Brief Communication
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Transmission Electron Microscope Study of Melanocytes on the Mongolian Blue Spot Region

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Objectives: We aimed to study the melanocyte and melanin granules of the blue spot skin. **Methods:** The skin specimens were taken from the sacral region of a 1 and an 8 month old child and a 35 year old man. We proceeded to examine the melanocytes of the blue spot and non-blue spot regions by light and transmission electron microscope analysis. **Results:** TThe melanocytes of the sacral skin of the adult man were found on the epidermal side of the epidermal-dermal junction. The cytoplasm of the melanocyte contained melanin granules of the dense type. The melanocytes of the blue spot skin of the 1 and 8 months old children were found at the uniformly thin basal membrane of the epidermis. The cytoplasm of these melanocytes contained melanin granules of both light and dense types. There were distinct differences in the thickness of the basal membrane, and in the size and composition of the melanin granules. **Conclusion:** Melanocytes of the sacral blue spot region were located on the basal membrane of the epidermis near to the epidermal-dermal junction. The cytoplasm of the melanocytes contained melanin granules of both dense and light types.

Keywords: Blue Spot Region, Melanin Granules, Melanocyte Cell

Introduction

The blue or slate-gray macular lesion commonly occurring on the sacral region skin is the so-called Mongolian spot [1]. The term for the sacral blue spot is variously called the blue spot or Mongolian blue spot depending on the research resource. Worldwide, over 90% of Native Americans and African descendants, 80% of Asians, 70% of Hispanics, 40% of Iranians, and fewer than 10% of Caucasians have Mongolian spots [2, 3].

Mongolian spots are benign skin markings, commonly appear at birth, and are not associated with any disorder. Generally, they fade in a few years and disappears by puberty [4, 5]. Therefore, Mongolian spots are a congenital dermatological condition believed due to neural crest-derived melanocytes normally migrating to the dermal-epidermal junction [6, 7] of the skin during embryogenesis.

Most of the research works reported that placing of melanocyte cells of Mongolian spots in the dermis [8-10] and dermal melanocytic areas occurs when the melanocytes fail to reach the epidermal basal cell layer/Malpighian layer [11]. In contrast, the histological data of the current study involving Mongolian children revealed that the number of melanocyte cells was much higher in the stratum basale of the epidermis when compared to non-blue spot regions [12]. The epidermal melanocyte is thought of as distinct from the Malpighian cell, both as regards to its morphology and its biochemical properties [13-15]. Obland et al. noticed that the melanocyte is free of fibrillar material (tonofibrils and tonofilaments) typical of the Malpighian cells and that intercellular bridges between them and the other cells of the epidermis are also absent [16, 17]. This raises the question of the mobility of the melanocyte [18]. The latter researchers described melanocytes which they interpret as passaging through the dermo-epidermal junction [19-23], however mobility was not eventually explained.

In Mongolian infants the prevalence of the blue spot was 91.3% and this is a very high prevalence compared to the world [24]. And there are a few research works of the blue spot morphology which involve Mongolian children. In the current work, we aimed to study the melanocyte and melanin granules in the sacral blue spot region of a one and an eight month old child's skin compared to the skin of the adult man in a non-blue spot region.

Materials and Methods

Skin specimens

The skin specimens of 5x5x2 mm^3 in size were taken from the sacral region of a one month old and an eight month child, and a 35 year old man respectively n=3 (total n=12) by biopsy after local anesthesia.

Light microscopy

The skin specimens were fixed in 4% paraformaldehyde in PBS for 4 hours at 4°C, and washed in PBS for 4 hours to be dehydrated in a graded series of ethanol 70-100% for each 1 hour. The samples were treated with xylene I and II for each 30 minutes, the mixture of xylene and paraffin 1:1 for 1 hour, soft and hard paraffin for each 1 hour, and finally embedded in paraffin. The embedded samples were consecutively cut into 4 µm sections with microtome (Microm, Walldorf, Germany) and stained with Hematoxylin and Eosin. The stained sections were examined with the light microscope (Olympus BX51, Tokyo, Japan).

