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# Expression and immunolocalization of the oxytocin receptor in human lactating and non-lactating mammary glands

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**The milk ejection reflex is mediated by the release of pituitary oxytocin and its interaction with specific receptors within the mammary gland. Although up-regulation of the oxytocin receptor during lactation has been shown for the rat mammary gland by ligand binding assay, investigation of the receptor expression in human breast at the molecular level has not yet been carried out in detail. Here we report the expression and immunolocalization of the oxytocin receptor in the human breast. It appears that the expression level of the receptor-specific mRNA is not significantly elevated during lactation and the protein remains at a relatively low level. However, this lack of increase may be only a dilution effect because of the high level of milk protein expression. Immunohistochemistry and immunoelectron microscopy using three anti-oxytocin receptor antibodies raised against different epitopes of the receptor indicated the presence of receptor immunoreactivity only to a very limited extent in the myoepithelial cells; more specific expression appeared to occur in the ductal/glandular epithelium in both the non-lactating as well as lactating breast. This finding was also confirmed in a New World monkey, the common marmoset (*Callithrix jacchus*). These results suggest that, at least for human and marmoset, in addition to—or even instead of—myoid cells, the ductal/glandular epithelium is also a target for oxytocin action, not only during lactation but also in the non-lactating breast. Thus, there may be other physiological effects of oxytocin besides direct myoid cell contraction in the breast.**

*Key words:* ductal glandular epithelium/lactation/mammary gland/myoepithelium/oxytocin receptor

## Introduction

Lactation in the mammary gland is essential for supporting the life of new-born young. This gland develops under the influence of a variety of hormones and growth factors. During puberty, steroid hormones (oestrogen, progestin and glucocorticoids) and growth factors (IGF, EGF) induce the growth and expansion of ductal and glandular epithelium, and after conception, prolactin and sex steroids prepare the gland for milk production (Hennighausen and Robinson, 1998). Following birth, the release of milk is controlled by an oxytocin-mediated reflex upon suckling. The nonapeptide hormone oxytocin (OT) plays a central role in the birth process through inducing uterine contraction (labour), mainly by the massive up-regulation of the specific oxytocin receptor (OTR) in the uterus (Fuchs *et al.*, 1995; Kimura *et al.*, 1996). However, this change in OTR does not appear to involve the mammary gland to any significant degree. On the other hand, in the postpartum period the mammary gland, besides having copious milk production, is now a target organ for OT. For laboratory animals such as the rat and rabbit, it is well documented that suckling or tactile stimulation of the nipples causes a signal input to the hypothalamus, and induces OT secretion from the posterior pituitary gland. The up-regulation of OTR is also observed in the rat lactating mammary gland (Soloff *et al.*, 1979). This OT–OTR system induces a rise of intramammary pressure and milk is released. This milk ejection reflex is considered to be the central physiological role for OT in the mammary gland (Lincoln and Paisley, 1982). In transgenic mice where the OT gene has been ablated by gene targeting, only this physiology appears to be disturbed (Nishimori *et al.*, 1996). No distinct effects of OT on the ductal/glandular epithelium have been described either for the lactating or the non-lactating (resting) mammary gland.

The recent molecular cloning of the OTR cDNA (Kimura *et al.*, 1992a) has made it possible to raise antibodies specifically recognizing the OTR molecule (Takemura *et al.*, 1994; Adan *et al.*, 1995; Ito *et al.*, 1996). In the rat mammary gland, OTR immunoreactivity has been observed exclusively in the myoepithelial cells (Adan *et al.*, 1995). However, in humans, several research groups have shown OTR to be present in breast cancer-derived cell lines (Taylor *et al.*, 1990; Cassoni *et al.*, 1994; Ito *et al.*, 1996). Since most breast cancer cells originate from ductal or glandular epithelium (Gusterson *et al.*, 1982), it is possible that, also in the normal breast, OTR might be present not only in the myoepithelium but also in the ductal/glandular epithelium, especially in humans. We report here the expression and immunolocalization of the human OTR in non-lactating and lactating tissue using three different anti-

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OTR antibodies. These results were corroborated by also examining the non-lactating gland, as well as the gland during early pregnancy, from a New World monkey, the common marmoset (*Callithrix jacchus*).

## Materials and methods

### *Tissue specimens*

Normal mammary gland tissues were obtained from surgical specimens of breast cancer at radical mastectomy. The ages of the patients undergoing operation were 32, 33, 35 and 58 years; the first three patients exhibited regular menstrual cycles. The sample of lactating mammary gland was obtained from a 29-year-old breast cancer patient, operated on 5 months after the birth of her child and while breast-feeding. Mammary tissue of normal appearance was dissected from the whole excised breast, leaving a margin of at least 1.5 cm from the border of the identified tumours. Mammary gland tissue from non-lactating or early pregnant marmosets (aged 3–5 years, and of proven fertility) was dissected immediately after their being killed by an overdose of halothane anaesthesia.

For RNA and protein extraction, samples were stored at –80°C, after snap freezing in liquid nitrogen. For immunohistochemical staining, two different procedures were used. In the first, tissues were soaked in diluted aldehyde solution (2% v/v formaldehyde, 0.05% v/v glutaraldehyde, 0.025% w/v $CaCl_2$, 0.05 M cacodylate buffer, pH 7.35) and then irradiated with 2450 mHz microwave energy at 500 W, twice for 10 s, with a 5-s interval (Login *et al.*, 1987). After dehydration with ethanol and xylene, tissues were embedded in paraffin wax. In the second procedure, tissues were frozen in liquid nitrogen and stored at –80°C until used.

