New Techniques for Biopsy and Culture of Human Olfactory Epithelial Neurons

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**Objective:** To improve the success of culturing olfactory neurons from human nasal mucosa by investigating the intranasal distribution of the olfactory epithelium and devising new techniques for growing human olfactory epithelium in vitro.

**Design:** Ninety-seven biopsy specimens were obtained from 33 individuals, aged 21 to 74 years, collected from 6 regions of the nasal cavity. Each biopsy specimen was bisected, and 1 piece was processed for immunohistochemistry or electron microscopy while the other piece was dissected further for explant culture. Four culture techniques were performed, including whole explants and explanted biopsy slices. Five days after plating, neuronal differentiation was induced by means of a medium that contained basic fibroblast growth factor. After another 5 days, cultures were processed for immunocytochemical analysis.

**Results:** The probability of finding olfactory epithelium in a biopsy specimen ranged from 30% to 76%, depending on its location. The dorsoposterior regions of the nasal septum and the superior turbinate provided the highest probability, but, surprisingly, olfactory epithelium was also found anteriorly and ventrally on both septum and turbinates. A new method of culturing the olfactory epithelium was devised. This slice culture technique improved the success rate for generating olfactory neurons from 10% to 90%.

**Conclusions:** This study explains and overcomes most of the variability in the success in observing neurogenesis in cultures of adult human olfactory epithelium. The techniques presented here make the human olfactory epithelium a useful model for clinical research into certain olfactory dysfunctions and a model for the causes of neurodevelopmental and neurodegenerative diseases.


The olfactory epithelium, in many ways, similar to the neuroepithelium of the embryonic neural tube. Within adult olfactory epithelium are stem cells that retain the capacity to divide and give rise to neuronal precursors that further divide and differentiate into mature sensory neurons.1,2 Olfactory neurogenesis also occurs in humans: we have shown that human olfactory epithelium retains the capacity for neurogenesis and neuronal differentiation at least until the age of 72 years and that this can be demonstrated in tissue collected up to 25 hours post mortem.4 Thus, the human olfactory epithelium has the potential to be used as a tool to examine certain human disorders resulting from abnormal development of the nervous system.

Neurogenesis has been shown to occur in the brain of the adult mouse,5 but this is not confirmed in humans. In any event, neurogenesis in adult human brain could only be studied post mortem. In contrast, olfactory neurogenesis can be studied in living patients and controls. The adult olfactory epithelium is therefore an accessible source of neuronal tissue in which to observe the early stages of neurogenesis and neuronal differentiation and the factors that control and regulate these processes.6

Although Murrell et al4 demonstrated the potential for the study of neurogenesis in human patients, there remain problems with the efficacy of the technique as applied to living patients, compared with postmortem studies. When nasal biopsy specimens are collected from subjects, it is ethically important to collect as little tissue as necessary and to avoid rebiopsy. It is therefore vital to maximize the efficacy of the culture technique.

In our previous study, only approximately 50% of explants produced viable cultures (cultures with at least an outgrowth of epithelial cells), and of these, only about 50% produced neurons after stimulation with basic fibroblast growth

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SUBJECTS AND METHODS

Human nasal mucosa was obtained by biopsy during routine nasal surgery with the patient under general anesthesia, with the use of an ethmoid forceps (No. 8211.551; Richard Wolf Medical, Hoyland Medical, Camp Hill, Australia). Ninety-seven biopsy specimens were obtained from 33 individuals (24 men and 9 women aged 21 to 74 years); of these, 10 had nasal abnormalities, such as polyps. The patients were undergoing surgery for septoplasty or turbinate surgery. All biopsy tissues were obtained with the informed consent of the patients, and the studies were carried out in accordance with the guidelines of the National Health and Medical Research Council of Australia.

Six areas of collection were chosen: the dorsomedial and dorsoposterior areas of the superior turbinate, the dorsanterior and the ventroposterior areas of the middle turbinate, and the dorsomedial and the dorsoposterior areas of the septum. The approximate location of each biopsy was noted and marked on standardized “maps” of the septum and the medial face of the lateral wall of the nasal cavity. To aid localization of specimens collected from the septum, reference points to adjacent landmarks were noted. In some cases, the luminal surface of the nasal epithelium was stained with methylene blue before collection to assist in orientation of the excised pieces during explant culture.

