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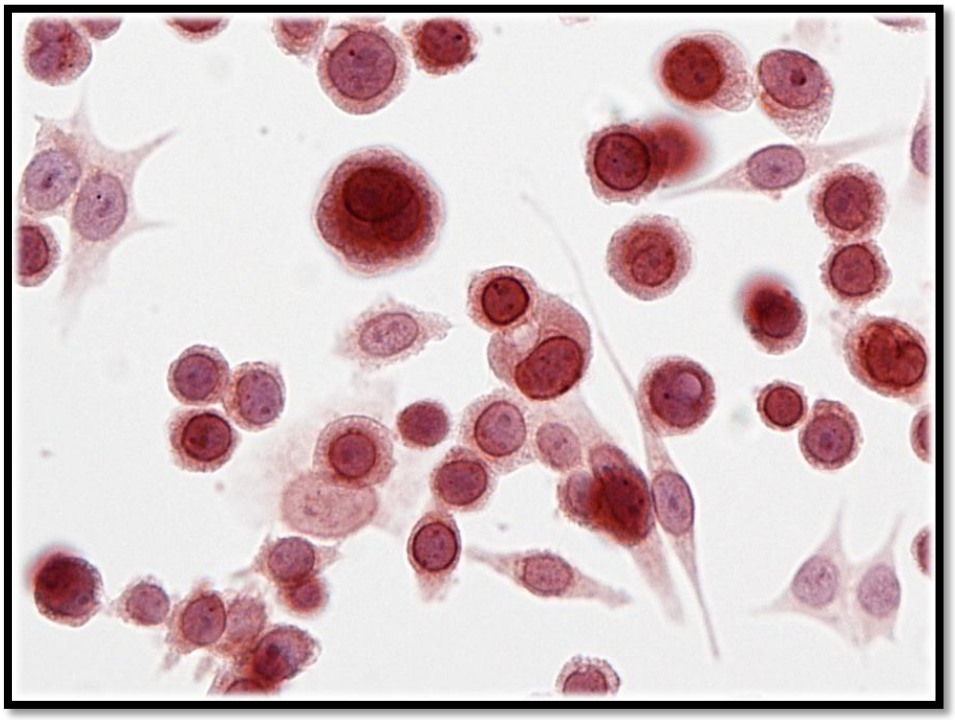

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Betydelsen av oxytocin vid juvertumörer hos hund

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Key Words: canine mammary carcinoma cells, oxytocin, oxytocin receptors

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ABSTRACT

Oxytocin is a hormone which plays a crucial role in many reproductive and behavioural functions. It affects many organs and the classical peripheral targets are the mammary glands during lactation and uterus during labour. Oxytocin receptors have recently been described in a variety of normal tissues and primary cell cultures, but also in neoplastic tissues and established neoplastic cell lines, as breast cancer cell lines. The signalling pathways and biological effects of the oxytocin/oxytocin receptor system seem to depend on species, type of tissue, physiological versus neoplastic state and receptor location within the cell membrane.

The aim of this project was to find out if the cell proliferation in the canine mammary carcinoma cell line CMT-U27 was affected upon stimulation of oxytocin, and to investigate the presence of oxytocin receptors in these cells. The cell proliferation was examined by using an ELISA-kit, where the absorbance measured is relative to the amount of living cells. Immunocytochemistry was used to detect possible oxytocin receptors.

The results showed that there was significant inhibition of the cell proliferation with the addition of oxytocin. In three different assays at least one cell concentration showed significant inhibition of the cell growth. The immunocytochemistry analysis showed the presence of oxytocin receptors and based on the location of the receptors it seemed to be at least two groups within the cell line. Some of the cells show marked staining in the nuclear membrane and some seemed to be unstained or had weak staining in the cell membrane or cytoplasm.

Since the location of the oxytocin receptors show that they have been activated, further studies can find out which of the cells that produce oxytocin, and if the possible synthesis leads to paracrine or autocrine stimulation.

Keywords: canine mammary carcinoma cells, oxytocin, oxytocin receptors

SAMMANFATTNING

Oxytocin är ett hormon som spelar en viktig roll i många funktioner för reproduktion och beteende. Det påverkar många organ och vävnader, de klassiska perifera målorganen är mjölkkörtlarna vid laktation och livmodern under förlossning. Oxytocinreceptorer har nyligen beskrivits i en mängd normala vävnader och primära cellkulturer, men även i neoplastisk vävnad och etablerade neoplastiska cellinjer, som cellinjer från bröstcancertumörer. Signalvägarna och de biologiska effekterna av oxytocin/oxytocinreceptor-systemet verkar bero på art, vävnadstyp, fysiologisk kontra neoplastisk status och receptorns plats i cellmembranet.

Syftet med det här projektet var att ta reda på om celltillväxten i cellinjen CMT-U27 från juvercarcinom hos hund påverkades av oxytocinstimulering samt att undersöka förekomsten av oxytocinreceptorer i dessa celler. Celltillväxten analyserades med hjälp av ett ELISA-kit, där den uppmätta absorbansen är relativ mot antalet levande celler. Immunocytokemi användes för att undersöka förekomsten av oxytocinreceptorer.

Resultaten visade på signifikant inhibering av celltillväxten vid tillsats av oxytocin. I tre olika analysomgångar visade minst en cellkoncentration på signifikant inhibering av celltillväxten. Immunocytokemianalysen påvisade förekomst av oxytocinreceptorer och baserat på receptorernas placering verkade det finnas minst två grupper inom cellinjen. Några celler var starkt infärgade i kärnmembranet och några verkade helt ofärgade eller hade svagare infärgning i cellmembranet eller cytoplasman.

Eftersom oxytocinreceptorernas position visar att de blivit aktiverade kan ytterligare studier ta reda på vilka celler som producerar oxytocin, samt om det leder till parakin eller autokrin stimulering.

Nyckelord: juvercarcinomceller hos hund, oxytocin, oxytocinreceptorer

INTRODUCTION

Mammary tumours in dogs are the second most common group of neoplasms, following skin tumours (McCarthy *et al.*, 2003). In areas where early ovariectomy of bitches is not routinely done, the incidence is high. For intact dogs, middle-aged bitches (nine to eleven years) are primarily affected, and an increase in incidence begins at approximately six years of age (Alenza *et al.*, 2000). In the case of breast cancer in human there is a correlation between the age of a woman's first child and the chance of developing breast cancer. Earlier childbirth gives a lower chance of developing breast cancer. It is thought that the first full-term pregnancy changes the state of differentiation of the cells in the breast, which alters their subsequent hormonal responses (Alberts *et al.*, 2002).

Oxytocin receptors (OTRs) have been detected in several human cancer tissues and cell lines, including human breast cancer. OTRs were found in over 80% of the breast cancers, but no apparent relationship between OTR expression and other clinical variables was found (Zingg & Laporte, 2003). Cassoni *et al.* (2006) has detected OTRs in contractile myoepithelial cells, in primary breast carcinomas and in breast carcinoma cell lines. Oxytocin (OT) regulates cell proliferation in breast carcinoma cells via OTRs. The study shows local synthesis of OT within the human mammary gland under both normal and neoplastic conditions. Normal myoepithelial cells were able to synthesize and secrete OT and different carcinoma cell lines were able to synthesize and release OT. Both normal epithelial and myoepithelial cells contain mRNA for OT, but only myoepithelial cells actively produce and release OT in the culture medium. This suggests the possibility of a local autocrine loop involving myoepithelial cells, but has not yet been confirmed. In the referred study normal mammary epithelial cells did not show active synthesis and release of OT in the culture medium.

OT can have an inhibitory effect on cell growth. In studies of human breast cancer it has been found that OT also can inhibit breast cancer cell proliferation by down-regulating the mitogenic effects mediated by estrogens (Reversi *et al.*, 2006).