### *Antibodies*

Anti-human OTR monoclonal antibodies, 2F8 and 1-2, were raised against the N-terminal extracellular domain (amino acids 20–40 of the human OTR sequence PPGAEGNRTAGPPRRNEALAR; Takemura *et al.*, 1994) and the first extracellular loop (amino acids 102–119, TFRFYGPDLLCRLVKYLQ; Ito *et al.*, 1996), respectively. Anti-rat OTR polyclonal antibody, JV3580 (courtesy of Dr F.Van Leeuwen, Amsterdam, The Netherlands and Dr J.Verbalis, Washington DC, USA) was raised against a peptide from the third intracellular loop (WQNLRLKTAAA) of the rat receptor (Adan *et al.*, 1995). This sequence is perfectly homologous to the human OTR sequence at residues 228–238. Anti-human smooth muscle α-actin monoclonal antibody (1A4) was purchased from Dako (Tokyo, Japan).

### *Northern blotting*

Total RNA was extracted from frozen tissues using the 5.5 M guanidium isothiocyanate–5.7 M CsCl ultracentrifugation procedure as described by Kimura *et al.* (1992b). 20 μg of total RNA from non-lactating and lactating mammary gland, and from term human myometrium were electrophoresed in denaturing 2.2 M formaldehyde/1% agarose gel in 1× MOPS buffer and blotted onto nylon membrane (Biodyne A, Pall, New York, NY, USA). The filter was then hybridized with the 0.8 kb OTR cDNA *Bam*HI–*Pst*I fragment, previously labelled with [α-$^{32}$P]dCTP to high specific activity, in hybridization buffer (50% v/v formamide, 5× SSC, 5× Denhardt′s solution, 0.1% SDS and 100 μg/ml fragmented denatured salmon sperm DNA) at 42°C for 16 h. The filter was washed with 0.2× SSC, 0.1% SDS for 1 h at 50°C and exposed to Kodak X-Omat film at –80°C for 24 h.

### *Western blotting*

Frozen samples were pulverized in liquid nitrogen and lysed in 10 volumes of 1× SDS buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 100 mM DTT, 0.1% bromophenol blue, 10% glycerol). Either 5 μg (myometrium samples) or 5–200 μg (lactating mammary gland) of the lysates were resolved by electrophoresis on an 8% SDS–polyacrylamide gel (Sambrook *et al.*, 1989) and transferred to a nitrocellulose membrane (0.45 μm; Schleicher & Schuell, Dassel, Germany) using the mini-trans-blot electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA). The OTR-specific signal was detected using the ECL Western blotting analysis system (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. The primary antibody, 2F8, was used at a final concentration of 1.4 μg/ml.

### *Immunohistochemistry*

Microwave-fixed, paraffin-embedded tissues were sectioned (3–4 μm), dewaxed three times in xylene for 5 min each and rehydrated through graded ethanols (100% to 60%). Slides were then rinsed in phosphate-buffered saline (PBS), incubated in 0.3% (v/v) $H_2O_2$, 0.1% (w/v) sodium azide ($NaN_3$) for 30 min at room temperature (RT), and treated with 10% normal rabbit serum for 30 min at RT for blocking. The primary antibody (2F8 or 1-2, 10 μg/ml) was then applied to the slides and incubated at 4°C for overnight in a moist chamber. After rinsing with PBS, biotinylated rabbit anti-mouse immunoglobulin (Histofine SAB-PO kit; Nichirei Co., Tokyo, Japan) was applied to the slides, and incubated for 30 min at RT. The slides were then washed with PBS, and incubated with peroxidase-conjugated streptavidin (Histofine SAB-PO kit) for 30 min at RT. The signal was visualized by incubating slides in 50 mM Tris–HCl, pH 7.6, containing 0.02% (w/v) 3,3′-diaminobenzidine tetrahydrochloride and 0.01% (v/v) $H_2O_2$. For the determination of signal specificity, antigen peptide (5 mg/ml in PBS) was added to the primary antibody, and co-incubated with the neighbouring section overnight at 4°C. For double-staining immunohistochemistry, after the development of the first OTR-specific signal, antigen–antibody complex was removed by incubating in 0.1 M glycine–HCl buffer, pH 2.2, three times for 30 min each at RT. After washing with PBS, the slides were then incubated with anti-human smooth muscle α-actin antibody (1A4, diluted at 150 μg/ml). For visualizing the signal, we used 4-chloro-1-naphthol as a chromogen for the peroxidase reaction. For frozen tissues, cryostat sections (7 μm) were air-dried at RT for 1 h and fixed with 4% paraformaldehyde for 10 min. The endogenous peroxidase activity was quenched as above. 1:100 diluted normal human serum was used for blocking. Sections were incubated with the primary antibodies (2F8, 3 μg/ml or JV3580, 1:3000), diluted with PBSA [PBS plus 0.5% BSA (Sigma)], overnight at 4°C. These were then rinsed in PBS and reacted with the second biotinylated antibody (either biotinylated horse anti-mouse IgG or biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) at 5 μg/ml in PBSA for 30 min at RT. After rinsing in PBS, sections were incubated in the ABC complex reagent (Vector Laboratories), then rinsed briefly in PBS and the signal visualized using 3-amino-9-ethylcarbazol (AEC) substrate solution according to the manufacturer's instructions. Some sections were lightly counterstained with haematoxylin. As negative controls, the primary antibodies were replaced by an equivalent amount of pure mouse IgM (Dako) or pure rabbit IgG (Dako). Immunostaining for α-actin was performed as described above.

