, Lev & Spicer 1964) and the periodic acid-Schiff (PAS) method with or without prior digestion with diastase/saliva (Lillie & Greco 1947). In addition, the PAS technique was employed in combination with AB (pH 2.5) and vice-versa for neutral and acid mucins. Acid mucin was shown by metachromatic reactions with toluidine blue (Tock & Pearse 1965). Also, aldehyde fuchsin and with AB (Spicer & Meyer 1960), and high iron diamine (HID) and with AB (Spicer 1965) were used to study the nature of the acid mucins. Acetylation and deacetylation for the confirmation of hydroxyl group was performed following Lillie (1954). Methylation and demethylation (Spicer 1960) were undertaken to confirm the acidic nature of the mucins. Evaluations of the skin were made by light microscopy of the PAS entire mount preparations, and hematoxylin and eosin preparations.

Results

The integument of the *Iksookimia koreensis* could be divided into three layers: the epidermis, the dermis and the subcutis (Fig. 1A). The epidermis and the dermis was separated by a thin base membrane.

The epidermis can be divided into three layers-the stratum germinativum, the middle layer and the outermost layer (Fig. 1A). The average thickness of the epidermis was greatest at the abdominal (approximately 133.8 µm), and least in the lateral (approximately 89.5 µm) region (Table 1). The dorsal region was 110.7 µm and top of the head region 91.3 µm.

The outermost layer was composed of polygonal cells and a few flattened cells, which were arranged in two to six rows of cells (Fig. 1A). In between these cells, small spherical or flask-shaped unicellular mucous glands were discernible (Figs 1A, 1B and 1E). The mucous cells have a large spherical body and a short narrow neck that opens on the surface by a wide pore (Figs 1A, 1B and 1F). They have a basal, spherical or oval nucleus with

Table 1. Mean thickness of the epidermis in *Iksookimia koreensis*.

Sex/regions of body	Mean thickness of epidermis (µm)			
	Abdominal region	Lateral region	Dorsal region	Top of head
Male (n 2)	130.5	87.8	101.6	90.3
Female (n 10)	137.1	91.2	119.8	92.3
Mean value	133.8	89.5	110.7	91.3

a thin rim of slightly basophilic cytoplasm, pushed at the periphery of the cell due to the heavy accumulation of its basophilic secretions. The mucous cells were various, reaching from 7.5–42.5 μm in height. Its secretory matter was highly vacuolated and basophilic.