The aim of this project was to find out if the cell proliferation in the canine mammary carcinoma CMT-U27 cells was affected upon stimulation with OT, and to investigate the presence of OTRs in these cells.

BACKGROUND

The mammary gland is developed during life, particularly during puberty, pregnancy, and after parturition, and is not fully matured until the female has given birth to an offspring. During the foetal development the mammary gland develops the main large ducts and a nipple. In humans, the growth is isometric before puberty. With the onset of puberty (8-12 years) allometric growth of the stroma and epithelium begins. During puberty, increasing elongation and branching of the ducts creates a more extensive ductal network. The bud-like structures at the end of the ducts, which is the major site of growth, form the terminal duct lobular units. During the first half of the pregnancy intensified lobular–alveolar growth together with extension and branching of the ductal system occurs. The mammary gland growth is influenced by a number of hormones, e.g. oestrogen, progesterone, prolactin and growth hormone. By mid-pregnancy, there is some secretory development and in the last trimester, there is a further increase in lobular size (Geddes, 2007).

An essential role for OT is milk ejection from the mammary gland. It is critical for successful lactation, because only small volumes of milk can be removed from the lactating breast before milk ejection (Geddes, 2007). The release of milk is triggered by stimulation of the nipple, which generates sensory impulses that are transmitted via nervous impulses to the secretory oxytocinergic neurons in the hypothalamus. This stimulates the posterior pituitary gland to release OT into the bloodstream. OT causes contraction of the myoepithelial cells surrounding the alveoli, forcing milk into the ducts. In humans, it takes 30 s to 1 min from stimulation to milk ejection. This process continues to function until weaning (Geddes, 2007; Gimpl & Fahrenholz, 2001).

Experiments with OT-deficient mice, showed no obvious deficits in fertility, gestation, or parturition. The maternal behaviour both pre- and postpartum was normal as the females built a typical nest and the offspring was cleaned and present in the nest after delivery. Even though the maternal behaviour seemed normal, the offspring of the OT-deficient females died within 24 hours after delivery. Histological analysis of the breast tissue confirmed that there were no deficits in milk production. Postpartum injections of OT given to the OT-deficient females produced enough milk ejection to keep the offspring alive as long as the injections continued. This showed that OT is required for milk ejection (Nishimori *et al.*, 1996).

OT was the first peptide hormone to have its structure determined, and in 1953 the sequence was published. OT is a cyclic peptide consisting of nine amino acid peptide with a disulfide bond creating a ring structure. The hormone is shown to be a member of an old group of nine amino acid peptides of similar structure, with ancestral forms found in various vertebrates and even invertebrates, but OT is almost exclusively mammalian. Since it is involved in two particularly mammalian aspects of reproduction; uterine contraction during labour and milk ejection during nursing, this may not be a coincidence (Insel et al., 1997; Gimpl & Fahrenholz, 2001).

Like most neuropeptides, OT can exert both peripheral and central action. OT is synthesized in the hypothalamus, transported to the posterior pituitary and released to the blood stream in response to cervical dilation or suckling. The hormone also synthesizes in peripheral tissues

as the testis, ovary, uterus and placenta. Even breast carcinomas have been shown to produce OT. The main role for OT is in reproduction, as it regulates mating, pair bonding, pup care, nursing, learning, and memory. Research on OT-deficient mice has shown that OT may not be essential for all these functions, but the hormone is necessary for milk ejection (Insel *et al.*, 1997; Nishimori *et al.*, 1996; Reversi *et al.*, 2005).

Purified myoepithelial cells can express OT mRNA and use it actively to synthesize OT. The local source of peptide within the normal breast demonstrates the existence of a local autocrine/paracrine loop. This could participate in the differentiation process of the mammary gland at different steps of evolution. Also, since OT stimulates endothelial cell growth, the OT production in the mammary lobules may be involved in the vascular remodelling of the tissue (Reversi *et al.*, 2005).

In the human genome only one OTR gene has been found, and it is believed that all of the central and peripheral actions caused by OT are due to activation of this receptor subtype. In the mammary gland the mRNA of OTR has been found in the myoepithelial cells surrounding the alveoli and ducts (Reversi *et al.*, 2005).

In a study of mammary gland and uterine OTR gene expression during gestation, parturition, and lactation, it has been confirmed that OTR mRNA levels are differently regulated in the two tissues. Uterine OTRs are up-regulated before parturition and then down-regulated, whereas myoepithelial OTRs gradually increase during gestation and remain up-regulated during the lactation (Breton *et al.*, 2001; Reversi *et al.*, 2005).

The OTR contains seven transmembrane helices, which are highly conserved among the G protein-coupled receptor (GPCR) family (Gimpl *et al.*, 2001). The OTR binds to different G-proteins dependent on the cell conditions. This phenomenon is called receptor-G protein promiscuity. This enables OT to activate multiple responses at the same time in the same cell (Strunecká *et al.*, 2009).

G protein-coupled receptors (GPCRs) make up a great family of cell surface receptors with over 800 members. They are characterized by the presence of seven transmembrane α-helical segments separated by alternating intracellular and extracellular loop regions (Figure 1) (Jalink & Moolenaar, 2010; Rosenbaum *et al.*, 2009). Two requirements have to be fulfilled for a protein to be classified as a GPCR, the first one being seven sequence stretches of 25 to 35 succesive residues, believed to represent the seven α-helices, and the second requirement is the receptor's ability to interact with a G-protein. The functional couplings of the GPCRs are gr eatly diverse as they have a number of alternative signalling pathways, interacting directly with a number of other proteins (Fredriksson *et al.*, 2003).

The receptor family can be divided into five subfamilies on the basis of their structure and sequence similarity; rhodopsin (family A), secretin (family B), glutamate (family C), adhesion and Frizzled/Taste2 (Fredriksson *et al.*, 2003; Rosenbaum *et al.*, 2002). Although they have these similarities, individual GPCRs have unique combinations of signal-transduction activities involving multiple G-protein subtypes, as well as G-protein-independent signalling pathways and complex regulatory processes (Rosenbaum *et al.*, 2002).

The ligands for the GPCRs are very diverse: peptides, ions, lipids, nucleotids, proteins, ions, organic odorants, and even photons are able to mediate their message through these proteins (Fredriksson *et al.*, 2003). Because of this, the GPCRs are among the essential nodes of communication between the internal and external environments of cells. GPCRs connect the binding of agonists to the activation of specific heterotrimeric GTP-binding proteins (G-proteins), which leads to the modulation of downstream effector proteins (Rosenbaum *et al.*, 2002). GCPRs regulate the activity of a separate plasma-membrane-bound target protein, which can be either an enzyme or an ion channel, indirectly. A G protein, is mediating the interaction between the receptor and this target protein. If the target protein is an enzyme its activation can change the concentration of one or more intracellular mediators, and if the target protein is an ion channel, it can change the ion permeability of the plasma membrane. The intracellular mediators affected act in turn to alter the behaviour of yet other signalling proteins in the cell (Alberts *et al.*, 2002).

When stimulated by a ligand, GPCRs activate their respective G-protein-effector pathways instantly while they are recruited to specialized domains at the plasma membrane. Most GPCRs are desensitized after ligand stimulation (Jalink & Moolenaar, 2010). The process of desensitization involves multiple pathways, including phosphorylation of the receptor's cytoplasmic tail, arrestin-mediated internalization into endosomes, receptor recycling and lysosomal degradation (Pierce *et al.*, 2010).

The receptors undergo conformational changes that enable them to activate trimeric G-proteins when extracellular signalling molecules bind to them (Alberts *et al.*, 2002). Model GPCRs have shown that switching from inactive to active conformation is associated with a change in the relative orientation of transmembrane domains 3 and 6, which then unmasks G-protein binding sites (Gimpl *et al.*, 2001). The G-proteins are attached to the cytoplasmic face of the plasma membrane, functionally coupling the receptors to enzymes or ion channels in this membrane. There are various types of G-proteins, each specific for a particular set of serpentine receptors and for a particular set of downstream target proteins in the plasma membrane. All have similar structure, however, and they operate in a similar way (Alberts *et al.*, 2002).