Biopsy specimens were placed on ice in Dulbecco modified Eagle medium (Gibco-BRL, Gaithersburg, Md) supplemented with a serum supplement (Monomed, CSL, Melbourne, Australia), penicillin, streptomycin, gentamicin, and nystatin (Gibco-BRL). After 1 to 2 hours the specimens were bisected: 1 piece was processed for immunohistochemistry or electron microscopy, and the other was dissected further for explant culture. For culture, biopsy specimens were cut into pieces of approximately 1 to 2 mm² or, alternatively, sliced (200-µm thickness) by means of a McIlwain tissue chopper (Mickle Laboratory Engineering Company Ltd, Gomshall, Surrey, England) before being transferred to fibronectin-coated glass chamber slides and cultured in the conditions above, as previously described. Six to eight days later the cultures were exposed to FGF2 (50 ng/mL; Boehringer-Mannheim, South Brisbane, Queensland, Australia). Another 5 days later, cultures were fixed in methanol at −20°C and processed for immunocytochemical analysis.

Alternatively, some explants were cultured by a different technique. Instead of fibronectin-coated chamber slides, some explants were grown on small pieces of fibronectin-coated 0.4-µm nitrocellulose membrane (Millipore, North Ryde, Australia) at the interface of air and medium. The nitrocellulose membrane was placed on a transwell insert (Millipore) within a 6-well Costar plate (Trace Biosciences, Tingalpa, Queensland, Australia). The well was filled so that the medium just wetted the surface of the plastic membrane. With the methylene blue staining used to orient the tissue, some explants were plated lumen side down, while others were plated lumen side up. These cultures were otherwise treated identically to those grown on chamber slides, except that, after fixation and immunocytochemistry, the plastic membranes were mounted on slides before the coverslip was applied.

For electron microscopy, the tissue pieces were fixed for 2 hours in ice-cold fixative composed of 4% glutaraldehyde in 0.1-mol/L sodium cacodylate buffer (pH 7.4). Tissues were then washed in the same buffer, postfixed in 1% osmium tetroxide, washed, dipped in uranyl acetate, embedded in Spurr epoxy resin (ProSciTech, Queensland). Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and viewed on an electron microscope. For immunohistochemistry, the tissue pieces were fixed in Bouin fixative for 2 hours and washed in phosphate-buffered saline (pH 7.4). The tissues were embedded in paraffin, sectioned at 4 µm, and laid on slides coated with 3-aminopropyltriethoxy-silane (Sigma, Castle Hill, New South Wales, Australia).

Immunocytochemistry was performed with peroxidase-conjugated secondary antibodies (Sigma) with diaminobenzidine as the chromogen. The following polyclonal or monoclonal antibodies were used: anti-olfactory marker protein (goat polyclonal antibody, gift of Frank Margolis, PhD, University of Maryland, Baltimore), anti–neuron-specific β-tubulin type III (mouse monoclonal antibody, Sigma), and anti–microtubule-associated protein 5 (mouse monoclonal antibody, Sigma). Fixed cultures or deparaffinized sections were incubated overnight at 4°C followed by appropriate species-specific antibodies and peroxidase reagents. Extensive controls were used to exclude or determine the extent of nonspecific binding of secondary antibody and the presence of endogenous peroxidase.
RESULTS

After collection in operating theater, biopsy specimens were identified by 3 techniques: immunohistochemical analysis, electron microscopy, and in vitro explant culture.

To identify the olfactory epithelium inside the specimens, sections were immunostained with 3 neuron-specific antibodies: anti–neuron-specific β-tubulin type III, anti–microtubule-associated protein 5, and anti–olfactory marker protein, raised against a protein expressed only by mature olfactory neurons. The olfactory mucosa was easily identified under the light microscope by the presence of bipolar neurons in the epithelium and axon bundles in the lamina propria (Figure 1).

Samples visualized with transmission electron microscopy were screened with the use of 2 main criteria: the presence of tall columnar supporting cells and the shape of cilia-bearing cells. As shown in Figure 2, olfactory dendrites terminate in expanding knobs bearing immotile cilia, while the cells arising of the respiratory epithelium bear cilia whose parallel orientation indicates they are beating synchronously.

