Estrogen receptor-β is the predominant estrogen receptor subtype in human oral epithelium and salivary glands

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Abstract

Many studies have shown that the oral mucosa and salivary glands are sensitive to estrogen action. However, the expression of estrogen receptors (ERs) within these tissues is an area of controversy. ERs exist as two subtypes (ERα and ERβ), and we hypothesized that the incongruity between ER expression and estrogen sensitivity may result from differential expression of ER subtypes in oral tissues. To test this hypothesis, we analyzed oral mucosal and salivary gland samples for ERα and ERβ protein expression by immunohistochemistry from a cross-section of patients attending hospital for surgical problems of the head and neck. ERα was not detected in oral buccal and gingival epithelium or in salivary glands. In contrast, ERβ was widely expressed at high levels in all oral tissues studied. Within these tissues, ERβ was observed primarily in keratinocytes and salivary gland acinar and ductal cells. Our results demonstrating the expression of only the ERβ subtype within oral tissues may explain the contradictory results from previous studies investigating ER expression in these tissues. Importantly, these results suggest that estrogens may act via ERβ in oral tissues and explain the effect of hormonal changes on the oral mucosa as well as on saliva secretion and composition.

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Introduction

Estrogens are known to regulate cell growth, differentiation and function in reproductive as well as non-reproductive tissues. The effects of estrogens are mediated by estrogen receptors (ERs), and two different subtypes of ERs have been identified, namely ERα (Green *et al.* 1986, Greene *et al.* 1986) and ERβ (Kuiper *et al.* 1996). Although ERs are expressed in many different tissues, individual tissues differ dramatically in their expression of the two subtypes. ERα is expressed predominantly in classic estrogen-target tissues, such as mammary glands and the endometrium. In contrast, ERβ is mainly expressed in tissues that have only recently been identified as targets for estrogen – for example, in colonic (Campbell-Thompson *et al.* 2001) and prostatic (Kuiper *et al.* 1996) epithelia.

Sex steroid hormones appear to play a significant role in the physiology of the human oral cavity. A number of studies suggest that oral soft tissues are sensitive to changes in female sex steroid blood levels. Some diseases and disorders of the oral cavity, such as desquamative gingivitis (Nisengard & Rogers 1987), show a predilection for women and samples from these lesions appear to be ER-positive (Yih *et al.* 2000), supporting a role for estrogen in disease etiology. Similarly, during pregnancy, the severity of gingival inflammation is increased (Löe & Silness 1963, Hugoson 1971) and there is a heightened risk for development of gingival pyogenic granuloma (Daley *et al.* 1991). Estrogens are also known to modulate epithelial maturation in classic target organs, and similarly, the decrease in estrogen levels during menopause is thought to affect the oral epithelial maturation process, leading to thin, atrophic epithelium prone to inflammatory changes (Litwack *et al.* 1970, Forabosco *et al.* 1992). Clinically, menopausal women may exhibit symptoms of oral discomfort characterized by a burning sensation, sensation of oral dryness and decreased saliva secretion (Wardrop *et al.* 1989, Forabosco *et al.* 1992). Oral dryness

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can lead to considerable difficulty in speaking, eating and tasting, and predispose mucosa to wounds, abrasion and infection. A number of studies have shown that hormone replacement therapy (HRT) can relieve this oral discomfort in postmenopausal women, further suggesting a role for female sex hormones in the maintenance of oral tissues (Wardrop *et al.* 1989, Forabosco *et al.* 1992, Leimola-Virtanen *et al.* 1997, Eliasson *et al.* 2003).