The G-protein consists of three subunits (α, β and γ). Each of these subunits is a member of a gene family. The G-proteins are often referred to by their α-subunits. Like so, the G_s heterotrimeric complex contains $G\alpha_s$, G_q contains $G\alpha_q$ and so on. Four distinct α-subunit subfamilies are known: G_s-proteins interacts with adenylyl cyclase and stimulates the production of cyclic adenosine monophosphate (cAMP); G_i-proteins couple to inhibition of adenylyl cyclase and thereby production of cAMP, and also to activation of G-protein-coupled inwardly rectifying potassium (GIRK) channels; G_q-proteins couple to the activation of phospholipase Cβ; and G_{12}-protein couple to the activation of Rho guanine-nucleotide exchange factors (GEFs) (Pierce *et al.*, 2010; Strunecká *et al.*, 2009).The α-subunit is responsible for GTP and GDP binding and for GTP hydrolysis. When unstimulated the α-subunit has GDP bound and the G-protein is inactive. The α-subunit releases the bound GDP, which allows GTP to bind in its place, when stimulated by an active receptor. With this exchange, the heterotrimer dissociates into two activated components; an α-subunit and a βγ-

complex. The conformational change caused by GTP binding affects the surface of the α-subunit associated with the βγ-complex in the heterotrimer in two ways. It causes the release of the βγ-complex, and makes the α-subunit adopt a new shape that allows interaction with its target proteins. The βγ-complex does not undergo any conformational changes, but its surface is now available to interact with a second set of target proteins (Alberts *et al.*, 2002).

The superfamily of GPCRs is one of the largest families of proteins in the mammalian genome. The OTR belongs to the rhodopsin family, which is the largest and most diverse family within the superfamily. Four groups have been found in the rhodopsin family; OT belongs to the β-group. This group includes 36 receptors; all known ligands to these receptors are peptides. The closest neighbours to the OTR in the phylogeny relationship are the three arginine vasopressin receptors (Fredriksson *et al.*, 2003). In this family all except the V_2 receptor are coupled mainly to $G_{q/11}$ and activate phospholipase C (PLC) in response to agonist binding (Zingg & Laporte, 2003). OT can bind to two of the vasopressin receptors, V1a and V1b, although with low affinity. In particular tissues or under certain circumstances the actions of OT can be mediated by these vasopressin receptors (Reversi *et al.*, 2005). The OTR also couples to G_s and G_i. Several intracellular signalling pathways are activated via G_q (Viero *et al.*, 2010).

Only one OTR type exists, and can be found in as various places as the pituitary, kidney, testes, ovary and mammary glands (Zingg, H., 1996). The regulation of OTR expression is tissue-specific and the OTR expression can be up- or downregulated, unlike many other GPCRs. Examples are the uterus where the expression of OTR is upregulated during gestation which leads to a strong increase in uterus sensitivity towards OT. After parturition, the expression of OTR decreases rapidly in the uterine whereas OTR expression in the mammary gland remains raised throughout the lactation period. Since the OTR expression is tissue-specific regulated it enables circulating OT to switch its target organs and induce uterine contraction during parturition and milk ejection during lactation (Zingg & Laporte, 2003).

The cyclic part of the OT molecule is lodged in the upper one-third of the receptor-binding pocket and interacts with transmembrane domains 3, 4 and 6 when OT binds to the OTR (Figure 1 [1]). The linear C-terminal part of the OT molecule remains closer to the surface and interacts with the N-terminal domain and the first extracellular loop of the OTR (Zingg & Laporte, 2003; Postina *et al.*, 1996).

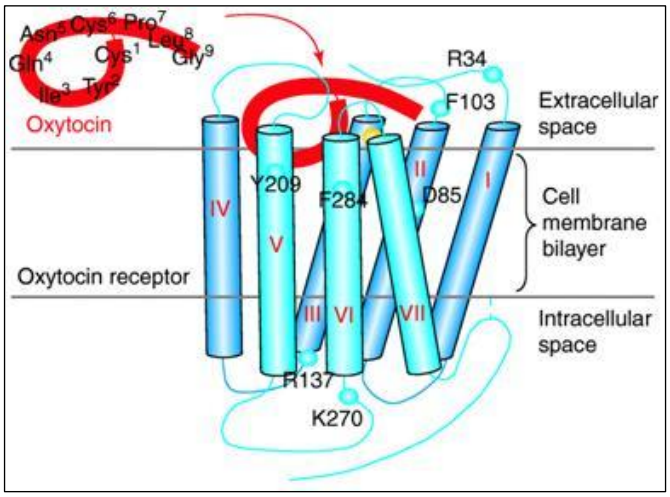

Figure 1. Schematic model of the structure of the oxytocin receptor and its interaction with the ligand (Zingg & Laporte, 2003)[1].

[1] Figure 1: Reprinted from Trends in Endocrinology and Metabolism 14 (5), Zingg, H. & Laporte, S., The oxytocin receptor, 222-227, Copyright (2003), with permission from Elsevier.

Evidence is provided for a specific contact site between an OT antagonist and residues 114-116 located at transmembrane domain 3, close to the extracellular surface (Breton *et al.*, 2001).

Oxytocin receptor signalling

OTR is able to couple to different G-proteins which give stimulation of various signalling cascades (Figure 3). Depending on the G-protein, OTR can give rise to opposite effects on the same cellular function (Strunecká *et al.*, 2009).

