〔*Original*〕

Stem cells of olfactory cells during embryonic development

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Abstract

Stem cells of olfactory (receptor) cells in the olfactory epithelium and vomeronasal organ during embryonic development of mice were investigated by double immunostaining using anti-neural cell adhesion molecule (NCAM) and anti-bromodeoxyuridine (BrdU) antibodies and by electron microscopy. The columnar-shaped cells which were negative for NCAM and located throughout the epithelium on embryonic day (E) 12 and then in the basal region after E 14, were numerously labeled with BrdU, indicating active division, when BrdU was injected 1 hr before sacrifice. These cells are presumed to develop into NCAM-immunoreactive olfactory (receptor) cells of the middle region of the epithelium since numerous labelings appeared in the middle region 24 hrs after injection of BrdU. Evidence of migration of the columnar cells from olfactory epithelium along the axons was observed. On E 17, the columnar cells in the vomeronasal organ became round-shaped, located above the processes of the supporting cells, and were stained with NCAM. On the other hand, the columnar cells in the olfactory epithelium differentiated into round-shaped cells and pyramidal-shaped cells on E 19, which respectively corresponded to globose basal cells and basal cells proper seen in postnatal epithelium, although the round-shaped cells were negative for NCAM. The result suggests that the columnar cells as stem cells of olfactory (recepetor) cells observed in embryonic days differentiate into round-shaped cells in late embryonic days and that cells in the olfactory epithelium mature at a later stage than those in the VNO.

key words : Bromodeoxyuridine (BrdU), Neural cell adhesion molecule (NCAM), Developing olfactory epithelium, Vomeronasal organ.

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Introduction

Our previous studies combining immunohistochemistry of keratin with the bromodeoxyuridine method to label dividing cells demonstrated that stem cells of olfactory cells of mice are globose basal cells and not basal cells proper during the early postnatal stage and adulthood[1,2]. The basal cells proper, which are in direct contact with the basement membrane, are positive for staining with antikeratin antibodies[3,4,5,6]; whereas globose basal cells, which lie between basal cells proper and the olfactory cells nuclei, or often close to the basement membrane, are positively stained by anti-neural cell adhesion molecule (NCAM) antibody[7,8] and are devoid of keratin[4]. These two types of basal cells have been shown to differentiate in the basal region of olfactory epithelium at birth[2]. Prior to the appearance of the basal cells in the olfactory epithelium, olfactory cells appear on embryonic day 12 in mice and increase in number during embryonic development[9,10]. Therefore, the stem cells of the olfactory cells during embryonic days are different from those observed in the postnatal days and adulthood; however, ultrastructural and immunohistochemical characteristics of stem cells in embryonic days have not been fully investigated.

On the other hand, it is known that the olfactory epithelium and vomeronasal organ both originate from the olfactory placode and that the vomeronasal organ is separated from the epithelium of the nasal septum, forming a tube during embryonic development[11]. In the mouse, it has been reported that there are no basal cells in the basal region of the neurosensory epithelium of the VNO but that precursor cells of receptor cells are present at the border between the respiratory epithelium and the neurosensory epithelium[12,13]. Therefore, the stem cells in the vomeronasal organ are different from those in the olfactory epithelium; however, the stem cells of receptor cells during the embryonic stage have not been investigated.

The aim of this study was to examine stem cells of olfactory (receptor) cells during embryonic development. The developing olfactory epithelium and vomeronasal organ of mice were examined by the combination of immunohistochemistry of NCAM with the BrdU method and by conventional light and electron microscopy.

Meterials and Methods

Dd-mice of embryonic days 12, 14, 17, and 19 were used. Bromodeoxyuridine (BrdU, Sigma, 50 mg/kg) was injected into the peritoneal cavity of pregnant mice and each animal was killed either 1 hr or 24 hrs after injection. The BrdU was detected by immunohistochemical method using anti-BrdU antibody. For immunohistochemistry, mice were sacrificed and their nasal cavities were fresh-frozen or fixed in 4% paraformaldehyde. Ten-micron-thick sections were prepared in a cryostat, incubated