Although many hormones are known to regulate saliva composition and secretion, the specific mechanism by which estrogens modulate human salivary gland function is poorly understood. Menstrual cycle, pregnancy and HRT have all been shown to affect saliva composition. During the menstrual cycle, specific changes can be observed in saliva composition, e.g. in the levels of salivary peroxidase (Tenovuo *et al.* 1981) and secretory IgA (Gómez *et al.* 1993). Similarly, the inorganic and protein composition of saliva changes during the course of pregnancy (Salvolini *et al.* 1998), and in late pregnancy the salivary buffer effect is significantly decreased (Laine & Pienihäkkinen 2000). HRT has been shown to improve the buffering effect of saliva (Laine & Leimola-Virtanen 1996) and, although evidence to the contrary does exist (Streckfus *et al.* 1998, Ghezzi *et al.* 2000), a number of studies (Laine & Leimola-Virtanen 1996, Eliasson *et al.* 2003) have reported an increase in salivary flow rate in association with HRT. Together, these observations suggest that estrogens may play an important role in oral mucosal and salivary gland physiology. However, the precise mechanism by which estrogens mediate these effects is unclear.

Although estrogens clearly modulate the physiology of the oral cavity, conflicting results exist regarding the expression of ERs in oral tissues. Human gingiva has been shown to contain receptors for estradiol by autoradiography using radioactively labeled estradiol (Vittek *et al.* 1982*a*) and oral mucosa has been shown to express ER mRNA (Leimola-Virtanen *et al.* 2000). In a single study, the presence of ERs in healthy gingiva has been demonstrated by immunohistochemistry (IHC), although the subtype was not determined (Forabosco *et al.* 1992). However, other IHC-based studies have been unable to confirm this finding (Ojanotko-Harri *et al.* 1992, Leimola-Virtanen *et al.* 2000). Studies investigating the presence of ERs in salivary glands have also given conflicting results. Low levels of ERs have been detected in salivary glands using an enzyme immunoassay (Wilson *et al.* 1993), and salivary gland ductal cells have been shown to be immunoreactive to anti-estradiol antibody (Ozono *et al.* 1992). Normal human salivary gland tissue and salivary gland tumors have also been shown to express ER mRNA (Leimola-Virtanen *et al.* 2000) and ER protein (Glas *et al.* 2002) and cytosolic fractions of these tissues bind estradiol (Dimery *et al.* 1987). However, several other studies have failed to demonstrate the presence of estrogen-binding activity or ER proteins by IHC in these tissues (Lamey *et al.* 1987, Shick *et al.* 1995, Leimola-Virtanen *et al.* 2000).

Recently, a number of non-classic estrogen-regulated tissues have been shown to express the ERβ subtype, suggesting that the function of these tissues may be controlled by the binding of estrogen specifically to this ER subtype. However, the expression of the ERβ subtype has not been specifically examined in any of the previous studies investigating ER expression in oral tissues. The present study was designed to determine whether the oral epithelium and salivary glands display a differential expression of the ERα and ERβ subtypes. Our results show that, although ERα was completely undetected in these tissues, ERβ was expressed at high levels in oral epithelium and salivary glands. This differential expression of ER subtypes may account for the conflicting results of ER expression in earlier studies. Importantly, the identification of ERβ in these tissues has significant clinical importance and suggests a direct role for estrogen in the physiology of oral mucosa and salivary gland function.

Materials and Methods

Subjects

Mucosal and minor salivary gland biopsies were obtained from patients attending the Department of Oral and Maxillofacial Surgery for surgical problems, and major salivary gland samples were donated by patients undergoing salivary gland surgery at the Department of Otolaryngology at Turku University Central Hospital, Turku, Finland. A total of 24 samples was analyzed from both women ($n=16$) and men ($n=8$) aged between 23 and 71 years (mean 49).

The study protocol was approved by the Ethical Committee of the Hospital District of South-Western Finland, and informed consent was obtained from subjects for the use of tissues in the present study. A brief medical history including medications and systemic diseases was recorded by a questionnaire completed prior to surgery.

Tissue samples

Mucosal samples were taken as punch biopsies from clinically healthy buccal mucosa or attached gingiva in the molar region adjacent to the surgical operation site. Minor salivary glands were excised from the lower lip or buccal mucosa, and major salivary gland samples consisted of clinically healthy region of the gland removed due to surgical indications. Tissue samples were immediately transferred to 10% phosphate-buffered neutral formalin for further routine histological processing.

