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Bcl-2 expression mediated by Cre/loxP system in olfactory epithelium

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Running Title: Bcl-2 in olfactory epithelium

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Abstract

Objective: To study the Bcl-2 expression mediated by the Cre/loxP recombination system and its effect on prevention of apoptosis in olfactory epithelium.

Methods: Adenoviral vectors with cassette for Bcl-2 (AxCALNLBcl-2) and Cre recombinase (AxCANCre) were applied to mouse olfactory epithelium by intranasal instillation. The effect of exogenous Bcl-2 expression on prevention of apoptosis of olfactory receptor neurons was investigated using an apoptosis model induced by bulbectomy.

Results: The Bcl-2 product was expressed not only in the olfactory receptor neurons but also in the supporting cells. Although statistical analysis did not show significant difference, the number of apoptotic cells in the infected olfactory epithelium on post-bulbectomy day 2 was lower than that of control and the number of survived mature olfactory receptor neurons in the infected olfactory epithelium on post-bulbectomy day 5 was higher than that of control.

Conclusions: Although further studies are required for clinical application, the results of our study suggest that this strategy may be able to deliver exogenous Bcl-2 for the treatment of degeneration of olfactory receptor neurons.

Key words: Cre/loxP, olfactory receptor neuron, adenoviral vector, Bcl-2

Introduction

The mammalian olfactory receptor neurons (ORNs), which harbor odorant receptors, are contained in the olfactory epithelium (OE) lining the upper area of the nasal cavity. ORNs have the unusual and characteristic ability to regenerate continuously throughout the lifetime, with dying olfactory neurons being replaced by newly generated ORNs [2]. However, it is thought that olfactory epithelium is degenerated due to toxic environmental effects, viruses, toxins, drugs, diseases or smoking [7] as well as ageing[18].

Various growth factors [16] and anti-apoptotic agents [4] have been reported to be essential for olfactory neurogenesis and have been proposed as potential candidate therapeutic drugs for olfactory dysfunction. However, while promising data have been reported based on *in vitro* experiments, only a few studies using *in vivo* experiments have been reported [20].

Bcl-2 was originally identified as a protein product of the proto-oncogene BCL-2 and increased Bcl-2 expression is a common feature of many types of human malignancies, indicating that Bcl-2 plays an important role in carcinogenesis[14]. The family of this proto-oncogene is vitally involved in the regulation of cell death, while Bcl-2 is widely expressed during embryonic development and has been shown to protect neurons from various types of cell death[9] [15]. Recent *in vivo* studies using transgenic mice have shown that Bcl-2 also has a neuro-protective effect in various pathological situations [8]. It has also been demonstrated that

Bcl-2 promotes regeneration of axons by an action occurring separately from its well-known anti-apoptotic activity [3].

The successful gene delivery to the ORNs in the olfactory system by adenoviral vector has been reported by us as well as others [6] [21] [10] [1] [13] [11] [5]. Encouraged by these results, we first investigated the potential of a novel strategy for introducing exogenous Bcl-2 into ORNs by using the Cre/loxP recombination system with an adenoviral vector[19]. The results presented here are expected to contribute to the development of clinical applications of the Bcl-2 gene transfer to the peripheral olfactory system for the treatment of olfactory dysfunction.

Materials & Methods

Recombinant adenovirus

The virus preparation of AxCALNLBcl-2 and AxCANcre has been described elsewhere [19]. Human Bcl-2 cDNA was kindly provided by Dr SJ Korsmeyer (Harvard Medical School, Boston, MA). The viruses were grown in HEK 293 cells and purified by CsCl gradient centrifugation. Virus titer was determined by plaque assay, and the concentrated virus was stored at -80°C.

Animals

Mice with a C57BL/6J background were originally purchased from the Jackson Laboratory (Bar Harbor, ME) and raised in our animal facility. Adult 35-day-old male animals were used in this study. All animals were housed in a temperature-controlled (22±1°C) colony room with a 12h/12h L/D cycle. They stayed in groups in acrylic cages with woodchip bedding and unlimited access to normal laboratory chow or food. All of the procedures were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised in 1996, and approved by the Animal Care Ethics Committee of Kobe University School of Medicine. Three mice were used in each group.