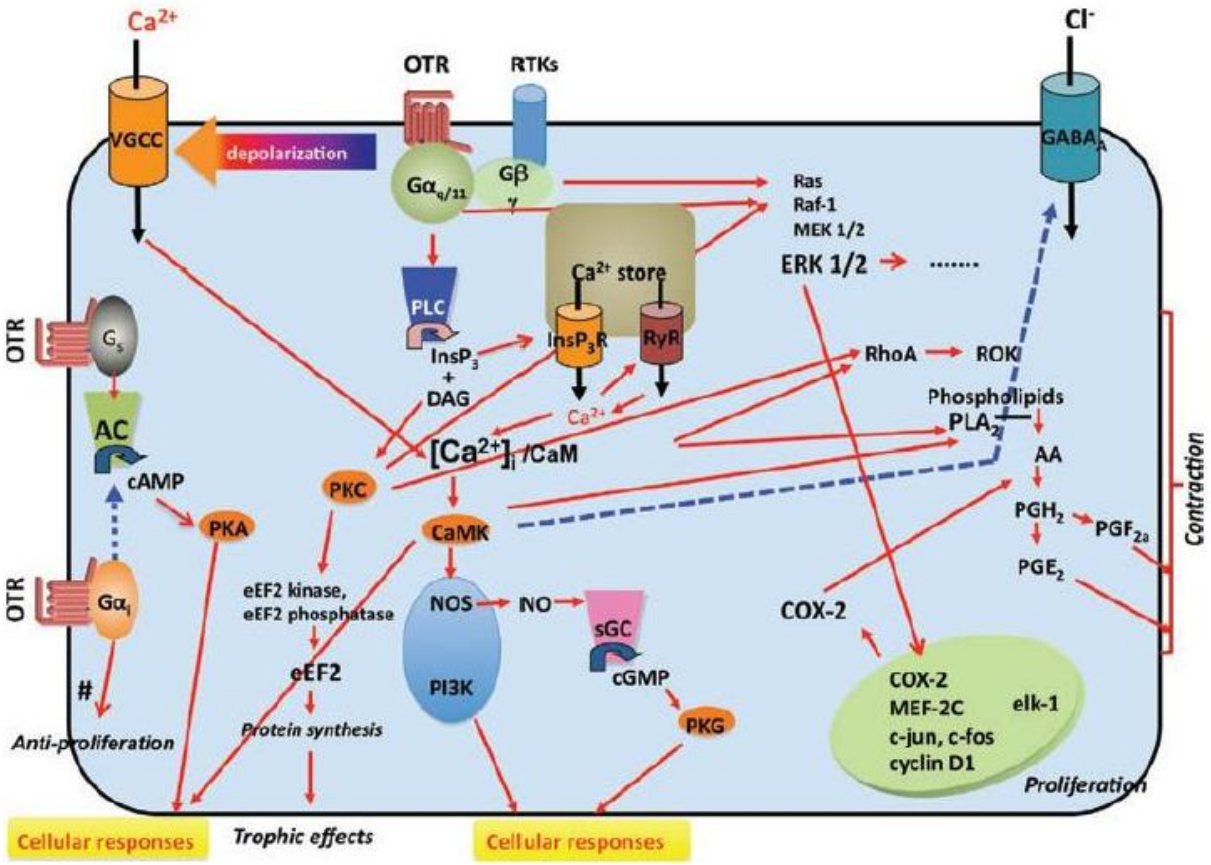

Figure 2. Schematic diagram of oxytocin receptor (OTR)-linked signalling pathways (Viero et al., 2010)[2].

OTR located on smooth muscle cells, such as uterine myometrial cells or mammary gland myoepithelial cells, induces contraction upon activation, which is triggered by an increase in intracellular Ca^{2+}. A $G\alpha_{q/11}$-mediated stimulation of PLC activity is involved in the increase in intracellular Ca^{2+} (Zingg & Laporte, 2003). Contractions induced by OT are also mediated via the activation of the Rho kinase pathway. The proliferative effects of OT appear to be G_q-linked and are probably involving mitogen-activated protein kinase (MAPK) activation, leading to c-fos and c-jun induction (Viero *et al.*, 2010). Anti-proliferative effects (# in Figure 2[2]) observed in certain cell types have been reported to be G_i-mediated, dependent on

[2] Figure 2: Reprinted from CNS Neuroscience & Therapeutics 16, Viero, C., Shibuya, I., Kitamura, N., Verkhratsky, A., Fujihara, H., Katoh, A., Ueta, Y., Zingg, H., Chvatal, A., Sykova, E. & Dayanithi, G, Oxytocin: Crossing the Bridge between Basic Science and Pharmacotherapy, 138-156, Copyright (2010), with permission from Elsevier.

epidermal growth factor receptors (EGFR) transactivation and MAPK activation via a PLC/PI3K/ cellular sarcoma tyrosine kinase (c-Src)-dependent pathway that ultimately leads to a sustained activation of the cell cycle inhibitor (Rimoldi *et al*, 2003).

The OT-mediated proliferative, trophic, contractile, and antiproliferative effects are supported by complex networks of signalling pathways. Which OT responses that occur in any given cell depends on the specific cell type as well as on the specific plasma membrane domains in which the receptor is located (Viero *et al*., 2010). OT may stimulate, inhibit or have no effect on cell proliferation, depending on the cell system (Guzzi *et al.*, 2002).

GPCRs are rapidly internalized and disappear from the cell surface after activation. This phenomenon also applies to the OTR. The OTR gathers with β-arrestin into defined punctuated regions of the plasma membrane, which suggests that OTRs are targeted into clathrin-coated pits for internalization. Internalization of the receptor and β–arrestin into large endocytic vesicles is induced by prolonged stimulation of the OTR (Zingg & Laporte, 2003).

Localization of human OTR in caveolin-1 enriched microdomains radically alters its regulatory effects on cell growth. A study has shown that OT inhibits cell proliferation when most of the OTRs are excluded from caveolin-enriched domains, but when OTRs are targeted to caveolin-enriched domains after being fused to caveolin-2, OT has a strong mitogenic effect. This suggests that the location of the receptor in the cell membrane may favour coupling to different G protein-mediated signalling pathways. Also the fraction of OTRs residing in caveolae domains may influence the proliferative or anti-proliferative effects of OT (Gimpl *et al.*, 2008; Guzzi *et al.*, 2002).

Since OTRs have the ability to couple to several G-proteins that exhibits opposite effects, the definition of "agonist" and "antagonist" is difficult to make. All OTR ligands presumably have the potential to stimulate dual signalling responses. Therefore, an "agonist" or "antagonist" should be defined based on e.g. cell type, stage of development, or phosphorylation level (Viero *et al*., 2010).

A study of human lactating and non-lactating mammary glands showed that OTR immunoreactivity was localized in the ductal and/or glandular epithelium rather than the myoepithelial as was previously thought. Both lactating and non-lactating tissue showed the same staining pattern (Kimura *et al.*, 1998). Another study showed that the major OTR immunoreactivity was localized in the outer, myoepithelial cell layer (Bussolati *et al.*, 1996).

A recent study shows that OTR localizes to several compartments within nuclei of cells derived from neoplastic breast epithelium (MCF7). An OTR-GFP plasmid, with green fluorescence protein (GFP) at the carboxyl terminus, was created, and the functionality was confirmed by cytological relocalization of the GFP following OT-treatment of cells. In the MCF7 cells OTR-GFP was translocated from cell membrane / cytoplasm to the nuclei after treatment with exogenous OT ($10^{-7}M$). Both internalization and nuclear localization of the OTR is strictly dependent on OT treatment of the cells, according to immunoflourescence studies in MCF7 cells. Only a few of the cytoplasmic OTRs entered the nucleus, but once

transported into the nucleus the OTR remained there regardless of the extracellular presence of OT for at least several hours (Kinsey *et al.*, 2007).

Oxytocin effects on cell proliferation and cancer

Nowadays it is known that women who have breast fed their babies run a lower risk of developing breast cancer, but the protective factors are not clearly identified. One early hypothesis states that breast cancer is caused by the action of superoxide free radicals released when acinar gland distension causes ischemia in the small blood vessels. The acinar gland distension would be relieved by OT production from nipple stimulation, causing contraction of the myoepithelial cells, aiding the active elimination of carcinogenic fluid from the breast (Murrell, T., 1995). More recently findings however, support the possibility of OT being a modulator of breast cancer progression since it may directly affect cell differentiation (Reversi *et al.*, 2005).

An early study on human breast cancer cell lines (MCF7, T47D and MDA-MB231) shows that OT plays a role in control of cell growth. The cells were treated with OT three times in three different doses ($10^{-9}M$, $10^{-8}M$, and $10^{-7}M$). $10^{-8}M$ and $10^{-7}M$ OT clearly reduced the cell proliferation rate in these three cell lines. The cells also showed a change in immunophenotype, possibly suggesting differentiation. OT has earlier been shown to induce cell differentiation in developing mouse mammary gland. This suggests that malignant cells can be compared to the small minority of undifferentiated stem cells which are detected within the population of the normal mammary gland (Cassoni *et al.*, 1994). An *in vivo* experiment shows that the results above are reproducible in living rodents. OT significantly reduced proliferation in implanted breast carcinomas (Cassoni *et al.*, 1996). Yet another study shows that cAMP increases significantly in OT treated breast carcinoma cells (MDA-MB231) while the inositol-Ca^{2+} pathway is not activated. Also, the anti-proliferative effect was inhibited by treatment of PKI (6-22) amide, a PKA inhibitor. Taken together, this indicates that the anti-proliferative effect of OT is strictly related to the cAMP-PKA pathway, in breast epithelial cells that lack contractile activity (Cassoni *et al.*, 1997).