Transmission electron microscopy

The skin specimens were fixed in 2.5 % glutaraldehyde in phosphate-buffered saline (PBS, pH=7.4) for 4 hours at 4°C. After fixation in 2.5 % glutaraldehyde, the specimens were washed in PBS for 4 hours and post-fixed in 2% OsO4 (Merck, Darmstadt, Germany) solution in PBS for 1 hour at 4°C, and then dehydrated in a graded series of ethanol (70-100%) twice each for 15 minutes, and immersed in a propylene oxide for 1 hour, in a mixture of ethanol and propylene oxide 1:1 for 1 hour, respectively (Sigma-Aldrich, Tokyo, Japan). The specimen was embedded with epon resin for 72 hours in a 60°C degree oven. The epon block was cut in 100-150 nm thin section by ultramicrotome and placed on a nickel grid, and observed on the transmission electron microscope (Hitachi H-800, Japan).

Statistical analysis

The length of the melanocytes and melanin granules were measured using the RS Image software (Media Cybernetics, Maryland 20910 USA) on the transmission electron micrographs. Because of the irregular shape of the melanocyte, we measured the shortest length of the cell body as the cell size and used the

dotted-analysis for counting of the melanin granules number. For the quantitative data, the mean, standard deviation, Kruskal-Wallis, and Mann-Whitney tests were calculated on a STATA 13 program. Statistical significance was considered as $p < 0.05$.

Ethical statements

The current study was approved by the Ethics and Research Committees of the Mongolian National University of Medical Sciences (№13/3/2016-13).

Results

In the light microscopy, the deep wavy epidermal-dermal junction was clearly observed in the blue spot region of the one-month-old infant skin. The basal cells were seen as a single row above the basal membrane of the epidermis. The spinous layer was composed of several cell rows. The dermal papillae composed of loose connective tissue was anchored with the epidermal inclusions forming the epidermal-dermal junction. The dark-colored melanocytes were observed in the Malpighian layer of the epidermis and at the dermal papillae around the epidermal-dermal junction (Figure 1).

