Molecular Characteristics and Site Specific Distribution of the Pigment of the Silky Fowl

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ABSTRACT. Silky fowl, a breed of chicken, is hyperpigmented in its various internal tissues. The pigment was extracted from various tissues of two strains of Silky fowl to determine its molecular structure and internal distribution. Analysis by infrared spectroscopy showed two spectrum patterns of the pigment in Silky fowl; one is from ovary and testis, the other is from periosteum and feather. The difference between the two spectra is possibly due to the sulfur contents of melanin. Especially, the spectra of the pigments from feather and periosteum shared the characteristics of synthesized melanin spectrum in common, which indicates that the melanocytes dispersed in these tissues were functionally the same. According to our quantitative analysis, the tissues examined were classified significantly in the order of the pigment content ($p<0.05$): periosteum > gonads (ovary or testis) = trachea $\geq$ heart, liver, gizzard, cecum, muscles (*Pectoralis* and *Supracoracoideus*) and skin. In addition, the specific regions of embryonic neural crest derived cells, such as cardiac artery and various parts of cephalic tissues, were found to be locally hyperpigmented. These data suggest that hyperpigmentation (fibromelanosis) in Silky fowl chicken occurs in a tissue- and organ-specific manner, which is strongly related to neural crest cell development. It is hypothesized that neural crest cells of the bird, containing melanocyte progenitors, acquire unusual ability to differentiate into melanocytes excessively, and to extend the distribution of their descendant along the destinations of neural crest derivatives.—KEY WORDS: hyperpigmentation, melanocyte, neural crest, pigment distribution, Silky fowl.
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Silky fowl (SF), a breed of chicken originated from China, has several feather color types, mainly white and black. Despite the feather color, both white and black type birds have notable morphological characteristics in common: fluffy feathers (silky), polydactyly and internal hyperpigmentation [7, 18]. The internal hyperpigmentation has been observed in various organs of the SF chicken: perimysium in muscle, the surface of bone (periosteum), trachea, mesentery, digestive canals, ovary and testis [9, 12, 13]. Similar phenotype has been observed in a few strain of mouse [14], however, such visceral pigmentation has been extremely rare in higher vertebrates, in which pigmentation usually occurs in the derivatives of epidermis, hair and feather, subcutaneous and optic dermis.

Although the mechanism of hyperpigmentation in the internal tissues is poorly understood, earlier genetic studies have suggested that this phenotype is induced by a sex-linked gene *id* and an autosomal gene *Fm* [4, 18]. However, gradual phenotypes from the lightest to the darkest in segregating populations were obtained in crossing experiments of SF and Leghorns, which suggests that the phenotypes are regulated by multiple genes.

According to a more detailed observation [9], a term, fibromelanosis, was suggested for the hyperpigmentation of internal connective tissue [7]. Several other anatomical studies have shown that numerous black granules and melanocytes were observed in the hyperpigmented tissues [6, 12, 13]. In addition, in our preliminary observation, the hyperpigmentation was limited to several specific organs. It is possible that SF melanocytes fail to be confined to epidermis during their migration. In order to elucidate the specific characteristics of SF, it is necessary to investigate the fibromelanosis, or the distribution and the characteristics of SF pigment and melanocyte.

The SF hyperpigmentation might also be caused by some uncontrolled biosynthesis of the pigment. Mammalian and avian melanocyte can produce two basic types of melanin; sulfur-free eumelanin and sulfur-containing pheomelanin [8]. It has been demonstrated that the SF pigment was a type of melanin [20]; however, it was isolated from a homogenate of various tissues. Some differences in the melanin molecule between the internal and the body surface tissues may occur, which might suggest that the melanin synthesis occurs by an unusual mechanism in the internally located melanocytes.

The present experiments were performed to determine the characteristics and the distribution of pigment in SF chickens.

MATERIALS AND METHODS

Animals and sample preparation: Six SF chickens (two white feather type hens, two white cocks, one black type hen and one black cock) and a White Leghorn (WL) chicken, aged 3 to 19 months, were obtained from National Institute of Animal Industry (NIAI; Kukisaki, Ibaraki, Japan). The birds were slaughtered by severing the jugular veins, bled for 3 to 5 min, and then were stored frozen at 20°C for several months before analysis. From each chicken, heart, liver, gizzard, cecum, trachea, *M. pectoralis* (MP), *M. supracoracoideus* (MS), skin, ovary or testis, and periosteum of the femur were cut out and minced for the present

experiments. For the observation of pigmentation in cephalic tissues, another three young white SF chickens, aged 0, 30, 90 days, and a black one aged 90 days were used.

