Olfactory Receptor Neurons in Partially Purified Epithelial Cell Cultures: Comparison of Techniques for Partial Purification and Identification of Insulin as an Important Survival Factor

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Abstract

Olfactory neurons have the rare property of being replaced throughout life. Factors regulating different developmental stages of olfactory receptor neurons (ORNs) are of great interest, because such factors might be used to extend regeneration in the post-developmental brain and spinal cord. Also, these factors may potentially be exploited to treat various smell disorders arising from changes in the olfactory epithelium. Characterization of trophic factors for ORNs requires cell culture systems that are simple and easy to manipulate. We have compared four different cell culture preparations, using two different enzymes and two different media to develop a simple culture system of olfactory epithelial cells. Our preferred preparation, which produces partially purified olfactory epithelial cultures, uses trypsin dissociation and a serum-free keratinocyte growth medium (KGM) supplemented with insulin. These conditions support ORN survival up to 1 week. They also supported other elements of the olfactory epithelium such as Bowman's gland cells and horizontal basal cells. Olfactory epithelial cells predominate, while contaminating mesenchymal cells (glia and fibroblasts) are present in low numbers. Using these cultures, it was determined that insulin was required for ORN survival *in vitro*. The simplicity of the epithelial cultures will be useful for further studies of insulin and other ORN trophic factors.

Introduction

The olfactory epithelium (OE) serves as a good model system for studying the regulation of different developmental events in the life of a neuron. Olfactory receptor neurons (ORNs) are continually replaced throughout life, originating from self-renewing cells (basal cells) lying at the base of the epithelium (Graziadei and Monti Graziadei, 1979; Caggiano *et al.*, 1994). These basal cells differentiate into immature ORNs, which eventually mature into olfactory marker protein (OMP) expressing neurons. Thus, due to the turnover of ORNs, neurogenesis, neuronal differentiation, neuronal maturation and neuronal death are occurring at all times in the epithelium (Graziadei and Monti-Graziadei, 1978; Miragall and Monti Graziadei, 1982; Schwartz Levey *et al.*, 1991).

Another interesting feature in the OE is that neurogenesis, neuronal survival, maturation and death, are all regulated. For example, removal of the olfactory bulb (bulbectomy), the target of ORNs, initially causes a dramatic increase in mature neuronal cell death, hence a decrease in the total number of neurons (Costanzo and Graziadei, 1983). This is followed by an increase in basal cell mitosis in the OE to replace the lost ORNs (Schwartz Levey *et al.*, 1991; Carr

and Farbman, 1992; Carr and Farbman, 1993). In the absence of the olfactory bulb, most newly generated ORNs do not live long enough to mature into OMP-expressing neurons (Monti Graziadei, 1983; Verhaagen *et al.*, 1990; Schwob *et al.*, 1992). Therefore, the neuronal cell death (Carr and Farbman, 1992) and basal cell mitosis rate remain chronically elevated (Schwob *et al.*, 1992). Also, OMP-expressing ORNs of the bulbectomized OE remain chronically reduced compared to the non-bulbectomized OE (Costanzo and Graziadei, 1983; Carr and Farbman, 1992). The above data indicate that the OE has intrinsic growth factors that can support genesis of new ORNs and their survival. However, optimal cellular maturation requires the olfactory bulb.

Neurogenesis, differentiation and maturation are rarely seen in the post-developmental brain and spinal cord, so the factors that regulate them in the olfactory system are of great interest. In addition, understanding these factors may also aid in development of treatments for smell disorders stemming from damage to the OE. To determine the identity and actions of these unknown factors, manipulation of the cellular environment is essential. Cell culture systems

provide this flexibility and are extremely helpful for characterizing trophic factors.

Previously, our laboratory developed an olfactory cell culture system that supported neurogenesis, survival and maturation of ORNs (Pixley, 1992a). This culture system was complex and required a central nervous system (CNS) derived astrocyte feeder layer to support the neurons. In addition to neurons and other olfactory epithelial cells, the cultures contained underlying connective tissue cells from the lamina propria, which include muscle, glial, fibroblast and cartilage cells (Pixley, 1996). Here we describe a way to prepare a simpler culture system that supports survival of ORNs in a serum-free basal medium supplemented with insulin. We compare separation of the epithelium from the lamina propria by thermolysin enzymatic treatment to the trypsin enzymatic dissociation currently used in our laboratory. We also compare the use of a keratinocyte growth medium to the use of the standard medium used in our laboratory (Pixley, 1992a). We characterize the cell types present in the cultures and the optimal conditions for ORN survival. Our results indicate that the partial purification achieved using a keratinocyte medium was comparable to the enzymatic purification; however, neither removed all connective tissue contaminants. The cultures have fewer non-epithelial cell types than our previously described cultures. Therefore, we anticipate easier growth factor analysis for ORNs using this system. Our initial finding is that insulin is a critical survival factor for ORNs and its use allows for elimination of all undefined medium components.