In a study ten breast carcinoma cell lines were examined regarding the presence of OTR and OT mRNA. All the cell lines expressed OTR mRNA, but only five of ten of the cell lines expressed OT mRNA. A local OT synthesis was found in both normal myoepithelial cells and different breast carcinoma cell lines; they were able to synthesize and secrete different amounts of OT. Normal epithelial cells isolated from human breast were found to contain mRNA for OT, but they did not actively produce and release OT in the culture medium. This suggests that an unexpected autocrine loop involving myoepithelial cells may exist. OT have been proved to induce myoepithelial cell proliferation and differentiation, which means that the presence of an OT synthesis within the breast could represent a local peptide source directly available for myoepithelial cell differentiating processes (Cassoni *et al.*, 2006a).

Tumour-derived endothelial cells obtained from breast carcinomas (B-TEC) have been used to verify some of the differences between “normal” and tumour-related endothelium. Tumour-derived endothelial cells differ from normal endothelial cells because of some morphologic, functional, and structural changes. B-TEC showed the characteristics to grow and to organize

in capillary like structures. In a study, mRNA for OTR, but not for OT was found in B-TEC, and about 80% of the cells were positive for OTR in the cell membrane. Also the cell proliferation was significantly increased after addition of OT, and the effect was dose dependent as a higher concentration of OT increased cell proliferation more. As of this result it is clear that the stimulating effects of OT on cell growth and migration are not limited to "normal" endothelial cells, but also effects endothelial cells derived form neoplastic tissue, specifically B-TEC. This gives OT a possible role in angiogenetic processes under non-neoplastic (i.e., wounding processes) and neoplastic conditions (Cassoni *et al.*, 2006b). Since studies have shown that OT is synthesized and locally released by breast carcinoma cells, a local source of OT within breast cancers could be effective in regulating the neoformed vessels even at low concentrations that previously resulted biologically ineffective on the neoplastic epithelial cells themselves (Cassoni *et al.*, 1994; Cassoni *et al.*, 1996; Cassoni *et al.*, 2006a).

MATERIALS AND METHODS

Cell line

The canine mammary carcinoma cell line CMT-U27 was used in this study (Hellmén, E., 1992). The cell line was routinely cultured in modified RPMI-1640 (without L-glutamine and phenol red) (Sigma-Aldrich, St Louis, MO, USA) supplemented with 200mM L-glutamine (National Veterinary Institute, [SVA], Uppsala, Sweden), 10% foetal bovine serum (Sigma-Aldrich) and antibiotics (6 mg/ml penicillin and 5 mg/ml streptomycin [SVA]). Cells were grown in 25 cm^2 Falcon flasks in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cell passages used and time-table for the cell culturing is shown in Table 1.

Table 1. List of passage number of the cells, number of days between the assays and reseeding cells and changing cell culture medium

Assay number	Cell passage number	No. of days from reseeding to assay	No. of days from change of cell culture medium to assay
Oxytocin1	145	8	1
Oxytocin2	146	7	2
Oxytocin3	146	6	3
Oxytocin4	149	5	5
Oxytocin5	149	5	5
Oxytocin6	150	7	7

Cell proliferation

The kit used to evaluate the cell proliferation was Colorimetric Cell Viability Kit I (CCVK-I) (PromoKine, Heidelberg, DE). CCVK-I-solution in the kit contains the tetrazolium salt WST-8 that are reduced to water-soluble, orange formazan dyes by dehydrogenases present in viable cells. The absorbance of the formazan dye is proportional to the number of metabolically active cells and can be measured directly from a 96-well plate without additional processing.

To decide which cell concentrations that should be used to get a good standard curve a cell proliferation assay was performed three times before the addition of OT was done. In the first test the concentrations was ranging from 3 750 to 60 000 cells/ml, and in the two following tests the concentrations were ranging from 15 000 to 90 000 cells/ml. Cells were seeded in triplicates in 96-well plates. After incubation at 37°C for 22 hours CCVK-I was added and the plate was incubated for another two hours. The absorbance was read in a microplate reader (Infinite M1000; Tecan Group Ltd., Männedorf, CH) at 405 nm after totally 24 and 48 hours incubation.

To decide if the cell proliferation in the CMT-U27 cells was affected upon stimulation with the OT, three series of cells were seeded in triplicates in six concentrations, ranging from 15 000 to 90 000 cells/ml, in 96-well plates. $10^{-6}M$ and $10^{-7}M$ OT (Partoxin 10 IU/ml; Pharmaxim AB, Helsingborg, SE) was added after incubation at 37°C for 22 hours. CCVK-I-solution (PromoKine) was added after 20 minutes incubation with OT. The absorbance was

read in a microplate reader (Infinite M1000) at 405 nm after totally 24 and 48 hours incubation. The cell proliferation assay was repeated six times.

Three series were prepared for each plate (Figure 3); one standard series without addition of OT, one series with addition of $10^{-6}M$ OT and one series with addition of $10^{-7}M$ OT (Bogacki *et al.*, 2002; Cassoni *et al.*, 1996). For each series a set of blanks was also prepared.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	ST2	ST5	SM1_1	SM1_3	SM1_6	SM2_2	SM2_4				
B	Blank	ST3	ST5	SM1_1	SM1_4	SM1_6	SM2_2	SM2_5				
C	Blank	ST3	ST6	SM1_1	SM1_4	Blank3	SM2_2	SM2_5				
D	ST1	ST3	ST6	SM1_2	SM1_4	Blank3	SM2_3	SM2_5				
E	ST1	ST4	ST6	SM1_2	SM1_5	Blank3	SM2_3	SM2_6				
F	ST1	ST4	Blank2	SM1_2	SM1_5	SM2_1	SM2_3	SM2_6				
G	ST2	ST4	Blank2	SM1_3	SM1_5	SM2_1	SM2_4	SM2_6				
H	ST2	ST5	Blank2	SM1_3	SM1_6	SM2_1	SM2_4					

Figure 3. The plate was prepared in this manner, with all samples in triplicates.

*ST: Standard samples, SM1: Samples with addition of 10^{-6}*M *oxytocin (OT), SM2: Samples with addition of 10^{-7}*M *OT.*

The results from the microplate reader were run through MultiCalc and then statistical analysis on growth curves was carried out with GraphPad Prism software version 5.02 for Windows, using one-way analysis of variance (ANOVA, Bonferroni's Multiple Comparison Test).

Immunocytochemistry

The OTR antibody used ([A-16]: sc-34078) was a goat polyclonal anti-human antibody from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA). It has been proved to detect OTR of human, rat and mouse origin and to determine if the antibody could detect OTR of canine origin, resting canine mammary gland was used.

Sections of resting and lactating canine mammary gland tissue were used as positive controls. Five µm sections of the lactating mammary gland were cut in a cryostat at -25°C, and either stained immediately or stored at -70°C until used, in the project.

The cells were grown on cover slips for the immunocytochemistry. The cover slips were rinsed in 70% alcohol, PBS and cell culture medium (RPMI-1640; Sigma-Aldrich). The cover slips were then placed in 2.5 ml Petri dishes. The cells were diluted to appropriate concentrations (about 80% confluence is desired) and 2.5 ml cell suspension was added to each Petri dish, and incubated at 37°C overnight.

The sections and cells were fixated in methanol, and then blocked in avidin and biotin (Vector Laboratories Inc., Burlingame, CA, USA) for 15 minutes each. Normal horse serum (diluted 1:50 in 0.05 M Tris-HCl pH 7.6 (Tris-buffer)) was followed by overnight incubation of the OTR antibody (diluted in Tris-buffer) at 4°C. Negative controls were incubated with only Tris-buffer. Biotinylated anti-goat IgG (diluted 1:200 in Tris-buffer) was subsequently incubated for 30 min and then the ABC-Elite system (Vector Laboratories) was used. Nova RED or 3,3'-diaminobenzidine (Vector Laboratories) was used as chromogen for both tissue and cells. After rinsing in distilled water, the slides were counterstained with Mayer´s haematoxylin. Finally, the slides were rinsed in tap water, dehydrated and the cover slips were mounted onto slides, while the slides were covered with cover slips. The immunocytochemistry was repeated three times.