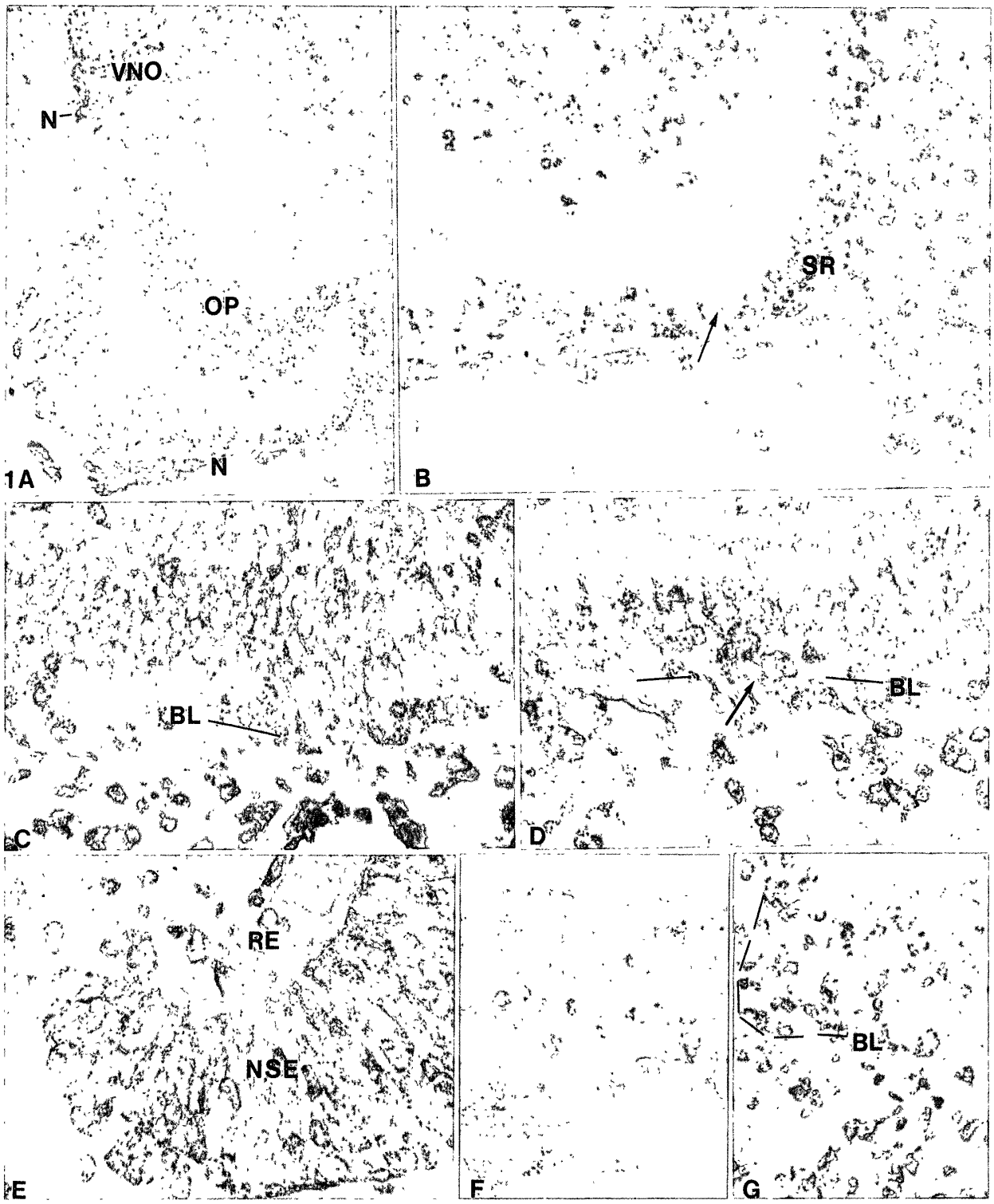
for 1 hr at room temperature with monoclonal anti-neural cell adhesion molecule (NCAM) antibody (Immunotech), and stained by the PAP method using a PAP kit (Dako). For NCAM/BrdU staining, the sections were initially incubated with monoclonal anti-NCAM antibody for 1 hr at room temperature, followed by staining using HRP-labeled anti rat IgG2 antibody; and the immunoreactive product was colored brown by DAB (diaminobenzidine). The sections were then rinsed in PBST (0.1M phosphate-buffered saline containing 0.3% Triton X100) overnight at 4°C, and subsequently incubated in 2N HCl for 20 min at room temperature to denature the DNA. After neutralization by a wash in 0.1M tetraborate, they were next incubated with anti-BrdU antibody (Becton-Dickinson), stained with a PAP kit, and colored violet with 4-chloro-1-naphtol. As a control, normal ascites was used instead of monoclonal antibodies. The number of BrdU-labeled cells along a 180μm length of the olfactory epithelium was counted under a light microscope (400x magnification) equipped with an ocular micrometer(cf. Suzuki and Takeda, 1993).