Measurement of Infrared (IR) spectrum: Samples from the ovary, testis or periosteum of white SF chickens were individually sonicated in phosphate-buffered saline (PBS) on ice to soften tissue structure. Collagenase (Type II) (Sigma Chem., St. Louis, MO, U.S.A.) solution was added to the sample suspension to a final concentration of 0.2%, followed by incubation at 37°C for 30 min, then 0.25% trypsin (Type III) (Sigma) solution was added. The sample was digested at 37°C for 1 hr and centrifuged at $9,500 \times g$ for 5 min. The precipitate was dissolved in 1 N NaOH and boiled for 20 to 30 min, depending on the solubility. To remove lipids, an equal volume of chloroform was added to the solution, mixed, and centrifuged at $9,500 \times g$ for 3 min.

Feather samples were prepared as follows: the barbs and the barbules of the black feathers were boiled in denaturing solvent consisting of 8 M urea (Nacalai Tesque, Kyoto, Japan), 1% 2-mercaptoethanol (Wako Pure Chem., Osaka, Japan) for 30 min and then sonicated to disrupt the feather structure. After separation at $9,500 \times g$, the precipitate was washed with PBS and incubated in 0.25% trypsin at 37°C for 1 hr. The sample was then centrifuged at $9,500 \times g$, washed with 0.1 N HCl, and dissolved in 1 N NaOH by boiling for 20 min. The solution was centrifuged at $9,500 \times g$ for 5 min and lipids included in the supernatant were removed with chloroform as described above.

The sample solution was neutralized by adding 5 N HCl and centrifuged at $9,500 \times g$ for 5 min. The precipitate was washed briefly with acetone and dried. The sample, mixed with finely ground potassium bromide (Japan Spectroscopic, Tokyo, Japan) powder was dried at 60°C for 2 hr and stored in a desiccator overnight. Disc samples were made by pressing the powder and were applied to an IR spectrophotometer (FT-IR 5MP) (Japan Spectroscopic). As a control, synthesized melanin (Sigma) was used.

Measurement of the pigment content of tissue: For apparently hyperpigmented organs (cecum, trachea, ovary, testis, periosteum of femur), the pigment solution of each tissue was prepared by the same method described in the "Measurement of IR Spectrum" section. For the other more lightly pigmented organs, collagenase treatment was preceded by trypsin treatment followed by centrifugation.

The concentration of brown-black pigment was determined by measuring the absorbance at 540 nm using the aqueous solution following chloroform treatment. In the preliminary experiment, no specific absorption signal was present in ultraviolet (UV) and visible region spectrum of SF black pigment and, at 540 nm, the absorption level of the WL muscle sample was extremely low compared to that of SF muscle. The pigment content of each tissue for each chicken was measured in duplicate.

Because there was no difference in UV and visible region spectrum between SF black pigment and synthesized melanin, the latter was used for drawing a calibration curve

for the black pigment. The pigment content of each tissue was calculated from the absorbance of the solution at 540 nm, based on the calibration curve.

Statistical analysis: The data obtained in the evaluation of the pigment content were analyzed by a three-way classification with the factors of sex, feather color and tissue, using the General Linear Models (GLM) procedure of SAS® [17]. Means for tissues were separated using the Tukey's significant difference test with confirmation of no interaction among the three factors. The probability level of p=0.05 was accepted as significant.

RESULTS

To examine the molecular characteristics of the internally expressed pigment, we obtained the IR spectra of the pigment expressed in ovary, testis, periosteum of the femur. Feather of black SF chicken was also used as a control because it is a typically pigmented body surface tissue in many higher vertebrates. The black-brown pigment finally obtained was insoluble in acid and scarcely soluble in alkali at room temperature. Figure 1 shows that there are two spectra patterns of the pigment extracted from the SF. All spectra had four main absorption bands in common, with peaks at 3,457 to 3,408 cm^{-1}, 2,963 to 2,922 cm^{-1}, 1,645 to 1,622 cm^{-1} and 1,400 to 1,395 cm^{-1}, respectively. These bands show the presence of CH-CH, N-H, C=C, C-O and C-N bonds, all of which constitute hetero-aromatic ring, pyrrole or indole ring. The broad band at 3,500 to 3,200 cm^{-1}, especially at the proximity of 3,400 cm^{-1}, is characteristic of stretching of N-H bond of pyrrole or indole ring. In this region, a strong band attributed to O-H stretching of H$_2$O molecule often appears under humid conditions, as shown in the spectra of testis and feather. The band at the proximity of 1,700 cm^{-1} can be attributed to the stretching of the C=O bond that is also included in melanin structure. These functional groups and rings are typical molecular structure of melanin. All of these spectra are roughly similar to those of synthesized as well as previously reported melanin [20].