### *Immunoelectron microscopy*

Dissected pieces of fresh tissue were fixed in 10 mM $NaIO_4$, 75 mM L-lysine, 37.5 mM phosphate buffer, 2% (w/v) paraformaldehyde, pH 6.2, for 6 h at 4°C, further incubated in PBS containing 10% (w/v) sucrose for 4 h, PBS plus 15% sucrose for 4 h, PBS plus 20% sucrose overnight at 4°C, and then embedded in OCT compound and frozen

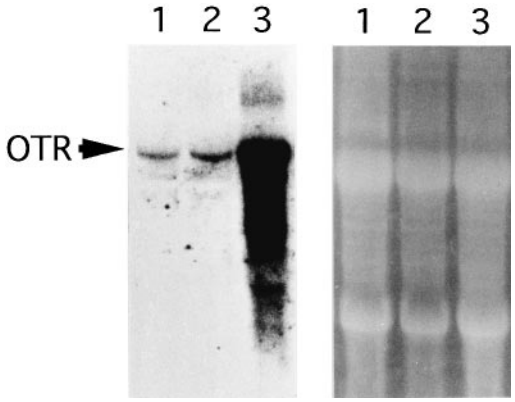


**Figure 1.** Northern blotting of the oxytocin receptor (OTR) in human mammary gland. 20 μg of total RNA samples from human non-lactating (lane 1), lactating (lane 2) mammary gland or from human term myometrium (lane 3) were electrophoresed in 2.2 M formaldehyde/1% agarose gel and blotted onto nylon membrane. The filter was hybridized with 0.8 kb OTR cDNA *Bam*HI–*Pst*I fragment, labelled with [α-$^{32}$P]dCTP to high specific activity, and exposed to film. Left: the result of hybridization. Right: the photograph of the gel stained by ethidium bromide before blotting. The signal of OTR mRNA could be detected both in non-lactating and lactating mammary glands, of which the same size observed in the term myometrial tissue (4.4kb). However, no obvious up-regulation of the receptor message was detected in the lactating mammary gland.

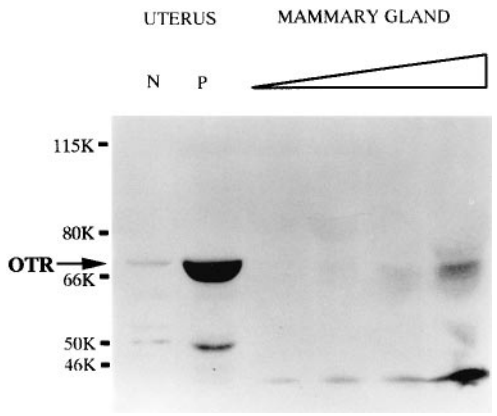


**Figure 2.** Western blotting of the oxytocin receptor (OTR) protein. 5 μg of tissue lysates from non-pregnant (N) or term myometrium (P), and 5, 20, 50 and 200 μg of lysate from lactating mammary gland (left to right) were separated by 8% SDS–polyacrylamide gel and transferred onto a nitrocellulose membrane using an electrophoretic blotter. The OTR-specific signal was detected using ECL Western blotting analysis system (Amersham), with the primary antibody, 2F8, at 1.4 μg/ml. The OTR-specific 70 kDa signals were detected in both uterine tissues (N, P), and were massively up-regulated in term myometrial lysate (P). In the lactating mammary gland, the 70 kDa signal could be detected when >50 μg of lysate was loaded. As 5 μg of lysate from non-pregnant myometrium already gave a significant OTR signal, the concentration of the OTR protein in the lactating mammary gland must be low.

at –70°C. Cryostat sections (4 μm) were incubated in 0.3% (v/v) $H_2O_2$, 0.1% (w/v) $NaN_3$ for 30 min at RT, and incubated with 10% normal rabbit serum for 30 min at RT for blocking. The sections were then incubated with 2F8 (100 μg/ml) at 4°C overnight. After washing with ice-cold PBS, the sections were incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulin (13 μg/ml; Dako) at 4°C overnight. After washing with PBS, the sections were immersed in 50 mM Tris–HCl, pH 7.6, 0.02% (w/v) 3,3′-diaminobenzidine tetrahydrochloride for 30 min and developed by incubating the same solution containing 0.03% (v/v) $H_2O_2$ for 5 min. Sections were post-fixed in 1% glutaraldehyde for 20 min, embedded in epoxy resin, sliced with an ultramicrotome (Ultracut N; Reichert-Nissei, Tokyo, Japan), and examined under a JEM-1200EX electron microscope (JEOL, Tokyo, Japan) with no staining.

### *Chemicals and solutions*

Chemicals were purchased from local suppliers and were of analytical grade. PBS was prepared as 10 mM phosphate buffer, pH 7.5, 150 mM NaCl and contained neither $Ca^{2+}$ nor $Mg^{2+}$.

## Results

### *Northern blotting*

Northern blotting using 20 μg of total RNA from non-lactating (Figure 1, lane 1), lactating (lane 2) and term myometrium (lane 3) indicated that mammary glands express significant amounts of OTR mRNA, although we could not detect any obvious message induction in the lactating mammary gland. The size of the signal in mammary glands was the same as the major band expressed in term myometrium (4.4 kb). Previously, we had reported that the mammary gland might express OTR mRNA of a different size (Kimura *et al.*, 1992a). However, this appears to have been due to an artefact created by electrophoresing poly($A^+$)-RNA (myometrium) and total RNA (mammary gland) on the same gel; the migration rate of the different RNA types proved to be slightly disparate.

### *Western blotting*

The OTR protein in human breast tissue could only be visualized using a tissue lysate from a lactating mammary gland (Figure 2). When 200 μg of lysate were applied, a clear signal was detected for the OTR protein at 70 kDa, the same size as observed in the uterus. No signals were seen using 5 μg or 20 μg of lysate, whereas 50 μg gave only a faint band. As 5 μg of tissue lysate from non-pregnant uterus already gave a significant OTR signal, that was then massively up-regulated at parturition, the concentration of OTR protein in the lactating mammary gland must be very low indeed.