Biopsy cultures provided complementary information. Cilia of respiratory cells continued to beat in vitro and were easily identified; thus, we could estimate the proportion of respiratory and olfactory tissues on the whole explant. Furthermore, using a medium containing FGF2, we tested the capability of explants to produce new neurons. Figure 3 shows neurons induced by FGF2 and immunolabeled with antitubulin antibody, 10 days after plating. When “pure” respiratory mucosa (judged by the abundance of beating ciliated cells) was plated, no neurogenesis was observed after FGF2 treatment.

In total, 97 biopsy specimens were collected from 33 patients: 15 of these were not cultivated in vitro and only observed by either immunochemistry or electron microscopy. Of the others (82 specimens), 1 piece was plated in a culture dish and induced to differentiate, while the other was processed for either immunohistochemical analysis or electron microscopy. After comparison of the histological findings and cultures, the explants were classified into 1 of the 4 following groups: pure olfactory mucosa, pure respiratory mucosa, mixed tissue (olfactory and respiratory mucosae), and unidentified tissue.

The results are summarized in Table 1. In the whole population under study (33 patients), olfactory tissue (alone or with respiratory mucosa) was found in one half of the biopsy specimens (53%). This percentage decreased to 34% in the subpopulation (10 patients) of people with nasal abnormalities and increased to 59% in the healthy individuals (23 patients). When polyps were present, the ratio of pure olfactory mucosa decreased substantially while the percentage of unidentified tissue increased dramatically. Furthermore, pure respiratory mucosa was found in the dorsoposterior area of the septum, even in the younger patients.

The probability of obtaining olfactory tissue was calculated with only biopsy specimens collected from normal individuals (71 specimens from 23 patients). Figure 4 shows that olfactory tissue is more likely to be found further back in the nasal cavity on the septum as well as on the turbinate. However, on the posterior septum and on the posterior superior turbinate, pure olfactory mucosa was found in only 1 of 4 cases. In half of the cases, specimens contained interspersed patches of respiratory epithelium. A greater proportion of pure olfactory mucosa (40%) was detected on the posterior area of the medial turbinate.
There were no obvious age-related differences in our sample. In fact, examination of the specimens of the 7 youngest healthy individuals (all younger than 30 years) allows us to assert that interindividual variations are probably as important as age-dependent differences in the relative distribution of respiratory and olfactory epithelium.

Human biopsy specimens were first plated as previously described in the mouse, but only a third of them remained attached to the substrate (Table 2) whereas the attachment ratio was 90% in mouse experiments (unpublished observations, July 1996). We attempted to avoid detachment by using a technique in which explants were not able to float in the medium: they were plated on a nitrocellulose membrane and fed with an underlying medium. The use of large membranes (30-mm diameter) also gave us the opportunity to demonstrate that the attachment success rate was dependent on the way the explant was plated. After the luminal side of the nasal tissue was stained in the operating theater by means of methylene blue, the explant was seeded lumen side-down (the epithelium in contact with the insert membrane) or lumen side-up (the lamina propria in contact with the membrane). In the first case, the attachment ratio was very poor, and almost no cellular outgrowth was observed. In the second case, the attachment ratio was 66%. Nevertheless, the neuronal outgrowth ratio remained low (Table 2).

Light microscope observations of the explants suggested that epithelial cells and neurons were contained

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**Table 1. Tissues Obtained by Biopsy**

<table>
<thead>
<tr>
<th>Biopsy Tissue</th>
<th>All Subjects (N = 33)</th>
<th>Normal Subjects (n = 23)</th>
<th>Patients With Nasal Disease (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olfactory</td>
<td>20 (21)</td>
<td>18 (25)</td>
<td>2 (7)</td>
</tr>
<tr>
<td>Olfactory plus respiratory</td>
<td>31 (32)</td>
<td>24 (34)</td>
<td>7 (27)</td>
</tr>
<tr>
<td>Respiratory</td>
<td>29 (30)</td>
<td>21 (30)</td>
<td>8 (31)</td>
</tr>
<tr>
<td>Unidentified</td>
<td>17 (17)</td>
<td>8 (11)</td>
<td>9 (35)</td>
</tr>
<tr>
<td>Total</td>
<td>97 (100)</td>
<td>71 (100)</td>
<td>26 (100)</td>
</tr>
</tbody>
</table>
within the explant but were unable to migrate from it. The human lamina propria is thicker than that in the rodent, especially on the turbinates, and the human olfactory epithelium is thinner. For this reason, outgrowing cells may have been unable to reach the substrate and were prevented from proliferating and differentiating on the membrane, away from the explant.

