

# The Effects of Sialoadenectomy and Exogenous EGF on Taste Bud Morphology and Maintenance

J. Morris-Wiman, R. Segó, L. Brinkley and C. Dolce

Department of Orthodontics, University of Florida Dental College, Gainesville, FL, USA

Correspondence to be sent to: Joyce Morris-Wiman, Department of Orthodontics, Box 100444, JHMHC, University of Florida Dental College, Gainesville, FL 32610-0444, USA. e-mail: morris-wiman@dental.ufl.edu

## Abstract

Taste buds on the dorsal tongue surface are continually bathed in saliva rich in epidermal growth factor (EGF). In the following experiment, taste bud number and morphology were monitored following submandibular and sublingual salivary gland removal (sialoadenectomy), to determine if EGF plays a role in the maintenance and formation of taste buds. Adult male rats were divided into four groups: sialoadenectomized (SX,  $n = 4$ ); sialoadenectomized with EGF replacement (SX + EGF,  $n = 5$ ); sham-operated (SH,  $n = 4$ ); and sham-operated with exogenous EGF (SH + EGF,  $n = 5$ ). After a 3 week recovery, SX + EGF and SH + EGF animals were given 50  $\mu\text{g/day}$  EGF in their drinking water for 14 days. At day 14, saliva was collected, the animals were killed and the presence of EGF determined by radioligand-binding assay. Tongues were removed and histologically examined for the presence and morphology of taste buds on fungiform and circumvallate papillae, or immunostained for the presence of EGF, TGF $\alpha$  (transforming growth factor  $\alpha$ ) and EGFR (EGF receptor). The removal of submandibular and sublingual salivary glands resulted in the loss of fungiform taste buds and normal fungiform papillae morphology. These effects were reversed by EGF supplementation, indicating a role for EGF in fungiform taste bud maintenance. In addition, supplementation of EGF to sham-operated animals increased the size of fungiform taste buds. In contrast, removal of salivary glands had no effect on the size, numbers, or morphology of circumvallate taste buds, suggesting that the formation and maintenance of taste buds in fungiform and circumvallate papillae may involve different and distinct processes. EGF, TGF $\alpha$  and EGFR were localized to distinct layers of the dorsal epithelium and to within both fungiform and circumvallate taste buds. Their expression within the epithelium or taste buds was not altered with sialoadenectomy, indicating that the actions of endogenous EGF and TGF $\alpha$  are distinct and not regulated by exogenous EGF and TGF $\alpha$  supplied in saliva.

## Introduction

Patients with oral dryness such as those with Sjogren's Syndrome (Byyny *et al.*, 1974; Kalmus and Farnsworth, 1998) or those subjected to therapeutic doses of oral radiation (Weiffenbach *et al.*, 1986) are reported to have changes in taste acuity, suggesting that saliva may play an important role in the maintenance of normal taste function. The oral epithelium of Sjogren's syndrome patients is characterized by decreased numbers of circumvallate papillae and ultrastructural changes in taste bud morphology, indicating that salivary components may have an important function in taste bud maintenance (Kalmus and Farnsworth, 1998). Previous studies examining the effects of salivary gland removal in rats support this idea. Salivary deficit in rats was correlated with an increased keratosis of the dorsal tongue and shrunken and disorganized taste buds within circumvallate papillae (Nanda and Catalanotto, 1981). Although these studies have established a role for saliva in maintenance of normal taste bud morphology and function, it remains to be determined what factor(s) in saliva may be responsible for taste bud maintenance.

Alternatively, alterations in taste bud structure may merely be the result of the decreased lubrication of the dorsal tongue that results with desalivation.

A large amount of EGF (epidermal growth factor) is secreted into the saliva in an exocrine manner and the saliva contains a high concentration of this growth factor (Byyny *et al.*, 1974). The removal of the submandibular salivary glands has been shown to abolish salivary EGF and cause a decrease in plasma EGF levels (Noguchi *et al.*, 1991). TGF $\alpha$  (transforming growth factor  $\alpha$ ) is homologous to EGF in sequence, has identical activity to EGF and is also produced in the salivary glands, although in lesser amounts (Shultz *et al.*, 1991; Humphreys-Beher *et al.*, 1994). The effects of both EGF and TGF $\alpha$  are triggered through their binding to a membrane receptor, EGFR, which activates an intrinsic tyrosine kinase in the cytoplasmic domain of the receptor (Yarden and Ullrich, 1988). The significance of the large amount of excess EGF in the saliva is not known. It has been shown that salivary EGF effects wound closure, mediated through licking (Hutson *et al.*, 1979). It has been

suggested that the males of certain species produce more salivary EGF as an evolutionary response to increased fighting, resulting in a higher frequency of wounding. Indeed, EGF/TGF $\alpha$  can stimulate many of the essential processes in wound healing, including neovascularization, chemotaxis of wound cells and keratinocyte proliferation and maturation (Shultz *et al.*, 1991). However, another possibility is that the excess of EGF in the saliva is necessary to maintain the integrity and normal function of the oral cavity.

As taste buds on the dorsal tongue surface are continually bathed in saliva rich in EGF, it would seem likely that this growth factor plays a role in normal taste bud maintenance. This study was done to examine the effects of removal of the submandibular salivary glands on taste bud morphology and to determine if supplementation with EGF could reverse these effects. We also examined by immunofluorescent staining the distribution of EGF, TGF $\alpha$ , and their receptor EGFR within fungiform and circumvallate taste buds to see what effect, if any, sialoadenectomy might have on their expression. The results of this study suggest that salivary EGF does play a major role in the maintenance of taste buds.

## Materials and methods

### Animals

Adult Sprague–Dawley rats, 180–200 days old and 400–450 g in weight were obtained from Charles River and immediately upon arrival randomly assigned to experimental groups. They were housed in individual cages in a temperature-controlled environment (21°C) with a 12:12 h light–dark cycle and were maintained on ground Purina Rodent Chow and tap water. To monitor the effects of EGF depletion on taste papillae and taste bud morphology, the rats were divided into the following groups: sialoadenectomized (SX,  $n = 4$ ); sialoadenectomized with EGF replacement (SX + EGF,  $n = 5$ ); sham-operated (SH,  $n = 4$ ); and sham-operated with EGF addition (SH + EGF,  $n = 5$ ). These animals also served as controls in a study conducted concomitantly with this one examining EGF's role in orthodontic tooth movement (Dolce *et al.*, 1994).

### Surgical procedures

For all surgical procedures the rats were anesthetized with an intramuscular injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). To remove the salivary glands, a 15 mm incision was made below the mandible, the salivary duct was ligated, and the submandibular and sublingual salivary glands removed. The incision was closed with sutures and the animal allowed to recuperate for 3 weeks. In sham-operated rats, the salivary gland was merely exposed.

### EGF administration and analysis of EGF in saliva

Appropriate groups were given 50  $\mu$ g/day EGF (human recombinant EGF, Upstate Biotechnology, Lake Placid,

NY) in their drinking water. This concentration was chosen because it had been shown previously to produce a biological response (Noguchi *et al.*, 1991). It has been shown previously that EGF is fairly stable in drinking water, since by radioimmunoassay 93.1% of the EGF was intact in drinking water after 24 h (Noguchi *et al.*, 1991). The solution was made fresh nightly and given to the rats during the 12 h dark cycle. The rats were monitored to verify that they consumed all of the EGF-containing water within the 12 h dark period. During the 12 h light period they had *ad libitum* access to tap water.