Materials and methods

Newborn rat nasal cultures

Newborn Sprague–Dawley rats (P1–P3) were cold anesthetized and decapitated. Cartilage and the soft mucosal nasal tissues of the posterior nasal cavity (the olfactory area) were dissected (Pixley, 1992a; Pixley *et al.*, 1997) and prepared either by trypsin or thermolysin treatment. Trypsin treatment was as described by Pixley (1992a). For thermolysin enzyme treatment, dissected tissues were washed five times with Spinner's minimal essential medium (SMEM) containing 2.2 g/l sodium bicarbonate and 13 mM HEPES (pH 7.3). Then, the tissues were incubated in 250 µg/ml thermolysin (Sigma) in Dulbecco's modified Eagle's medium (DMEM) + 13 mM HEPES (pH 7.3) for 60 min at 37°C (6 ml per 10 pups). To stop the enzyme activity, fetal calf serum (FCS) was added to the tissues to a final concentration of 10%. The epithelium was pulled away from the underlying tissue under a dissecting microscope. Epithelial tissues were washed twice with SMEM, and then cells were physically dissociated.

Cells were resuspended in either DSN1 (Pixley, 1992a; Pixley *et al.*, 1997) or a fully supplemented keratinocyte growth medium (KGM) + 10% fibroblast conditioned medium (FCM). Fully supplemented KGM consisted of KBM (Clonetics, MCF 153 or Cascade Biologics, Medium 154), 54 µg/ml bovine pituitary extract (BPE), 10 µg/ml bovine insulin, 1 µg/ml hydrocortisone, 200 pg/ml epidermal growth factor (EGF), 50 µg/ml gentamicin and 50 ng/ml amphotericin-B. Supplements were purchased from Clonetics. Cells were plated onto mouse laminin (20 µg/ml, Upstate Biotechnology) coated glass coverslips (10 mm diameter rounds, Dynalab) in a 48-well tissue culture plate (Corning) at 2.7×10^5 cells/cm^2. Medium changes were performed every 3 days.

All chemicals, unless otherwise noted, were from Sigma (St Louis, MO).

Production of fibroblast conditioned medium (FCM)

After trypsin preparation of the nasal mucosa (described above), nasal cells were plated in DMEM with 10% FCS in T75 tissue culture flasks. After 2 weeks, the fibroblasts were confluent and 99% pure, as determined by morphology and scarcity of GFAP immunopositive cells. Three-day conditioned medium was collected, spun to remove debris, and frozen until use.

Immunocytochemistry of cultured cells

Cells were fixed in 4% paraformaldehyde (Fisher, Cincinnati, OH) in 0.1 M phosphate buffer (PB, pH 7.4) for 15 min at room temperature, then washed three times with phosphate buffered saline (PBS; 0.01 M phosphate, pH 7.3, 0.15 M NaCl). Next, cells were incubated in blocking buffer (PBS with 0.02% sodium azide, 0.2% Triton X-100 and 10% serum from host of secondary antibody) for 1 h at room temperature. For immunoperoxidase labeling, cells were incubated in primary antibody overnight at room temperature, in secondary biotinylated antibody (1:500; Vector Laboratories, Burlingame, CA) for 2 h at room temperature, and in ABC Elite Reagent (avidin DH-biotinylated horseradish peroxidase, 1:500 in 1× PBS; Vector Laboratories) for 2 h at room temperature. The chromagen was diaminobenzidine (DAB; 0.5 mg/ml in 0.1 PB) with 0.12% H$_2$O$_2$. For immunofluorescent labeling, the fluorescent secondary antibodies (1:200 in PBS) were incubated with cells for 2.5 h at room temperature then washed twice with PBS. All reagent dilutions were in PBS with 0.2% Triton X-100 unless otherwise noted.

The primary antibodies used were anti-NST [monoclonal antibody against the Class III β isoform (neuron specific) tubulin, 1:3000; Sigma], anti-GFAP (rabbit serum, 1:1000; Dako, Carpinteria, CA), anti-OMP (goat antiserum, 1:30 000; gift from F. Margolis, University of Maryland at Baltimore, MD), anti-Olf-1 (rabbit antiserum, 1:10 000; gift from R. Reed, Johns Hopkins University, Baltimore, MD), anti-PAX-6 (rabbit antiserum, 1:500; gift from R. Reed), anti-GLA-2 (monoclonal Ab, 1:200; gift from J. Morgan, St Jude's Hospital, Memphis, TN), anti-keratin (rabbit serum, 1:2000; Dako), anti-CK 5/6 (monoclonal Ab, 1:50;

Boehringer Mannheim, Indianapolis, IN). After immuno-staining, cells on coverslips were mounted cell side down on microscope slides with Gelvatol (Harlow and Lane, 1988), 2× DABCO concentration.