Infection of mouse olfactory epithelium with AxCALNLBcl-2 and AxCANcre by nasal instillation

The animals were anesthetized by an intraperitoneal injection of 3.5% chloral hydrate (3.5mg/10g body weight) and clamped in a stereotactic apparatus (Narishige, Tokyo). The concentrated

virus suspension (approximately 3.94 ×10¹⁰plaque forming units (PFU)/ml of AxCALNLBcl-2 or 3.94 ×10¹⁰ PFU/ml of AxCANCre) was instilled during a 5-minute period into the nasal cavity through the right nostril to a depth of 8 mm using a 10 μl Hamilton microsyringe. The volume of the suspension was 4 μl per instillation, which was determined by referring our previous study. To examine the expression of bcl-2, mice were sacrificed at 1, 2, 5, and 8 days after injection.

Bulbectomy

For unilateral bulbectomy, the animals were anesthetized by an intraperitoneal injection of 3.5% chloral hydrate (3.5mg/10g body weight) and clamped in a stereotactic apparatus (Narishige, Tokyo). The bone over the right olfactory bulb was removed and the ipsilateral bulb was totally removed by gentle suction as previously described[5]. Care was taken to avoid damage to the contralateral olfactory bulb. The animals were then sutured and allowed to recover from anesthesia on a warming pad. The extent of each lesion was verified by microscopic examination of fixed specimens. After recovery from anesthesia, mice were maintained on a normal diet until sacrifice at 2 and 5 days after the surgery. Control and surgically lesioned mice were re-anesthetized and transcardially perfused with a solution of 4% paraformaldehyde, buffered to PH 7.4 with 0.1M phosphate buffer (PB). After perfusion, the nasal cavity containing the olfactory turbinates and septum was dissected and post-fixed in the same fixative solution for 2h.

Immunohistochemical analysis

Seven-micron-thick serial sections were cut from formalin-fixed paraffin-embedded specimens and mounted on silan coated slides (Dako Japan, Tokyo). Deparaffinized and rehydrated tissue was placed in a citrate-buffered solution (pH 6.0) and microwaved to 100°C for 20 min (antigen retrieval) as previously described. Endogenous peroxidase and nonspecific binding was blocked with 3% hydrogen peroxide and 10% normal serum respectively. To detect the expression of bcl-2, the mouse monoclonal antibody against human Bcl-2 (Dako, Denmark) was diluted to 1:200 and incubated for one hour at room temperature. To identify the mature olfactory receptor neurons, the goat polyclonal antibody against OMP (Wako Pure Chemical Industries, Osaka) was diluted to 1:4000. Immunoreactivity was detected with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Normal serum was substituted for the primary antibody in negative controls. Diaminobenzidine (DAB) was used as the chromogen and nuclei were counterstained with hematoxylin.

Detection of Apoptotic Cells (TUNEL method)

Cell death resulting from apoptosis was measured using the terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling (TUNEL) method. Briefly, 7μ-thick serial sections were cut from formalin-fixed paraffin-embedded specimens and mounted on silan coated slides (Dako, Japan). Sections were deparaffinized and rehydrated through graded xylene and alcohol series followed by incubation with proteinase K (20 mg/mL in 10mM

Tris/HCl, pH 7.6 for 60 min at 37°C). The TUNEL method was performed with the aid of the In Situ Cell Detection Kit, AP (Boehringer Mannheim Biochemicals, Mannheim, Germany), according to the manufacturer's protocol. Reactions were visualized by using Fast Red (Boehringer Mannheim Biochemicals) as the substrate. Specimens were briefly counterstained with hematoxylin and mounted.

Quantitative analysis of the effect of bcl-2 delivered by adenoviral vector to prevent apoptosis of olfactory epithelium induced by bulbectomy

To quantify the effect of bcl-2 on the prevention of apoptosis induced by bulbectomy, the numbers of cells positive for TUNEL or OMP in the olfactory epithelium were counted. Since there were variations in the shapes of turbinates, the olfactory epitheliums overlying the nasal septum of the sections which included the maximum olfactory epithelium area were used as representative. At least six fields per each section were examined at a magnification of x 400 under a Nikon microscope. Statistical analysis was performed using Student's t-test. $P<0.05$ was considered statistically significant.

Results

Exogenous expression of Bcl-2 gene

Microscopic examinations disclosed positive staining for Bcl-2 protein in the OE from post-infection day 1. The staining was predominantly observed in the upper layer of the OE (Fig. 1). Close observation identified positive staining not only in the ORNs and globose basal cells but also in the supporting cells. However, no expression was detected in the horizontal basal cells on the basal lamina or in the olfactory nerve bundles in the lamina propria (Fig.2). In addition, no toxic effects of adenoviral vectors were observed as they were in normal control. The expression of Bcl-2 was observed at least until post-infection day 8.