### Saliva collection and analysis

On the day that they were killed, after 14 days of EGF replacement, the animals were anesthetized and given an injection of pilocarpine (0.25 mg/100 g body wt) to stimulate salivary flow. The saliva was collected from the oral cavity using a micropipette and was stored at  $-70^{\circ}\text{C}$  until analyzed. The presence of EGF in the saliva was determined using a radioligand-binding assay (DeLeon *et al.*, 1986). The details of this assay have been reported elsewhere (Dolce *et al.*, 1994). Briefly, in the receptor-binding assay, 10  $\mu$ l saliva was diluted to 100  $\mu$ l in phosphate-buffered saline (PBS) containing 0.2 mg/ml bovine serum albumin (BSA). Human placenta microvillar membrane (100  $\mu$ l) and 100  $\mu$ l  $^{125}\text{I}$ -labelled Human EGF were added and incubated at  $4^{\circ}\text{C}$  for 24 h. The mixture was then diluted with 3.5 ml ice-cold PBS containing BSA solution, centrifuged for 20 min at 7000 g in an RC-3B Sorvall centrifuge and the radiolabel associated with the membrane pellet was determined using a Beckman gamma counter. The concentration of EGF was determined by a standard curve generated by a dilution of a series of known quantities of EGF.

### Histological methods

After 14 days of EGF replacement, rats were killed by decapitation and their tongues removed. The tongues were cut transversely into anterior (fungiform) and posterior (circumvallate) portions; these were then halved longitudinally. One half of the tongue was fixed in acid alcohol at  $4^{\circ}\text{C}$  and embedded in paraffin for immunofluorescent staining. Half tongues from two sialoadenectomized animals, two shams and two controls were sectioned in a frontal plane at 7  $\mu$ m and mounted on four sets of slides. Alternative sections were blocked with normal goat serum for 20 min at room temperature and then incubated in primary antibody for 3 h at  $37^{\circ}\text{C}$ . Primary antibodies used were: rabbit anti-rat EGF IgG; rabbit anti-rat TGF $\alpha$  IgG; or sheep anti-rat EGFR IgG (Simms *et al.*, 1991), a kind gift of N. Chegini, Department of Obstetrics and Gynecology, University of Florida. After rinsing in PBS, sections were incubated with appropriate secondary antibodies (goat anti-rat IgG or goat anti-sheep IgG, Biosource, International; diluted 1:100 in 10% normal goat serum in PBS) conjugated to fluorescein for 1 h at  $37^{\circ}\text{C}$ . Negative controls were pre-

formed by substituting the primary antibodies with normal serum. Sections were mounted in glycerol containing an anti-bleach reagent and examined under epifluorescence with a Nikon FXA Photomicroscope.

The other tongue half was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4°C and embedded in glycol methacrylate (JB4 Plus, PolySciences). Tongues from age-matched male rats were also fixed and embedded in glycol methacrylate to act as unoperated controls for fungiform taste bud counts. Two micrometer, parasagittal sections were collected at 10 µm intervals and mounted on subbed slides. Sections were examined for the presence of taste papillae and taste buds. Fungiform papillae were identified in sections from SX, SH, SX + EGF, and SH + EGF animals based on size and shape. As each fungiform papilla spanned several sections, all sections with papillae were first circled on the slides. Then each papilla was coded on consecutive sections as to its location to ensure it was counted only once. Taste buds were identified in parasagittal sections from SX, SH, SX + EGF, SH + EGF, and unoperated control animals as 'ball-like' collections of lightly staining cells within the apical epithelium of fungiform or circumvallate papillae. From sections of each tongue, ten fungiform taste buds and ten circumvallate taste buds sectioned in a longitudinal plane were selected at random. Since the average diameter of taste buds is ~30 µm, profiles of individual taste buds spanned three or four sections. The profile of each selected taste bud at its widest diameter was digitized using an Optronics 470E camera. Each digitized taste bud was given a number code and then traced manually. The extent of the taste bud was delimited by, but did not include, dark edge cells. The individual doing both the tracing and measurements was blinded as to which experimental group the tongue belonged to, in order to eliminate bias. The area of each taste bud was then calculated from the number of pixels contained within the tracings using an image analysis program, Image Pro Plus (Media Cybernetics). Statistically significant differences among SX, SH, SX + EGF, and SH + EGF taste bud areas and fungiform papillae number were assessed using an ANOVA for repeated measures and, when significant, multiple *post hoc* comparisons to determine which groups were different.

## Results

### Effect of sialoadenectomy on salivary EGF levels

The absence of salivary EGF in sialoadenectomized animals was confirmed by radioligand-binding assay (Table 1). Sialoadenectomy (SX) decreased salivary EGF to barely detectable levels ( $P \leq 0.05$ ). Oral administration of EGF (SX + EGF) partially restored salivary EGF levels, but did not reach sham levels ( $P \leq 0.05$ ). Supplemental EGF to sham operated animals (SH + EGF) increased salivary

**Table 1** EGF concentrations (mean  $\pm$  SD) in saliva collected from sialoadenectomized (SX), sialoadenectomized with EGF supplementation (SX + EGF), sham-operated (SH) and sham-operated with EGF supplementation (SH + EGF) rats

Group	Mean $\pm$ SD (ng/ml)
SX ( $n = 2$ )	2.55 $\pm$ 0.64 <sup>a</sup>
SX + EGF ( $n = 3$ )	880 $\pm$ 69.3 <sup>b</sup>
SH ( $n = 2$ )	1800 $\pm$ 848.5
SH + EGF ( $n = 6$ )	3000 $\pm$ 1587.5

EGF was barely detectable in saliva from SX rats (<sup>a</sup> $P = 0.05$ , ANOVA, repeated measures). Oral administration of EGF restored EGF salivary levels, but did not reach sham levels (SX + EGF; <sup>b</sup> $P = 0.05$ ).

**Table 2** Number of fungiform papillae and taste buds (mean  $\pm$  SD) in SX, SX + EGF, SH and SH + EGF animals

Group	No. (mean $\pm$ SD) of fungiform papillae	No. (mean $\pm$ SD) of fungiform taste buds
SX ( $n = 2$ )	49.5 $\pm$ 13.44 <sup>a</sup>	13.5 $\pm$ 0.7 <sup>a</sup>
SX + EGF ( $n = 5$ )	92.5 $\pm$ 16.4	81.8 $\pm$ 38
SH ( $n = 2$ )	101.5 $\pm$ 10.61	97.6 $\pm$ 3.5
SH + EGF ( $n = 10$ )	105.7 $\pm$ 15.6	99.3 $\pm$ 12.8

