Chem. Senses 30 (suppl 1): i125–i126, 2005 doi:10.1093/chemse/bjh146

Expression of bHLH Transcription Factors and IGFs in the Non-sensory Patches, Olfactory Epithelium and Vomeronasal Organ

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Key words: *IGFBP-5*, *Mash1*, *Neurogenin 1*, non-sensory patch, olfactory epithelium

Introduction

Non-sensory patches in the posterior dorsal fossa of the rodent nasal cavity are formed between postnatal (P) days 10 and 21 due to programmed death of ORNs and GBCs (Suzuki *et al.*, 2000). In a previous study, we found bHLH transcription factors *Hes6* and *NeuroD* to be expressed in the GBC layer in the olfactory epithelium (OE) of mice. During embryonic days, there were no regional differences in the expression of these two genes, whereas at postnatal days 3–7, expression disappeared in the region corresponding to the presumptive non-sensory patches (Suzuki *et al.*, 2003). *Hes 6* and *NeuroD* are known to promote neuronal differentiation, *NeuroD* acting downstream of *Mash1* and *Neurogenin 1*, which are determination genes for ORNs. *Mash 1* null mutant mice failed to produce progenitor cells and *Neurogenin 1* null mutant mice, progenitors of ORNs are generated but their differentiation is blocked (Cau *et al.*, 2002). It is not yet known whether *Mash1* and *Neurogenin1* are related to the formation of non-sensory patches. On the other hand, insulin-like growth factor (IGF) family and related molecules were reported to be expressed in the rat OE where IGFs may have functions of differentiation and survival of ORNs (Suzuki and Takeda, 2002). Six high-affinity IGF binding proteins (IGFBP-1 to -6) modulate IGF effects. In the present study, we examined whether the expression of bHLH transcription factors and IGFBPs changes in the region where the programmed death of ORNs and GBCs occurs.

Materials and methods

Timed pregnant and postnatal ddY mice were obtained from Sankyo Labs. All animals were maintained in a heat- and humidity-controlled vivarium on food and water provided *ad libitum*. Tissue preparation and *in situ* hybridization methods are described elsewhere (Suzuki *et al.*, 2003). For the synthesis of cRNA probes, cDNA fragments of *Mash1*, *Neurogenin 1*, *IGFBP-2* and *IGFBP-5* were cloned by RT-PCR using the total RNA extracted from the olfactory mucosa of adult mice. Each resulting fragment was cloned into *Hin*dIII/*Eco*RI sites of pT7/T3 α 18 and sequenced. DIG-UTP-labeled RNA probes were synthesized by use of RNA transcription kit (Roche Diagnostics, Mannheim, Germany). For double labeling of *NeuroD* and BrdU, sections were immersed with a mixture of goat anti-*NeuroD* antibody (Santa Cruz) and monoclonal anti-BrdU antibody (Becton-Dickinson) for overnight at 4°C. The immunoreacted sections were reacted with Alexa fluor 488-conjugated donkey anti-goat IgG (Molecular Probes, Eugene, OR) and rhodamine-conjugated donkey anti-mouse IgG (Santa Cruz) for 2 h at room temperature, and then examined with a Leica confocal laser scanning electron microscope.

Results

At E12–18, *Mash1* was expressed in both apical and basal regions of the OE and VNO as determined by *in situ* hybridization. *Neurogenin 1* was expressed in a single layer of the cells just above the basement membrane, termed basal progenitors. At P1, GBCs differentiated in the basal region of the OE, and *Mash1* and *Neurogenin 1* were expressed in these cells. At P 3, the expression of *Mash1* in GBCs disappeared from the dorsal fossa of the posterior nasal cavity (Figure 1). At the same time, *Neurogenin1* expression in that region also disappeared. Since BrdU is a marker for proliferating GBCs, immunohistochemistry using anti-*NeuroD* and BrdU antibodies was performed. In the basal region of the OE, some *NeuroD*-immunoreactive cells overlapped with BrdU-immunoreactive cells, however, most of the *NeuroD*-immunoreactive cells were located above the BrdU-positive cell layer. From embryonic days to P1, the expression of *NeuroD* and BrdU labeling showed no regional differences. At P3, *NeuroD*-immunoreactive cells decreased in number in the dorsal fossa. At that time, BrdU-immunoreactive cells were present in that