Quantification of cell staining

Cells were counted in ten microscope fields per coverslip at 10× magnification, using a pre-set counting pattern of three rows of sequential, non-overlapping fields. This constitutes 32% of the coverslip area. For quantification of keratin area, a Photometrics ImagePoint cooled CCD video camera coupled to a Sony video monitor was used with Metamorph software (Universal Imaging Corp., Tucson, AZ). After thresholding images, the total area was measured in 15 coverslip fields at 20× magnification (12% of the coverslip). Two or more coverslips were counted per condition per experiment, with the observer blinded as to the conditions. At least two experiments (separate litters) were carried out per test and coverslip values were used as individual points.

Results

Thermolysin treatment does not enrich for olfactory neurons over trypsin treatment when cells are grown in DSN1 medium

Initially, to determine if a different enzyme treatment would allow enrichment of epithelial cells, the newborn rat olfactory mucosa was prepared with either trypsin (TY) or thermolysin (TH) and plated in our standard serum-free DSN1 medium (Figure 1a, c). The trypsin preparation contained the olfactory epithelium, lamina propria and perichondrial cells. The thermolysin preparation allowed crude separation of the olfactory epithelium from the underlying lamina propria (Warchol, 1995). The thermolysin treatment was expected to provide enrichment of epithelial components and reduction of non-epithelial components compared to the trypsin treatment, since a higher percentage of the cells were expected to be from the epithelium. Glial fibrillary acidic protein (GFAP; which labels the olfactory nerve glia found in the lamina propria) (Pixley, 1992b) was used as a non-epithelial marker, and NST was used as a neuroepithelial cell marker (Lee and Pixley, 1994). Cells were fixed, stained and counted after 5 days *in vitro* (5 DIV) for each enzyme treatment. Even though there was a trend toward lower numbers of glial cells with the thermolysin preparation, neither the NST positive (NST+) nor the GFAP positive (GFAP+) cell numbers were statistically different between the thermolysin and trypsin cultures in DSN1 medium (Figure 1a,c).

KGM medium enriches for olfactory neurons over DSN1 medium

In another attempt to purify olfactory epithelial cells *in vitro*, we chose a different medium for cell growth. This was keratinocyte growth medium (KGM; a modification of
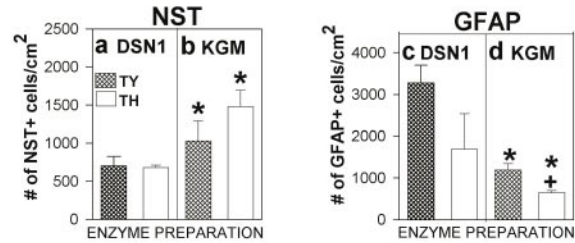
Figure 1 Purification of epithelial cells by enzymes and a selective media. **(a, b)** Neuronal numbers were significantly higher, at 5 DIV (Student's *t*-test, *$P < 0.05$, $n = 3$), when cells were grown in KGM (panel b) compared with DSN1 (panel a), with either trypsin (TY, white bars) or thermolysin (TH, black bars) enzyme treatments. The type of enzyme used did not significantly alter numbers of neurons in either medium. **(c, d)** Glial numbers—in KGM medium (panel d), glial cell numbers were significantly decreased by using thermolysin (panel d, white bar) instead of trypsin (panel d, black bar) (+ $P < 0.05$, $n = 3$). However, in DSN1, both enzyme treatments resulted in comparable glial numbers ($P > 0.05$). For either enzyme treatment, glial cell numbers were significantly decreased by KGM use (*$P > 0.05$, $n = 3$). Thus, the optimal reduction of glial cells was with KGM and thermolysin treatment. However, in KGM, thermolysin (panel b, white bar) use did not significantly increase neuronal numbers over those seen with trypsin (panel b, black bar).

MCDB 153), consisting of keratinocyte basal medium (KBM) plus supplements, which is normally used to grow human skin keratinocytes (Boyce and Ham, 1983). This medium was chosen because it was designed to support epithelial cells, and a previous study using a similar medium [with added fibroblast conditioned medium (FCM)] supported the growth of keratin positive (keratin+) cells from both newborn and adult olfactory tissues (Pixley, 1992c). The composition of KBM is not available, but it does not contain any other proteins (i.e. transferrin). We added 10% FCM to the KGM prior to plating because we found that the FCM greatly facilitated cell plating (data not shown). Without the FCM, very few cells attached to the laminin substrate.

Growth in KGM significantly increased the number of neurons in the cultures compared to growth in DSN1 (Figure 1a,b). However, in KGM the thermolysin treatment did not increase the number of neurons over trypsin (Figure 1b). Growth in KGM decreased the number of glial cells in the cultures significantly compared to growth in DSN1 (Figure 1c,d). Thermolysin use in KGM further significantly decreased the number of glial cells compared to the trypsin preparation in KGM (Figure 1d).

The enzyme and medium combination that supported the highest neuronal numbers and lowest glial numbers was the thermolysin and KGM combination. Using KGM instead of DSN1 medium increased neurons ~1.4-fold and decreased glial cells by ~3-fold. Using thermolysin instead of trypsin enzymatic treatment gave no increase in neurons, but decreased glial cells ~2-fold in KGM. However, the cell yields for the thermolysin preparations were very low, ~1/10 that of the trypsin preparation. This put constraints on the

information that could be obtained from one litter of rat pups. The thermolysin preparation did not eliminate GFAP+ cells and did not increase the number of NST+ cells over the trypsin preparation, so we chose to use the trypsin and KGM combination for subsequent cell culture studies.