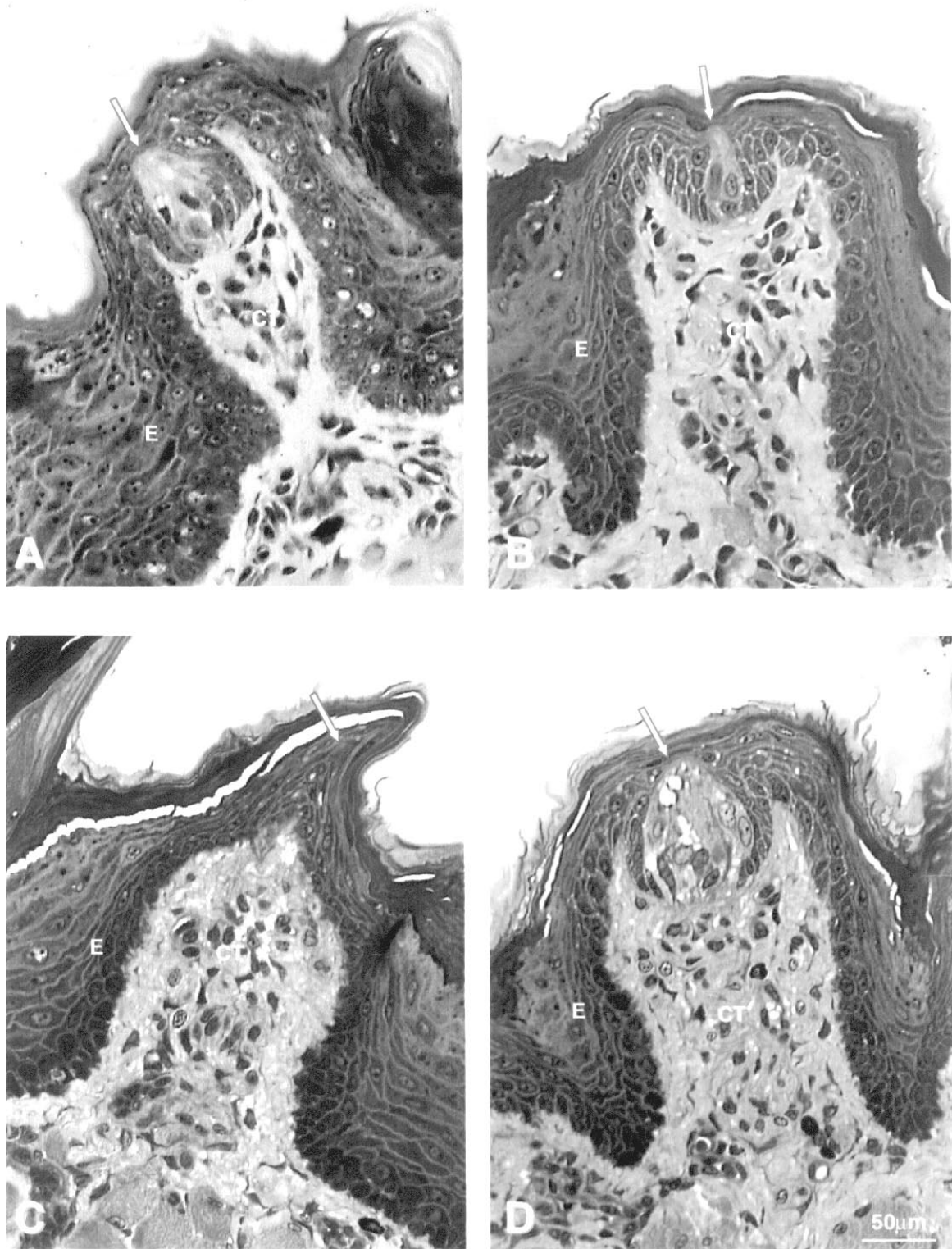
Fungiform papillae and taste bud numbers in SX animals were significantly smaller than in all other groups (<sup>a</sup> $P = 0.05$ , ANOVA, repeated measures).

levels above those measured in controls, but the increase was not statistically significant.

### Effects of sialoadenectomy on taste bud morphology and size

Few fungiform taste buds were identified in SX animals (Table 2). Those observed were smaller than controls, comprised of only a few cells (compare Figure 1B with control in Figure 1A). Many appeared atrophic. Morphometric measurements confirmed this observation. Fungiform taste bud area in SX animals was significantly decreased from that measured in controls or sham-operated animals (Table 3). The anterior tongue of SX animals also had fewer fungiform papillae (Table 2). These tongues did possess several papillae that were distinguished from adjacent filiform papillae by their enlarged bases and their increased apical protrusion; these papillae were presumed to be fungiform papillae (Figure 1C). They lacked taste buds, but did contain keratinized spines similar to those normally observed on filiform papillae.

Fungiform taste buds in the tongues of SX animals given supplemental EGF (SX + EGF) were morphologically identical to those in sham and in controls (data not shown)



**Figure 1** Toluidine-blue-stained sections of fungiform papillae from unoperated control (A) and SX (B, C) and SH + EGF (D) rats. Few fungiform taste buds were identified in the SX rats; those present were often atrophic (arrow, B). Several fungiform papillae in SX animals had apical spines characteristic of filiform papillae (arrow, C). Taste buds identified in SH + EGF animals (arrow, D) appeared to be larger than in controls (arrow, A). E, epithelium; CT, connective tissue.

**Table 3** Taste bud area (mean  $\pm$  SD)

Taste bud area	Group	Mean $\pm$ SD ( $\mu\text{m}^2$ )
Fungiform	control ( $n = 30$ )	$486 \pm 100$
	SX ( $n = 20$ )	$339 \pm 81^a$
	SX + EGF ( $n = 50$ )	$426 \pm 191$
	SH ( $n = 20$ )	$429 \pm 149$
	SH + EGF ( $n = 100$ )	$538 \pm 143^b$
Circumvallate	SX ( $n = 20$ )	$618 \pm 208$
	SX + EGF ( $n = 50$ )	$755 \pm 289$
	SH ( $n = 20$ )	$757 \pm 309$
	SH + EGF ( $n = 100$ )	$773 \pm 267$

The fungiform taste bud area in SX animals was significantly smaller than in all other groups ( $^aP = 0.001$ , ANOVA, repeated measures) whereas that in SH + EGF animals was significantly greater than in all other groups ( $^bP = 0.001$ ). No significant differences were found between groups for the circumvallate taste bud area (ANOVA, repeated measures).