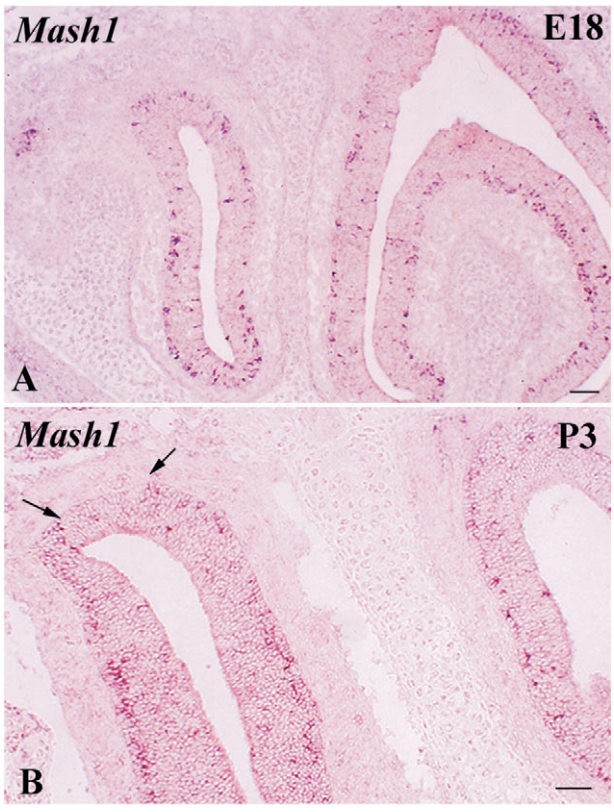

Figure 1 *In situ* hybridization with RNA probes for *Mash1* in the posterior regions of the nasal cavity. **(A)** At E18, *Mash1* is expressed in both the apical and basal regions of the OE. **(B)** At P3, *Mash1* is absent in the dorsal fossa formed between the third and the fourth nasal turbinates (between arrows). Bars = 50 μm.

region. At P7, *NeuroD*-immunoreactive cells were not observed. BrdU-immunoreactive cells were decreased in number as development proceeded further.

Among IGFBPs, IGFBP-2 and -5 were found in the OE and the VNO by *in situ* hybridization.

During embryonic stages, specific *IGFBP-5* signals were detected in some of the ORNs and receptor neurons of the VNO. Strong expression was observed in ORNs of the developing nasal turbinates and at the boundary of the respiratory epithelium. From P1, the expression was restricted to the apical layer of presumptive non-sensory patches in the dorsal fossa. This expression pattern persisted up to P14. Moreover, strong expression was observed in the mitral cells of the olfactory bulb.

Discussion

Mash1 and *Neurogenin 1* are determination genes for ORNs (Cau *et al.*, 2002). The results of the present study suggest that loss of expression of *Mash 1* and *Neurogenin 1* cause the formation of non-sensory patches. This loss may consequently prevent the activation of the downstream genes *NeuroD* and *Hes6*. The lack of these gene functions may stop differentiation and may cause apoptosis of GBCs and ORNs in the presumptive non-sensory patches. A similar finding was reported in the case of the non-sensory epithelium of the VNO (NS-VNO), which contains receptor neurons during embryonic stages: neither *NeuroD* nor *Hes6* was expressed before apoptosis of the receptor neurons (Suzuki *et al.*, 2003).

IGFBPs have been shown to inhibit or potentiate IGF actions. Furthermore, some IGFBPs have been demonstrated to have IGF-independent actions (Zhou *et al.*, 2003). In the present study, a change in the expression pattern of *IGFBP-5* during development of the nasal cavity was observed. During embryonic stages, *IGFBP-5* may affect growth of the OE and differentiation or maintenance of ORNs and receptor neurons of the VNO. Postnatally, *IGFBP-5* may influence growth and survival of non-sensory patches.

Summary

Molecules related to the formation of non-sensory patches in the rodent nasal cavity were examined. During embryonic stages, bHLH transcription factors *Mash1* and *Neurogenin 1* were expressed in the progenitor layers of the olfactory epithelium (OE) and vomeronasal organ (VNO). After birth, in the basal region of the OE, *Mash 1* and *Neurogenin 1* were expressed in globose basal cells (GBCs). In the posterior nasal cavity, the expression of *Mash1* and *Neurogenin 1* disappeared from GBCs of the region corresponding to the presumptive non-sensory patches. Downstream genes *Hes6* and *NeuroD* were also absent in that region as described previously (Suzuki *et al.*, 2003). The loss of these genes may stop the differentiation and may cause apoptosis of olfactory receptor neurons (ORNs) and GBCs. Moreover, the expression pattern of insulin-like growth factor binding protein (*IGFBP*)*-5* was unique: it was expressed in ORNs and vomeronasal receptor neurons during embryonic stages. At postnatal days 1, 3, 7 and 14, the signals of *IGFBP-5* were detected in the apical layer of the presumptive non-sensory patches. *IGFBP-5* may influence the growth and survival of non-sensory patches.

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