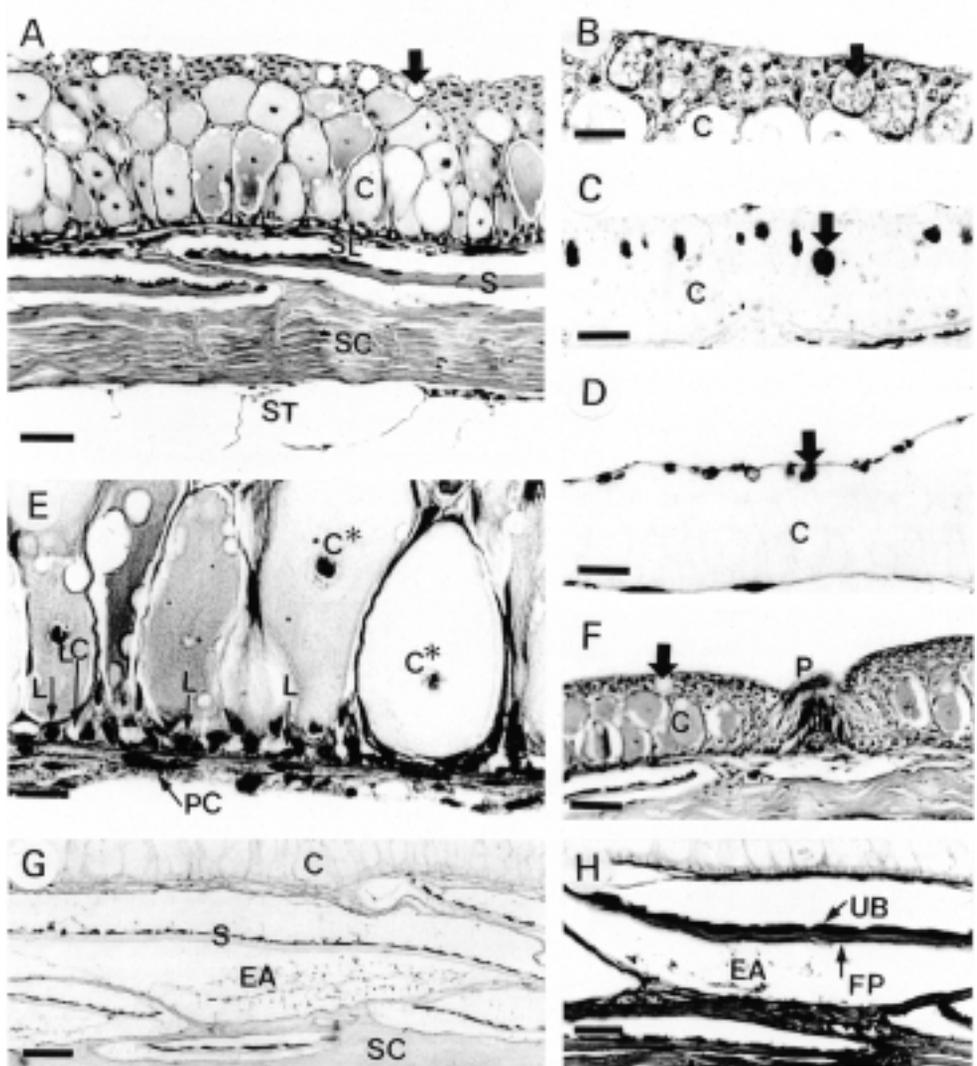


Fig. 1. Transverse sections of skin of *Iksookimia koreensis*. A, The dorsal skin with haematoxylin and eosin. The skin is composed of epidermis, dermis and subcutis (ST). C, club cell; S, scale; SC, stratum compactum; SL, stratum laxum; arrow, mucous cell. Bar indicates 24 μm . B, The ventral skin with haematoxylin and eosin. Note mucous cells (arrow). Bar indicates 24 μm . C, The abdominal skin with AB (pH 1.0) stain. Arrow, mucous cell; C, club cells. Bar indicates 24 μm . D, The dorsal skin with AB-PAS reaction. Arrow, mucous cell; C, club cell. Bar indicates 24 μm . E, The dorsal skin with haematoxylin and eosin. Note Club cells with sometimes binucleate (C*). L, lymphocytes; LC, lymphatic space; PC, pigment cells. Bar indicates 8 μm . F, The dorsal skin with haematoxylin and eosin. Note pit organ (P), club cell (C) and mucous cell (Arrow) of the epidermis. Bar indicates 24 μm . G, The dorsal skin with AB (pH 2.5)-PAS reaction. C, club cell; EA, definite areas; S, scale; SC, stratum compactum. Bar indicates 24 μm . H, The dorsal skin with AB (pH 2.5)-PAS reaction. Note an upper bony layer (UP) and inner fibrillary plate (FP) of the scale. EA, a define area. Bar indicates 24 μm .

These cells gave a deep red color reaction with PAS technique, which is diastase resistant, γ -metachromasia with toluidine blue, blue with the AB at pH 1.0 and 2.5 (Table 2). The mucous cells, giving a red color with aldehyde fuchsin and black color with high iron diamine, were likely to be sulfomucins. In mild methylation/AB and acetylation/PAS techniques they were negative, and in methylation /saponification-AB they were stained blue. Their nuclei were purple or red color with AB-PAS reaction and Masson trichrome stain. A few lymphocytes were present between epidermal cells and mucous cells.

The middle layer was composed mainly of voluminous club cells and a few flask or spherical mucous cells (Figs 1A, 1E and 1F). In between these skin glands, the elongated spindle shaped epidermal cells were found to be vertical (Fig. 1E). The cell boundary of these cells was usually not clear, as it greatly expanded or stretched out due to the collateral pressure of these glands.

The club cells arranged in three to four layers were oval or spherical in shape (Figs 1A, 1E and 1F). They were more numerous. They were usually uninucleate of an oval nucleus, sometimes binucleate, two nuclei very close to each other (Fig. 1E). Their cytoplasm was finely granular or more or less homogeneous in nature and invariably showed some shrinkage due to fixations. Some had a few vacuoles in their cytoplasm. Club cells were approximately 30.0–65.0 μ m in height and were arranged in two to three rows of cells. The main thickness of the epidermis is due to the middle layer, skin glands, particularly. With hamatoxylin/eosin stained preparations, the nuclei of club cells showed blue and the cytoplasm was stained light pink. However, they did not give any histochemical tests for mucosubstances (Table 2). The club cell took a greenish color with Masson trichrome staining and orthochromasia with toluidine blue.

