Histopathological and Immunohistochemical Studies of Intramucosal Nevus in the Oral Mucosa: With Special Reference to “Maturation” and “Origin” of the Nevus Cells

Toshio Nakajima, Kayo Kuyama, and Yan Sun

Department of Oral Pathology, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

Correspondence to:
Kayo Kuyama
E-mail: kuyama.kayo@nihon-u.ac.jp

Abstract
Histopathological and immunohistochemical studies were conducted to elucidate the characteristics of nevus cells that accumulate in intramucosal nevi in the oral mucosa, primarily focusing on the maturation and origin of these cells. Twelve cases (5 men, 7 women; mean age: 29.8 years) of intramucosal nevi were examined, with the most frequent site being the gingiva. Macroscopically, Miescher's nevi were seen more frequently than Unna's nevi. Intramucosal nevi were characterized by a proliferation of nevus cells only within the underlying connective tissue, exhibiting maturation but not neuroid differentiation. From the immunohistochemical analysis of the nevus cells, staining for S100, Melan-A, and MITF was stronger in A-type cells than in deeper B-type cells, although B-type cells along the edge of the nevus cell nest exhibited moderate positive for S100, Melan-A, and MITF. Positive cytoplasmic expression of c-Kit was scattered in B-type cells deep within the lesion and in mast cells surrounding the blood vessels around the nevus cell nest. These results and a review of the literature suggest that nevus cell formation begins with the proliferation of melanocytes and their migration into the submucosa with a concomitant loss of cell adhesion. Nevus cells exhibited decreased melanocytic development with the depth of the lesion. However, along the edge of the lesion, B-type cells exhibited melanocytic but not neuroid differentiation. Thus, maturation of nevus cells could be a form of adaptation to the surrounding environment.

Keywords: intramucosal nevus, maturation, nevus cells

Introduction
A pigmented nevus is a tissue malformation resulting from an excessive proliferation of nevus cells. Pigmented nevi in cutaneous layers are classified into three groups on the basis of the histological location of the nevus cells. Junctional nevi are located in the basal epithelial layers, intradermal nevi are located in the connective tissue, and compound nevi are located in both the basal epithelial layers and connective tissue (1). Oral pigmented nevi follow the same classification; however, the term intradermal is replaced with intramucosal. While histopathological findings of intradermal nevi have already been described (2), pigmented nevi are rarely encountered in the oral mucosa.

Histologically, intramucosal nevi comprise an accumulation of nevus cells in the connective tissue. Nevus cells are confined to the submucosa, where they are arranged in nests and cords, and multinuclear nevus cells are frequently present. Nevus cells actually show considerable variation in their appearance, depending on their localization in the connective tissue. This difference in their appearance, depending on whether they are located superficially or in the deep dermis, has been referred to as nevus cell maturation (1, 3).

Nevus cells are supposedly derived from cells that migrate from the neural crest to the epithelium and intramucosa; however, their true origin has not yet been clarified. The theory of Abtropfung, which
states that nevus cells migrate from the epidermis to
the dermis and proliferate during the development of
melanocytic tumors, is widely accepted (1). Another
widespread belief is that nevus cells have a dual
origin. More specifically, nevus cells located with the
region from the basal cell layer to the upper part of
the dermis are derived from melanocytes, while those
in the lower part of the dermis are derived from
neural structures; specifically, Schwann’s cells (4).
Furthermore, the theory of Hochspringer states
that melanocytes derived from the neural crest
migrate up from the dermis and into the epidermis (5,
6). The theory of Hochspringer is more congruous
with current research than theories involving an
epidermal origin.

The purpose of the present study was to compare
the histopathological findings between intramuscosal
and intradermal nevi, and to clarify the maturation
and origin of nevus cells.

Materials and Methods

Histopathological specimens

Twelve consecutive cases (5 men, 7 women; mean
age: 29.8 years; range: 5–68 years) of intramuscosal
nevus were selected from the pathology files of the
Department of Oral Pathology, Nihon University
School of Dentistry at Matsudo. The specimens were
stained with hematoxylin and eosin and toluidine
blue (pH 4.1) and reviewed by two oral pathologists
to confirm the diagnosis and observe the histological
characteristics. The protocol was approved by the
Committee on Studies Involving Human Beings of
Nihon University School of Dentistry at Matsudo (E
–5,002). Informed consent was obtained from all
patients before retrieving the pathological speci-
mens.