To provide direct contact of the epithelium with the substrate, biopsy specimens were sliced (200 µm) and cultured either in chamber slides or on inserts. In both cases, attachment and neuronal outgrowth ratios increased dramatically. Because no significant difference was observed between the 2 culture techniques (immersed slices in chamber slides or nonimmersed slices on nitrocellulose membranes), both can be routinely used. However, culturing on chamber slides provides superior visualization compared with the membrane technique.

This study explains some of the variability in the efficacy of observing neurogenesis in explants of human olfactory epithelium. Two key issues were addressed: the location of the nasal region from which the biopsy specimen was taken and the method of explant culture. It is evident that the success rate for observing olfactory neurogenesis in healthy persons can be increased by (1) taking biopsy specimens only from the dorsoposterior regions of the nasal septum and the surface of the superior turbinate opposite and (2) culturing the epithelium in thin slices. The application of these methods should reduce the numbers of biopsy specimens necessary to demonstrate olfactory neurogenesis in each individual.

In accordance with the accepted location of the olfactory epithelium,11 the highest probability of finding olfactory epithelium was in the dorsoposterior region. Accordingly, we recommend taking biopsy specimens on the most posterior areas of the septum and the superior turbinates only, in contrast to Strahan et al,7 who suggest taking 4 specimens from each patient, 1 anterior and 1 posterior from each side of the nasal septum.

Our observations confirm previous reports that the distribution of the olfactory epithelium in adult humans is frequently disrupted with interspersed patches of respiratory epithelium. In contrast, the olfactory epithelium in the human fetus has been reported to be uniformly distributed without interruption by respiratory epithelium, extending from the roof of the nasal cavity onto the superior turbinate and to the middle of the septum in a continuous pattern.13

Even in the dorsoposterior regions of the adult nasal cavity, where the probability of finding olfactory epithelium was highest (Figure 4), only 40% of the specimens contained olfactory epithelium exclusively. Considering the small surface area within each biopsy specimen (1 mm²), it is apparent that the olfactory and respiratory tissues are intimately dispersed in the adult nasal cavity. Comparison of fetal and adult tissues suggests that invasion of respiratory tissue into olfactory epithelium increases with age, as suggested in a previous study of adult olfactory epithelium.8

The observations of this study raise issues related to the etiology of the distribution of olfactory tissue. It is often assumed that the appearance of respiratory epithelium within olfactory epithelium represents an “invasion” of the former into the latter, or a replacement of the latter by the former after damage to the olfactory epithelium by environmental insults, such as viruses and other diseases or head trauma.13,14 However, the wide distribution of the olfactory epithelium within the nasal cavity of the adult also raises the possibility that the olfactory epithelium may invade, replace, or migrate into regions of respiratory epithelium. This issue is unlikely to be resolved without a systematic mapping of the olfactory epithelium throughout the nasal cavity in hu-
mans of different ages, particularly of the ages between birth and adulthood, for which there are no data at all.

It is now possible to efficiently cultivate human olfactory epithelium. The optimal technique (slice culture, Table 2) differs significantly in efficacy from our previous method (whole explant culture, Table 2) by improving the success rate for attachment of the tissue to the substrate and by improving the success rate for the outgrowth of neurons. For attachment, the important factor appears to be that the lamina propria is in contact with the substrate (here the fibronectin-coated dish or membrane instead of the underlying cartilage). For neuronal outgrowth, the factor appears to be that the migrating neurons are able to contact the substrate. Both of these conditions are satisfied in the slice preparation. These results differ from our observations in the mouse, for which high success rates for attachment and neuronal outgrowth can be achieved when whole explants are cultured, without slicing them. We ascribe these species differences in efficacy to differences in the thickness of the lamina propria. The human lamina propria is thicker than in mouse and may provide a bigger barrier to neuronal migration from the epithelium to the substrate.

The success rates of the biopsy and culture techniques presented here make them particularly useful for further research on olfactory disorders as well as comparative studies on putative neurodevelopmental diseases (eg, schizophrenia) or neurodegenerative diseases (eg, Alzheimer or Parkinson disease).

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