Table 2. A summary of the histochemical tests performed to show the nature of the gland cell of the skin in *Iksookimia koreensis*.

Techniques employed	Gland cells	
	Mucous cell	Club cell
Hematoxylin & eosin	\pm B	+++ PN
Weigerts iron	+++ B	
Haematoxylin		-
Masson trichrome		++ G
PAS	++ R	-
PAS after digestion in malt diastase /PAS	++ R	-
Alcian blue(1.0)	+ B	-
Alcian blue(2.5)	++ B	-
Alcian blue/PAS	++ B, BR, R	-
PAS/Alcian blue	++ B, BR, R	-
Toluidine blue	γ -meta	Ortho
Acetylation/PAS	-	-
Methylation/AB	-	-
Methylation	+ B	
/Saponification /AB		
Aldehyde fuchsin	+ R	
Aldehyde fuchsin/ Alcain blue (2.5)	++ BR	
High iron diamine	+ N	
High iron diamine /alcian blue	+++ BN	

B, blue; BN, bluish black; BR, bluish red; G, green; N, black; PN, pink; R, red; +, increasing intensity of reaction; \pm , fairly present; -, absent.

The stratum germinativum layer was composed of a single layer of cuboidal cells on a thin basement membrane, which was PAS positive, AB positive, took green color in Masson trichrome and gave γ -metachromasia with toluidine blue. Each basal cell had a prominent, lightly stained, centrally placed spherical or oval nucleus, and their cytoplasm was homogeneous (Fig. 1E). There were small oval or round lymphatic spaces in between the cuboidal cells (Fig. 1E). Inside these spaces, we observed one or two small lymphocytes and they had deeply stained nuclei, surrounded by small amounts of faintly stained cytoplasm. They were stained purple in AB and blue in PAS. The lymphocytes penetrated the middle layer, often reaching up to the outer cell layers of the epidermis.

The pit organs, single or in groups of two to three, were distributed on the surface of the skin (Fig. 1F). Each pit organ had a pear-shaped structure, sunk below the epithelial cells, lying directly on the basement membrane. In the intraepithelial layer, there were blood vessels serving the sense organs of the epidermis.

The dermis consisted of a relatively thin upper layer of loose vascular connective tissue, stratum laxum, and a thick lower compact layer, stratum compactum (Figs 1A, 1G and 1H).

The stratum laxum layer contained loose connective tissues consisted of coarse collagenous fibers, blood capillaries, thin scales and pigment cells (Figs 1A, 1G and 1H). A definite area was present between the basement membrane and the scales (Figs 1G and 1H). The area was PAS positive and diastase resistant, AB positive, giemsa positive, and it gave γ -metachromasia with toluidine blue. These materials were present in some free space in which scales were lodged.

The scales consisted of two distinct layers-the upper bony layer and the inner fibrillary plate (Figs 1A and 1H). The bony layer carried concentrically arranged ridges of striae on its free surface, whereas the lower fibrillary plate was mainly composed of parallel collagen fibers. The upper bony layers of the scale were basophilic in nature, and gave red colour with PAS technique and γ -metachromasia with toluidine blue. The lower fibrillary plate was eosinophilic, weakly PAS positive, alcian blue negative and gave strong orthochromasia with toluidine blue.

The stratum compactum layer consisted of bundles of coarse collagenous fiber arranged compactly in several layers (Fig. 1A). This layer was weakly PAS positive and took a deep green color in Masson trichrome stained preparations for collagen. There were a few pigment cells and blood capillaries. Pigment cells are distributed sparsely on the inner part of this layer.

The subcutis layer was situated in between the stratum compactum and the muscle (Fig. 1A). Fine collagen fiber bundles from the stratum compactum traversed this layer. The main branches of the nerves and blood vessels were found in this layer. In hematoxylin and eosin preparations, this layer had numerous empty spaces, which were occupied by fat cells (Fig. 1A).

Discussion

The general structure of the skin of *I. koreensis* was similar to that of other teleosts (Zaccone et al. 2001). But there were histological characteristics as follows: a thick epidermis, two types of gland cells, a well-developed lymphocytes of epidermis, presence of definite area and reduction of scale. These skin features of *I. koreensis* may be closely related to respiration through skin for their oxygen uptake (Jakubowski 1958, Liem 1967, Johansen 1970, Mittal & Munshi 1971, Mittal & Banerjee 1974, Mittal et al. 1980, Whitear 1986, Park & Kim 1999).