RESULTS

Cell proliferation standard curve

In each well 100 µl of the cell suspension was added, which means 375 to 6 000 cells/well for the first test. In the lowest two concentrations there were too few cells for the microplate reader to detect properly. In the two following tests the concentrations were ranging from 15 000 to 90 000 cells/ml, which means 1 500 to 9 000 cells/well, which gave two nice curves (Figure 4). The recommendations for the kit was that 1 000 to 25 000 adhesive cells/ml should be used in the assay.

Effect of oxytocin addition on cell proliferation

The aim of the cell proliferation assays was to determine if addition of OT has any effect on the cell growth. Indeed, there was a significant inhibition of the cell proliferation in four cell concentrations in three of the assays (Figure 5). In five of the six assays there seems to be inhibition in cell growth, although not significant (Figure 6). In two assays both OT concentrations inhibited the cell proliferation and only $10^{-6}M$ OT acted inhibiting in the last assay performed.

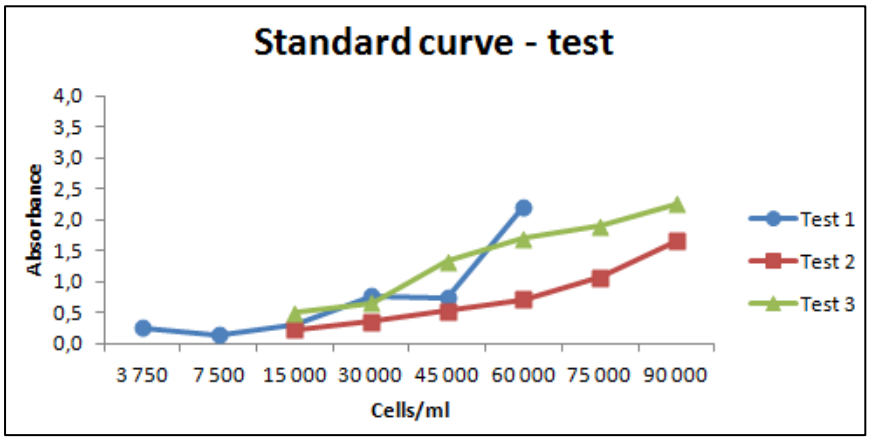

Figure 4. Assay for standard curve was performed three times to determine the most suitable cell concentrations to use in the cell proliferation assay.

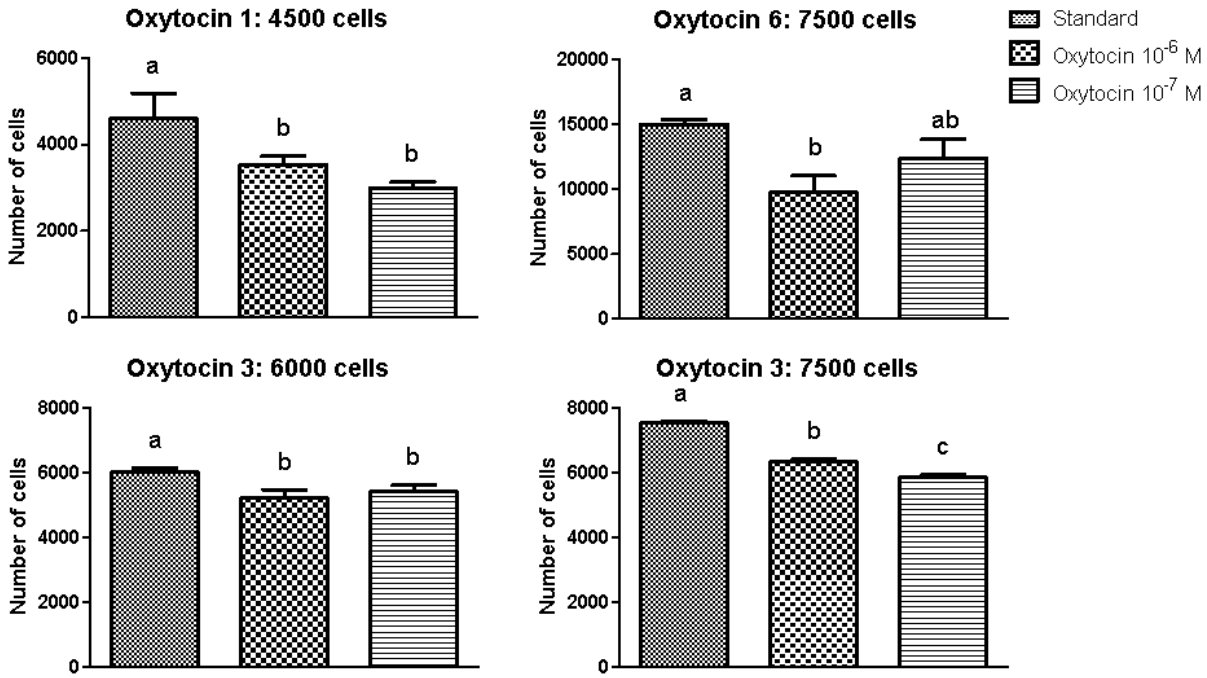

Figure 5. Diagrams of the significant inhibition of cell growth in three assays. Bars with different superscripts within each diagram are different (P < 0.05). In Oxytocin 1 significant inhibition of cell proliferation was achieved at the cell concentration of 45 000 cells/ml. In Oxytocin 3 significant inhibition of cell proliferation was achieved at the cell concentrations 60 000 cells/ml and 75 000 cells/ml. In Oxytocin 6 significant inhibition of cell proliferation was achieved at the cell concentration 75 000 cells/ml.
Note: The unit for cell concentration is cells/ml and the result was calculated as cells/well from the plate. Each well contained 100 ul cell suspension, therefore the cell concentration is ten times higher than the number of cells in the results.

In the first, third, and sixth assay a significant decrease in cell proliferation was observed, compared with the control samples (Figure 6). Both $10^{-6}M$ and $10^{-7}M$ oxytocin was significantly inhibiting the cell proliferation in the first and third assay. In the sixth assay only $10^{-6}M$ oxytocin was significantly inhibiting the cell proliferation.

The significant decrease in cell proliferation observed was; in the first assay at 45 000 cells/ml with $10^{-6}M$ OT (*, P < 0,05) and $10^{-7}M$ OT (**, P < 0,01). In the third assay at 60 000 cells/ml with $10^{-6}M$ OT (**, P < 0,01) and $10^{-7}M$ OT (*, P < 0,05) and 75 000 cells/ml with $10^{-6}M$ OT (***, P < 0,001) and $10^{-7}M$ OT (***, P < 0,001). In the third assay at 75 000 cells/ml there was also a significant difference in cell proliferation between $10^{-6}M$ and $10^{-7}M$ OT (*, P < 0,05). In the sixth assay at 75 000 cells/ml with $10^{-6}M$ OT (**, P < 0,01).