### *Immunohistochemistry*

As the 2F8 and 1-2 monoclonal antibodies had already been used successfully to detect OTR in breast cancer tissues (Ito *et al.*, 1996) and pregnant myometrium (Kimura *et al.*, 1996), the same antibodies were used initially with microwave-fixed and paraffin-embedded mammary tissue. As mentioned previously, 2F8 and 1-2 gave identical staining patterns, though the signal was weaker with the 1-2 antibody in all breast specimens (data not shown). Therefore, only results obtained using the 2F8 antibody are illustrated. As shown in Figure 3A–D, OTR immunoreactivity was localized predominantly in the ductal and/or glandular epithelium, and not in the myoepithelial layer which should surround these epithelial cells. The same staining pattern was obtained in both non-lactating (Figure 3A,B) and lactating tissues (Figure 3C,D); in the latter, the ducts and acini were dilated and the cells flattened. This result

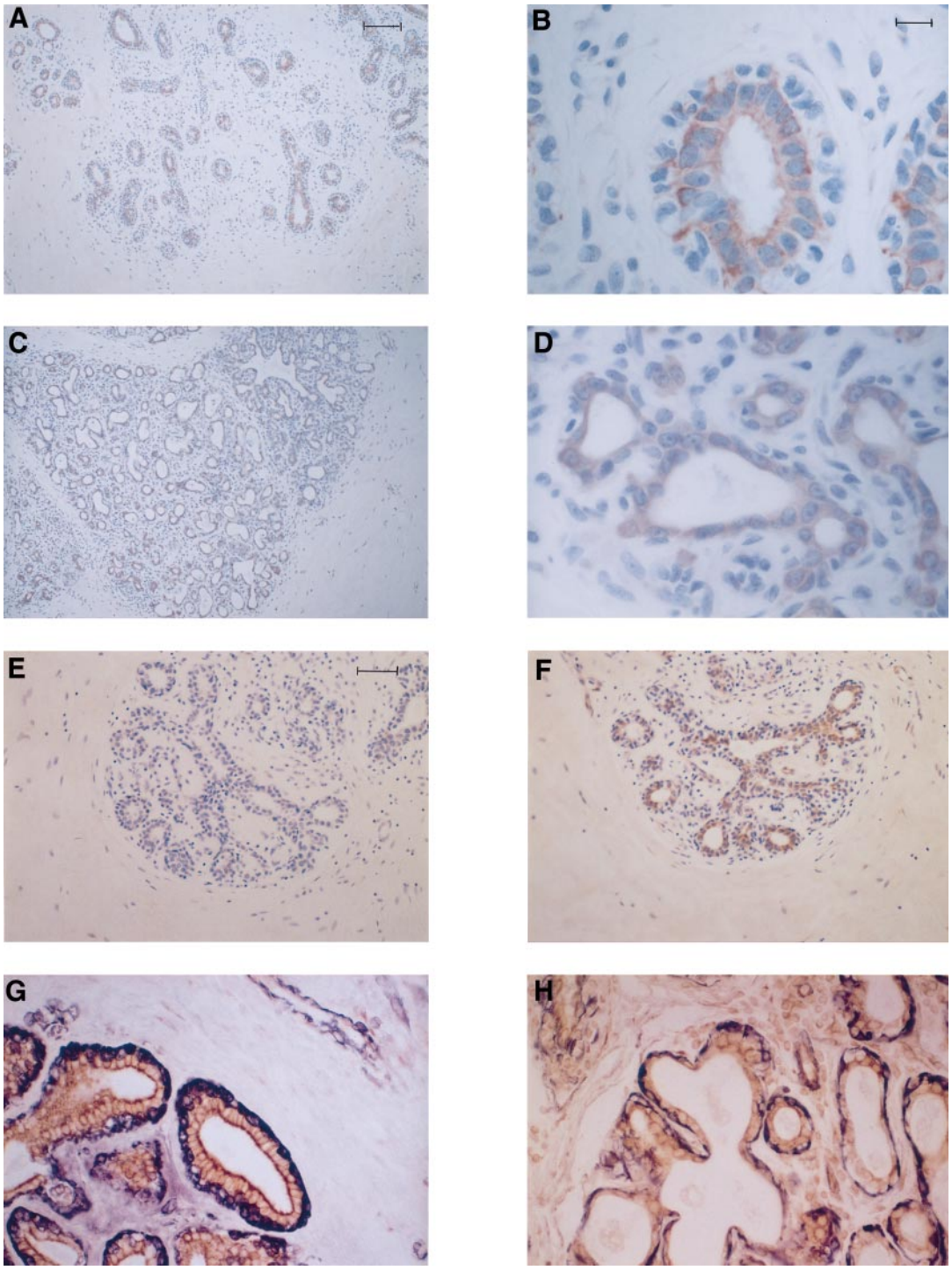


**Figure 3.** Immunohistochemistry of microwave-fixed section from human mammary gland. Non-lactating mammary gland (**A**, **B**, **E**–**G**) and lactating mammary gland (**C**, **D**, **H**) were fixed with microwave irradiation and stained with 2F8 or 1-2 (data not shown) at 10 μg/ml. OTR immunoreactivity was localized predominantly in the inner cell layer, which is equivalent to the ductal/glandular epithelium both in non-lactating mammary gland (**A**, **B**), and lactating mammary gland (**C**, **D**). (**E**, **F**) The specificity of the OTR immunoreactivity was shown by presence (**E**) or absence (**F**) of co-incubated antigen peptide with the primary antibody (2F8) in the non-lactating tissue. The excess amount of the antigen peptide (5 mg/ml) completely suppressed the OTR immunoreactivity (**E**), indicating that the immunostaining of the 2F8 is antigen-specific. (**G**, **H**) Double staining of the OTR and smooth muscle α-actin. After the development of the OTR signal, primary antigen–antibody complex was removed and stained with anti-human smooth muscle α-actin antibody (150 μg/ml). Both in non-pregnant (**G**) and lactating (**H**) mammary gland, myoepithelium which is stained by anti-α-actin antibody (dark blue) is clearly distinguishable from the OTR-positive ductal/glandular epithelium (brown). Original magnification: (**A**, **C**) ×120; (**B**, **D**, **G**, **H**) ×500; (**E**, **F**) ×250. Scale bars: (**A**) 60 μm; (**B**) 14 μm; (**C**) 28 μm.