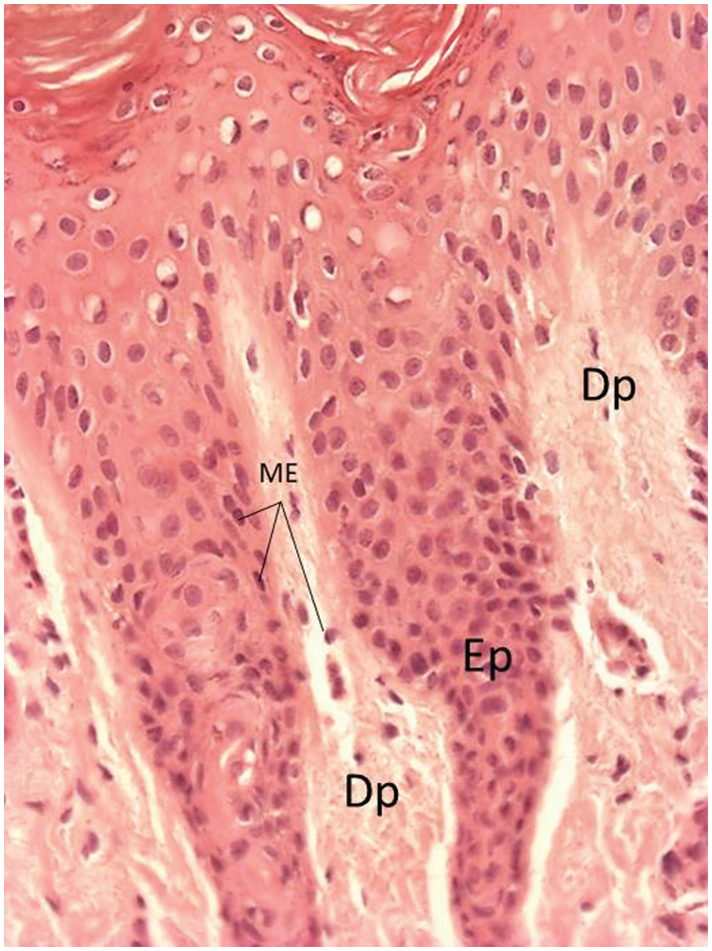

Figure 1. Light micrograph of the sacral blue spot region of the 1month old child skin, HE, x200. The wavy epidermal-dermal junction is shown between the epidermis (Ep) and dermal papillae (DM). Melanocytes (ME) are observed in the Malpighian layer of the epidermis and around the epidermal-dermal junction..

The melanocyte in the sacral region of the adult human skin

The melanocytes of the sacral region of the adult human skin were regularly arranged on the epidermal side of the epidermal-dermal junction. Melanocytes (ME) bulging deeply into the epidermal basal cells (MC) were always found to be separated from the dermal connective tissue by the basal membrane (DM). The cytoplasm of the melanocyte contained mitochondria (mi), oblique sections of filaments (ft) close to the nucleus, and melanin granules of the dense type (mg-2) and formed irregular extensions (CE) resembling pseudopodia. Lastly, slender dendrites (DE) extending from the cell body of the melanocytes were observed between two Malpighian cells identified by the presence of tonofilaments in their cytoplasm. In such dendrites, the melanin granules (mg-C) accumulated at the periphery of the limiting cell membrane close to the extension (Figure 2).

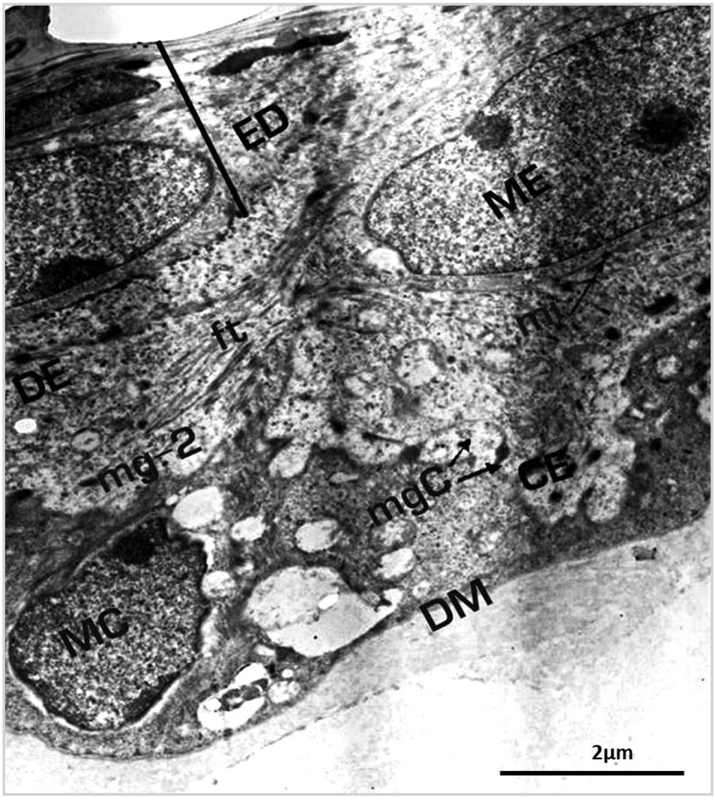

Figure 2. Transmission electron micrograph of a melanocyte in the sacral region of the adult human skin, x2000. The melanocyte (ME) is located on the basal membrane of the epidermis (DM). The cytoplasm of the melanocyte contains mitochondria (mi), oblique sections of filaments (ft) close to the nucleus, and the melanin granules of the dense type (mg-2). CE-cytoplasmic extensions, DE-dendrites.