Immunohistochemical assessment

Immunohistochemical studies were conducted
using formalin-fixed, paraffin-embedded tissue from
all cases. Sections (4 μm thick) were deparaffinized
in xylene, and hydrated in graded ethanol solutions.
The EnVision+ Polymer System (Dako, Glostrup,
Denmark) was used for antigen detection. Primary
antibodies used were directed against the following
antigens: polyclonal antibody S100 (1:1,000; Dako);
Ki–67 (MIB-1, 1:50; Dako); microphthalmia transcrip-
tion factor (MITF, D5, 1:100; Dako); Melan–A (A103,
1:50; Dako); c–Kit (CD117, 1:100; Dako); and CD68 (pG–M1, 1:100; Dako). To
improve detection, deparaffinized sections were
pretreated by microwave heating. Peroxidase
activity was visualized using histogreen (Cosmo Bio Co.,
Ltd., Tokyo, Japan), except for that of Ki–67.
Diaminobenzidine was used for visualizing Ki–67
activity. Positive cytoplasmic expression of S100,
Melan–A, and c–Kit and nuclear expression of MITF
were observed as a blue–green color. Positive
nuclear expression of Ki–67 was a brown color.
Finally, all sections were counterstained with
Mayer’s hematoxylin. Positive controls were speci-
mens of skin tissue from healthy individuals. For
evaluation of the immunostaining technique, as a
negative control, mouse and rabbit universal nega-
tive controls (Dako) were used during the staining
procedure instead of the primary antibodies.

Results

Clinical findings

The locations with the highest frequencies of
intramuscosal nevi were the gingiva (n=6, 50.0%), the
palate (n=3, 25.0%), the cheek (n=2, 16.7%), and the
lip (n=1, 8.3%). All cases had clinical pigmentation
and an extroversion or flat appearance similar to
intradermal nevi in the skin.

Histopathological findings

Representative images of intramuscosal nevi are
shown in Figs. 1a–c, 2a–c, and 3a and the histopa-
thological findings are summarized in Table 1. Macro-
scopic findings show two nevi patterns. In Unna’s
nevus, nevus cells grew with a papillar or nodular
appearance (Fig. 1a); in Miescher’s nevi, nevus cells
diffusely infiltrated under the epithelium and appear-
ed flat (Fig. 1b). There was a 1:2 ratio in the distri-
bution of these patterns, Unna’s nevi (n=4, 33.3%;
mean patient age: 31.0±3.2 years) and Miescher’s
nevus (n=8, 66.7%; mean patient age: 29.3±21.3

89
years). The Grenz zone, the border between the lesion and the epithelium, was rarely seen (16.7%).

Intramucosal nevi were characterized by a benign, unencapsulated proliferation of nevus cells only within the underlying connective tissue. Microscopic findings of nevus cells showed maturation; superficial nevus cells with clusters tended to be organized in a small and scattered distribution (Fig. 1b). The superficial cells appeared larger and epithelioid, with round nuclei and abundant cytoplasm, frequent intracellular melanin, and a tendency to form clusters (A-type cells, Fig. 2a). Nevus cells in the intermediate layer of the lesion were smaller with dark-stained nuclei and less cytoplasm, resembling lymphocytes or fibroblasts (Fig. 2b), and were seldom pigmented, especially in the deep layer (B-type cells, Fig. 2c). Nevus cells in all cases lacked the spindle cells seen in Meissner's corpuscles. There were more A-type cells than B-type cells, except in two cases. Multinuclear giant cells were often scattered, mainly in the superficial lesion (50.0%; Fig. 1c, arrowhead), and there was pseudo-inclusion in the nuclei of nevus cells (66.7%; Fig. 1c, arrow). Toulidine blue (pH 4.1) staining revealed metachromasia cytoplasmic granules of mast cells near the edge of the nevus cell nest (Fig. 3a, arrow).

**Immunohistochemical staining**

Representative examples of staining are shown in Figs. 3b–7c and the immunohistochemical findings are summarized in Table 2.

Intramucosal nevi exhibited only rare staining of cells for Ki-67, 0.2% in A-type cells and 0.01% in B-type cells. There was no difference of proliferative activity between the basal epithelial layer of the intramucosal nevi (5.5%) and that of healthy tissue (6.8%) (Fig. 3b).

Intramucosal nevi exhibited strong cytoplasmic expression of S100 and Melan-A in the superficial lesion that decreased with depth (Figs. 4a, b and 5a, b). The positive expression of Melan-A in B-type cells clearly decreased as lesion depth increased (Fig. 5b). However, B-type cells along the edge of the nevus cell nest exhibited moderate positive expression of S100 and Melan-A (Figs. 4c and 5c).