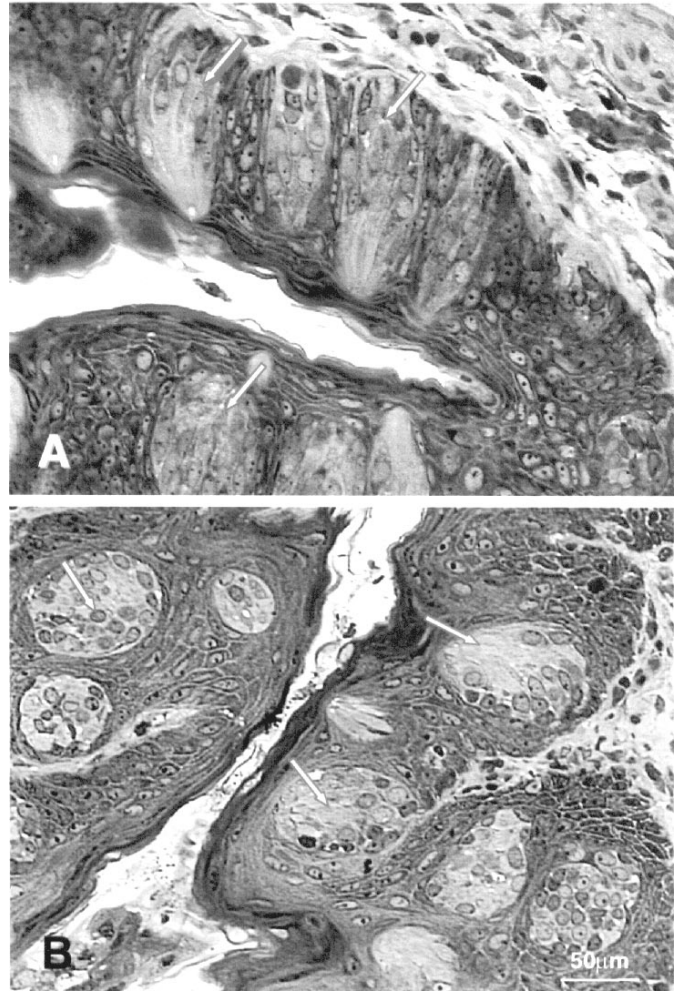
and fungiform numbers were restored (Table 2). Oral administration of EGF to sialoadenectomized animals also restored normal taste bud area to control and sham operated values (Table 3). Supplemental EGF to sham-operated animals (SH + EGF) resulted in enlarged fungiform taste buds (Figure 1D) with a significant increase in area over unoperated controls and shams (Table 3). The striking differences observed in fungiform taste bud morphology, size and numbers between sham, EGF-deficient (SX) and EGF-supplemented (SH + EGF) animals were not observed for circumvallate taste buds. The area of circumvallate taste buds showed no statistically significant differences between SX, SX + EGF, SH and SH + EGF (Table 3). Circumvallate taste buds in SX tongues showed no morphological peculiarities (compare Figure 2B to Figure 2A). There was, however, a noticeable increase in keratinization of the lingual epithelium and an increased infiltration of inflammatory cells within the mucosa of SX tongues.

#### Immunolocalization of EGF, TGF $\alpha$ and EGFR in taste buds of sialoadenectomized and sham-operated animals

Using standard immunofluorescent staining methods, the distributions of EGF, EGFR and TGF $\alpha$  were examined in fungiform and circumvallate taste buds from SX, SH and control animals. No discernible differences in the distribution or the intensity of staining for EGF, EGFR or TGF $\alpha$  were observed between the dorsal tongue epithelium and papillae of SX, SH or control animals. In addition no differences were seen between the distribution of these factors in taste buds identified in SX, SH or controls.

#### EGF

EGF was diffusely distributed throughout all layers of the stratified epithelium of the dorsal tongue in SX, SH and

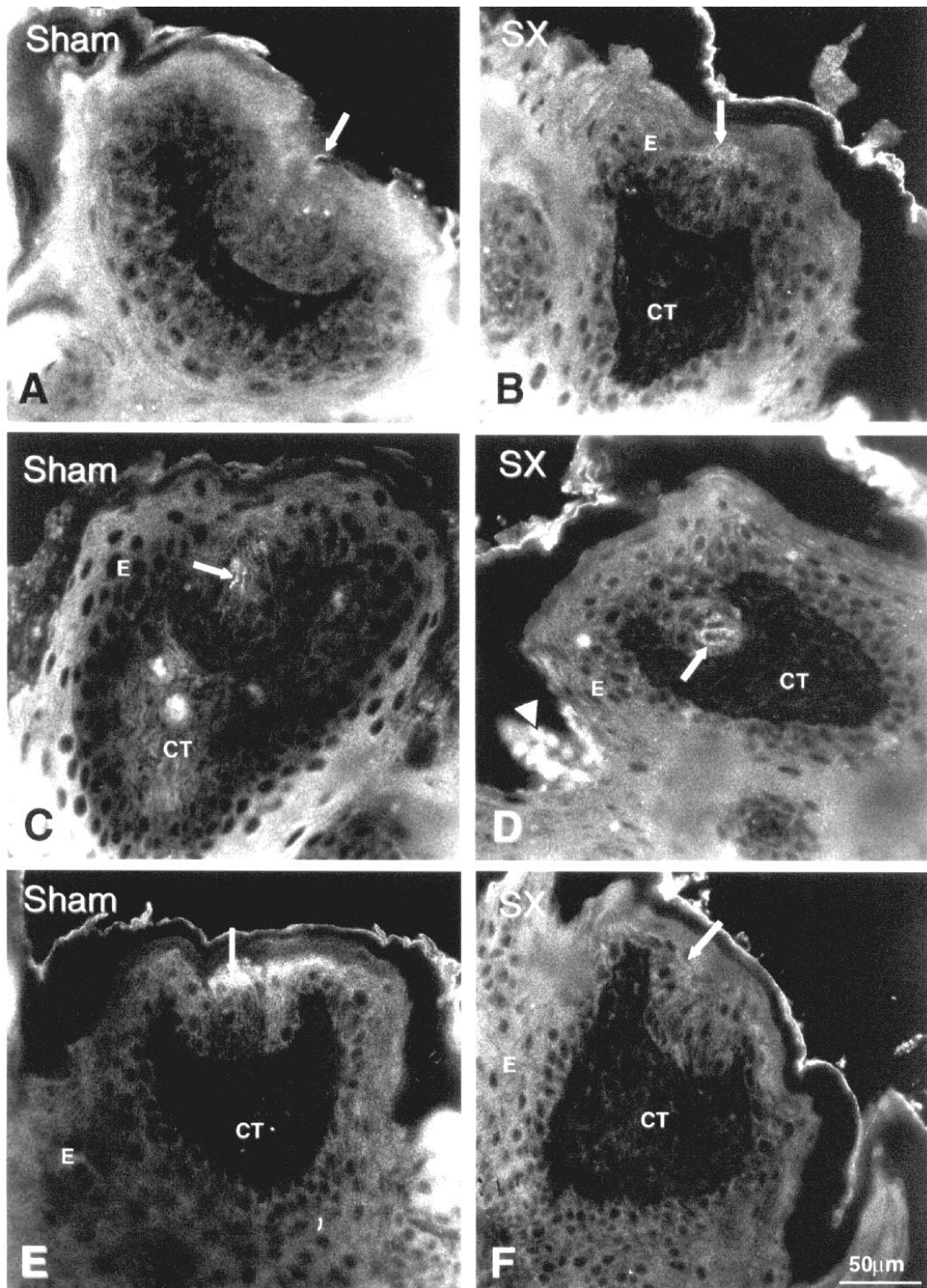


**Figure 2** Toluidine-blue-stained sections of circumvallate papillae from SH (A) and SX (B) rats. The numbers and morphology of circumvallate taste buds (arrows) were not affected by sialoadenectomy.