The melanocyte in the sacral region of the 1 month old infant skin

The pyramid-shaped melanocyte (ME) lined the uniformly thin basal membrane of the epidermis (DM) near its dermal surface. The cytoplasm of the melanocyte contained vesicles (v) and melanin granules of the light type (mg-1) distinguishable from the melanocyte of the adult man skin. The melanocyte was close to the base of the epidermal basal cell (MC) on its epidermal side. Numerous irregular cytoplasmic extensions (CE) containing melanin granules and vesicles between the basal cells were observed. It was interesting that the basal membrane appeared uniformly thin when in contact with the melanocytes more than in the sacral region of the adult man skin (Figure 3).

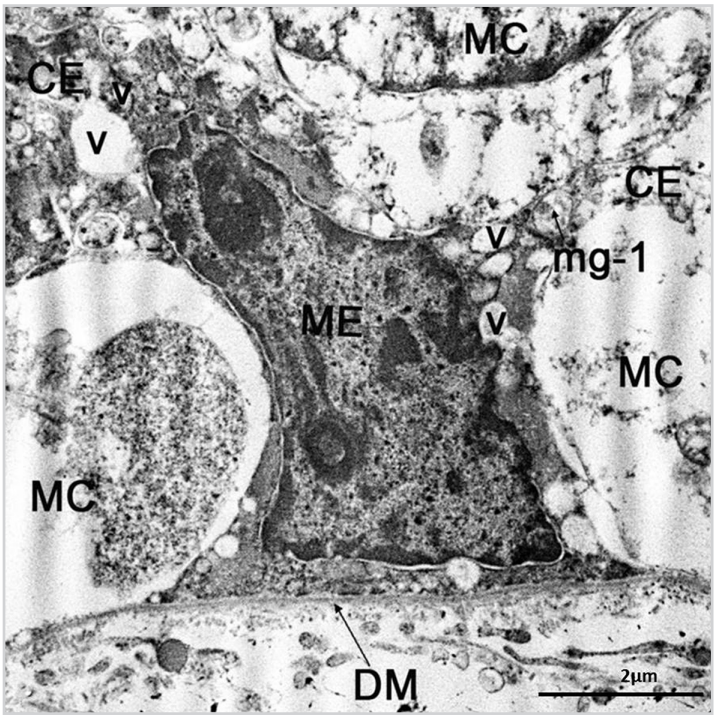

Figure 3. Transmission electron micrograph of the sacral blue spot region of the 1 month old child, x2000. The pyramid-shaped melanocyte (ME) line the uniformly thin basal membrane (DM). The cytoplasm of the melanocyte contains vesicles (v) and melanin granules of the light type (mg-1). The melanocyte is close to the base of the epidermal basal cell (MC) on its epidermal side. CE- cytoplasmic extentensions.

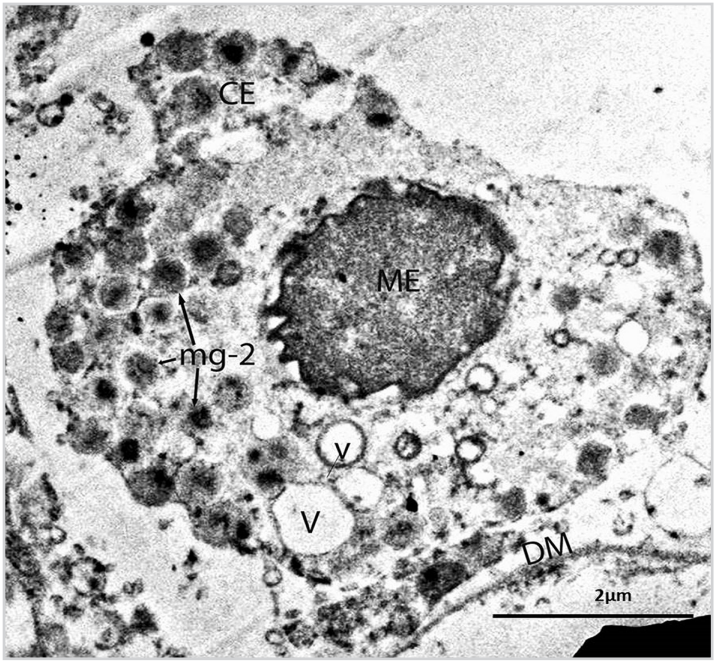

Figure 4. Transmission electron micrograph of the melanocyte in the sacral blue spot region of the 1 month old child, x2500. The round-shaped melanocyte (ME) lines the uniformly thin basal membrane of the epidermis (DM). The cytoplasm containes melanin granules of the light and the dense type (mg-2), and vesicles (V).