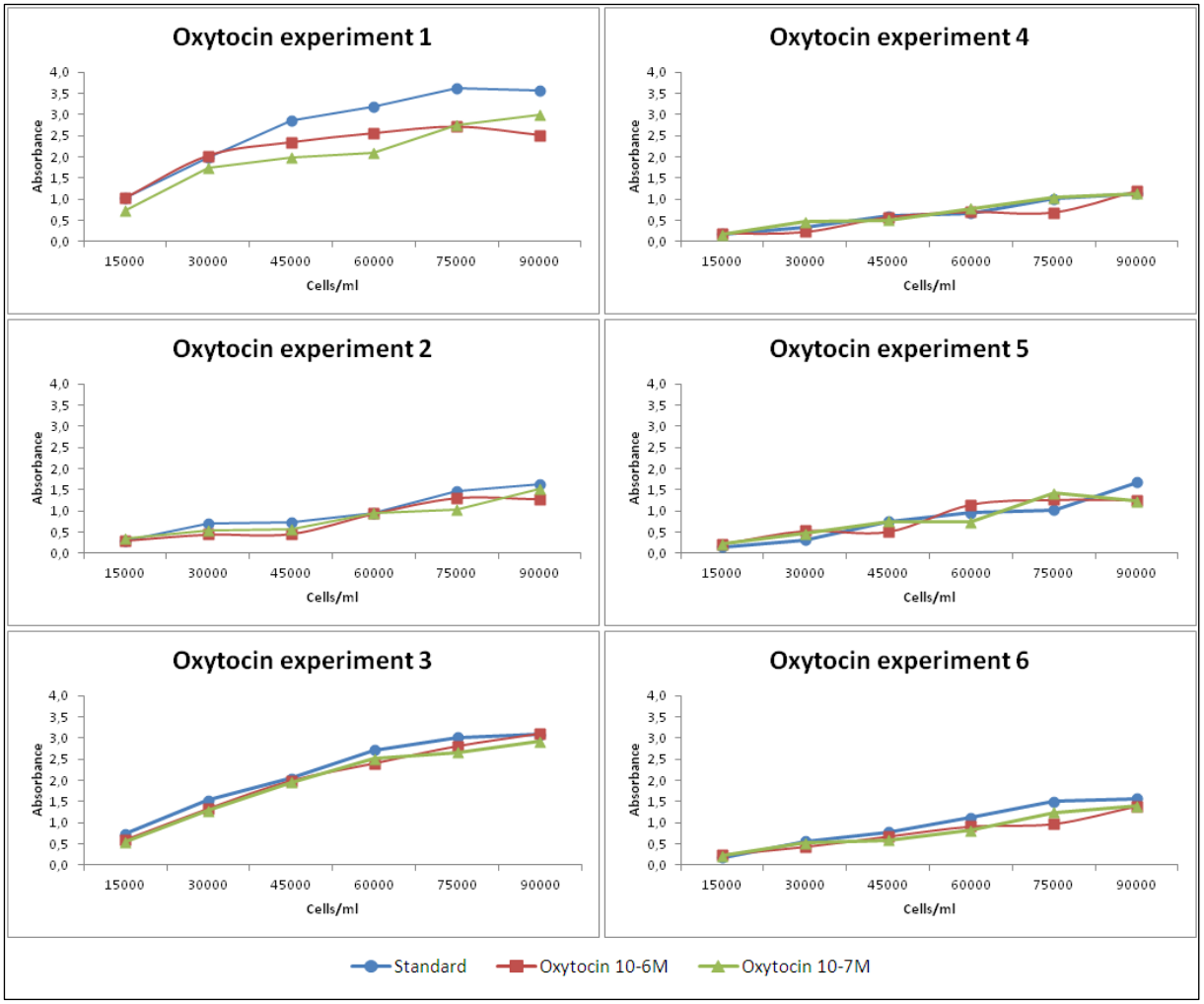

Figure 6. Cell proliferation curves, where absorbance are relative to proliferating cells.

Immunocytochemistry

Immunocytochemistry analysis on cells was repeated three times, with cell passage no. 149 twice and 150 once. The first analysis showed no staining in cells or controls, probably due to a technical problem. The two following analyses showed staining in the cell membrane and cytoplasm in some cells, in the cytoplasm, nuclear membrane and in the nucleus in some cells, and no staining at all in some cells (Figure 7).

Resting mammary gland was used as control in the first two analyses, and in the third analysis lactating mammary gland was used as control. Staining of the resting mammary gland shows that the receptors are located in the cell membrane, whereas they are located in the cytoplasm and nucleus in the lactating mammary gland (Figure 7).

The oxytocin receptor antibody was tested in four concentrations; 1:50, 1:100, 1:250 and 1:1000, where 1:200 proved to give best staining.

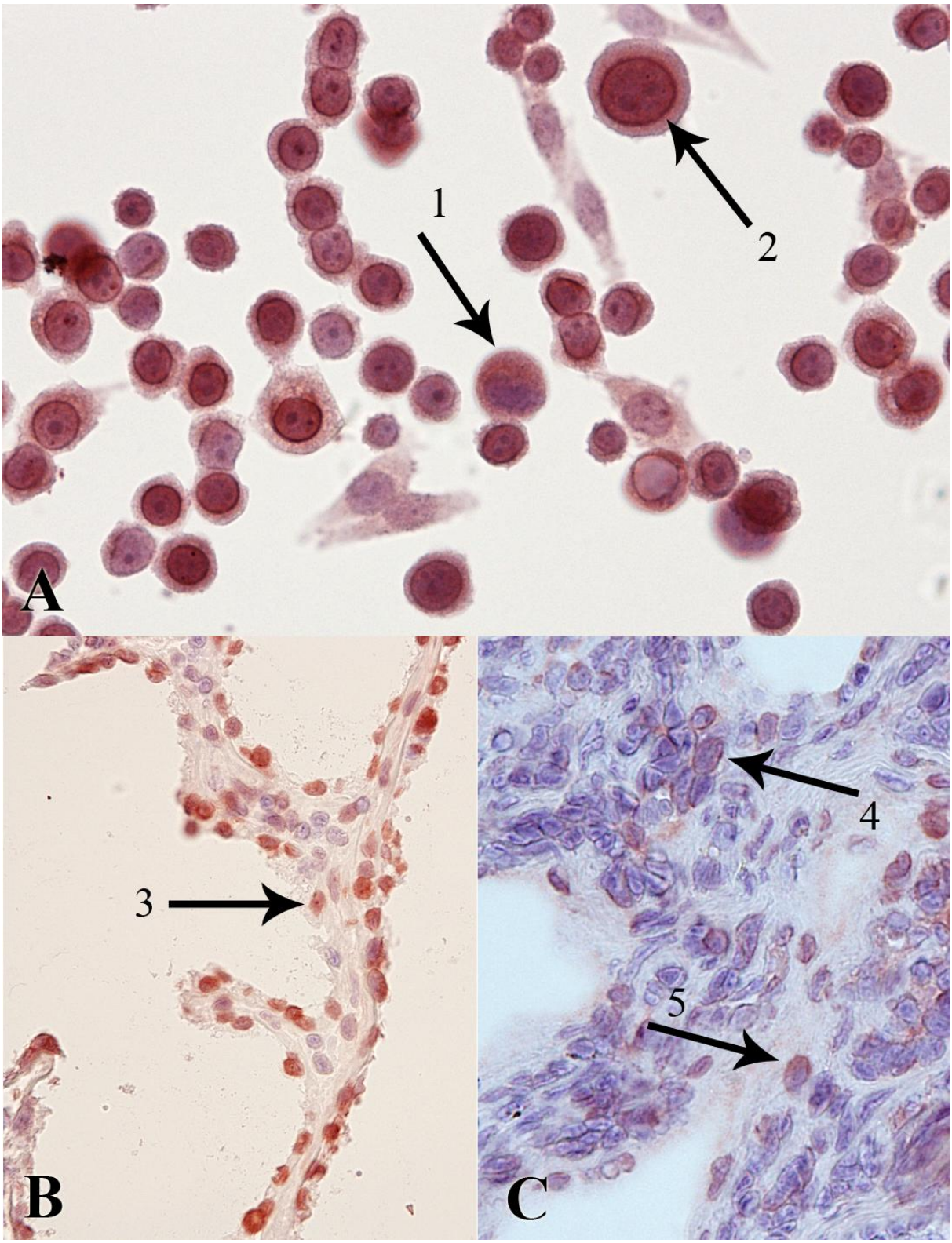

Figure 7. Immunocytochemistry of the cell line CMT-U27 and the controls – lactating and resting mammary gland demonstrating expression of the oxytocin receptor(40x objective). The cells (A) show different patterns of staining; some (1) show staining in the cytoplasm of the cell and the nucleus is unstained. Some (2) show marked staining in the nuclear membrane and the nucleus. Some elongated cells seem to be unstained. Lactating mammary gland (B) shows staining in the nuclei in many cells surrounding the alveoli (3) and some cells are weakly stained. Resting mammary gland (C) shows staining in the cell membrane (4) and the cytoplasm (5) of some cells surrounding intralobular ducts.

