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Participation of Overlying Layers in Determining the Shape of Melanophores on *Oryzias latipes* Scales

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Abstract Scale melanophores of the medaka, *Oryzias latipes*, were examined to determine whether their overlying layers affect their morphology and melanosome distribution, especially upon treatment with cytochalasin B or hypotonic saline. Observations were conducted with both light and scanning electron microscopy. The overlying layers were mechanically removed with fine forceps after collagenase-treatment. Melanophores with intact overlying layers responded to cytochalasin B (1 to 10 μg/ml) or hypotonic saline (M/15) by undergoing remarkable super-dispersion of their melanosomes. The central part of the melanophores became concave. On the other hand, melanophores without the overlying layers showed a tendency for melanosome aggregation, although the aggregation was incomplete. Their central part swelled. It was concluded that the overlying layers affected the shape of the melanophores and the melanosome distribution within them.

Generally fish chromatophores are covered with collagen and epidermis. It has been pointed out in a pharmacological study (Fujii, 1973) that these layers covering the chromatophores interfere with rapid permeation of a reagent. In recent years, attempts have been made to isolate and culture chromatophores from fish (Obika, 1976; Ozato, 1976; Matsumoto et al., 1978; Wakamatsu, 1978). Scale chromatophores from which the overlying layers were mechanically removed, as well as cultured ones, have been used in experiments (Obika, 1975; Ohta, 1980). Since the surface of the chromatophores is exposed in both cases, they can respond rapidly to the action of drugs. The function of the overlying layers must be examined to learn whether they only prevent permeation or also affect the shape of cells and granular movements.

Ohta (1972, 1974) reported that cytochalasin B and a hypotonic solution induced distinct super-dispersion of melanosomes in melanophores. The present report examines the shape of melanophores in response to the action of cytochalasin B or a hypotonic solution and discusses the relationship between the shape of the cells and the presence of overlying layers.

Materials and methods

Melanophores of scales removed from the dorso-lateral part of the wild-type medaka, *Oryzias latipes*, which were collected in Toyohashi City, were used. The following melanophores with overlying layers were used: (1) those immediately after isolation, thus being regarded as innervated; (2) those incubated (20°C) in Eagle MEM for a day after isolation, thus being functionally denervated. Melanophores without overlying layers were obtained as follows. The overlying layers of the innervated melanophores were mechanically removed with fine forceps after treatment with 1% collagenase-10^{-4}M dibenamine-physiological saline for one hour (20°C). Dibenamine was used to prevent melanosome aggregation that would result from stimulation of melanophore-aggregating nerves when the overlying layers were removed. To prepare the denervated melanophores, the treatment with dibenamine was omitted. Incubation of melanophores on scales in Eagle MEM induced separation of the overlying layers from the scales (Ohta, 1980). Melanophores incubated for 5 days were used as specimens without overlying layers in addition to the above-mentioned denervated ones.

Yamamoto's (1941) salt solution (osmotically eq. to M/7.5 NaCl) adjusted to pH 7.2 with $NaHCO_3$ was used as a physiological saline. The concentration of cytochalasin B and the hypotonic saline was 1 ~ 10 μg/ml and M/15, respectively. Cytochalasin B was dissolved in dimethylsulfoxide (final concentration, 0.02 ~ 0.2