For electron microscopic observations, the mice were fixed by intracardial perfusion with a cacodylate-buffered mixture containing 2% glutalaldehyde and 0.8% paraformaldehyde. Pieces of olfactory mucosa were excised, postfixed in 1% OsO_4, and embedded in Epon 812. Ultrathin sections were cut, stained with uranyl acetate followed by lead citrate, and examined under a Hitachi H-500 electron microscope.

Results

Immunohistochemistry

In the 12-day-old embryonic mice (E 12), the olfactory pits and vomeronasal organ (VNO) were present. The lateral part of olfactory pits showed secondary recesses. At 1 hr after injection of BrdU, the immunolabeled cells with anti-BrdU antibody were numerously found throughout the olfactory epithelium and VNO, indicating active division. A few NCAM-immunoreactive cells were found among the BrdU-labeled cells, and NCAM-immunoreactive axons extended from the epithelium into the rostral forebrain. The axons were accompanied with many cells which were not labeled with BrdU (Fig. 1A). The secondary recesses of the lateral part of the olfactory pits, consisting of thin epithelium, were especially labeled with BrdU in the middle and basal regions, but NCAM-immunoreactive cells did not appear in these regions. The BrdU -labeled cells were also numerous in the underlying mesenchyme (Fig. 1B). The development of these regions was chronologically behind that of the other regions of the olfactory pits, indicating these regions to be probably in the placodal stage. On E 14, NCAM-immunoreactive cells, which were mostly negative for BrdU, increased in number and formed 6-7 layers in the middle region of olfactory epithelium. These cells corresponded to immature and mature olfactory cells. Most of the BrdU-labeled cells



were located in the apical and basal regions, which were negative for NCAM. There were mosaics of active or quiescent zones in the basal region where cells were labeled or unlabeled. In the lamina propria, axons were NCAM-immunoreactive and associated with a few BrdU-labeled cells (Fig. 1C). Observation of embryos sacrificed 24 hrs after injection of BrdU revealed many BrdU-labeled cells located in the middle region of

olfactory epithelium and along the axons in the lamina propria (Fig. 1D). In the VNO, NCAM-immunoreactive cells were located in the middle and apical regions of the neurosensory epithelium and in the respiratory epithelium. Most of the BrdU-labeled cells were located in the basal and midlle regions of the neurosensory epithelium, and a few of them were associated with NCAM-immunoreactive cells in the middle region (Fig. 1E). At 24 hrs after injection of BrdU, more labeled cells than at 1 hr after injection were observed in the middle region. At this stage, a group of the NCAM -immunoreactive cells was observed in the respiratory epithelium of the nasal septum, indicating the Masera organ. On E 17, the BrdU-labeled cells in the apical and basal regions of the olfactory epithelium were fewer than those observed on E 14. The NCAM-immunoreactive cells in the middle regions of the olfactory epithelium increased in number and formed 7-8 layers (Fig. 1F). The BrdU-labeled cells in the middle region of epithelium were few in number in most parts of the nasal cavity, but in the epithelium near the rostral forebrain a large number of BrdU-labeled cells was found in the middle and basal regions (Fig. 1G). The apical and basal regions of the olfactory epithelium were not stained with NCAM (Fig. 1F,G). In the VNO, BrdU-labeled cells in the middle region of the neurosensory epithelium decreased in number on E 17, and labeled cells were located mainly in the basal region. On E 19, the BrdU-labeled cells in the basal region of the olfactory epithelium decreased in number. NCAM -immunoreactive cells in the middle region were present in 7-8 layers, but the apical and basal regions were still unstained with NCAM (Fig. 2A). There were NCAM -negative areas occupied by the duct cells of Bowman's glands between NCAM-positive areas. In the VNO, all regions including the basal region were stained with NCAM (Fig. 2B).