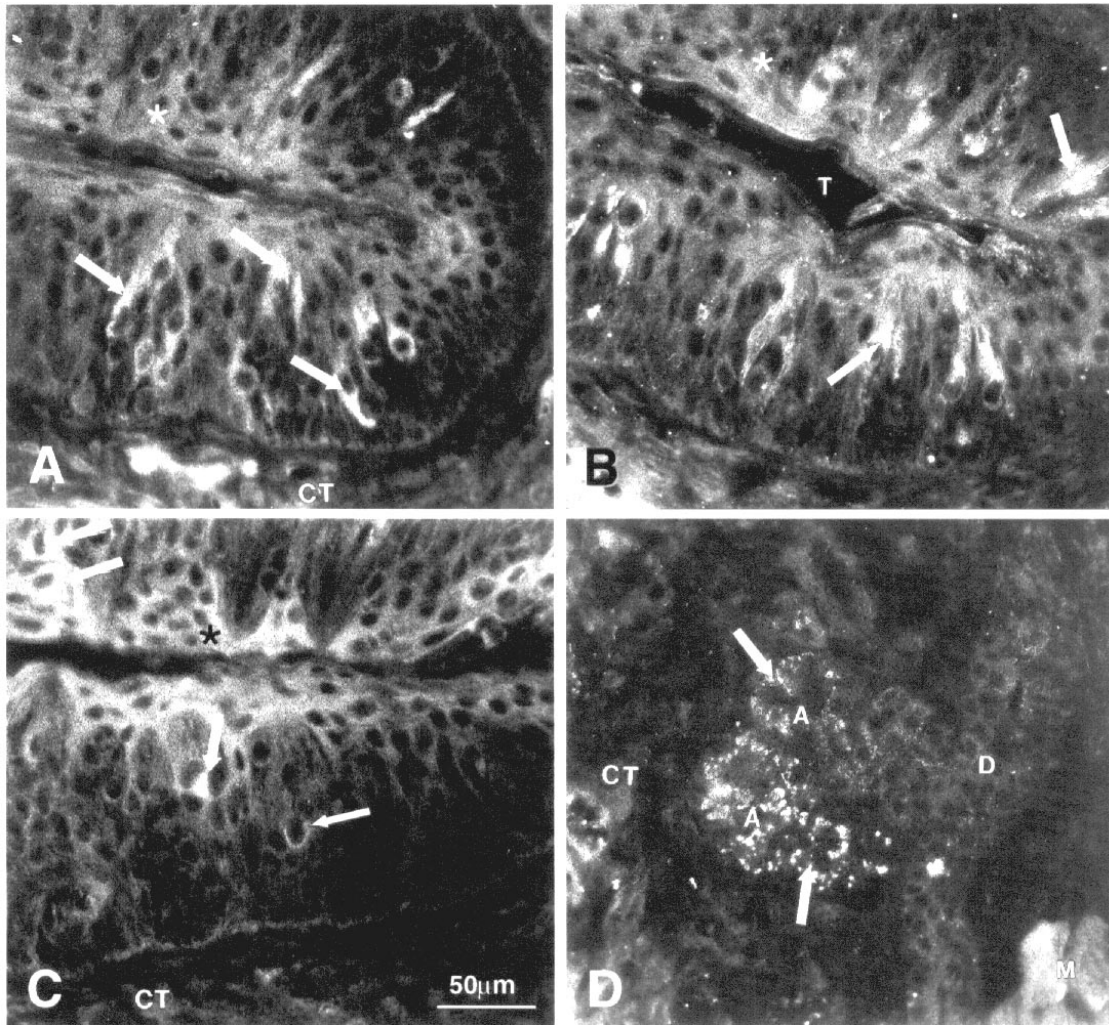
control animals, although immunostaining for EGF was slightly more intense in the suprabasal layers in the lingual epithelium (data not shown) and was enhanced in the superficial layers of the epithelium lining the circumvallate trench (Figure 4A). In general, immunostaining for EGF appeared less intense than staining for TGF $\alpha$  (Figures 3C, D and 4B) or for their receptor (Figures 3E,F and 4C). Within fungiform taste buds, EGF immunoreactivity outlined apical cell membranes forming the taste pore (Figure 3A,B). In contrast, in circumvallate taste buds, a subpopulation of taste buds cells was intensely immunopositive for EGF, with this factor distributed throughout their apical, perinuclear and basal regions (Figure 4A). The intensity of immunostaining for EGF was significantly greater in these immunopositive circumvallate taste bud cells than was observed in fungiform taste bud cells.

#### TGF $\alpha$

As was observed for EGF, although TGF $\alpha$  was diffusely



**Figure 3** Immunolocalization of EGF (A, B), TGF $\alpha$  (C, D) and EGFR (E, F) in SH and SX fungiform papillae. In fungiform taste buds of both SX and SH tongues, EGF was localized to the apical surfaces of taste buds forming the taste pore (arrow, A and B). TGF $\alpha$  expression was also associated with apical cell surfaces forming the taste pore (arrow, C) as well as perinuclear cell regions (arrow, D). TGF $\alpha$  was also localized to granules within the apical spines of filiform papillae (arrowhead, D). EGFR expression is enhanced on the apical surfaces and adjacent papillary epithelium (arrow, E and F). E, epithelium; CT, connective tissue.



**Figure 4** Immunolocalization of EGF (**A**), TGF $\alpha$  (**B**) and EGFR (**C**) within an SX circumvallate papilla. A subpopulation of taste bud cells was demonstrated by immunofluorescent staining to express EGF (arrows, **A**). This expression of EGF within these cells was notably greater than that observed in fungiform taste bud cells. TGF $\alpha$  was localized to punctuate granules contained within the apical and perinuclear regions of a subpopulation of circumvallate taste bud cells (arrows, **B**). A few circumvallate taste bud cells also expressed EGFR (arrow, **C**). EGF, TGF $\alpha$  and EGFR expression was also enhanced within the superficial cell layer of the papillary epithelium (asterisks). (**D**) TGF $\alpha$  immunolocalization within von Ebner's glands of an SX rat. Punctuate granules (arrows) within acinar cells (**A**) of von Ebner's glands were immunopositive for TGF $\alpha$ . TGF $\alpha$  was also expressed in ductal cells (**D**). CT, connective tissue; M, muscle.

distributed throughout all layers of the stratified epithelium of the dorsal tongue in SX, SH and control animals, its expression was increased within the suprabasal layers of the lingual epithelium (data not shown) and was greatest within the superficial layers of the circumvallate trench epithelium (Figure 4B). Intensity of immunostaining for TGF $\alpha$  within the dorsal tongue epithelium, as well as within both fungiform and circumvallate taste buds, was significantly greater than that observed for EGF. In addition, TGF $\alpha$  showed a unique association with granules contained within the spines of filiform papillae (arrowhead, Figure 3D). In contrast to EGF distribution, immunostaining for TGF $\alpha$  was not restricted to apical cell regions forming the taste pore in fungiform taste buds, but was distributed through-

out the full extent of fungiform taste bud cells (Figure 3C, D). In circumvallate taste bud cells, TGF $\alpha$  was discretely expressed in what appeared to be almost punctuate granules, whose distribution extended from the perinuclear cell regions to the apical pore within immunopositive taste bud cells (Figure 4B). TGF $\alpha$  granules were not observed within the basal regions of circumvallate taste bud cells. TGF $\alpha$  was also present in punctuate secretory granules within acinar cells and outlining duct cells of the von Ebner's glands located under the circumvallate papilla (Figure 4D).