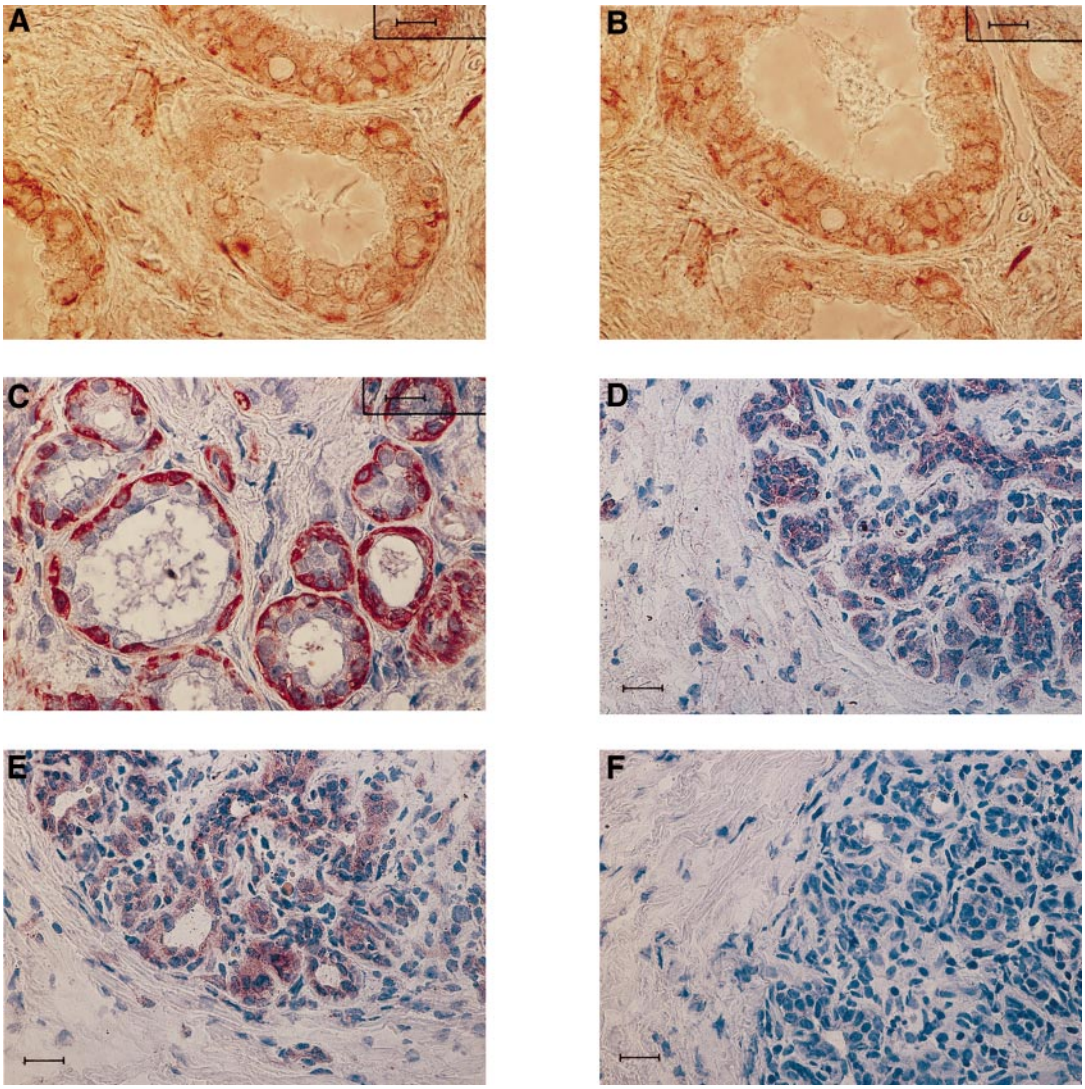


**Figure 4.** Comparison of 2F8 and JV3580 antibodies for OTR immunolocalization in frozen sections. Human non-lactating mammary gland (**A**–**C**) and lactating mammary gland (**D**–**F**) samples were incubated either with 2F8 [(3 μg/ml; (**A**, **D**)] or JV3580 [(1:3000; (**B**, **E**)] for the detection of OTR immunoreactivity. The staining pattern using both antibodies was identical. The OTR immunoreactivity was localized in ductal/glandular epithelium as well as, or to a lesser intensity, in myoepithelial cells. Myoepithelial cell layer was clearly identified by the immunostaining using anti α-actin antibody (**C**). The background staining with non-immune mouse IgM (**F**) or rabbit IgG (not shown) was negligible. Note that (**A**) and (**B**) were not counterstained in order to highlight the colour of immunostaining (brown). Original magnification: (**A**–**C**) ×551; (**D**–**F**) ×350. Scale bars: (**A**–**C**) = 12.6 μm; (**D**–**F**) = 20 μm.

was also reproducible in three different non-lactating tissues. To determine the specificity of the OTR staining, we used the antigen peptide to neutralize the antibody. As shown in Figure 3E, by addition of excess PPGAEGNRTAGPPRRNEALAR peptide (the antigen for 2F8), OTR immunoreactivity was completely suppressed in the inner cell layer, stained in the neighbouring section (Figure 3F). Anti-α-actin antibody (blue-black chromogen) clearly distinguishes the outer myoepithelial cell layer from the inner cell layer in both non-lactating (Figure 3G) and lactating (Figure 3H) tissues.