The changes in number and location of BrdU-labeled cells in the olfactory epithelium during the embryonic period are summarized in Fig. 3.

← Fig. 1. Double immunostaining for NCAM and BrdU in the olfactory epithelium and vomeronasal organ. The olfactory (receptor) cells and axons, stained by anti-NCAM antibody, are colored by DAB (brown). BrdU-immunolabeled products are stained by 4-chloro-1-naphtol (violet). Section thickness, 10 μm. A: A transverse section of the nasal cavity of on E 12 mouse embryo. BrdU -labeled cells are seen in the olfactory pits (OP) and vomeronasal organ (VNO) 1 hr after injection of BrdU. The NCAM-immunoreactive nerves (N) are arising from the epithelium. x120. B: At higher magnification of the olfactory epithelium containing the secondary recesses (SR) of olfactory pits. A few cells in the epithelium are weakly positive for NCAM (arrow). x250. C: The olfactory epithelium on E 14, 1 hr after injection of BrdU. Most of the BrdU -labeled cells are seen in the apical and basal regions, and NCAM-immunoreactive cells are seen in the middle region. BL, basal lamina. x340. D: The olfactory epithelium on E 14, 24 hrs after injection of BrdU. BrdU-labeled cells are seen in the middle region. The arrow indicates the cell migration along the olfactory nerves. BL, basal lamina. x340. E: The vomeronasal organ on E 14, 1 hr after injection of BrdU. BrdU-labeled cells are seen in the middle and basal regions of the neurosensory epithelium (NSE). RE, respiratory epithelium. x340. F,G: The olfactory epithelium on E 17, 1 hr after injection of BrdU. F:BrdU-labeled cells in the apical and basal regions are few in number. x250. G: BrdU-labeled cells are numerous in the NCAM-immunoreactive middle region of the epithelium near the rostral forebrain. BL, basal lamina. x250.

In control specimens incubated with mouse ascites instead of monoclonal antibodies, no staining was detected in the olfactory epithelium or in the VNO.

Ultrastructure

On E 12, the olfactory epithelium and VNO consisted of many columnar-shaped cells and a few spindle-shaped olfactory cells. On E 14, the columnar cells contained a small amount of rough endoplasmic reticulum (r-ER) and many free ribosomes, and

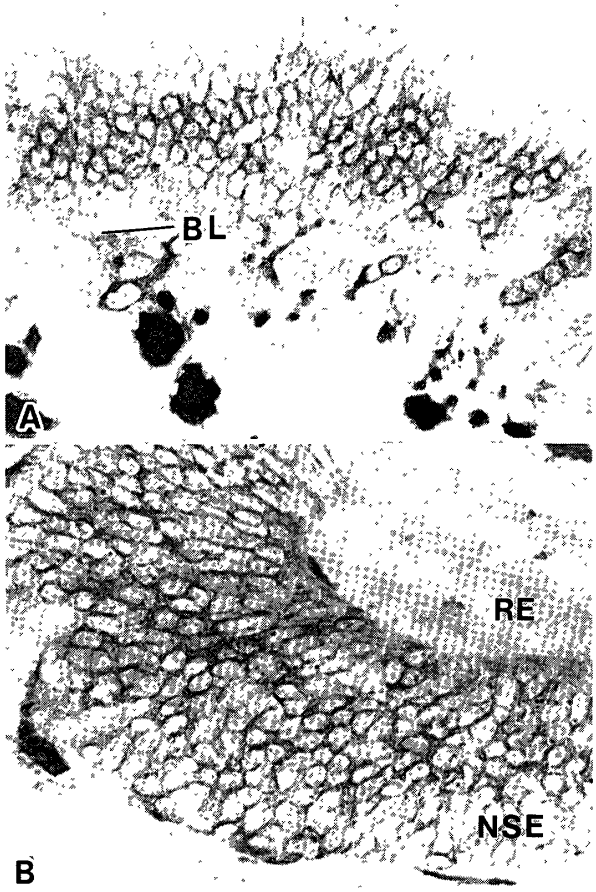


Fig. 2. Immunoreaction with anti-NCAM antibody in the olfactory epithelium (A) and vomeronasal organ (B) on E 19. A: Most cells of the middle region of the olfactory epithelium are stained with NCAM, and negative areas are occupied by the duct cells of Bowman's glands. BL, basal lamina. x350. B: NCAM -immunoreactive cells are seen in all regions of the neurosensory epithelium (NSE). RE, respiratory epithelium. x350.