Staining for MITF was strongest in the nuclei for A-type cells (Fig. 6a), and weak or absent in B-type cells (Fig. 6b). However, some B-type cells scattered deep within the lesion exhibited positive staining for MITF (Fig. 6c). Positive expression of c-Kit was not observed in any of A-type cells (Fig. 7a). However, positive cytoplasmic expression of c-Kit was scattered in the basal epithelial cells of the lesion (Fig. 7b), mast cells surrounding blood vessels near the edge of the nevus cell nest, and in B-type cells along the edge of the nevus cell nest (Fig. 7c).

In multinuclear giant cells, positive expression of S100 (Fig. 4a), Melan-A (Fig. 5a), and MITF (Fig. 6a)
Figs. 1a, b. Representative findings of Unna’s (1a) and Miescher’s (1b) nevi with papillary and flat appearance, respectively (×4, H·E staining).

Fig. 1c. Multinuclear giant cells (arrowhead) and pseudo-inclusion in the nuclear of nevus cells (arrow), (×60, H·E staining).

Figs. 2a-c. A-type cells with making cluster (2a), B-type cells with diffuse infiltration (2b) and B-type cells with scattered pigmentation in the deep layer (2c), (×40, H·E staining).

Fig. 3a. Metachromasia cytoplasmic granules of mast cells were observed near the edge of the nevus cell nest (arrow, ×60, Toluidine blue staining, pH 4.4).

Figs. 3b, c. Small proliferative activity with Ki 67 in A-type cells and the basal epithelial layer on the nevi (3b), and negative findings with CD68 in multinuclear giant cells (3c), (×40).

Figs. 4a-c. Strong cytoplasmic expression with S100 in A-type cells (4a) that decreased with depth (4b), although B-type cells along the edge of the nevus cell nest moderately exhibited (4c). Arrow shows multinuclear giant cell (4a), (×40).

Figs. 5a-c. Strong cytoplasmic expression with Melan-A in A-type cells (5a) that decreased with depth and almost lost in deep lesion (5b), but some positive findings were scattered along the edge of the nevus cell nest (5c). Arrow shows multinuclear giant cell (5a), (×40).

Figs. 6a-c. Strong expression for nuclei with MITF in A-type cells (6a), while expression was lost or scattered in B-type cells (6b). Some positive cells were scattered along the edge of the nevus cell nest (6c). Arrow shows multinuclear giant cell (6a), (×40).

Fig. 7a. Negative findings for c-Kit were shown in A-type cells including multinuclear giant cells (arrow, ×40).

Figs. 7b, c. Positive cytoplasmic expression of c-Kit was scattered in the basal epithelial cells of the lesion (7b, ×40), mast cells surrounding blood vessels and B-type cells near the edge of the nevus cell nest (7c, ×60).
was seen, but not for CD68 (Fig. 3c) and c–Kit (Fig. 7a).

**Discussion**

A pigmented nevus is generally interpreted as a hamartoma or neoplasm, in which nevus cells grow in basal epithelial layers, connective tissue, or both, although a precise definition has yet to be determined. There is ongoing discussion regarding the maturation and origin of nevus cells. Intramucosal nevus is the most common type of pigmented nevus, but it is rarely seen in the oral cavity. Consequently, few reports have described intraoral nevi in detail.

**Clinicopathological findings**

Oral mucosal nevi are more common in women than in men, and the mean age of patients is 35 years (2). In the present study, sex distribution was nearly identical, but the mean age was younger compared to previous reports (2, 7). Intramucosal nevus is the most common type among pigmented nevi and is seen most frequently on the hard palate (7) and buccal mucosa (2, 7), although the highest frequency was in the gingiva in the present study.

**Comparison to intradermal nevus**

Macroscopically, intradermal nevi are classified into either Unna’s or Miescher’s patterns (8). Yus et al. (9) reported that, on the entire body, Miescher’s nevi were almost the same prevalent as Unna’s nevi, although 91% of Miescher’s nevi were located on the face. There was no significant difference in patient age between the two types of nevi. Many histopathological characteristics of intradermal nevi have been described, including the Grenz zone, in-depth maturation including neuroid differentiation, multinuclear giant cells, pseudo-inclusion within the nuclei, and adipocytes within the lesion. Moreover, it is well known that nevus cells in the dermis appear as three types: A, B, and C (1). Histopathological findings of the intramucosal nevi in the present study were similar to those of intradermal nevi excluding the Grenz zone and exhibiting neuroid differentiation. C–type cells, which can form structures resembling Meissner’s corpuscles and never contain melanin (1), were not seen. Maturation of nevus cells has been described as occurring with increasing age of the lesion (3). It is expected that the period from discovery to excision is shorter for intramucosal nevi because, compared to the skin, recognizing a pigmented nevus in the oral cavity is easier. Therefore, it was supposed that intramucosal nevi in the present cases were excised before the formation of the Grenz zone and before the maturation of nevus cells was completed.