#### EGFR

EGFR was observed to be diffusely distributed throughout all layers of the dorsal tongue epithelium, although an increased expression was discernible within the basal cell

layer (data not shown). Within fungiform taste buds, immunostaining for EGFR extended throughout fungiform taste bud cells. However, increased expression of EGFR was associated with apical cell regions surrounding the taste pore and the adjacent papillary epithelium. In circumvallate papillae, immunostaining for EGFR was increased within superficial layers of the papillary epithelium adjacent to the pore regions of taste buds (Figure 4C). In circumvallate taste buds, EGFR was localized to the perinuclear region of a small population of taste bud cells.

## Discussion

The removal of submandibular salivary glands resulted in the loss of fungiform taste buds and normal fungiform papillae morphology. These effects were reversed by EGF supplementation, indicating a role for EGF in fungiform taste bud maintenance. In addition, supplementation of EGF to sham-operated animals increased the size of fungiform taste buds. In contrast, removal of salivary glands had no effect on the size, numbers, or morphology of circumvallate taste buds, suggesting that the formation and maintenance of taste buds in fungiform and circumvallate papillae may involve different and distinct processes. EGF, TGF $\alpha$  and EGFR were localized by immunofluorescent staining to distinct layers of the dorsal epithelium and to within both fungiform and circumvallate taste buds. Their expression within the epithelium or taste buds was not altered with sialoadenectomy, indicating that the actions of endogenous EGF and TGF $\alpha$  are distinct and not regulated by exogenous EGF and TGF $\alpha$  supplied in saliva.

Previous studies have shown that desalivated rats show significantly increased preferences for normally avoided solutions, indicating a generalized decrease in taste sensitivity related to the absence of some component of the saliva (Catalanotto and Sweeny, 1972; Catalanotto and Leffingwell, 1979; Nanda and Catalanotto, 1981; Brosvic and Hoey, 1990). Histological examination of dorsal tongue epithelium from rats desalivated for 95–110 days showed morphological changes, including an increased keratosis and qualitative changes in circumvallate taste buds with some appearing shrunken and disorganized (Nanda and Catalanotto, 1981). Cano *et al.* (Cano *et al.*, 1978) studied the effects of desalivation on taste bud maturation, as well as on continued taste bud maintenance, and observed that sialoadenectomy affected the formation of specific taste bud cell types. These observations have led investigators to postulate that factors within the saliva are responsible for the maintenance of normal taste function and morphology. However, what these factors might be was not determined.

In this study, sialoadenectomy was shown by radioligand-binding assay to reduce EGF levels in saliva to barely detectable levels. With the addition of EGF to drinking water, EGF was again detected in the saliva and the effects of desalivation on taste bud morphology reversed. The reversal

of the effects of desalivation by EGF supplementation demonstrates that alterations in taste bud and papillae structure were not the result of oral desiccation or the lack of some other salivary factor, such as NGF, but were mediated by EGF depletion. It has been previously shown that the oral administration of EGF does not elevate EGF levels in the peripheral and portal plasma (Noguchi *et al.*, 1991). Thus orally administered EGF is not absorbed through the intestine and the effects of its addition are attained by its absorption in the oral cavity. Therefore, the effects of sialoadenectomy on taste bud morphology in this study were the result of an EGF deficit. The effects of this deficit were mediated locally and were not the result of some systemically mediated change.

Although the results of this study indicate that EGF in saliva is responsible for the maintenance of normal taste bud morphology, it provides no clues as to how these effects might be mediated. EGF has been shown to affect a wide variety of cell types, but it was named for its first known action—epithelial growth and maturation. EGF was initially discovered by the ability of submandibular gland extracts to induce precocious eyelid opening and incisor tooth eruption when injected into newborn mice (Cohen, 1962). Subsequently, EGF and TGF $\alpha$  were shown to be mitogenic for epithelium, as well as for many other cell types (King *et al.*, 1990; Carpenter and Wahl, 1991; Dominey *et al.*, 1993). However, EGF/TGF $\alpha$  also mediate epithelial differentiation and maturation (Sakai *et al.*, 1994). It has been suggested that the main actions of EGF and TGF $\alpha$  on stratified epithelium are an increased stimulation of epithelial proliferation and keratinization (King *et al.*, 1990). However, these processes are often in conflict with each other and have made it hard to resolve these two activities of EGF/TGF $\alpha$  in epithelial morphogenesis and maintenance.

EGF and TGF $\alpha$  may effect increased epithelial proliferation through binding to their receptor EGFR. EGFR has been shown to be linked to the mitogenic pathway and the expression of EGFR has been shown to be enhanced in proliferating cells (Nanney *et al.*, 1984; Coffey *et al.*, 1987; Dominey *et al.*, 1993). In adults, tissues that are continuously undergoing proliferation and replacement, such as skin and oral and intestinal mucosa, are thought to depend on the interactions of EGF/TGF $\alpha$  with EGFR to provide the mitogenic signal (Yarden and Ullrich, 1988; Dominey *et al.*, 1993). In wound healing and regeneration, cells expressing EGFR are activated locally at wounds by platelets and macrophages shown to release EGF/TGF $\alpha$  (Shultz *et al.*, 1991). Responsiveness to the mitogenic effects of EGF is thereby regulated by availability of EGFR.

In stratified epithelium, EGFR immunoreactivity is identified within the proliferative cells of the basal epithelium (Nanney *et al.*, 1984; Coffey *et al.*, 1987). In contrast, studies have shown that local synthesis of EGF and TGF $\alpha$  occurs primarily in nondividing and maturing suprabasal cell layers and their expression occurs late in the program of epithelial



differentiation (Sakai *et al.*, 1994). These results indicate that in tissues such as the dorsal tongue epithelium, locally produced EGF/TGF $\alpha$  growth factors may function in more terminal aspects of epithelial differentiation. In suprabasal cell layers of the tongue, EGF is synthesized in a precursor form that is translocated to the cell membrane (Sakai *et al.*, 1994). This precursor has been shown not to provide a mitogenic stimulus within this cell layer, but may contribute to the differentiated phenotype by sequestering and regulating Ca<sup>2+</sup> influx (Handford *et al.*, 1991). An increase in Ca<sup>2+</sup> influx in keratinocytes *in vitro* has been linked to multiple events associated with terminal differentiation, including the activation of transglutaminase-catalyzed crosslinking of envelope proteins and the aggregation of keratin filaments (Hennings *et al.*, 1981).

The decrease in fungiform taste buds observed in sialoadenectomized rats could be the result of an EGF deficit affecting either the proliferation of taste bud cell precursors or stages in taste bud cell terminal differentiation, or both. Taste bud cells originate locally from the tongue epithelium (Stone *et al.*, 1995). In this study, as well as in previous studies, EGFR was localized to within the proliferative basal layer of the dorsal tongue epithelium. This observation indicates that in dorsal tongue epithelium EGF plays a role in modulating cell proliferation, as has been described for most other stratified epithelia (Nanney *et al.*, 1984; Coffey *et al.*, 1987). Thus, one consequence of a deficit in EGF would be decreased cell proliferation within the tongue epithelium. This could result in the formation of fewer taste bud precursor cells and thereby fewer taste buds. Taste buds in sham-operated rats given EGF supplementation appeared to be enlarged and when measured had a greater area than controls. This increase in taste bud size appeared to be the result of increased taste bud cell number and not cell size, although this was a qualitative observation. An increase in taste bud size could be the result of an EGF-mediated increased proliferation and recruitment of taste bud precursor cells. Alternatively, larger taste buds could be the result of a decreased cell turnover within the taste bud.