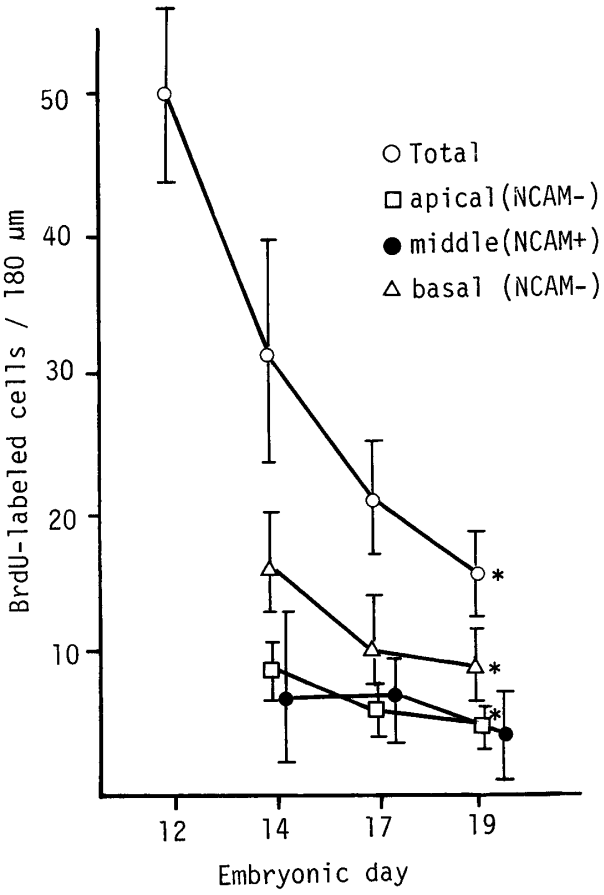


Fig. 3. Number of BrdU-labeled cells per 180 μm length of the olfactory epithelium on E 12, 14,17, and 19. Open circles, total number of BrdU-labeled cells. Rectangles, BrdU-labeled cells in the apical region. Closed circles, BrdU-labeled cells in the middle region. Triangles, BrdU-labeled cells in the basal region. Each value represents the mean and vertical bars, S.D. The total number of labeled cells on E 12 was high, and this number significantly decreased with age (t-test, *P <0.001). The labeled cells in the apical and basal regions significantly decreased in number from E 14 to E 19 (*P <0.001), whereas the number of those in the middle region did not change.

were located in the basal region of the olfactory epithelium, directly in contact with the basement membrane (Fig. 4A). In the middle and apical regions, olfactory and supporting cells were situated. In the VNO, similar columnar cells were located in the basal region of the neurosensory epithelium at this stage. On E 17, the columnar cells, processes of supporting cells, axons of olfactory cells, and round-shaped cells were present in the basal region of the olfactory epithelium. The round-shaped cells contained many r-ER and did not make contact with the basement membrane (Fig. 4B). The columnar cells disappeared on E 19 and pyramidal- and round-shaped cells occupied the basal region of ofactory epithelium. The pyramidal-shaped cells had