KGM cultures have neuronal and non-neuronal olfactory epithelial cells

The chosen culture conditions using KGM and trypsin supported most cell types found in the olfactory epithelium (Figures 2 and 3). The major elements of the cultures were epithelioid islands that were composed primarily of non-neuronal cells of the olfactory epithelium. An anti-keratin antibody that cross reacts with a wide range of cytokeratins stained the epithelial islands extensively (Figure 2a,b). This was probably due to antibody recognition of keratins expressed by sustentacular cells, Bowman's gland cells, HBCs and respiratory epithelial cells—see Pixley (Pixley, 1992c) and unpublished results. A subset of epithelioid cells was labeled with anti-cytokeratin 5/6 (Figure 2c), which stains HBCs specifically in the OE, but does not label cells in the respiratory epithelium (Suzuki and Takeda, 1991; Holbrook *et al.*, 1995). The islands also contained Bowman's gland cells, as shown by immunostaining with an antibody specific for these cells (GLA-2, Figure 2d) (Hempstead and Morgan, 1983, 1985). Immunostaining for sustentacular cells was unsuccessful, indicating that these cells do not survive in the cultures. Another marker of the non-neuronal components of the olfactory epithelium is an antibody against the transcription factor Pax-6, which stains HBCs, sustentacular cells and Bowman's gland cells *in vivo* (Davis and Reed, 1996). Almost all cells of the islands were Pax-6 positive (Pax-6+; Figure 2e).

Between the islands were cells that were spindle-shaped and non-epithelioid. Most of the cells between the islands were GFAP+ (Figure 2f). Other cells were negative for all of the above antibodies and were presumed to be fibroblasts.

Neurons in the culture were identified by NST. At 1 DIV, most neurons were round and had not extended processes. There were numerous OMP+ cells at this time (data not shown). During the first 2 days in culture most neurons existed as isolated cells or in small clumps with other neurons. Epithelioid cells at this time were observed as single cells or in tiny compact islands (data not shown). The neuronal numbers in the cultures were stable through 3 DIV.

At 3 DIV the epithelioid islands were larger, suggesting that epithelioid cells were dividing between day 1 and day 3. The neurons at day 3 became more spread out and many were extending long processes. After 5 DIV, the immature neurons were found throughout the cultures, but primarily in association with the epithelioid islands (Figure 3a). We have noticed that the neurons grow preferentially near other neurons and close to epithelioid islands. However, neurons can and do exist as single cells on the laminin substrate, with no other cells in close proximity (data not shown). Based on the location of neurons at 1–2 DIV versus their locations at 5 DIV, it seems as though they may be migrating to be near one another and/or other cell types, although we have not rigorously tested this hypothesis. Close inspection of neurons revealed the presence of dendritic knob-like structures on many neurons (Figure 3b). The neuronal numbers begin declining between 4 and 5 DIV, and continued declining through 9 DIV, when few neurons remained. However, the island size continued to expand and remained healthy up to 9 DIV, and the non-epithelioid cells between the islands increased and remained healthy. Most descriptions of cell types in this manuscript are at 5 DIV.

Neurons and some of their immediate precursors are labeled *in vivo* by antibodies to the transcription factor Olf-1 (Davis and Reed, 1996). In our cultures, we saw many Olf-1 labeled cells. Most cells were double-labeled with NST and Olf-1 (Figure 3c,d). However, there were a few cells that were Olf-1+ only (data not shown). This suggests that the cultures supported a small number of committed neuronal cells that were not yet expressing other neuronal markers.

Very few of the NST+ neurons were generated in the culture dish, as determined by bromodeoxyuridine (BrdU) uptake. Cells double-labeled with anti-BrdU and anti-NST were 1% or less of the total neurons (data not shown). This suggests that the neuronal precursors were present in the cultures at least transiently, but their division was significantly decreased compared to our previously described culture system (Pixley, 1992a). The total number of neurons was the same in cultures grown with or without BrdU, indicating that BrdU toxicity was not a factor in obtaining the small number of double-labeled cells.