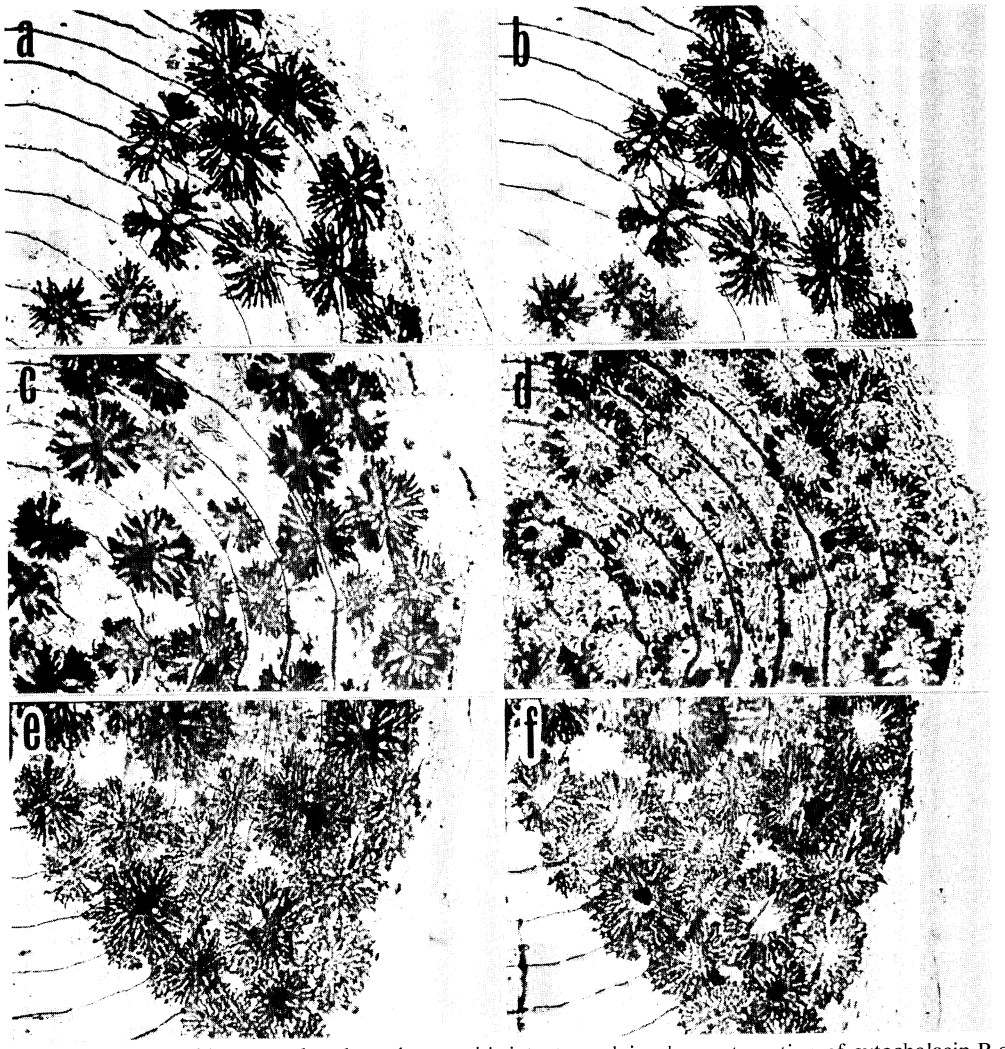

Fig. 1. Responses of innervated melanophores with intact overlying layers to action of cytochalasin B or hypotonic saline. a, c, e: melanophores in physiological saline. b: melanophores 30 min after immersion in physiological saline. d: melanophores 30 min after exposure to 10 μg/ml cytochalasin B. f: melanophores 30 min after immersion in hypotonic saline (M/15). Note the super-dispersion of melanosomes in d, f. ×150.

%) and diluted into the physiological saline.

The scale was fixed on a glass cover slip which was then inverted over a glass trough in such a way that the specimen could directly contact the surrounding solution. Responses and morphological changes in melanophores with or without the overlying layers were examined after cytochalasin B or the hypotonic saline had acted on them for 30 min. The specimens were examined with light and scanning electron microscopes.

For scanning electron microscopic observations, melanophores exposed to cytochalasin B or the hypotonic saline were fixed with 4% glutaraldehyde-0.1M phosphate buffer (pH 7.2). After they were rinsed in the buffer, they were postfixed with 1% osmium tetroxide for 1 hour (4°C) and then dehydrated with graded concentrations of ethanol, acetone and isoamylacetate. They were dried in liquid CO_2 (Hitachi, HCP-2 critical point drying apparatus) and coated with gold (JEOL, JFC-1200 fine coater). The preparations were observed with a JEM 100-B