At the proximity of 1,070 cm^{-1} and 485 cm^{-1}, signal bands that suggest presence of sulfur-containing groups were observed in the spectra of feather and periosteum, but were not in those of ovary and testis. Various sulfur-containing components, such as proteins and proteoglycans, should be included in the examined tissues to some extent. However, the process of pigment extraction used in this experiment may not have left these components in the final extract. These spectra suggest that the pigment molecule of feathers and periosteum have more sulfur-containing groups in its structure than that of ovary and testis.

We determined the pigment contents of the WL and SF organs or tissues described above, in order to investigate the distribution of pigment quantitatively (Table 1). No statistically significant difference in the content was found between hen and cock or between white and black SF types. Therefore, to investigate the tissue specificity of the
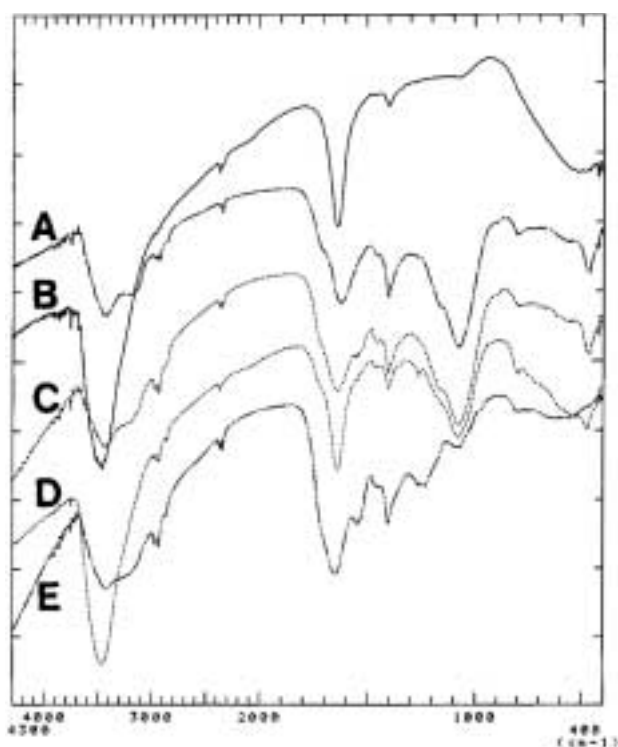
Fig. 1. Infrared (IR) spectra of melanins extracted from various tissues of Silky fowl. The black-brown pigment of testis (B), periosteum (C), feather (D) and ovary (E) of white Silky fowl were extracted by treatment with trypsin and collagenase followed by solubilization in 1N NaOH. The dried pigment samples and potassium bromide powder were mixed together to prepare disc samples for IR spectroscopic analysis. Synthesized melanin (A) was used as a control.

Table 1. Melanin contents of various tissues of Silky fowl chicken and White Leghorn chicken

Organ (Tissue)	Melanin content (mg/g)*	
	Silky Fowl	White Leghorn
Periosteum (Femur)	21 ± 2.2[a]	0.27
Ovary or Testis	9.7 ± 4.2[b]	0.14
Trachea	8.6 ± 0.94[bc]	0.60
Skin	0.944 ± 0.289[cd]	0.012
Cecum	0.889 ± 0.283[cd]	0.053
Heart	0.124 ± 0.033[d]	0.112
Liver	0.072 ± 0.024[d]	0.092
Supracoracoideus	0.067 ± 0.007[d]	0.009
Pectoralis	0.050 ± 0.014[d]	0.010
Gizzard	0.039 ± 0.004[d]	0.046

*: Six Silky fowls and one White Leghorn were used. Pigment content of each organ for each chick was measured in duplicate. Means with no common superscripts (a-d) among Silky fowl tissues differ ($p<0.05$).

shown in Table 2, though the pigmentation level differed by individual SF chickens. Heavy pigmentation occurred especially around neural crest (NC) derivatives, but not the organs derived from mesoderm, such as lingual muscles, and the other embryonic tissues. The pigmentation pattern of cephalic tissues was similar among the four young chickens at different growth stages, though viscerocranium of the youngest one was slightly less pigmented. Although the pigment content of SF heart was at a low level as that of the WL, the inner wall of cardiac artery and the surrounding tissues were locally pigmented in all SF chickens examined.

DISCUSSION

The IR spectra obtained in the present experiment resembled those shown by a previous study [20] and all of the pigments from examined tissues were found to be melanin, although they seemed to be classified into at least two subtypes, eumelanin and pheomelanin. The spectra of feather and periosteum showed the signal bands suggesting involvement of sulfur containing groups which is a structural characteristic of pheomelanin, but none of such signal was observed in the cases of ovary and testis. This result does not contradict a recent study of element content of SF chicken [15]. According to these data, it is suggested that the ratio of the two types of melanin may vary from organ to organ in the SF chicken. The tissue specificity of the type of melanin molecule is possibly due to some disturbance of organ-specific factors that encourage melanocyte to synthesize pheomelanin, such as α-melanocyte stimulating hormone.