The round-shaped melanocyte (ME) also was found on the dermal side of the basal membrane (DM) of the epidermis. Also the cytoplasm and cytoplasmic extensions (CE) contained the melanin granules of both the light and dense types. Some of the melanin granules were a mixture of light and dense types (mg-2). It seemed that the dense granules are developing inside of the light granules (Figure 4).

The melanocyte in the sacral region of the 8 months old child's skin

Melanocytes were found also in the epidermis and moreover they bulged into the stratum spinosum (SS) of the epidermis. The polygon-shaped melanocytes (ME) formed long dendritic processes (DP) located in the stratum spinosum. The melanocyte cytoplasm contained the vesicles (v) and the melanin granules of the light type (mg-1). Irregular cytoplasmic extensions (CE) containing melanin granules and vesicles were observed (Figure 5).

Three-angled Merkel cells (MeC) lined the basal membrane (DM) on its dermal surface. The cytoplasm of the Merkel cells contained numerous neurosecretory granules and vesicles (V). The Merkel cells were close to the base of the epidermal basal cell (MC) on its epidermal side. Irregular cytoplasmic extensions as melanocyte dendrites containing melanin granules of the dense type (mg-2) were observed in the intercellular spaces between the Merkel cell and basal membrane (DM) (Figure 6).

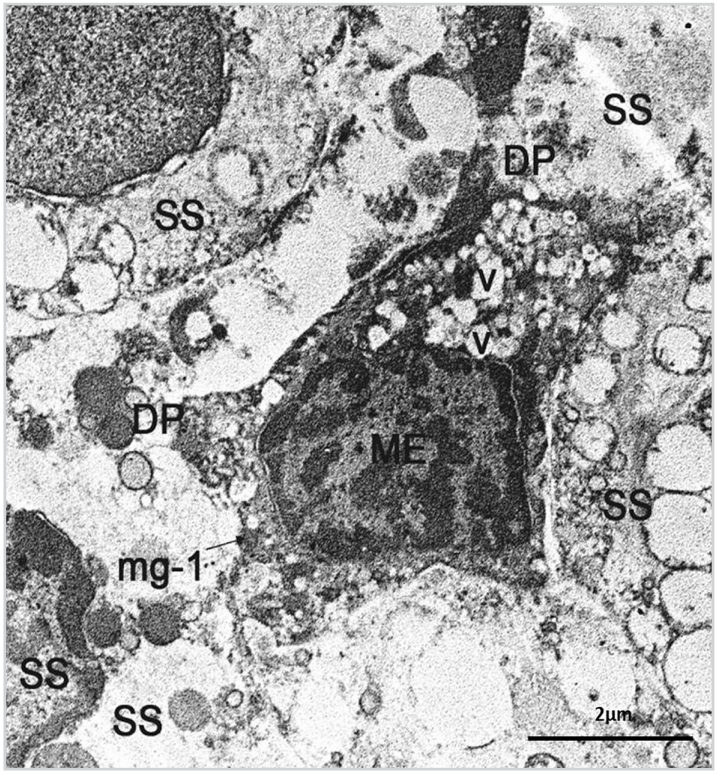

Figure 5. Transmission electron micrograph of the melanocyte in the sacral blue spot region of an 8 month old child, x2000. The polygon-shaped melanocyte (ME) forms the long dendritic processes (DP) located in the stratum spinosum (SS). CE-cytoplasmic extensions, mg-1-melanin granules of the light type, v-vesicles.