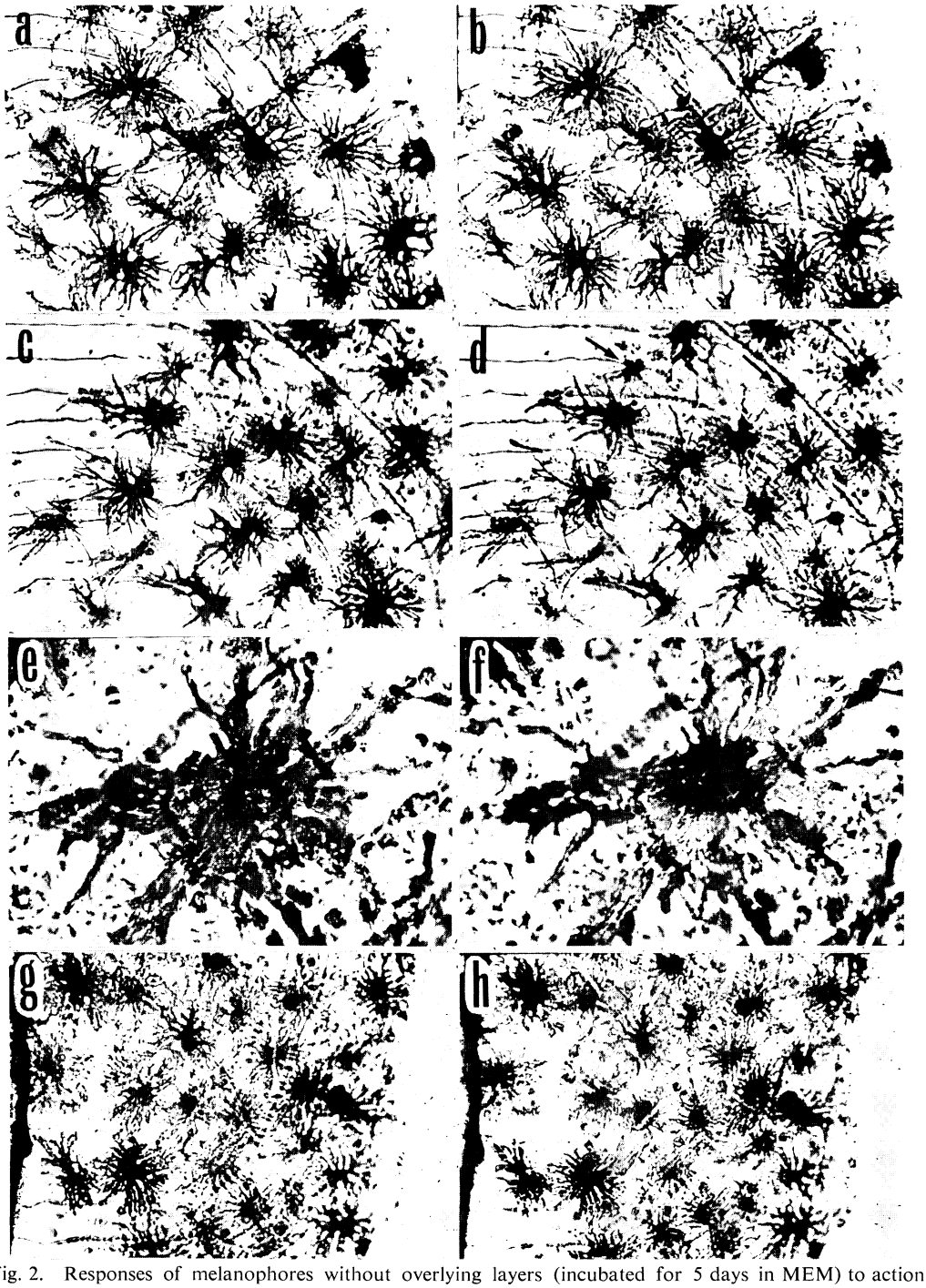

Fig. 2. Responses of melanophores without overlying layers (incubated for 5 days in MEM) to action of cytochalasin B or hypotonic saline. a, c, e, g: melanophores in physiological saline. b: melanophores 30 min after immersion in physiological saline. d: melanophores 30 min after exposure to 10 μg/ml cytochalasin B. f: high magnification of a melanophore under the condition of d. h: melanophores 30 min after immersion in hypotonic saline. Note the slight aggregation of melanosomes in photos d, f and h. Completely aggregated melanophores are seen in photo d (arrows). a～d, g, h: ×150; e, f: ×600.