As this result using the 2F8 antibody and microwave-fixed tissues was unexpected, we decided to perform immunohistochemistry by applying a different procedure. Frozen pieces of non-lactating and lactating tissues were sectioned and fixed with 4% paraformaldehyde, and then tested using both 2F8, as well as a polyclonal antibody, JV3580, which had been raised against an intracellular epitope of the rat OTR polypeptide sharing 100% homology with the human molecule. As shown in Figure 4A (using 2F8) and Figure 4B (using JV3580), OTR immunoreactivity was localized in both the ductal/glandular epithelial, as well as myoepithelial layers of non-lactating mammary gland, using the α-actin-positive staining to identify the myoepithelial cells (Figure 4C). In the lactating gland, the immunoreactive OTR was mainly detected in the ductal/glandular cell layer using both 2F8 (Figure 4D) and JV3580 (Figure 4E) antibodies, although a few myoepithelium-like cells also appeared to be stained (Figure 4D,E). Neither non-immune mouse IgM nor rabbit IgG gave significant background staining in both tissues (Figure 4F).

In order to examine whether the localization of OTR immunoreactivity in the ductal/glandular epithelium was

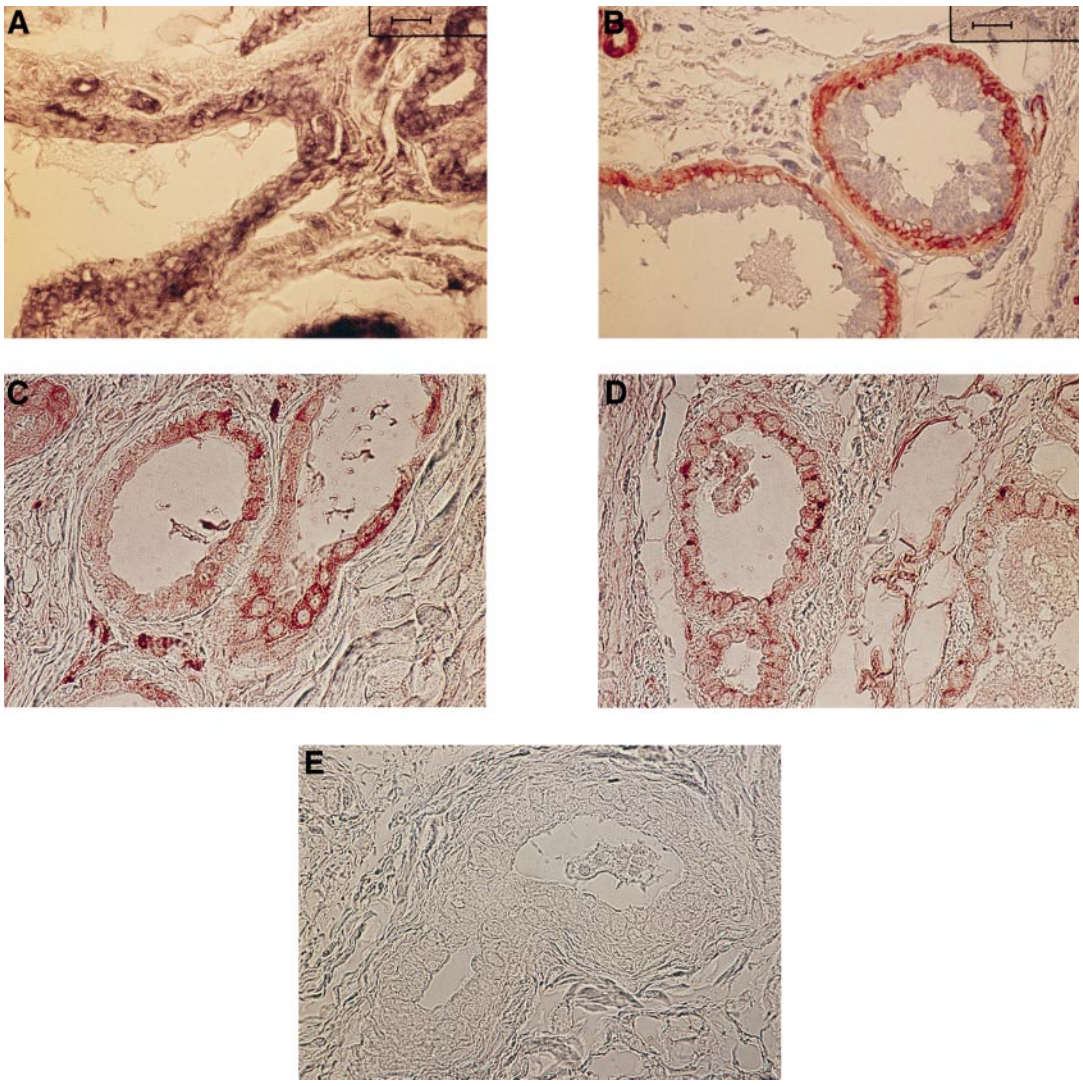


**Figure 5.** Immunohistochemistry of OTR in marmoset mammary gland. Mammary gland tissues from non-pregnant, cycling (**A**, **B**) and early pregnant (**C**–**E**) marmoset (*C.jacchus*) were frozen, and treated as described in the legend for Figure 4. (**A**, **C**) Stained with 2F8 (3 μg/ml). (**B**) Stained with anti-α-actin antibody (150 μg/ml). (**D**) Stained with JV3580 (1:3000). (**E**) Purified non-immune mouse IgM was used as a primary antibody for negative control of 2F8. In both non-pregnant and early pregnant samples, OTR immunoreactivity was localized mainly in ductal/glandular epithelial layer, as well as weakly in a few myoepithelial cells. Negative controls stained with non-immune mouse IgM for 2F8 (not shown) and non-immune rabbit IgG for JV3580 (**E**) showed no reaction in these frozen tissues. Original magnification: (**B**) ×350; (**A**, **C**–**E**) ×551. Scale bars: (**A**) = 12.6 μm; (**B**) = 20 μm.