The role of exogenous Bcl-2 in protecting the ORNs against apoptosis induced by bulbectomy

To determine whether adenovirus-mediated overexpression of Bcl-2 induced by Cre/loxP system could protect ORNs against apoptotic stimuli, we examined the OE, which had already been infected with AxCALNLBcl-2 and AxCANCre, for bulbectomy-induced apoptosis.

Administration of adenoviral vectors was performed as described above. Bulbectomy was performed 7 days after the infection, and mice were sacrificed at post-bulbectomy days 2 and 5. The apoptotic cells on post-bulbectomy day 2 were detected using TUNEL method. As shown in Fig. 3, marginal significance was

observed in the number of TUNEL positive cells between control and infected mouse (51.2 vs. 41.0 per field, p=0.09)

To quantify the survived mature OMP at post-bulbectomy day 5, the numbers of cells positive for OMP in the olfactory epithelium overlying the nasal septum were counted as above described. Although statistical analysis did not show significant difference, the number of survived olfactory receptor neurons in the infected mouse was higher in comparison with control (12.0 vs. 8.8 per field, p=0.18).

Discussion

The successful gene delivery to the ORNs in the olfactory systems by means of adenoviral vector has been reported by several investigators [6] [21] [10] [1] [13]. In these studies, the adenoviral vectors were administered into the nasal cavity, followed by the observation of the expression of the transgene products in olfactory epithelial cells, such as mature and immature ORNs and supporting cells, and tracing of the olfactory axons to their targets in the glomerular layer of the olfactory bulb. Most recently, Isoyama et al succeeded in inducing BDNF expression in the OE with the aid of this system[11].

Currently, the generation of replication-defective recombinant adenoviruses is commonly based on the propagation of viruses in host cells, such as human embryonic kidney (HEK) 293 cells, which express the E1 region that is essential for virus replication. However, some foreign genes, including Bcl-2, have been found unsuitable for producing recombinant adenoviruses in this manner [17]. To address this issue, we have recently used the Cre/loxP system to generate replication-defective recombinant adenoviruses carrying the bcl-2 gene [19].

Recombinase Cre derived from bacteriophage P1 mediates excisional deletion of a DNA sequence flanked by a pair of loxP sites, known as the Cre-specific recognition sequence. Further, the spacer DNA flanked by a pair of loxP sites that lies between the promoter and the coding region of genes of interest is excisionally deleted by Cre. Thus, the target gene in this

expression cassette is expressed only when this adenovirus vector is co-infected with the adenovirus expressing recombinase Cre [12].

The Cre/loxP recombination system has been successfully utilized in a gene activation and inactivation strategy not only *in vitro* but also *in vivo*. In fact, we previously used the Cre/loxP recombination system to demonstrate *in vivo* Bcl-2 expression in motor neurons in mice. However, the applications of this system are still limited because gene activation by Cre recombinase could be attained in only part of the targeted cells [19]. In fact, the number of ORNs expressing bcl-2 was significantly fewer than that of ORNs expressing LacZ in our previous study using the standard adenoviral vector[5]. In terms of its effect of neruoprotection, the survival benefit of exogenous Bcl-2 was expected from the findings at post-bulbectomy day 2 and 5, while statistical analysis did not reach to significance. These results suggest that bcl-2 might have a neuro-protective effect on the ORNs, but, as of now, the Cre/loxP system is not yet capable of delivering sufficient amounts of this gene to the peripheral olfactory system.

Conclusions

Although further studies are required for clinical application, the results of our study suggest that this strategy may be able to deliver exogenous Bcl-2 for the treatment of degeneration of olfactory receptor neurons.

Legends

Fig. 1 Immunohistochemical detection of exogenous Bcl-2 protein in the olfactory epithelium.

The concentrated virus suspensions of AxCALNLBcl-2 and AxCANCre were instilled during a 5-minute period into the right nasal cavity through the nostril to a depth of 8 mm using a 10 μl Hamilton microsyringe. Positive staining for Bcl-2 was observed predominantly in the upper and middle layers of the olfactory epithelium of the right nasal cavity. R: rihgt, L:left. X 40

Fig.2 Exogenous Bcl-2 protein in olfactory receptor neurons and supporting cells.