Cascade Biologics KGM did not require serum for cell plating

We noted a large variation in NST numbers from lot to lot

Figure 2   Olfactory cell cultures prepared with trypsin and KGM contained most of the supporting cell types of the olfactory epithelium, as well as connective tissue cell types. Cells were fixed at 5 DIV and stained with indicated antibodies. **(a, b)** Anti-pankeratin staining of epithelial cultures stained most of the cells in the islands. Panels (a) and (b) are pictures of the same field except (a) is viewed at a 10× magnification and (b) is viewed at a 20× magnification. The arrow indicates a keratin+ cell and the arrowhead indicates a cell not stained for keratin. **(c)** Anti-cytokeratin 5/6 is a specific antibody for HBCs in the olfactory epithelium and stained a subset of cells in the epithelioid islands. **(d)** Anti-GLA-2, a specific marker for Bowman's glands stained a subset of cells in the islands. **(e)** An anti-Pax-6 antibody stained most of the nuclei of the cells in the islands, but not surrounding non-epithelial cells. **(f)** Cells not in association with the islands stained positive for GFAP (arrow). Cells not stained for GFAP and with flat triangular morphology were presumed to be fibroblasts or single keratin+ cells (arrowhead). **(g)** No primary antibody control for mouse primary antibodies (arrowhead indicates an epithelioid island). **(h)** Normal rabbit serum control for rabbit primary antibodies. Measure bar in (b) applies to (d–h) also.
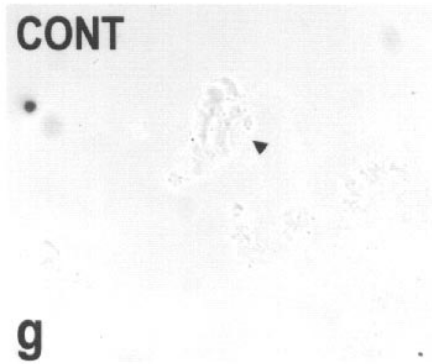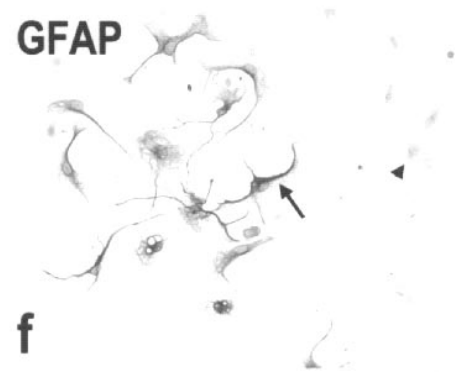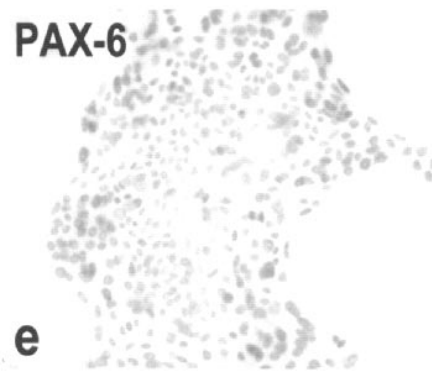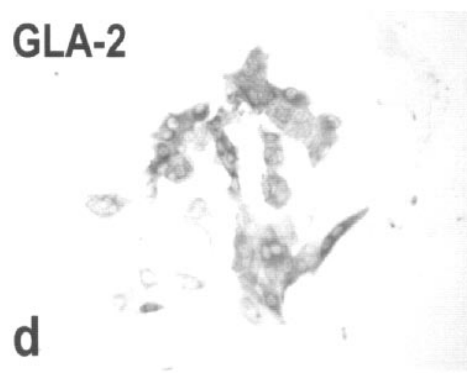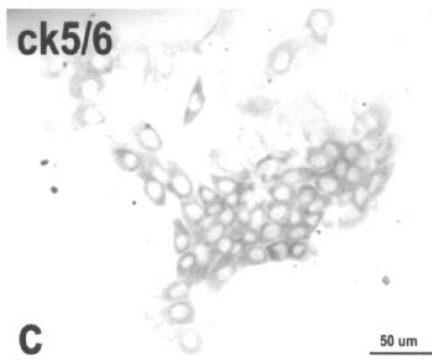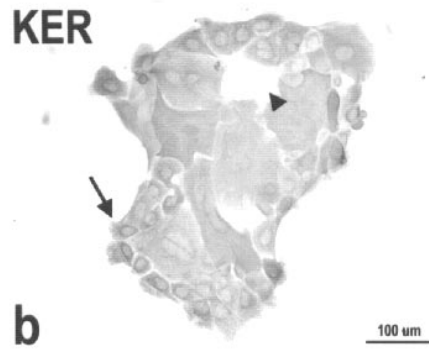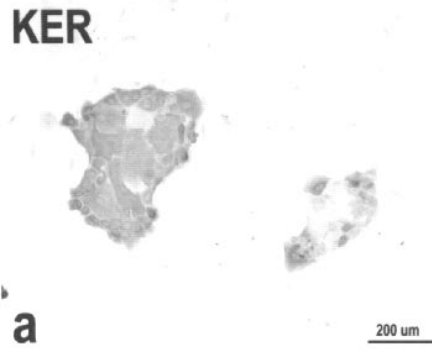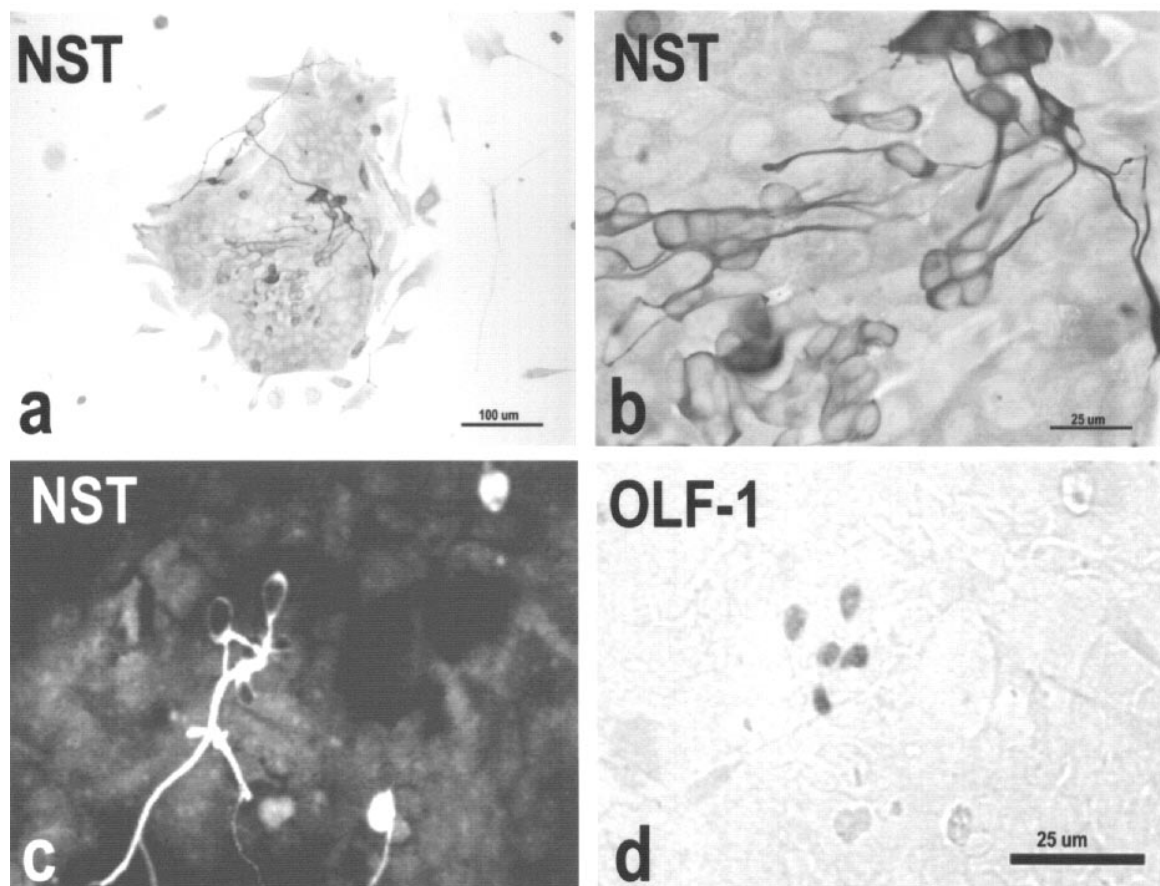
Figure 3 Olfactory cell cultures prepared with trypsin and KGM contained ORNs. Cells prepared using trypsin and KGM were fixed at 5 DIV and stained with anti-Olf-1 and/or anti-NST. **(a, b)** Pictures are from the same field taken at 20× for (a) and 40× for (b). **(a)** Most neurons were associated with the epithelioid islands. **(b)** Many neurons had processes with structures that resembled dendritic knobs. **(c, d)** Picture of the same field double labeled with anti-NST **(c)** and anti-Olf-1 **(d)**. Most Olf-1+ cells were also NST+. Measure bar in (d) applies to (c) also.