Salivary EGF may also exert its effects through modulating the maturation and maintenance of taste bud cells. In this study EGF, TGF $\alpha$  and EGFR were all localized by immunofluorescent staining to within the taste bud. As these cells are non-proliferative, the presence of EGF/TGF $\alpha$  suggests a role other than mitogenic and an exogenous source of EGF from the salivary glands could facilitate this role. Interestingly, EGFR, EGF and TGF $\alpha$  were co-localized within the taste bud on apical surfaces of taste buds. EGFR expression was also pronounced within the adjacent epithelium. The presence of its receptor indicates that salivary EGF could bind and mediate its effects at these locations. The distribution of EGF, TGF $\alpha$  and EGFR within the taste bud or within the dorsal tongue epithelium was not altered by sialoadenectomy, indicating that local

expression of these factors is not regulated by the availability of salivary EGF. The results of a study examining the effects of supplemental EGF on wound healing in the tongue also support the conclusion that endogenous EGF levels are not affected by sialoadenectomy (Noguchi *et al.*, 1991).

In the tongues of sialoadenectomized rats, fungiform papillae often had spines reminiscent of the apical spines of filiform papillae. EGF supplementation restored papillae to their normal morphology. The maintenance of normal morphology in fungiform papillae has been shown to be also dependent on an intact innervation. If sensory innervation to these papillae is interrupted in the adult rat, taste buds are lost and papillae acquire apical spines (Oakley *et al.*, 1990; Oakley, 1993). Oakley and colleagues have postulated that one role innervation may play in the development of taste papillae and their maintenance may be to specify keratin type or to prevent the formation of inappropriate types that may disrupt or prevent taste bud formation (Oakley *et al.*, 1990). Atypical forms of fungiform papillae were also observed in mice lacking the neurotrophin BDNF, and BDNF knockout mice also had a decreased innervation of taste buds and papillae and decreased numbers of taste buds (Nosrat *et al.*, 1997; Zhang *et al.*, 1997; Oakley *et al.*, 1998; Cooper and Oakley, 1998; Ringstedt *et al.*, 1999; Mistretta *et al.*, 1999). The results of BDNF knockout studies suggest that when taste buds do not produce the proper taste neurotrophic factor—BDNF—innervation to taste buds and papillae is not established or is lost. The trophic function of the nerve disappears and taste buds and papillae are not maintained. Thus, the presence of spines on fungiform papillae in sialoadenectomized rats might be a consequence of the loss of taste buds on these papillae. Such a loss could result from a decrease in taste cell precursor recruitment mediated by the EGF deficit. An alternative explanation for the presence of apical spines would entail a role for EGF in controlling the differentiation of the apical epithelium of fungiform papillae. A deficit in EGF could effect the normal maturation of the apical papillary epithelium and cause it to default to a filiform phenotype. In particular, the perturbation of EGF levels could affect the normal programming of keratinization, resulting in the formation of the highly keratinized apical spines. Taste bud loss would then be the sequel to spine formation.

A third possible explanation for the presence of apical spines and the loss of fungiform taste buds in the tongues of sialoadenectomized animals could be that the chorda tympani nerve was damaged during sialoadenectomy. Large intact profiles of the lingual nerve could be identified in sections of SX and SX + EGF tongues; however, it was not possible to provide an independent measure of innervation of papillae in this study. As fungiform papillae numbers and taste bud numbers and area were not affected in sialoadenectomized rats that received supplementation with EGF, this explanation would require that exogenous EGF

either promoted the reinnervation and subsequent regeneration of taste buds or maintained fungiform papillae and taste bud morphology in the absence of innervation in SX + EGF animals. EGF, TGF $\alpha$  and EGFR have been localized in the peripheral nervous system (Vega *et al.*, 1994) and evidence does exist for a role for EGF in peripheral nerve repair (Toma *et al.*, 1992). Thus the possibility that the observed morphological changes were due to chorda tympani damage during sialoadenectomy and that restoration of normal taste papillae and taste bud morphology by EGF supplementation was through the promotion of reinnervation of papillae cannot be ruled out. A closer evaluation of both innervation of papillae after sialoadenectomy and the effects of EGF deficit on innervation and regeneration is needed.

Sialoadenectomy had no effect on the number or morphology of circumvallate taste buds. This lack of effect may be due to the increased local presence of EGF and TGF in circumvallate taste bud cells, as shown by immunofluorescent staining. In circumvallate taste buds, EGF and TGF $\alpha$ , as well as EGFR, were expressed by a subpopulation of cells. Immunostaining for these factors within circumvallate taste bud cells was greatly increased over that observed in fungiform taste buds. This enhanced expression within circumvallate taste buds may prevent the effects caused by fluctuations in salivary EGF on taste bud numbers and morphology observed in fungiform taste buds. TGF $\alpha$  is also localized to acinar cells within von Ebner's glands subjacent to the circumvallate papilla. The ready availability of TGF $\alpha$  produced by von Ebner's glands to circumvallate taste bud cells may also offset the effects of salivary gland ablation. Our results are in contrast to those reported by Nadana and Catalinotto who reported that sialoadenectomy resulted in an increased keratosis of the dorsal tongue epithelium and shrunken and disorganized circumvallate taste buds. However, these investigators examined the long-term effects of surgical desalivation, examining rats 95–110 days post-operative. Many of the changes observed in these long-term studies may have been the result of the continuous lack of salivary factors, including EGF, necessary for epithelial maintenance, and the accompanying desiccation of the lingual epithelium.

## Acknowledgements

This study was supported in part by a USPHS grant DC01657 from the National Institute of Deafness and Communication Disorders