In this paper, we also showed the difference in the pigment content among various organs of the SF chicken. The pigment distribution pattern shown in Table 1 was consistent well with previous anatomical observation of the melanocyte distribution [9, 12, 13]. Especially, the contents of periosteum and gonads were significantly higher than those of the other organs. However, melanocyte distribution

hyperpigmentation of SF chickens, we considered the white and black types as the same phenotype samples in the experiment. The pigment content of periosteum severed from femur was 21 mg per one gram of tissue and was the highest of all parts examined ($p<0.05$). Pigmentation in the gonads (ovary or testis) and trachea (9.7 and 8.6 mg/g, respectively) were significantly lower than in the periosteum, but significantly grater than in the other tissues. No significant difference in the pigmentation was observed among the other organs. The pigment contents of heart, liver, gizzard, cecum, skin, MS and MP were evaluated to be less than 1.0 mg/g. The contents of skin and cecum (0.944 mg/g and 0.889 mg/g, respectively) were higher than those of heart, liver, gizzard, MP and MS. When we used the WL organs as the control of this experiment, the pigment contents evaluated from the spectroscopic absorption at 540 nm were virtually at the background level.

The spatial expression pattern of the pigment shown in Table 1 was consistent with the results of previous reports [6, 9, 12, 13] as well as our recent anatomical observation of pigmentation. In the macroscopic observation, several cephalic tissues, such as viscero- and neurocranium, connective tissue, and peripheral nerves, were pigmented as

Table 2.   Pigmentation of cephalic and cardiac tissues of Silky fowl chicken

Organ (Tissue)[a]		Developmental origin[b]	Pigmentation[c]
Neurocranium	Pareital bone	NC	+
	Frontal bone	NC	+
Viscerocranium	Palatine bone	NC	+
	Maxilla	NC	+
Dura mater		NC	+
Brain Cerebrum		–	–
Cerebellum	White matter	–	–
	Gray matter	–	+
Medulla oblongata		–	–
Optic chiasma		–	–
Lingual muscles		–	–
Cranial nerves		–	–
Cephalic connective tissues		NC	+
Cardiac artery		NC	+

a) Four and six chickens were used for the obsevations of cephalic and cardiac tissues, respec-
   tively.
b) NC and – indicate neural crest and the other tissues, respectively [2, 10].
c) + and – indicate pigmented and unpigmented, respectively.

to gonads is not unique to SF; pigmentation in gonads has also been observed in quail (*Coturnix coturnix japonica*) [5], *Saxicoloides fulicata* and *Bubulcus ibis* [3], *Tetrao urogallus* and *Lyrurus tetrix* [19]. The pigment expression was also observed in other specific organs of SF chicken, and apparently the internal hyperpigmentation does not occur at random, which suggests that the SF melanocytes are distributed in a tissue- and organ-specific manner. Periosteum hyperpigmentation is one of the most SF specific phenotype and reflects typical fibromelanosis. As our IR data shows, the pigment accumulated in the connective tissue of SF periosteum is possibly the same as that accumulated in the epidermis or the feather. Therefore, although tissue specificity of the ratio of melanin types was found in gonads, we concluded that, at least in the periosteum, the hyperpigmentation depends on the expansive distribution process of the melanocyte but not on the unusual biosynthesis and characteristics of the melanin.

SF specific structural constituents of the loose connective tissue around blood vessels and nerves of periosteum, mesentery and surface of trachea may cause the unique melanocyte distribution. On the other hand, the peculiar pattern of pigment distribution suggests that the SF uniqueness is due to an unusual development of the NC cells. In our anatomical observation, cranial peripheral nerves, viscerocranium, dura mater and inner wall of cardiac artery were the specific regions of SF hyperpigmentation (Table 2), which is partly supported by previous observations [12, 13]. Morphogenesis of these tissues largely depends on the cephalic NC derived cells in bird, mouse and human [1, 2, 10, 11]. The distribution of the other pigmented regions was also coincident with developmental destinations of NC cells. Embryonic branchial arches originated from NC contribute to the formation of the wall of digestive canals and trachea [11], which are quantitatively determined as a significantly

hyperpigmented organs. Since the developmental origin of melanocytes is embryonic NC in higher vertebrates [2, 10], from these observations, it is hypothesized that SF melanocyte progenitors fail to be spatially confined, keeping the NC derived migratory behavior.

In SF embryos, melanocyte progenitors are thought to undergo unique migration and differentiation process [5, 16]. When quail NC with neural tube was explanted into SF embryo, the distribution of quail melanoblasts derived from the explant was influenced by the environmental factors of the host SF embryo [5]. Further study for the SF fibromelanosis should be performed with the viewpoint of molecular constituents of hyperpigmented loose connective tissue, as well as factors regulating SF melanogenic differentiation and migration.

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