of the Clonetics KGM medium, so we tested another brand of KGM medium. We chose the KGM medium from Cascade Biologics, termed Medium 154 (modification of MCDB 151). The 'new' medium introduced less variability from lot to lot and did not change neuronal numbers or any other aspect of the cultures so far examined, so we continued with the Cascade Biologics medium (Figure 4). We would like to state that neither KGM media were designed to support neurons, so our results do not mean that Clonetics is not a quality keratinocyte growth medium. In addition, the fibroblast conditioned medium (FCM) used to facilitate plating in the Clonetics medium was removed from the Cascade medium with no change in the number of neurons (data not shown). Thus, the Cascade Biologics KGM used allowed for a completely serum-free environment.

Insulin is important for neuronal support

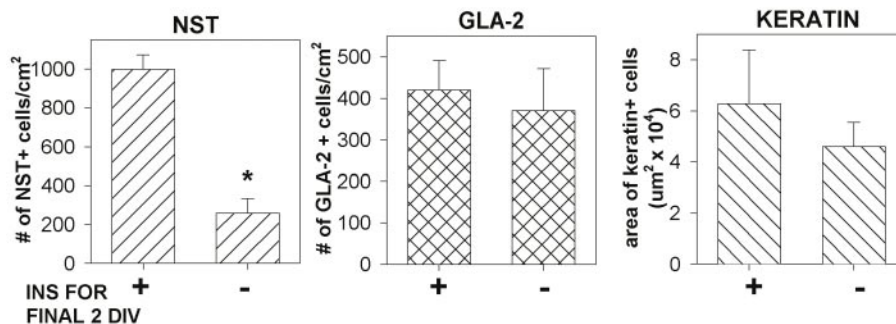Next we removed all three of the added supplements from the KGM (EGF, BPE and insulin) in a quest to get medium conditions as defined as possible. Fully supplemented medium (+SPL) contained more neurons than without supplements (–SPL; Figure 4). Addition of each individual supplement alone back to the cultures resulted in insulin as the only supplement that increased neuronal numbers over non-supplemented medium (Figure 4). In fact, addition of insulin (+INS) increased neuronal numbers back to fully supplemented medium conditions. Addition of BPE did not effect the neuronal numbers significantly and EGF seemed to decrease neuronal numbers. With insulin as the only supplement, the fibroblasts and glial cells seemed to be greatly reduced compared with fully supplemented medium (data not shown), probably due to the removal of the undefined BPE component.

Insulin addition could have increased plating efficiency of the olfactory epithelial cells, which would explain the higher neuronal numbers after 5 DIV. Also, insulin is important for glucose uptake in most cells. Absence of insulin in the medium may have caused all cells in the culture to die or become unhealthy. To address both of these questions, we grew all cells for the first 3 DIV with insulin. At day 3, we removed the insulin-containing medium and added medium

with or without insulin for the final 2 DIV. After 5 DIV cells were fixed and stained for NST, GLA-2 and keratin. The number of NST or GLA-2 positive cells was determined. Keratin**+** cells were assayed (using the polyspecific antibody) by determining the area covered by the keratin+ cells. The total area was used because the keratin+ cells were in large islands and individual cells were, in many instances, difficult to count. Keratin+ cells were similar in size (Figure 2b) and in a monolayer. The measure of area allowed for relative determination of the number of keratin+ cells. By growing the cells in insulin for the first 3 DIV, we eliminated the effect of insulin use on plating. By counting other cell types, we could determine if insulin was supporting all cells or more specifically the ORNs. Removal of insulin from the cells at 3 DIV decreased only the number of NST+ cells (Figure 5). GLA-2+ cells and the area of keratin+ cells were statistically unchanged 48 h after the removal of insulin. Therefore, insulin appears to have had a very specific trophic effect for ORNs in the epithelial cultures, and was not facilitating plating or causing cell death in general.
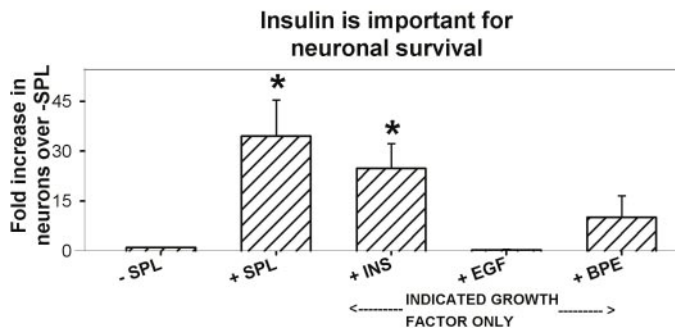
Figure 4 Insulin is necessary and sufficient for ORN survival in olfactory epithelial cultures. Cells were grown in Medium 154 (Cascade Biologics) with or without supplements for 5 DIV. Fully supplemented medium conditions (+SPL) contained significantly more neurons than non-supplemented medium conditions (–SPL). Of the three supplements (insulin, EGF and BPE), only insulin addition to non-supplemented medium made a significant difference. Insulin significantly increased neuronal numbers over –SPL and was equivalent to +SPL. (*) = Statistically different from –SPL. (Student's t-test, $P < 0.05$, $n = 2$).

We concluded that Medium 154 supplemented with 10 µg/ml insulin was sufficient to support neuronal survival. This serum-free system contains few non-epithelial cells and will provide a means with which to further study the effects of growth factors on ORNs.

Discussion

To facilitate the study of the effects of growth factors and cell–cell interactions on olfactory epithelial cells, we have created partially purified olfactory epithelial cell cultures. These cultures contained most components of the olfactory epithelium as well as some lamina propria contaminants. The cultures were organized as epithelioid islands that contain the non-neuronal components of the olfactory epithelium. The islands were positive for GLA-2, cytokeratin 5/6 and Pax-6 which mark Bowman's gland cells, HBCs, and both of these respectively (as well as sustentacular cells). The neuronal component, the ORN, was found most often in association with the islands. Most neurons grew on top of the islands and extended long processes, while some neurons had very short processes. Some non-epithelial components, usually found in the lamina propria (fibroblasts and glia), were usually found between the islands. We removed all undefined components from the media and, in the presence of insulin, found no effect on the neuronal numbers. However, the removal of EGF and BPE, which are two of the three growth factors recommended for growing keratinocytes in KGM, appeared to reduce glia and fibroblast-like cell numbers. With insulin, EGF and BPE, after 5 DIV, the epithelioid islands and the fibroblast-like cells were almost confluent (data not shown). Removal of EGF and BPE made for much sparser cultures, without effecting neuronal numbers. Removal of insulin at 3 DIV resulted in significantly fewer neurons at 5 DIV, while Bowman's gland numbers and the area covered by keratin+ cells did not change.