The dorsal epidermis of *I. koreensis* was thicker, approximately 110.7 μm . In well known fishes with cutaneous respiration, the thickness of the epidermis was generally thicker: *Misgurnus fossilis* has 392 μm (J a k u b o w s k i 1958), *M. anguillicaudatus* 162.8 μm (P a r k & K i m 1999), *Heteropneustes fossilis* 98 μm , *Mastacembelus pancalus* 44 μm , *Amphipnous cuchia* 119 μm (M i t t a l & M u n s h i 1971), and *Monopterus albus* 75 μm (L i e m 1967).

The epidermis of *I. koreensis* was composed of two types of glands, a small mucous cell and a large club cell. These epidermal glands are related to their main thickness of the epidermis as in other fishes with cutaneous respiration (J a k u b o w s k i 1958, M i t t a l & M u n s h i 1971, P a r k & K i m 1999, 2000). These skin glands contained a lot of water, and oxygen may easily penetrate them towards the deeper layers of the skin (J a k u b o w s k i 1958, M i t t a l & M u n s h i 1971). The mucous cell of *I. koreensis* was acidic sulfomucins (some sialomucins) in nature. The acidic nature of the mucous cell was reported in other fishes with cutaneous respiration: *Monopterus*, *Mastacembelus*, *Amphipnous*, and *Misgurnus* of strongly acidic sulphated mucopolysaccharides (M i t t a l & M u n s h i 1971, M i t t a l & B a n e r j e e 1974, M i t t a l et al. 1980, P a r k & K i m 1999, 2000) and *Heteropneustes* of weakly acidic sulphated mucopolysaccharides (M i t t a l & M u n s h i 1971). This mucus had great ability to bind a large amount of water (L e t t e r e r 1959, R o g e r s 1961), and the mucus secreted by the skin in air breathing fishes may also be used to keep the skin clear for respiration (H o r a 1934, M i t t a l et al. 1980).

There was a definite area with acid mucopolysaccharides in the stratum laxum of the dermis of *I. koreensis*. The existence of mucopolysaccharides in the stratum laxum may be related to the semiterrestrial ecological habits of fish or was of characteristic of fishes with cutaneous respiration (M i t t a l & M u n s h i 1971).

A well-defined lymphatic system with a series of lymphatic spaces containing small lymphocytes was present in the stratum germinativum layer of the epidermis of *I. koreensis*. The lymphatic system was known in other for use of cutaneous respiration, functioning in the supply of nutrition to the stratum germinativum for cell proliferation and to protect the epidermis from microorganisms or foreign proteins for protection of the epidermis (M i t t a l & M u n s h i 1971).

I. koreensis also had small scales embedded in the superficial layer of the dermis. The small scales, rudimentary scales or absence of scales were found in a burrowing and a mud-dwelling fish (*Amphipnous*, *Monopterus*) and were considered as an adaptation to its peculiar mode of life (L i e m 1967, M i t t a l & M u n s h i 1971, W h i t e a r 1986).

Well-developed vascularization of the skin is characteristic of fishes with cutaneous respiration. Amphibious fishes, *Periophthalmus cantonensis* and *Periophthalmus modestus* have intraepithelial capillaries (T a m u r a et al. 1976, P a r k & K i m 2000), and the skin of *M. fossilis*, *M. anguillicaudatus*, *Anguilla*, *Amphipnous*, and *Monopterus* has the blood vessels in the dermis (J a k u b o w s k i 1958, L i e m 1967, L e t h b r i d g e & P o t t e r 1982, P a r k & K i m 1999). Also, the skin of *I. koreensis* has blood vessels between the basement membrane and the dermis, which means that diffusion of oxygen takes place readily across the mucous coat of the epithelium, though the blood vessels were situated at the deep dermis (M i t t a l & M u n s h i 1971, P e r r y & M c D o n a l d 1993).

A c k n o w l e d g e m e n t s

We wish to thank Professor K. C. R i c h a r d s o n, Laboratory of Anatomy, Murdoch University, Australia for comments and revision of the manuscript, and Professor R. A l l e n, Language Education Center, Chonbuk National University, Korea for correcting of English of the manuscript.

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