Positive staining for Bcl-2 was observed in the mature olfactory receptor neurons (white arrow) and globose basal cells (white arrow head) as well as in the supporting cells (black arrow)(A). Mature olfactory receptor neurons were identified as cells positive for OMP (B). No positive staining was observed in the olfactory epithelium of mice not infected by means of the Cre/loxP recombination system (data not shown). Black arrow head: basal lamina.

Fig.3 Olfactory **epithelium of the septum after bulbectomy.**

Apoptotic cells were stained red as detected with the TUNEL method. Survived mature olfactory receptor neurons were identified as OMP-positive cells. On post-bulbectomy day 2, the number of

apoptotic cells in the infected olfactory epithelium (A) was fewer than that of control (B). While statistical analysis did not show significance, the number of survived mature olfactory receptor neurons of the infected epithelium was higher than that of non-infected olfactory epithelium on post-bulbectomy day 5.

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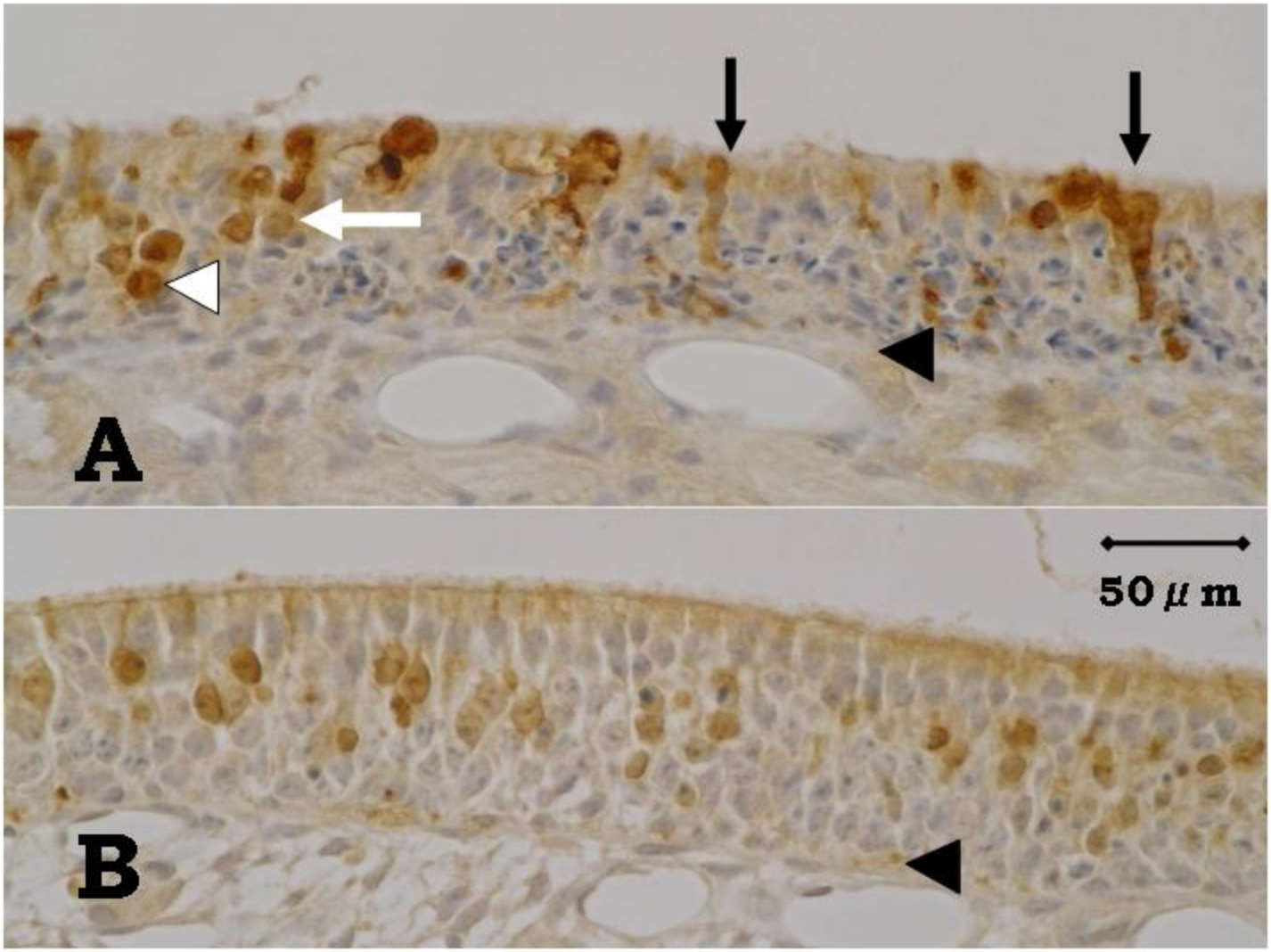
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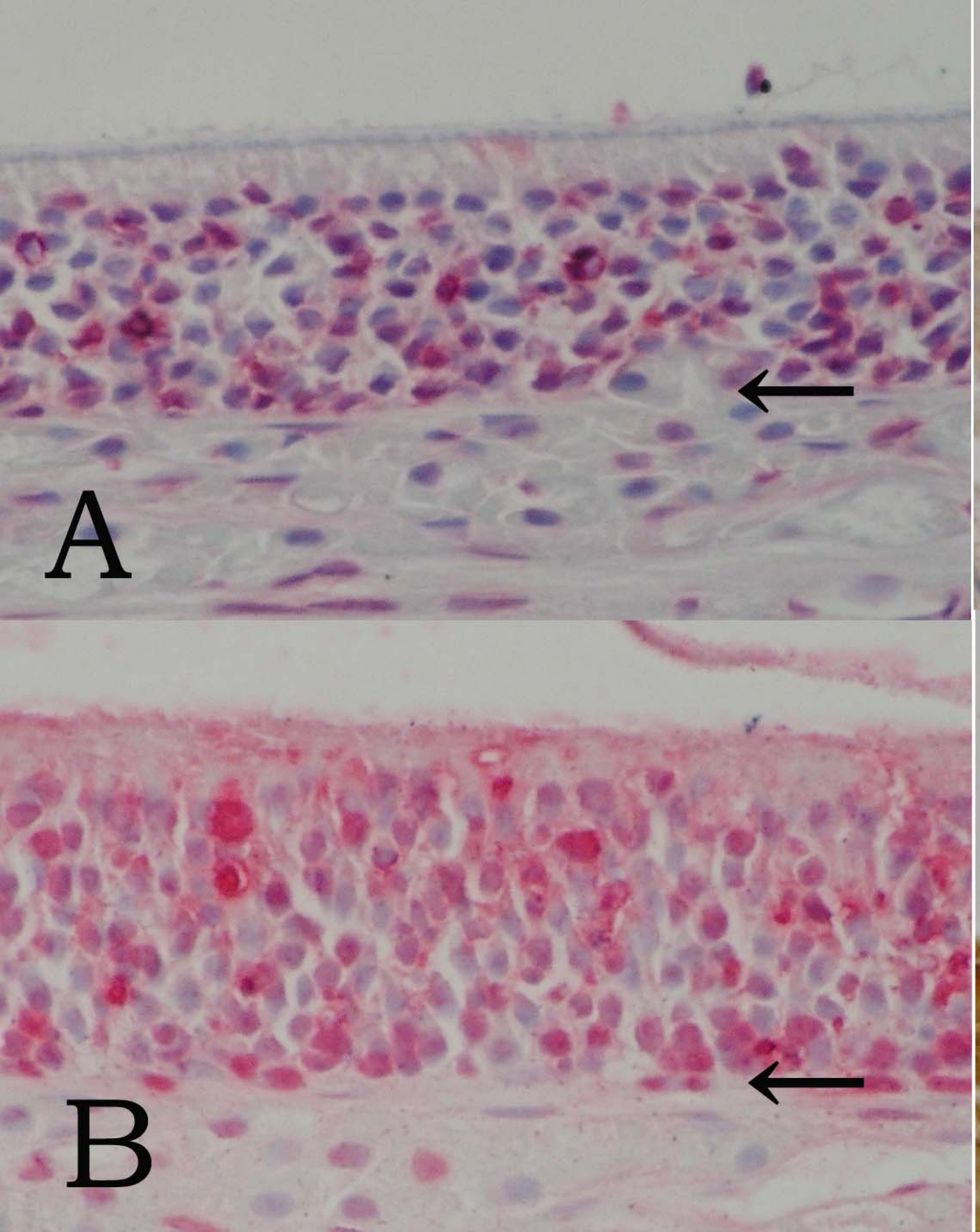
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