**Characteristics of nevus cells**

1) Nevus cell origin

Previous reports suggested that a melanocytic nevus is a benign tumor with a proliferation of nevus cells that originated from epidermal melanocytes (5, 10). The results of the present study indicate that these intramucosal lesions exhibit non-tumorous characteristics; a similar conclusion has been made regarding intradermal lesions (11). Melanocytes are derived from the neural crest before migrating to the oral mucosal membrane during embryogenesis (5), and they share morphological and functional features with both neurons and Schwann cells. Nevus cell formation likely begins with the proliferation of melanocytes within the basal cell layer and it may be associated with elongation of the rete ridges. Presumably, nevus cells migrate into the submucosa by losing cell adhesion when the proliferation of melanocytes begins. The maturation of nevus cells begins after they migrate and proliferate into the submucosa.

2) Maturation of nevus cells

Pigmentation was decreased in B–type cells, although a large amount of melanin was observed in A–type cells in the present study. These results agreed with those from a previous study indicating that A–type cells exhibited melanosomes with a larger number of electron-dense particles than B–type cells (12). Cytomorphologically, the characteristics of nevus cells changed from A–type to B–type cells in the present study. In the literature, a decrease of E–cadherin expression has been attributed to this
cytological change; namely, the maturation of nevus cells (13, 14). Additionally, Goovaerts et al. (15) showed that there was a decrease in number and size of all organelles other than mitochondria and microfilaments in nevus cells with maturation, consistent with changes seen during atrophy.

In addition to cytomorphology, there are identifiable differences in protein expression. S100 is an acidic protein present in the nervous system, dendritic cells, and melanocytes (16). Melan–A is a transmembrane protein composed of 118 amino acids (17), and is expressed in more than 90% of melanomas as well as in melanocytes (17, 18). The function of Melan–A, however, remains unclear (19). MITF is a nuclear protein that acts as a transcription factor to regulate the development and survival of melanocytes (20). MITF has been shown to be phosphorylated by MAP kinase in response to c–Kit activation, resulting in the upregulation of MITF transcriptional activity (21, 22). The patterns of MITF expression in the present study resembled maturation; A-type cells exhibited a strong expression, while staining of MITF was weak or absent in B-type cells. This result corresponds to those of previous studies on lesions in the skin (23, 24). Apparently, B-type cells lose their melanocytic development and survival ability.

Pigmentation and positive expression of S100, Melan–A, and MITF were scattered in B–type cells along the edge of the nevus cell nest. It was thought that these cells possessed melanocytic but not neur- oid ability. The c–Kit ligand, also known as stem cell factor (SCF), which is produced in keratinocytes, endothelial cells, and fibroblasts, stimulates differentiation, migration, survival, and proliferation of the melanocytes in the skin (25). The SCF/c–Kit ligand is expressed in a membrane-bound form in epidermal keratinocytes and in a soluble form secreted by fibroblasts and endothelial cells (26, 27). SCF/c–Kit plays an important role in the paracrine regulation of melanocytes and subsequent pigmented disorders (26). In relation to this, skin with clusters of mast cells is also characterized by increased epidermal melanin produced by melanocytes (28). The abnormal pigmentation and emergence of mast cells in lesions of the skin imply the involvement of local factors; both melanocytes and mast cells express c–Kit and respond to SCF. In the present study, c–Kit was observed in mast cells and B–type cells in deep lesions as a response to SCF expressed in the endothelial cells and fibroblasts, subsequently resulting in the expression of MITF in nevus cells. In contrast, positive expression of c–Kit in basal cells indicate that epithelium having the membrane–type c–Kit ligand (26) was stimulated by c–Kit. However, intramucosal nevus cells may have lost their dependence on the SCF/c–Kit ligand for survival and proliferation.

From these results and a review of the literature, the maturation of nevus cells could be considered an adaptation to the surrounding environment; for example, a lack of contact with keratinocytes or new contact with the dermal extracellular matrix (14).

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