IHC

Tissue sections (5 μm) were mounted on poly-L-lysine-coated slides. For staining, slides were dewaxed with

xylene, rehydrated with descending grades of ethanol and rinsed with distilled water. For ERα and ERβ staining, tissue sections were pretreated by microwaving for 15 min in 10 mM citrate buffer, pH 6·0, and allowed to cool at room temperature (RT) for 20 min. After washing with PBS, endogenous peroxidase was blocked by incubating sections with 1% hydrogen peroxide at RT for 20 min. Slides were then washed with PBS and blocked for 1 h at 4 °C with either goat serum (ERα and smooth muscle cell (SMC) α-actin) or rabbit serum (ERβ). SMC α-actin staining was performed to enable discrimination between myoepithelial and acinar cells in salivary gland tissue. After washing with PBS, primary antibodies were diluted in diluent buffer (3% BSA in PBS) and applied to slides followed by incubation overnight at 4 °C. Antibodies used were a mouse monoclonal anti-human ERα antibody diluted 1:200 (Clone 1D5; Dako A/S, Glostrup, Denmark), a chicken anti-human ERβ 503 antibody diluted 1:1000 and a mouse monoclonal anti-SMC α-actin antibody diluted 1:20 000 (Sigma-Aldrich, St Louis, MO, USA). ERβ 503 antibody was raised by immunizing laying hens with ERβ 503 protein. ERβ 503 protein is human ERβ1, that has been modified in its ligand-binding domain by insertion of the rat 18 amino acid sequence (Ogawa *et al.* 1998) resulting in a protein equivalent of human ERβ2. This modified protein used for immunization was expressed in SF9 cells by KaroBio (Huddinge, Sweden). The chicken polyclonal ERβ 503 IgY was isolated from egg yolks by polyethylene glycol precipitation and DE52 cellulose chromatography. The specificity of ERβ 503 antibody for ERβ has been previously described (Saji *et al.* 2000). For ERβ staining, an absorption control was included in which ERβ antibody was preabsorbed with an excess of purified human ERβ protein (Panvera, Madison, WI, USA). Negative control slides were incubated in diluent buffer alone. Slides were then washed with PBS and incubated for 1 h at RT with either biotinylated goat anti-mouse secondary antibody diluted 1:200 (Dako) (ERα and SMC α-actin detection) or peroxidase-conjugated rabbit anti-chicken secondary antibody diluted 1:1000 (Sigma-Aldrich) (ERβ detection). After washing with PBS, peroxidase standard VectaStain ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA) was applied to slides containing the biotinylated secondary antibody according to the manufacturer's instructions. All slides were then incubated with 3,3′-diaminobenzidine chromogen substrate (Zymed, San Francisco, CA, USA) supplemented with hydrogen peroxide, washed with distilled water, counterstained with Mayer's hematoxylin, dehydrated and mounted. Human endometrium and prostate tissues were used as positive control tissues for ERα and SMC α-actin or ERβ staining respectively (Fig. 1). Slides were microscopically evaluated in a blinded analysis by two independent investigators; staining intensity was expressed on a following scale: no visible staining (−), mild staining (+), moderate staining (++) and strong staining (+++).

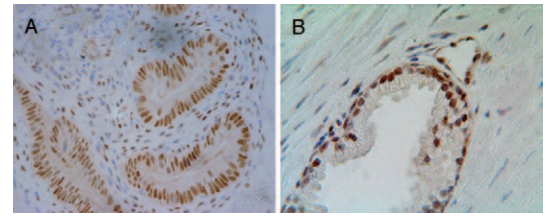

Figure 1 Human endometrium and prostate tissues were used as positive controls for ERα and ERβ staining respectively. In the endometrium (A), both glandular and interstitial cells are positive for ERα (brown). The prostatic epithelium and endothelial cells (B) displayed strong immunoreactivity for ERβ (brown).