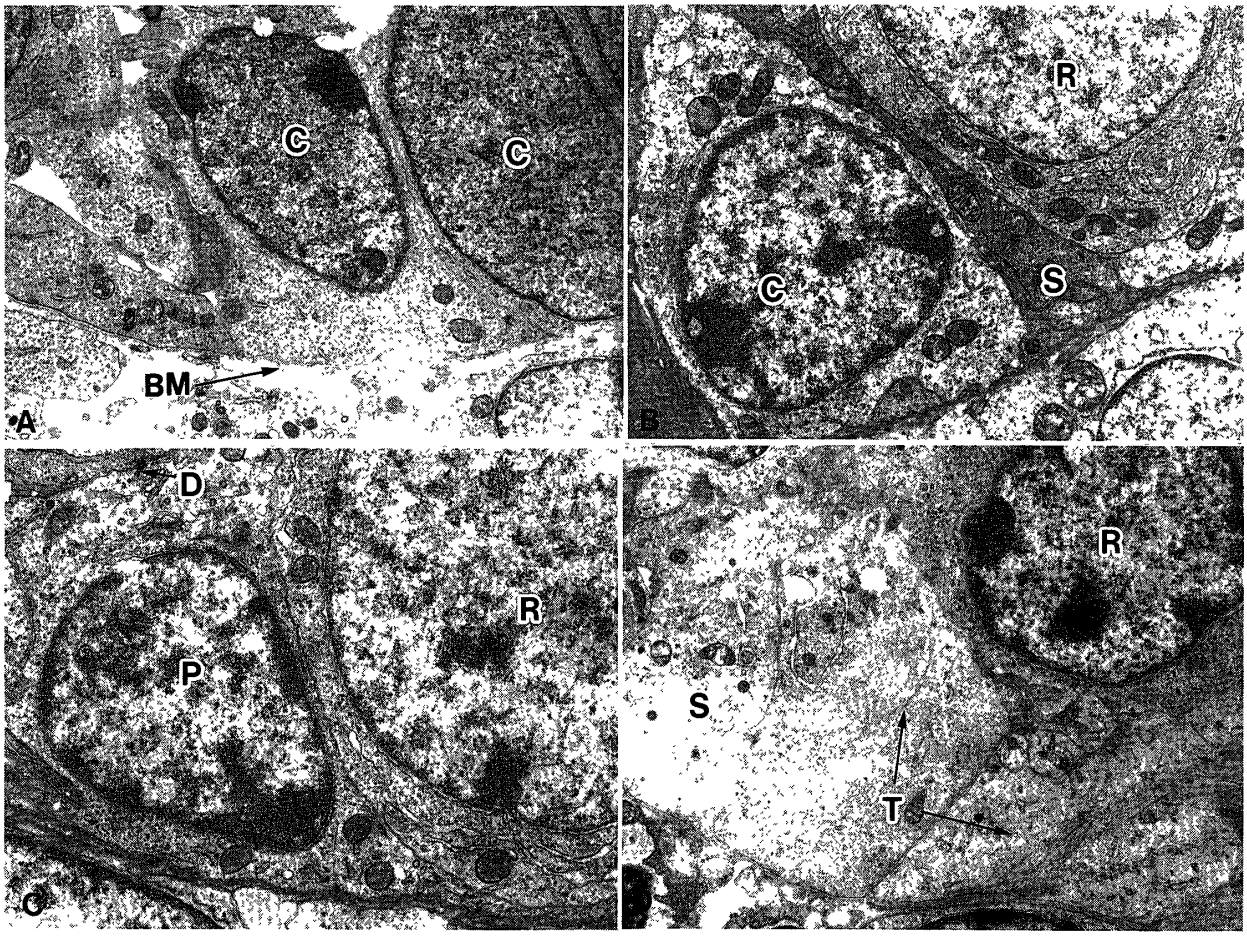


Fig. 4. Electron micrographs of the basal region of the olfactory epithelium and vomeronasal organ. A: The olfactory epithelium on E 14. The columnar cells (C) are seen in the basal region, in direct contact with the basement membrane (BM). x5500. B: The olfactory epithelium on E 17. The columnar cells (C), round-shaped cells (R), and processes of supporting cells (S) are seen in the basal region. x4900. C: The olfactory epithelium on E 19. Pyramidal-shaped cells (P) located adjacent to round-shaped ones (R) in the basal region. D, desmosome. x6800. D: The neurosensory epithelium of the vomeronasal organ on E 17, showing round-shaped cells (R) located in the basal region above the processes of supporting cells (S). T, tonofilaments. x6000.

desmosomes and tonofilaments in their cytoplasm (Fig. 4C). In the VNO, the basal region of the neurosensory epithelium was occupied by the processes of supporting cells, and round-shaped cells which were devoid of tonofilaments located above the processes of soupporting cells, after E 17 (Fig. 4D).