specific to human tissue, we also examined specimens from another primate, *C.jacchus*. In both non-pregnant, cycling (Figure 5A,B) and early pregnant (Figure 5C–E) animals, OTR immunoreactivity was located mainly in the ductal/glandular epithelial layer, as well as weakly in a few myoepithelial cells, identified by α-actin staining (Figure 5B). Controls performed with non-immune IgM or IgG showed no reaction in these frozen sections (Figure 5E). In all samples, neither fat tissue nor blood vessels were immunoreactive for OTR. Taken together, all results indicated that the immunolocalization of OTR was predominantly within the ductal/glandular epithelium rather than, or in addition to, the myoepithelium.

### *Immunoelectron microscopy*

To examine the OTR localization more closely, we performed immunoelectron microscopy using non-lactating human tissue and the 2F8 antibody. In this procedure, the myoepithelium could be clearly distinguished from the ductal/glandular epithelium on the basis of morphology. As shown in Figure 6A, myoepithelial cells have restricted cytoplasm and are located directly at the basal lamina. Most immunoreactivity for OTR is localized in the ductal/glandular epithelium. Both in the basal and apical portions of the epithelium (Figure 6A,B), both the cell surface membrane and vesicles near the surface were stained by the antibody. There are several high-contrast, amorphous particles near the apical

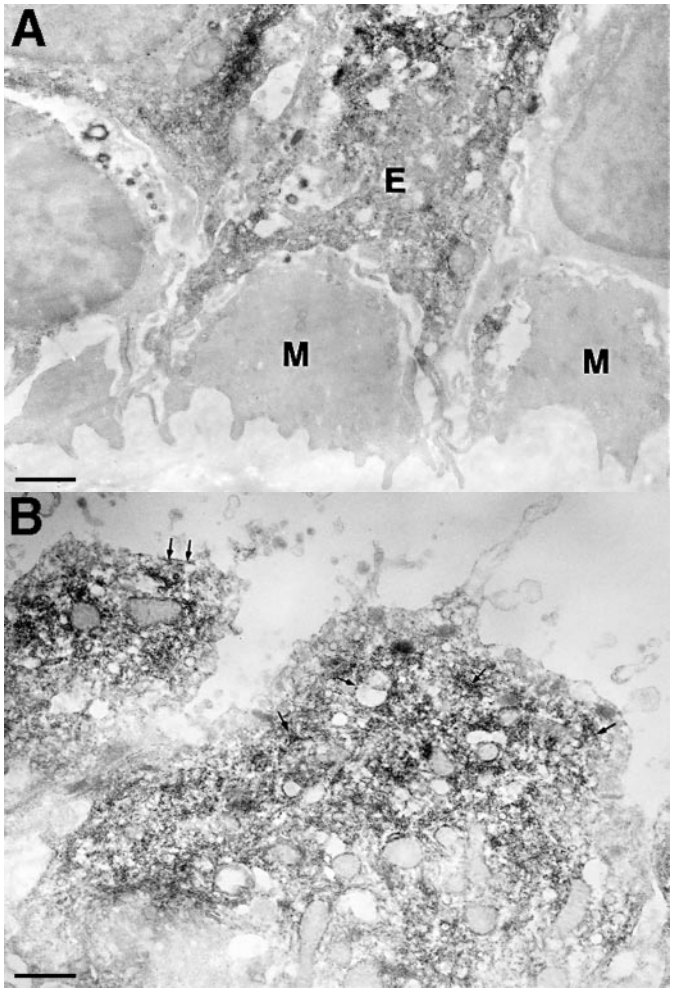


**Figure 6.** Immunoelectron microscopy of OTR in human non-lactating mammary gland. Fresh non-lactating mammary gland was fixed and frozen in OCT compound as described in the text. After blocking endogenous peroxidase activity, the section was incubated with 2F8 (100 μg/ml), then treated with peroxidase-conjugated rabbit anti-mouse immunoglobulin. The section was then developed, post-fixed in 1% glutaraldehyde, embedded in epoxy resin, sliced with an ultramicrotome and examined under an electron microscope without staining. (**A**) A field indicating myoepithelial cells (M; lower part, cells with restricted cytoplasm and attached to the basal lamina) and basal part of ductal/glandular epithelium (E). (**B**) Apical part of ductal/glandular epithelium. Most OTR immunoreactivity was localized in the cytoplasm, surface membrane and vesicles near the cell surface of ductal/glandular epithelium (arrows). Original magnification: (**A**) ×8300; (**B**) ×10 900. Scale bars: (**A**) = 1.2 μm; (**B**) = 0.9 μm.

surface membrane (Figure 6B). These appear to be lipid droplets which preferentially adsorb osmium. The staining pattern is consistent with the result obtained in immunohistochemistry, in which mainly the ductal/glandular epithelium stains for OTR, not only at the cell membrane but also within the cytoplasm.