Results

ERβ, but not ERα, is expressed in oral mucosa

Mucosal biopsies were taken from either buccal mucosa or attached gingiva in the molar region. Ten of eleven patients were immunopositive for ERβ, and staining intensity ranged from mild to strong (Table 1). ERβ immunoreactivity was exclusively nuclear, and was identified in all cell layers of the stratified squamous epithelium including basal cells, but excluding the nuclei of the flattened cells in the surface layer (Fig. 2B–D). Although, positive nuclei were detected in all layers of the epithelium, not all nuclei within these layers were positive for ERβ. The overall staining intensity appeared to be stronger in the gingival epithelium than the buccal epithelium. No apparent sex differences were observed in the pattern or intensity of staining. Mucosal samples from all of these patients were negative for ERα staining (Table 1, Fig. 2A).

Within this patient population, all men and women of reproductive age were positive for ERβ staining. Neither of these patient groups had any complaint of oral discomfort. Among postmenopausal women, a single individual was negative for ERβ (Table 1: no. 3). Interestingly, this patient was a 71-year-old female complaining of continuous gingival and palatal irritation, who was not receiving any medication, including HRT. Of the remaining ERβ-positive samples from this group, two of four were obtained from postmenopausal women using HRT. One of these women complained of tongue irritation (Table 1: no. 2). However, this patient was using antidepressive medication in addition to HRT, which may be the possible cause of oral discomfort due to the reduced salivary secretion associated with this medication. The remaining ERβ-positive sample was obtained from a patient who neither had feelings of oral discomfort nor was on HRT.

Table 1 Immunohistochemical staining (negative (−), positive (+) scale) of oral epithelium with ERα and ERβ antibodies

	Age	ERα	ERβ	Diseases, HRT and oral symptoms: medication
Sample no.				
Women				
Buccal epithelium				
1	44	−	+++	None
2	62	−	+	Depression: SSRI HRT: combined estrogen and medroxyprogesterone Continuous tongue irritation
3	71	−	−	None Continuous gingival and palatal irritation
Gingival epithelium				
4	31	−	+++	None
5	37	−	+++	Mitral prolapse Frequent sinusitis: glucocorticoids Frequent aphtae
6	53	−	++	Allergy: antihistamines HRT: estrogen
7	67	−	+++	Hypothyroidism: thyroxine Rheumatoid arthritis: sulfasalazine Hypercholesterolemia: statin
Men				
Buccal epithelium				
8	48	−	+	Allergy
Gingival epithelium				
9	23	−	+++	None
10	55	−	+++	Asthma: glucocorticoids, β_2-agonist
11	63	−	++	Hyperplasia of the prostate: α_{1A}-receptor antagonist

ERβ, but not ERα, is expressed in salivary glands

Salivary glands from 12 of a total of 13 patients were immunopositive for ERβ. ERβ positivity was observed at varying intensity in both mucous and serous acinar cells as well as in intercalated, striated and excretory ductal cells (Table 2, Fig. 3). ERβ immunoreactivity was mainly localized to the nucleus, although weak staining was also observed in the cytoplasm. The staining intensity of both acinar and ductal cell nuclei varied from mild to strong (Table 2). As observed within the oral epithelium, some ERβ-negative cells were distributed among the ERβ-positive cells. In general, the immunoreactivity for ERβ was strongest in minor salivary gland samples and minimal differences were observed in staining intensity and pattern between salivary gland samples from women and men. Myoepithelial cells appeared to be mostly ERβ-negative. Although acinar cells of one patient (Table 2: no. 7) were ERβ-negative, the ductal cell nuclei from this patient were mildly positive. The parotid sample from one patient (Table 2: no. 6) was totally negative for ERβ. This sample was, however, positive for SMC α-actin and can therefore be considered as a true negative instead of being inadequately processed. Importantly, samples from all patients were completely negative for ERα (Table 2).

Discussion

Recent studies have revealed a significant tissue-specific difference with regard to the distribution of ER subtypes, ERα and ERβ, with classic estrogen-target tissues expressing primarily the ERα subtype. In the present study, our results show that a tissue-specific ER subtype distribution is also observed in oral tissues – with ERβ, but not ERα, being widely expressed in both the oral epithelium and the salivary glands. This finding may serve to resolve the current controversy associated with the contradictory reports on ER expression in these tissues. Importantly, the identification of ERβ in oral epithelium and salivary glands suggests that estrogens may directly regulate the physiology of oral tissues by binding to the ERβ subtype.