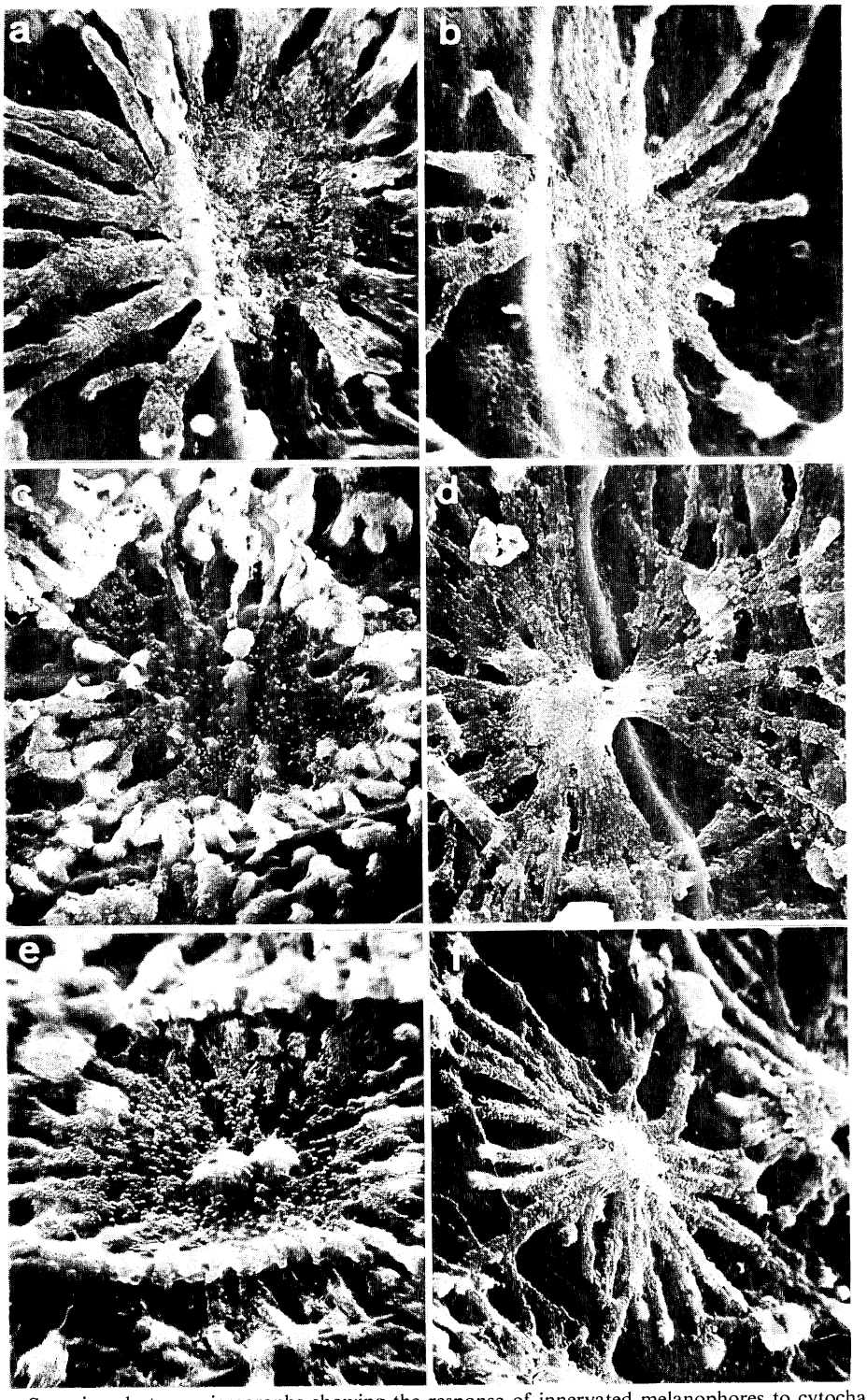

Fig. 3. Scanning electron micrographs showing the response of innervated melanophores to cytochalasin B or hypotonic saline. a: a melanophore in physiological saline immediately after removal of overlying layers. ×1,050. b: a melanophore in physiological saline 30 min (continued on p. 66)

electron microscope with a scanning attachment.