## Discussion

Northern and Western blotting indicated that the expression level of OTR mRNA and its encoded protein were not high, even in the lactating human mammary gland, as compared with the myometrium. On the other hand, the physiological sensitivity to OT in human lactating mammary gland is very high; as little as 1 mU OT can cause a rise of the intraductal pressure measured by cannulation (Cobo *et al.*, 1967). However, the lactating gland produces an enormous amount of milk proteins and their corresponding mRNAs, hence diluting parallel changes in other gene products. It might be for this reason that an up-regulation of the OTR mRNA or its protein was not evident in the lactating gland.

The cellular localization of OTR immunoreactivity in human breast tissues was unexpected. We would have predicted that the major OTR immunoreactivity should be in the outer, myoepithelial cell layer, and that the inner cell layer might also be weakly positive by analogy with data from human breast cancer. Indeed, Bussolati *et al.* (1996) recently reported OTR immunolocalization exclusively in the myoepithelium using a novel anti-OTR antibody. Nevertheless, the results presented in the present article could be reproduced using three different antibodies, 2F8, JV3580 and 1-2 (data not shown), raised against three different epitopes, employing three different fixation protocols, and in two different species (human and marmoset). Bussolati and colleagues also established different antibodies against the N- and C-terminal regions of the OTR sequence, and examined the specificity of these antibodies by Western blotting. The reacting bands indicated similar molecular weights of 53 kDa, substantially less than the 70 kDa determined using the 2F8 antibody. The latter, however, is in better agreement with a previous report using a photoaffinity labelling procedure (Kojro *et al.*, 1991). The 2F8 antibody could also detect the massive up-regulation of the OTR protein in term myometrium as compared with non-pregnant uterus. An independent polyclonal antibody, JV3580 (Adan *et al.*, 1995) clearly showed OTR immunoreactivity to be in the myoepithelium of the rat mammary gland using frozen tissue, and both antibodies (2F8 and JV3580) recognized OTR immunoreactivity in identical cellular locations in the male reproductive tract of the marmoset (Einspanier and Ivell, 1997). It therefore seems very unlikely that the results presented here, using a combination of validated antibodies, represent an artefact of the immunohistochemical procedure.

It is evident that the myoepithelium is a contractile tissue in the breast (Linzell, 1955), and a cell-line established from human myoepithelium was able to respond to OT, measured by a rise in intracellular calcium concentration (Seitz *et al.*, 1993). This cell-line was obtained by serial subculture of primary myoepithelial cells (Hackett *et al.*, 1977). Using a similar procedure to generate a human myometrial cell-line, it was shown that there was a concurrent spontaneous up-regulation of OTR expression (Phaneuf *et al.*, 1993). It might, therefore, be possible that the OTR was originally expressed at a very low level in the primary myoepithelial cells, but became similarly up-regulated after serial subculture *in vitro*. It is also possible that the detection of low-level OTR immunoreactivity in myoepithelial cells could be more difficult than detection in the ductal/glandular epithelium. For example, in uterine samples from pregnant cows, the 2F8 antibody initially failed to detect OTR immunoreactivity in the myometrium, although this tissue shows significant ligand-binding ability,

whereas the endometrium indicated the expectedly strong immunohistochemical signals (Bathgate *et al.*, 1995). However, subsequent protocol modifications, such as those applied here, indeed revealed weak albeit positive immunostaining also in the bovine myometrium using the 2F8 antibody (R.Ivell, unpublished results). The milk ejection reflex is well described also in the human, and not only from the viewpoint of the serum OT concentration (Weitzman *et al.*, 1980; Dawood *et al.*, 1981; Johnston and Amico, 1986) but also by the direct measurement of intramammary pressure (Cobo *et al.*, 1967).

What could the function be for OT on the ductal/glandular epithelial cells? It has been shown that exogenous prostaglandin (PG) $F_{2\alpha}$ can elevate intraductal pressure in the human lactating breast *in vivo* (Cobo *et al.*, 1974). By analogy to the uterine endometrium, which secretes $PGF_{2\alpha}$ in the ruminant (Roberts *et al.*, 1976) or in pregnant human (Fuchs *et al.*, 1981), the ductal/glandular epithelium might secrete local prostaglandins in response to OT in order to facilitate myoepithelial contraction. Local PGs could also affect the mammary blood flow which itself is an important regulator of milk production (Nielsen *et al.*, 1995). In fact, it has recently been reported that Chinese hamster ovary cells transfected with recombinant OTR cDNA are able to secrete $PGE_2$ in response to OT (Jeng *et al.*, 1996). Thus, in several different tissues, OT-induced PG secretion might be a relatively common pathway for OT action and, at least in human and marmoset, the milk ejection reflex might not be a simple straightforward response of the myoepithelium to OT.

For the non-lactating mammary gland, the role of OT is poorly described. A single report indicates that OT can enhance growth and differentiation of mouse myoepithelial cells (Sapino *et al.*, 1993). It is possible, however, that this could be a response to an indirect, paracrine factor from the ductal/glandular epithelium. Several reports have shown OTR expression in human breast cancer tissues, as well as in several breast cancer cell-lines (Taylor *et al.*, 1990; Cassoni *et al.*, 1994; Ito *et al.*, 1996). However, the effect of OT on their growth is controversial. OT would appear to activate mitogen-activated protein (MAP) kinase activity (Ohmichi *et al.*, 1995), a major mediator of cell growth and differentiation. Thus, OT might also play a role in the morphogenesis and turnover of breast epithelial cells.

In conclusion, we have detected OTR expression in the human mammary gland using several different procedures. In lactating or non-lactating tissues, OTR immunoreactivity was localized predominantly in the ductal/glandular epithelium as well as, or rather than, in the myoepithelium. These results were confirmed also in the mammary tissue of cycling and pregnant marmoset monkeys. Together, these data highlight the potential functional importance of the ductal/glandular epithelium as a target tissue for OT, a function which should now be investigated in greater depth.

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