The evidence for the presence of ERs in normal human oral mucosa and salivary glands by IHC has been

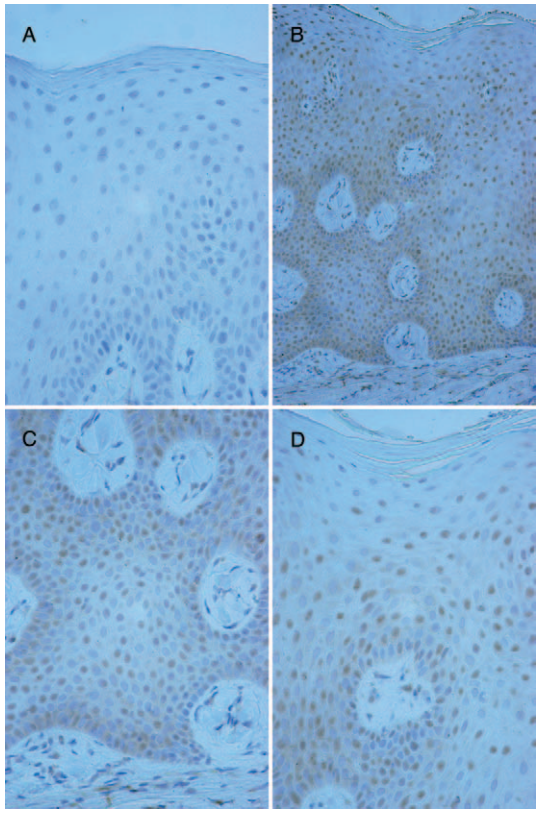

Figure 2 Immunostaining for ERα and ERβ in oral mucosa. All mucosal samples were ERα-negative (A). In contrast, nuclear ERβ positivity (brown) was detected in all cell layers of the stratified squamous epithelium excluding flattened cells in the surface layer (B–D). Objective magnification ×40 (A, C and D), ×20 (B).

inconclusive (Forabosco *et al.* 1992, Ojanotko-Harri *et al.* 1992, Shick *et al.* 1995, Leimola-Virtanen *et al.* 2000, Glas *et al.* 2002). In contrast, a number of studies using non-IHC-based detection methods have suggested the presence of ERs within these tissues. For example, both gingival tissue (Vittek *et al.* 1982*a*) and salivary glands (Dimery *et al.* 1987) have been shown to bind estradiol by autoradiography and a ligand-binding assay respectively. In other studies, binding of anti-estradiol antibody has identified major salivary gland ductal cells as predominant estradiol-binding sites (Ozono *et al.* 1992) and oral mucosa and salivary glands have been shown to express ER mRNA in the absence of positive staining for ER by IHC (Leimola-Virtanen *et al.* 2000).

Consistent with the results from the majority of earlier studies, we were unable to detect ERα by IHC in the oral epithelium or salivary glands. However, in these earlier studies, ERβ subtype expression was not specifically examined. The antibodies used for IHC in these previous studies were ER-ICA (Abbott Laboratories, North Chicago, IL, USA) and ER1D5 (Coulter-Immunoteck, West Brook, ME, USA). ER-ICA antibody was made using MCF-7 human breast cancer cell cytosolic fraction as an immunogen and it has been shown to recognize an epitope in the C-terminal ligand-binding domain of ERα, whereas ER1D5 was raised against recombinant ERα and shown to react with the A/B region in the N-terminal domain of the receptor. Since both ERα and ERβ can bind 17β-estradiol (Kuiper *et al.* 1997), we hypothesized that the discrepancy between earlier results from IHC and ligand-binding assays regarding the expression of ER could be explained by tissue-specific expression of ER subtypes in oral tissues. Consistent with this idea, we identified high levels of ERβ in both oral gingival and buccal epithelium as well as in the salivary glands by IHC. In contrast, ERα was not identified in these tissues. However, it cannot be excluded that under certain conditions (e.g. inflammation) the expression of ERα may be induced within oral tissues. A recently described controversial finding of ER-positive cells by RT-PCR in oral tissues in the absence of ER by IHC (Leimola-Virtanen *et al.* 2000) can be further explained by the insensitivity of IHC compared with RT-PCR or by the use of homogenized tissues for the RT-PCR analysis. Oral tissues contain cells that are well established to express high levels of ERα in other tissues, e.g. the vascular endothelial and SMCs (for review see Mendelsohn & Karas 1999) and it is likely that this ER-positive finding by RT-PCR may have originated from cells other than keratinocytes or salivary gland cells within these tissues. Together, our results strongly suggest that the inconsistency in the detection of ER in oral epithelium and salivary glands in the earlier studies is due to expression of ERβ, but not ERα, in these tissues.