DISCUSSION

The aim of this project was to find out if the cell proliferation in CMT-U27 cells was affected upon stimulation with the OT, and given the results it is clear that OT has an inhibiting effect on this cell line. The presence of oxytocin receptors was also investigated and staining of the oxytocin receptor showed staining in the cell membrane, cytoplasm and also in the nuclear membrane and in the nuclei of the cells. Since there was staining in the nuclear membrane and in the nuclei, the OTRs have been activated (Kinsey *et al.*, 2007).

The cell proliferation assay was repeated six times and the proliferation curves show both similarities and differences. Two and four curves show similar cell numbers and growth rates, while the two groups compared to each other show different cell numbers and growth rates (Figure 7). This can be due to when the cell culture medium was changed in relation to the assay, when the cells last were reseeded before the assay (Table 1), or in which stadium of the cell cycle the cells are. It is difficult to say which stadium of the cell cycle the cells were since that was not a part of this project, but it is probable that a larger portion of the cells were due to divide in the first and third assay. Cell culture medium was changed at almost the same intervals, one to three days before the first three assays and five to seven days before the last three. This does not seem to correlate with the cell proliferation. The cells were reseeded at five to eight days before the assays, and the only correlation between differences in cell proliferation and the reseeding is that the first and third assay was done after an even number of days, six and eight, after the reseeding. The cell line has been shown to have a growth rate of 48h, (Hellmén, E., 1992). All cells do not divide at the same time, so it is possible that more cells in a dividing state have been collected in the first and third assay.

The statistical analysis in GraphPad Prism was performed when all assays were done and that revealed that the oxytocin had had more inhibiting effect than indicated in the curves (Figure 6). When the third assay was done it seemed that there had been no inhibition at all in the cell growth, so when performing the fourth and fifth assay two different flasks of OT was used, in order to investigate if the first flask had expired. No significant inhibition was achieved in either the fourth or the fifth assay. In fact it seems as if the cell proliferation was low over all. In the sixth assay, where the second flask of OT also was used, inhibition in cell growth was achieved.

The cell culture medium was changed to RPMI-1640 without phenol red (Sigma-Aldrich) before the cell proliferation assays were performed, because phenol red can have the same effect as oestrogen and stimulate proliferation within oestrogen-positive cells (Berthois *et al.*, 1986). The cell line CMT-U27 can grow in serum-free medium (Hellmén, E., 1992) and maybe that would have given different results when adding the oxytocin, but that was not done in this project. It could also have given a better understanding of what activated the oxytocin receptors. The cells have been tested for oxytocin synthesis (personal communication, E. Hellmén), but if the amount of oxytocin they produce is enough to activate the receptors is not clear. It is possible that some ingredients in the cell medium stimulate the activation of the oxytocin receptors. That might be of interest to find out in further studies of this cell line. Another study has shown that myoepithelial cells from normal breast tissue synthesize and release OT in cell medium. Also some breast carcinoma cell lines can

synthesize OT (Cassoni *et al.*, 2006a). An earlier study has shown that OT stimulates differentiation in myoepithelial cells within organotypic cultures of mouse mammary gland and *in vivo* in non-lactating mouse mammary gland. This means that an OT synthesis within the mammary gland could represent a local peptide source directly available for myoepithelial cell differentiation (Sapino *et al.*, 1993).

As previously shown growth in breast cancer cell lines is inhibited with the addition of OT. The canine mammary tumour cell line used in this project shows similar characteristics. In some cases a significant inhibition was reached. Both $10^{-6}M$ and $10^{-7}M$ OT have an inhibiting effect on the cell proliferation. In a previous study on breast cancer cell lines as low doses of OT as $10^{-9}M$ have been shown to have an inhibiting effect (Cassoni *et al.*, 1994).

Further studies of the cell line CMT-U27 could include another measure of the amounts of OT synthesized. If the doses are high enough to affect the cell proliferation it would be interesting to examine how it is affecting the cells. It would also be interesting to find out which of the cells that synthesize OT, and if it is those with OTRs a possible autocrine loop could be investigated.

The immunocytochemistry showed some interesting results regarding the location of the OTRs in the cells. No OT was added to the cells prior to the staining, but the cells showed staining in the cell membrane, cytoplasm and nucleus nevertheless. There were three groups of cells of which some showed staining in the cell membrane and cytoplasm, some showed staining in the nuclei and nuclear membrane, and some showed no staining at all. This cell line has been shown to produce OT itself (personal communication, E. Hellmén), and with that in mind the behaviour of the receptors may not be that strange. If the amount of OT is enough for the cells to stimulate themselves, it can induce translocation of the OTRs from the cell membrane and cytoplasm to the nuclear membrane or the nuclei (Kinsey *et al.*, 2007).

Further studies of the receptor location in the cell membrane could give an indication of which signalling pathways are used in the cells studied. It has been proposed that OT inhibits cell proliferation when OTRs in mouse mammary carcinomas are not targeted to lipid rafts enriched in caveolin-1 (Cassoni *et al.*, 1996). Also it has been observed that the cAMP-PKA pathway was involved in the antiproliferative effect in breast, endometrial, bone and nervous tumours. It seems that the intracellular cAMP levels are dose-dependent to the levels of OT added. The highest anti-proliferative effect was reached when adding $10^{-7}M$ OT, and so was the highest concentration of cAMP (Amico *et al.*, 2002; Cassoni *et al.*, 1997; Cassoni *et al.*, 1998).

Two interesting things were also noted in the control sections in the immunocytochemistry analysis. Resting and lactating mammary gland tissue were used as controls, but not simultaneously. When studying the slides microscopically, it was clear that the OTRs were located in different parts of the cell in the different tissues. Receptors are located in the cell membrane and cytoplasm in the resting mammary gland, but in lactating mammary gland receptors are also visible in the nuclear membrane and in the nucleus of the cell. This has been reported for a breast cancer cell line, where OT treatment induced translocation of the OTR from the cell membrane and cytoplasm to the nuclei (Kinsey *et al.*, 2007). Resting

mammary gland should not be a target tissue for OT, whereas the lactating mammary gland should be. This can explain the difference in the location of the receptors.

The function of OTR within mammary carcinomas is not yet understood. It is thought that the effects of OT are mediated via the OTRs, since OT has been reported to inhibit the growth of human breast cancer cells and mouse mammary carcinoma cells (both *in vitro* and *in vivo*), and to decrease the rate of tumour formation. The location of the OTR in the cell membrane, changes in the amount or binding of the OTRs may regulate mammary differentiation or neoplasia, since OT has been reported to inhibit growth and promote differentiation in human breast cancer cells (Amico *et al.*, 2002; Cassoni *et al.*, 1994; Cassoni *et al.*, 1996; Cassoni *et al.*, 2002).

CONCLUSION

OT proved to inhibit the cell proliferation in the studied CMT-U27 cell line. The immunocytochemical analyses also revealed that some of the cells have OTRs. The localization of the receptors indicates that the cells synthesize OT themselves since the receptors had been internalized although no OT was added prior to the immunocytochemistry analyses. Further analyses need to be conducted to determine which of the cells that produce the OT.

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