Results

1. Light microscopic observations on the response of melanophores with intact overlying layers to action of cytochalasin B or hypotonic saline.

Innervated melanophores were treated for 30 min with cytochalasin B or hypotonic saline and changes in their morphology were observed. As controls, melanophores were treated with physiological saline only. The results are shown in Fig. 1. Innervated melanophores remained dispersed in the physiological saline (20°C) for about two hours after scale-isolation. Most of the melanophores had a uniform distribution of melanosomes for 30 min (Fig. 1a, b). On the other hand, several minutes after the exposure to 10 μg/ml of cytochalasin B, the melanosomes moved back and forth vigorously in the cells and gradually migrated from the centrosphere to the periphery of the branches of the cell. Consequently, remarkable super-dispersion of melanosomes resulted (Fig. 1c, d).

The innervated melanophores did not show an aggregation response in M/15 saline, but underwent the same super-dispersion of melanosomes as with cytochalasin B action (Fig. 1e, f). Particularly vigorous Brownian movement of melanosomes was observed in this case.

2. Light microscopic observations on the response of melanophores without overlying layers to actions of cytochalasin B or hypotonic saline.

When the melanophores on the scales were incubated in MEM (20°C), melanophore-aggregating nerves lost their function within about a day after isolation of the scales. Moreover, separation of the overlying layers from the scales proceeded with the elapsing days so that the surface of the melanophores was exposed in 5 or 6 days of incubation (Ohta, 1980). After 5 days of incubation, the response of the melanophores without overlying layers to cytochalasin B or hypotonic saline was observed (Fig. 2). Fig. 2a and b shows dispersed melanophores in physiological saline. Melanosomes were distributed evenly in the cells and their distribution did not change for 30 min. In contrast to the response of melanophores with overlying layers, the melanosomes in exposed melanophores tended to aggregate in response to 1～10 μg/ml cytochalasin B. There were a few melanophores that aggregated completely (Fig. 2d, arrows). In most melanophores, the melanosomes moved to the centrosphere, but their branches still remained (Fig. 2d, e).

In the M/15 saline, most of melanophores underwent a slight aggregation of melanosomes, which moved to the centrosphere of the cell. The aggregation was similar to that induced by cytochalasin B (Fig. 2g, h).

3. Scanning electron microscopic observations on the response of melanophores to cytochalasin B or hypotonic saline.

Melanophores with or without overlying layers were treated for 30 min with cytochalasin B or hypotonic saline, and then examined with a scanning electron microscope. Fig. 3a illustrates a melanophore immediately after removal of the overlying layers, while Fig. 3b shows one that had been immersed in physiological saline for 30 min after the removal. The surface of each melanophore with or without overlying layers was flat when the melanophores were immersed in saline.

The melanophores with intact overlying layers responded to cytochalasin B by a remarkable super-dispersion of their melanosomes. A few melanosomes remained in the centrosphere of the cell but the branches were filled with a number of melanosomes (Fig. 3c). It was clearly observed that in the melanophores without overlying layers, melanosomes aggregated at the centrosphere and the central part of the cell swelled (Fig. 3d). One cannot observe the surface of the melanophores if the overlying layers remain on them even slightly. The overlying layers of these samples (Fig. 3) were completely removed.

The melanophores with overlying layers responded to hypotonic saline by undergoing remarkable super-dispersion, as with cytochala-

after the removal of overlying layers. ×1,050. c: a melanophore with intact overlying layers after 30-min treatment with 10 μg/ml cytochalasin B. ×700. d: a melanophore without its overlying layers after 30-min treatment with 10 μg/ml cytochalasin B. ×1,050. e: a melanophore with intact overlying layers that has been exposed to hypotonic saline (M/15) for 30 min. ×1,050. f: a melanophore without its overlying layers that has been exposed to hypotonic saline for 30 min. ×700.

sin B. Conversely, the central part of the melanophores without overlying layers swelled after treatment in hypotonic saline (Fig. 3e, f). Similar results were obtained with the denervated melanophores incubated in Eagle MEM for a day.