ERβ was detected in all layers of the gingival and buccal mucosal epithelium excluding the squamous cell layer. This observed distribution of ERβ is distinct from the distribution of other sex steroid receptors, for example androgen receptors, which are restricted predominantly to the basal epithelial cells (Ojanotko-Harri *et al.* 1992). This difference may reflect responsiveness of oral keratinocytes to estrogens early as well as late in the process of epithelial maturation compared with androgens that appear to function only at early stages. Human oral mucosa is known to bind progesterone (Vittek *et al.* 1982*b*), but the exact localization of progesterone receptors (PRs) in oral mucosa is not known. Identification of ERs in oral epithelium suggests that estrogens have an important role in the maturation of oral epithelium. Therefore, the lack of estrogens in postmenopausal women may account for the atrophic oral epithelium and substitution of this deficiency with HRT may restore, at least in part, normal epithelial proliferation and maturation thereby decreasing oral discomfort.

Saliva composition shows hormone-related changes, suggesting that hormones may have a role in the control of salivary gland function. Previous studies have shown that secretion of proteins and the inorganic components of

Table 2 Immunohistochemical staining (negative (−), positive (+) scale) of salivary glands with ERα and ERβ antibodies

		ERα		ERβ		
	Age	Ducts	Acini	Ducts	Acini	**Diseases, HRT and oral symptoms: medication**
Sample no.						
Women						
Buccal minor salivary gland						
1	31	−	−	++	++	None
Labial minor salivary gland						
2	31	−	−	+++	+++	None
3	44	−	−	+++	+++	None
4	59	−	−	+	+	HRT: estrogen
Parotid gland						
5	34	−	−	++	++	Pleomorphic adenoma
6	42	−	−	−	−	Pleomorphic adenoma
7	54	−	−	+	−	Pleomorphic adenoma Pernicious anemia: vitamin B12 injections
8	67	−	−	++	++	Warthin tumor Non-insulin-dependent diabetes mellitus: metformin Hypertension and angina pectoris: nitrates, ASA, β1-selective adrenergic receptor antagonist, diuretics
Submandibular gland						
9	71	−	−	++	++	Chronic sialadenitis Hypertension: diuretics Status post infarctus cerebralis: ASA, dipyridamole Epilepsy Overactive bladder: anticholinergic drug Benzodiazepine derivative
Men						
Labial minor salivary gland						
10	41	−	−	+++	+++	None
11	44	−	−	+++	++	None
Parotid gland						
12	54	−	−	+++	+++	Warthin tumor
Submandibular gland						
13	55	−	−	+	+++	Dissection of submandibular gland due to attached lipoma Hypercholesterolemia: statin

ASA, acetylsalicylic acid.