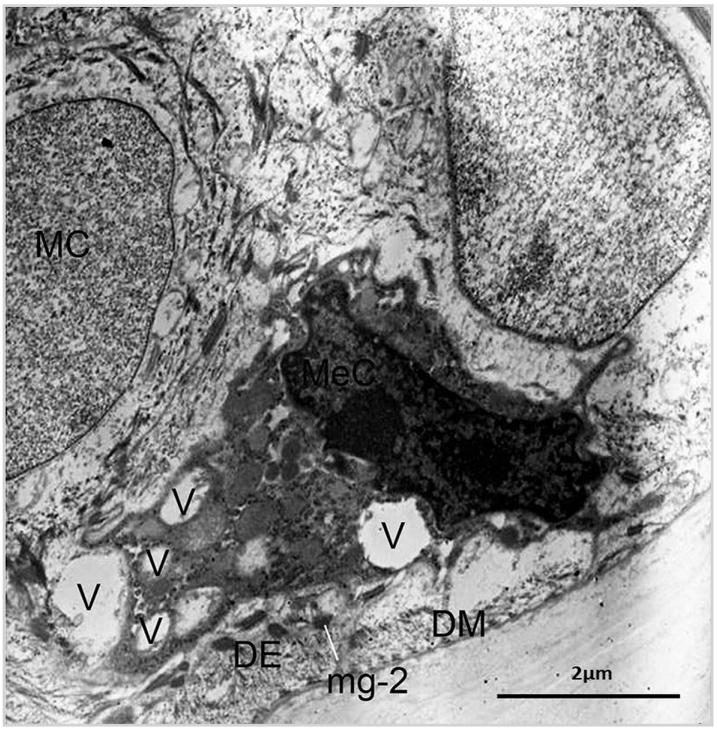

Figure 6. Transmission electron micrograph of the sacral blue spot region of an 8 month old child, x2000. Three-angled Merkel cells (MeC) line the basal membrane (DM). The cytoplasm of the Merkel cell contains numerous neurosecretory granules and vesicles (V). MC-epidermal basal cell, mg-2 -melanin granules of the dense type, DE-dendrite.

Comparison of the melanocytes and the melanin granules of the sacral region

The melanocyte size and location were similar in the sacral region of the adult man's skin and blue spot region of the 1 and 8 months old children's skin. But there was a distinct difference in the thickness of the basal membrane where it contacts the melanocyte cells. In the blue spot region of 1 month child's skin it was significantly thinner than in the adult man's skin (*** $p < 0.001$, Table 1).

In the melanocyte cells of the adult man skin, only the dense type of the melanin granules were found. However, the number and size of the dense melanin granules were higher than in the children's blue spot region. In the melanocytes of the blue spot region of the 1 and 8 months old children, both of the dense and light types of melanin granules were found. In the 8 month old child, the number of the dense and light granules were decreased compared with the 1 month, but the size of the granules slightly increased becoming similar to the adult skin sample, especially in the dense granules (Table 1).

Table 1. The measurement of the melanocyte and melanin granules.

Variables	Adult	1 month	8 months	p-value
	Mean ± SD	Mean ± SD	Mean ± SD	
ME size (μm)	4.17 ± 0.38	4.71 ± 0.20	4.07 ± 0.26	*0.113
Dense granule (nm)	176.5 ± 43.01	117.75 ± 22.04	169.33 ± 41.00	*0.177
Light granule (nm)	-	263.16 ± 20.84	280.7±59.34	§0.827
DM thickness (nm)	511 ± 71.09	117.65 ± 18.61	-	§0.001

Note: ME-melanocyte cell; DM-basal membrane of epidermis; *Kruskal-Wallis test; §Mann-Whitney test

Discussion

The pigment cells referred to as the melanocyte are endowed with the capacity to produce melanin pigment, and the property associated with the presence of oxidative enzymes (tyrosinases) in loci appears to correspond to the melanin granules [25-28]. The melanocyte body and its dendrites always appeared to be located above the dermal membrane as a rule among the Malpighian cells of the basal layers. Charles et al. suggested the possibility that melanocytes may pass through the dermal membrane [18]. However this conception has not been confirmed in this study.