Discussion

Melanophores on fish scales are covered with overlying layers (epidermis and collagen). Examination of the role of these overlying layers should be conducted by using melanophores with or without them. The overlying layers could be mechanically removed with fine forceps after collagenase treatment, and this did not affect the physiology of the melanophores. In the present experiment, the complete removal of the overlying layers, which could be ascertained with a scanning electron microscope, was a focal point.

Generally, fish melanophores are controlled by adrenergic nerves, which have an aggregating action on the melanosomes (Fujii, 1961). Immediately after isolation of a scale, the aggregating nerves remain functional for a few hours. Mechanical stimulation of the nerves cannot be avoided upon removal of the scale. Therefore, we prevented aggregation of melanosomes by using dibenamine, an adrenergic α-receptor blocking agent. On the other hand, in the melanophores immersed in physiological saline (Iwata et al., 1959) or Eagle MEM (Ohta, 1980), the nerves ceased functioning within about a day (20°C). Therefore, the use of dibenamine could be omitted in the denervated melanophores. The results obtained were the same for both innervated and denervated melanophores; that is, the presence of nerves and the use of dibenamine did not affect on the results in the experiment.

Ohta (1974), employing fish melanophores with intact overlying layers, investigated the effects of cytochalasin B on melanosome movement and the shape of melanophores. He clarified that cytochalasin B-sensitive microfilaments did not play a significant role in melanosome movement and that melanophores subjected to cytochalasin B underwent a remarkable super-dispersion in which melanosomes were almost nonexistent in the centrosphere of the cell, having dispersed to the branches. On the other hand, Obika (1975) observed melanosome aggregation in fish melanophores without overlying layers. Although both Ohta and Obika used the melanophores of medaka scales as materials, their results did not agree. The present data demonstrate that the response of melanophores to cytochalasin B action is completely reversed by the presence or absence of the overlying layers. This should explain the difference in the results obtained by the two investigators. Direct action of cytochalasin B on melanophores may induce melanosome aggregation. However, melanophores of fish are usually covered with overlying layers. In addition, the overlying layers exert an influence on the shape of the melanophores. Therefore, we have to take the participation of the overlying layers into consideration when studying the mechanism of melanosome movement.

Although cytochalasin B has some obscure actions, it is known for its action upon microfilaments in cells (Carter, 1967; Wessells et al., 1971). The rounding up of cells in the presence of cytochalasin B is not peculiar to melanophores since other cultivated cells are rounded up by its action (Croop and Holtzer, 1975). Melanophores with overlying layers underwent super-dispersion. We have no conclusive explanation for this phenomenon. Cytochalasin B may act upon not only the melanophores but also the overlying layers. As a result, changes in the overlying layers may affect the melanophores. For clarification of this point, it may be necessary to examine the morphological relationship between the overlying layers and the melanophores.

Ohta (1972) found that melanosomes in melanophores did not aggregate, but underwent super-dispersion in M/15 saline. Therefore, this concentration of saline was used as a hypotonic solution in the present experiment. In this saline water should permeate into cells and consequently the cells should swell. Transformation of the overlying layers by permeation of water may induce the super-dispersion of melanosomes. The morphological changes in the melanophore caused by the saline were almost the same as those obtained with cytochalasin B.

These results in the present study demonstrate that the overlying layers affect the melanosome distribution and shape of the melanophores. However, the detail mode of their effects

on the melanophores is unclear and remained as a future problem.

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メダカ鱗上の黒色素胞の形態と上皮組織との関連性

太田忠之

メダカ鱗上の黒色素胞はその周囲をコラーゲンや上皮組織（以下，両者を OL と略す）によっておおわれている．OL と黒色素胞の形態・顆粒の分布との関係をサイトカラシン B (CB) 及び低張溶液 (HS) を用いて調べた．OL の存在する黒色素胞は CB 及び HS の作用により著しい顆粒拡散を示し，細胞の中心部は扁平となった．一方，OL を除去した黒色素胞は逆にそれらの作用により顆粒の凝集を示し，細胞の中心部の盛り上がりがみられた．これらの結果は黒色素胞凝集神経の存否と無関係であった．以上のことから OL は黒色素胞の形態や顆粒の分布に影響を及ぼしているものと思われる．

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