Discussion

The origin of olfactory and supporting cells in the developing olfactory epithelium of the mouse has been examined by many investigations using light and electron microscopy. The first olfactory cells are found in the middle region of the olfactory epithelium on E 12[10]. Mitotic figures are found only in the apical region of the developing olfactory epithelium up to E 13 and subsequently appear at the base and increase in number postnatally[14]. Mitotic figures in the apical region of the olfactory epithelium is thought to be related to the differentiation of stem cells into supporting cells, whereas those in the basal region are considered to be related to stem cells of olfactory cells[10,14]. Our present study combining immunohistochemistry of NCAM with BrdU identified which types of cells were dividing in the developing olfactory epithelium. The columnar cells of the embryonic stage were numerously labeled with BrdU, indicating active division. The data suggest that columnar cells, which are located throughout the olfactory epithelium on E 12 and then in the basal region after E 14, are stem cells of olfactory cells during the embryonic period, since numerous labeled cells were found in the olfactory cell layer of the middle region 24 hrs after injection of BrdU. Also in the VNO, the columnar cells in the neurosensory epithelium are suggested to be the stem cells of receptor cells and to become the round-shaped cells observed near the border with the respiratory epithelium in the adult[12,13].

The migration of columnar cells from the olfactory epithelium is suggested, since the BrdU-labeled cells along the axons increased in number on E14, 24 hrs after the injection of BrdU. This movement is also suggested by the appearance of numerous BrdU-labeled cells in the middle and basal regions of the olfactory epithelium near the rostral forebrain on E 17. The migrating cells are thought to develop into lutenizing hormone- releasing hormone (LHRH) neurons, into sheath cells of olfactory axons, into cell bodies of the ganglion of the terminal nerve, and into precursor cells of periglomerular cells of the central nervous system[15,16,17,18].

The differentiation of columnar cells in the olfactory epithelium into two types of basal cells was observed in late embryonic development. The round-shaped cells, which contain many r-ER and are devoid of tonofilaments, are regarded as globose basal cells of postnatal days, and the pyramidal-shaped cells possessing tonofilaments and desmosomes as basal cells proper. The columnar cells resemble globose basal cells, which are stem cells of the olfactory cells in early postnatal and adult stages, rather

than basal cells proper, because the globose basal cells have been shown to actively divide and to be devoid of tonofilaments in their cytoplasm[2,19]. Although the globose basal cells in the adult have been shown to express NCAM[8], the columnar cells and newly-differentiated round-shaped cells during embryonic days were negative for NCAM. In the VNO, columnar cells were negative for NCAM but round-shaped cells were positive on E 17. Thus, round-shaped cells in the olfactory epithelium are supposed to be more immature than those in the VNO in later embryonic days, mature into NCAM -positive globose basal cells after birth. It appears that in the late embryonic period the stem cells of olfactory cells are NCAM-negative round cells in the olfactory epithelium and NCAM-positive round ones in the VNO. Finally, in view of their ultrastructural and immunohistochemical characteristics, the stem cells of olfactory cells in the olfactory epithelium may mature at a later stage than those in the VNO.

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抄 録

胎生期のマウスの嗅上皮と鋤鼻器の嗅細胞の幹細胞についてブロモデオキシウリジン（BrdU）と神経細胞接着分子（NCAM）の抗体を用いた二重染色と電顕により調べた。妊娠マウスにBrdUを投与し1時間後屠殺すると，胎生12日目の嗅上皮と鋤鼻器では上皮全体にBrdU標識細胞が多数みられ，活発に分裂していることが示唆された。これらの細胞は円柱形で，少数のリボゾーム，粗面小胞体を有しNCAM陰性であった。これらの細胞の間にNCAM陽性の嗅細胞が少数みられた。胎生14日目には，円柱形細胞は上皮の基底側に限局するようになった。円柱形細胞のBrdU標識は，BrdU投与24時間後には大部分，上皮中央の嗅細胞層へ移動することから，この円柱形細胞が嗅細胞へ分化することが示唆された。胎生17～19日目には，円柱形細胞は，鋤鼻器ではNCAM陽性の丸い細胞に，嗅上皮では二種類の基底細胞（globose basal cells, basal cells proper）に置き代るようになる。