saliva is hormone-related (Tenovuo *et al.* 1981, Gómez *et al.* 1993, Salvolini *et al.* 1998, Laine & Pienihäkkinen 2000) and HRT appears to increase saliva flow rate, buffer effect and protein content of saliva in menopausal women (Laine & Leimola-Virtanen 1996, Leimola-Virtanen *et al.* 1997, Eliasson *et al.* 2003). In our present study, we identified ERβ in both mucous and serous acinar and ductal cells in minor salivary glands, and in parotid and submandibular glands. Since growth factors have been shown to use nuclear receptors in their signaling pathways (Driggers & Segars 2002) and estrogens have been shown to modulate the expression of critical growth factors (e.g. nerve growth factor) (Bjorling *et al.* 2002), ERβ may play an important role in the maintenance and function of salivary glands. This distribution of ERβ may explain the effect of estrogens on the inorganic composition of saliva and the positive effect of HRT on saliva secretion. Earlier studies have demonstrated PRs in ductal cells (Ozono *et al.* 1992) and androgen receptors in both ductal and acinar cells (Laine *et al.* 1993). Consequently, sex steroid hormones appear to play an important, but complex, role in the regulation of salivary glands, especially ductal cells, which are known to have a significant role in modulating the inorganic composition of saliva. The differential

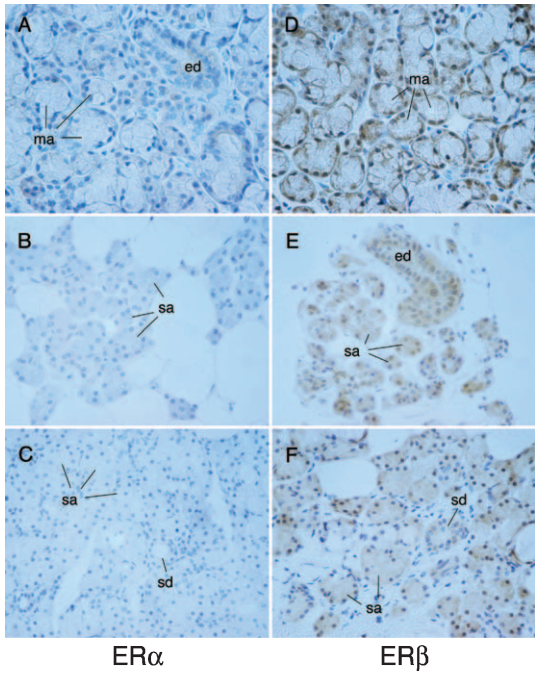

Figure 3 Immunostaining for ERα and ERβ in salivary glands. Acinar and ductal cells displayed immunoreactivity (brown) for ERβ (D–F), but were negative for ERα (A–C). Top: labial minor salivary gland, middle: parotid gland, bottom: submandibular gland. Objective magnification × 40 for all panels. Mucous acini (ma), serous acini (sa), excretory duct (ed), striated duct (st).

expression of ER subtypes observed in our study also suggests that ERβ, rather than ERα, is the predominant inducer of PR within these tissues.

There was no apparent difference between the sexes in the ERβ staining pattern or intensity in the oral epithelium or the salivary glands. However, the number of patients in this study was limited and further studies are required to elucidate the possible effect of sex and age on the ERβ expression pattern in oral tissues. In addition, ERβ-negative cells were observed interspersed within ERβ-positive cells in both the epithelium and salivary glands. The presence of these ERβ-negative cells may reflect cell cycle and differentiation-dependent differences in ER expression. In the endometrium the expression of ERs has been shown to be influenced by the fluctuation in hormone levels (Lecce *et al.* 2001), and in the prostate, developmental stage and age are associated with ERβ expression in a defined cell population (Adams *et al.* 2002). Since a particular staining pattern was not discovered to be associated with either sex or age in our study, other factors such as growth factors may play an important role in the regulation of ER expression in oral tissues.

In conclusion, we have identified ERβ as the predominant ER subtype in the human oral epithelium and salivary glands. The identification of ERβ in the oral tissues may explain the contradictory results from a number of studies investigating the presence of ERs in these tissues using IHC compared with ligand-binding assays and mRNA expression analysis. Importantly, the expression of ERβ in oral epithelial cells and salivary gland acinar and ductal cells suggests that estrogens may regulate the physiology of these tissues through the ERβ subtype. These findings may also serve to explain clinical observations of sensitivity of oral tissues to estrogens and the beneficial effects of HRT on oral symptoms in postmenopausal women.

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