The morphological information seemed to reinforce the view that the melanocyte is an integral constituent of the epidermis. Formerly it was suggested that the melanocyte cells of the blue spot/Mongolian spot were located within the dermis [8-11]. Further it was explained that Mongolian spot arises due to the failure of melanocytes to reach the epidermis and hence their sequestration in the dermis layer of the epithelium [29, 30]. But Baigalmaa et al. reported that the melanocyte cells of the blue spot skin of Mongolian children were located in both of the epidermal basal layer and dermis, and the ratio of melanocyte to keratinocyte was higher in the epidermal basal layer compared with the non-blue spot region [31]. Consistent with this, the current study revealed that the melanocyte cells of the blue spot region were found in the epidermis, especially on the epidermal basal membrane near the epidermal-dermal junction. Therefore we considered it is possible that the melanocyte cells of the blue spot can be located in both epidermis and dermis layers of the skin reflecting the motility of this cell. In addition, the thickness of the epidermal basal membrane was significantly thinner in contact with melanocyte cells than in the adult man skin. So, the epidermal melanocyte might be specific in the blue spot of Mongolian children. We will have to check whether the melanocyte cells of the blue spot have the mutual property of distribution on both epidermis and dermis using the primary cell culture and immunohistochemical analysis in the near future. On the other hand, the tissue specimen of the transmission electron microscope is very small and thin as measured in nanometers. For this reason, we might not find the dermal melanocyte cell clearly on ultrathin sections.

The melanocyte size was smaller in our study than it was recorded in literature, approximately 7 μm, Because of the irregular shape of this cell we only measured the shortest length. Okawa et al measured the sheath thickness of the dermal melanocyte as 0.5-1.5 μm [10]. Similarly, the thickness of basal membrane of the adult skin epithelium where it contacts the melanocyte cells was 0.5 μm (Table 1). Also, there was a distinct difference in the thickness of the basal membrane where it contacts the melanocyte cells between the adult man skin and the blue spot region of 1 month old child's skin. It was significantly thinner in the blue spot region of the 1 month old child's skin making us to think about epidermal melanocyte cell motility near the epidermal-dermal junction. We will have to describe it precisely in subsequent works.

Miroslawa et al. summarized that the melanogenetic process has four different stages of melanin granule development. At first it develops a vesicle (Stage I) which builds inside a fibrillar matrix formed by glycoproteins and accumulates tyrosinase and other enzymes of melanogenesis (Stage II). The melanosome then produces melanin, which polymerizes and settles on the internal fibrils (Stage III). In the last stage (Stage IV), the melanosome is filled with melanin [32]. In the current work, we cannot distinguish melanosome development along these stages. But morphologically two different types of melanin granules were found that we named the light and dense type. The dense type of the melanin granules was found in the melanocyte cells of the adult man's skin, and both of the dense and light types of melanin granules were found in the blue spot region of the 1 and 8 month old children. Furthermore, the number of dense and light granules was decreased in the 8 month old child compared with the 1 month old, and the size of the granules slightly increased becoming similar to the adult skin sample, especially in the dense granules. However, there was no statistical significance. In addition, a lot of vesicles were found inside of the melanocyte cytoplasm. This might reflect the various stages of melanin granules. In this study, we only used the morphological analysis in the light and transmission electron microscopy because of the very small number of samples. Fortunately, we hope this current study will be the beginning of further blue spot ultrastructure research, and to answer the unsolved questions around this field in our country. To make it clear, in the near future, we need to use the immunohistochemical method with melanosome stage-specific biomarkers.

Conclusion

The melanocytes of the sacral blue spot region were located on the basal membrane of the epidermis near to the epidermal-dermal junction. The cytoplasm of the melanocytes contained the melanin granules both of the dense and light types.

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