## References

- Brosvic, G.M.** and **Hoey, N.E.** (1990) *Taste detection and discrimination performance of rats following selective desalivation.* *Physiol. Behav.*, **48**, 617–623.
- Byyny, R.L., Orth, D.N., Cohen, S.** and **Doyno, E.S.** (1974) *Epidermal growth factor: effects of androgens and adrenergic agents.* *Endocrinology*, **95**, 776–782.
- Cano, J., Roza, C.** and **Rodriguez-Echandia, E.L.** (1978) *Effects of selective removal of the salivary glands on taste bud cells in the vallate papilla of the rat.* *Experientia*, **34**, 1290.
- Carpenter, G.** and **Wahl, M.I.** (1991) *The epidermal growth factor family.* In Sporn, M.B. and Roberts, A.B. (eds), *Peptide Growth Factors and their Receptors.* Springer, New York, pp. 69–171.
- Catalanotto, F.** and **Leffingwell, C.** (1979) *Early effects of desalivation upon fluid consumption and taste acuity in the rat.* *Behav. Neural Biol.*, **25**, 190–205.
- Catalanotto, F.** and **Sweeny, E.A.** (1972) *The effects of surgical desalivation of the rat upon taste acuity.* *Arch. Oral Biol.*, **17**, 1455–1465.
- Coffey, R.J., Derynck, R., Wilcox, J.N., Bringham, T.S., Goustin, A.S., Moses, H.L.** and **Pittelkow, M.R.** (1987) *Production and auto-induction of transforming growth factor alpha in human keratinocytes.* *Nature Lond.*, **328**, 817–820.
- Cohen, S.** (1962) *Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal.* *J. Biol. Chem.*, **237**, 1555–1562.
- Cooper, D.** and **Oakley, B.** (1998) *Functional redundancy and gustatory development in BDNF null mutant mice.* *Dev. Brain Res.*, **105**, 79–84.
- DeLeon, F.D., Vijayakumar, R., Brown, M., Rai, V., Yussman, M.A.** and **Schultz, G.** (1986) *Peritoneal fluid volume, estrogen, progesterone and epidermal growth factor concentrations in patients with and without endometriosis.* *Obstet. Gynecol.*, **68**, 189–194.
- Dolce, C., Anguita, J., Brinkley, L., Karnam, P., Humphreys-Beher, M., Nakagawa, S.** and **King, G.** (1994) *Effects of sialoadenectomy and exogenous EGF on molar drift and orthodontic tooth movement in rats.* *Am. J. Physiol.*, **266**, E731–E738.
- Dominey, A.M., Wang, X.-J., King, L.E., Nanney, L.B., Gagne, T.A., Sellheyer, K., Bundman, D.S., Longley, M.A., Rothnagel, J.A., Greenhalgh, D.A.** and **Roop, D.R.** (1993) *Targeted overexpression of transforming growth factor in the epidermis of transgenic mice elicits hyperplasia, hyperkeratosis, and spontaneous squamous papillomas.* *Cell Growth Dev.*, **4**, 1071–1082.
- Handford, P.A., Mayhew, M., Baron, M., Winship, P.R., Campbell, I.D.** and **Brownlee, G.G.** (1991) *Key residues involved in calcium-binding motifs in EGF-like domains.* *Nature Lond.*, **351**, 164–167.
- Hennings, H., Steinert, P.** and **Buxman, M.M.** (1981) *Calcium induction of transglutaminase and the formation of (gamma-glutamyl)-lysine crosslinks in cultured epidermal cells.* *Biochem. Biophys. Res. Commun.*, **102**, 739–745.
- Humphreys-Beher, M., Macauley, S.P., Chegini, N., van Setten, G., Purushotham, K., Stewart, C., Wheeler, T.T.** and **Schultz, G.** (1994) *Characterization of the synthesis and secretion of transforming growth factor-alpha from salivary glands and saliva.* *Endocrinology*, **134**, 963–970.
- Hutson, J.M., Niall, M., Evans, D.** and **Fowler, R.** (1979) *Effect of salivary glands on wound contraction.* *Nature Lond.*, **278**, 793–795.
- Kalmus, H.** and **Farnsworth, P.** (1998) *Impairment and recovery of taste following irradiation of the oropharynx.* *J. Laryngol. Otolaryngol.*, **73**, 180–182.
- King, L.E., Gates, R.E., Stoscheck, C.M.** and **Nanney, L.B.** (1990) *The EGF/TGF $\alpha$  receptor in skin.* *J. Invest. Dermatol.*, **4**, 164S–170S.
- Mistretta, C.M., Goosens, K., Farinas, I.** and **Reichardt, L.F.** (1999) *Alterations in size, number, and morphology of gustatory papillae and taste buds in BDNF null mutant mice demonstrate neural dependence of developing taste organs.* *J. Comp. Neurol.*, **409**, 13–24.

- Nanda, R.** and **Catalanotto, F.** (1981) *Long-term effects of surgical desalivation upon taste acuity, fluid intake, and taste buds in the rat.* J. Dent. Res., 60, 69–76.
- Nanney, L.B., Magid, M., Stoscheck, C.M.** and **King, L.E.** (1984) *Comparison of epidermal growth factor binding and receptor distribution in normal human epidermis and epidermal appendages.* J. Invest. Dermatol., 83, 385–393.
- Noguchi, S., Ohba, Y.** and **Oka, T.** (1991) *Effect of salivary epidermal growth factor on wound healing of tongue in mice.* Am. J. Physiol., 260, E620–E625.
- Nosrat, C.A., Blomlof, J., ElShamy, W.M., Ernfors, P.** and **Olson, L.** (1997) *Lingual defects in BDNF and NT3 mutant mice leading to gustatory and somatosensory disturbances, respectively.* Development, 124, 1333–1342.
- Oakley, B.** (1993) *The gustatory competence of the lingual epithelium requires neonatal innervation.* Dev. Brain Res., 72, 259–264.
- Oakley, B., Wu, L.H., Lawton, A.** and **deSibour, C.** (1990) *Neural control of ectopic filiform spines in adult tongue.* Neurosci., 36, 831–838.
- Oakley, B., Brandemihl, A., Cooper, D., Lau, D., Lawton, A.** and **Zhang, C.** (1998) *The morphogenesis of mouse vallate gustatory epithelium and taste buds requires BDNF-dependent taste neurons.* Dev. Brain Res., 105, 85–96.
- Ringstedt, T., Ibanez, C.F.** and **Nosrat, C.A.** (1999) *Role of brain-derived neurotrophic factor in target invasion in the gustatory system.* J. Neurosci., 19, 3507–3518.
- Sakai, Y., Nelson, K.G., Snedeker, S., Bossert, N.L., Walker, M.P., McLachlan, J.** and **DiAugustine, R.P.** (1994) *Expression of epidermal growth factor in suprabasal cells of stratified squamous epithelia: implications for a role in differentiation.* Cell Growth Different., 5, 527–535.
- Shultz, G.S., Rotatori, D.S.** and **Clark, W.** (1991) *EGF and TGF alpha in wound healing and repair.* J. Cell Biochem., 45, 346–352.
- Simms, J.S., Chegini, N., Williams, R.S., Rossi, A.M.** and **Dunn, W.A., Jr** (1991) *Identification of epidermal growth factor, transforming growth factor-alpha, and epidermal growth factor receptor in surgically induced endometriosis in rats.* Obstet.-Gynecol., 78, 850–857.
- Stone, L.M., Finger, T., Tam, P.P.L.** and **Tan, S.-S.** (1995) *Taste receptor cells arise from local epithelium, not neurogenic ectoderm.* Proc. Natl Acad. Sci. USA, 92, 1916–1920.
- Toma, J.G., Pareek, S., Barker, P.A., Mathew, T.C., Murphy, R.A., Acheson, A.** and **Miller, F.D.** (1992) *Spatiotemporal increases in epidermal growth factor receptors following peripheral nerve injury.* J. Neurosci., 12, 2504–2515.
- Vega, J.A., Vazquez, E., Naves, F.J., Calzada, B., del Valle, M.E.** and **Represa, J.J.** (1994) *Expression of epidermal growth factor receptor (EGFr) immunoreactivity in human cutaneous nerves and sensory corpuscles.* Anat. Rec., 240, 125–130.
- Weiffenbach, J.M., Fox, P.C.** and **Baum, B.** (1986) *Taste and salivary function.* Proc. Natl Acad. Sci. USA, 83, 6103–6106.
- Yarden, Y.** and **Ullrich, A.** (1988) *Growth factor receptor tyrosine kinases.* Annu. Rev. Biochem., 57, 443–478.
- Zhang, C., Brandemihl, A., Lau, D., Lawton, A.** and **Oakley, B.** (1997) *BDNF is required for the normal development of taste neurons in vivo.* Neuroreport, 8, 1013–1017.

Accepted July 20, 1999