Over the past 10 years there have been several serum-free olfactory epithelial culture systems described in the literature. Calof and Chikaraishi (Calof and Chikaraishi, 1989) described removing the epithelium from the nasal tissues of

Figure 5 Insulin removal at 3 DIV reduced neuronal cell numbers but not GLA-2+ or keratin+ cells found at 5 DIV compared to culturing with insulin for the full 5 days. Cells were grown in Medium 154 containing 10 µg/ml insulin until 3 DIV, when the medium was replaced with (+) or without insulin (–). Cells were grown an additional 2 days before fixation and immunostaining were performed. Removal of insulin significantly reduced only NST+ (*) cells and not GLA2+ or keratin+ cells compared to continual incubation in the presence of insulin. (Student's t-test, $P < 0.05$, $n = 3$).

embryonic mice, then culturing the sheets of epithelium in a serum-free medium. These sheets produce migratory dividing cells that were shown to differentiate into neurons, and were therefore called 'immediate neuronal precursors'. The neurons in the cultures described by Calof and Chikaraishi have a similar life span to those we have described (~7 days). This system was subsequently used to study neurogenesis and the factors that regulate division of the precursors. FGF-2 was found to be important for neuronal precursor division (DeHamer *et al.*, 1994). The system we describe here differs because there is very little neurogenesis and it contains mostly immature ORNs. In fact, addition of FGF-2 (up to 100 ng/ml in serum-free as well as fully supplemented plus FCM-containing conditions, data not shown) to our cultures did not change neuronal numbers, supporting the idea that immediate neuronal precursors may only be transiently present in small numbers. Thus, we are able to focus on factors that influence survival and apoptosis of neurons, without also having to consider effects on survival and apoptosis of neuronal precursors. The Calof system has a large population of neuronal precursors, so it is best suited for studies of factors that influence those cells.

Mahanthappa and Schwarting (Mahanthappa and Schwarting, 1993) described a culture system that is quite similar to what we have described here. Their cultures contained keratin+, epithelioid islands that had NCAM+ neurons associated with them. In their studies, they made a correlation between EGF and increased [3H]thymidine incorporation into keratin+ cells and decreased neuronal numbers. TGF-β2 increased incorporation of [3H]thymidine into neurons, while keratin+ cell numbers decreased. They suggested that EGF acted to stimulate HBC mitosis, while TGF-β2 caused differentiation of basal cells into neurons.

The cultures described by Mahanthappa *et al.* only lived for ~4 DIV. In our cultures, neuronal numbers were relatively stable through 5 DIV, and then declined until 9 DIV (the longest assayed). The epithelioid islands were very healthy and robust until at least 9 DIV (data not shown). Consistent with Mahanthappa *et al.* we have found in our cultures that EGF had a tendency to decrease neuronal numbers. We have not attempted to treat the cells with TGF-β2 in the serum-free conditions; however, we have tested TGF-β2 (1–100 ng/ml) in the presence of supplements and FCM, and saw no increase in the number of NST+ cells. Another advantage of our cultures is that we used a lower plating density, but obtained much higher neuronal numbers per field.

We used these cultures to explore the effects on ORNs of those few growth factors present in the KGM medium on ORNs. Insulin was necessary and sufficient for ORN survival. Interestingly, removal of insulin had no detectable effects on the cells of the epithelioid islands. They did not show signs of degeneration with or without insulin. Counting Bowman's gland cells and determining the area covered by keratin+ cells demonstrated no change after removal of insulin at 3 DIV. However, insulin removal at 3 DIV resulted in a significant decrease in the number of neurons at 5 DIV. We would next like to determine how insulin is supporting the ORN. Insulin is a common medium additive that increases glucose uptake by most cells. However, insulin does not increase glucose uptake in neurons (Heidenreich *et al.*, 1989). Therefore, the support role insulin plays may be very different for neuronal cell types. By using the KBM from Cascade Biologics, which contains no other growth factors, hormones or proteins, and by not adding the recommended supplements, EGF and BPE, we have developed a defined medium that can now be used to examine further the actions of insulin. Insulin at the concentrations used may be cross-reacting with the homologous IGF-1 receptor, or working indirectly through another cell type in the culture. Further investigation into this area is necessary to determine the mechanism of survival we have observed with insulin. This culture system can now also be used to explore the effects of the very large number of potential neurotrophic factors. Thus we conclude that the culture method described herein will be useful for ORN trophic factor analysis, particularly those factors involved in prolonging survival of immature ORNs.

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