Oxytocin Induces Proliferation and Migration in Immortalized Human Dermal Microvascular Endothelial Cells and Human Breast Tumor-Derived Endothelial Cells

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Abstract

Oxytocin either increases or inhibits cell growth in different cell subtypes. We tested here the effect of oxytocin on cell proliferation and migration of human dermal microvascular endothelial cells (HMEC) and tumor-associated endothelial cells purified from human breast carcinomas (B-TEC). Oxytocin receptors were expressed in both cell subtypes at mRNA and protein levels. Through oxytocin receptor, oxytocin (1 nmol/L-1 μmol/L) significantly increased cell proliferation and migration in both HMEC and B-TEC, and addition of a selective oxytocin antagonist fully reverted these effects. To verify whether a different expression of adhesion molecule-related genes could be responsible for the oxytocin-induced cell migration, untreated and treated cells were compared applying a microarray technique. In HMEC, oxytocin induced the overexpression of the matrix metalloproteinase (MMP)-17, cathepsin D, and integrin β_6 genes. In B-TEC, oxytocin significantly switched on the gene profile of some MMP (MMP-11 and MMP-26) and of integrin β_6. The up-regulation of the integrin β_6 gene could be involved in the oxytocin-induced cell growth, because this subunit is known to determine activation of mitogen-activated protein kinase-extracellular signal-regulated kinase 2, which is involved in the oxytocin mitogenic effect. In B-TEC, oxytocin also increased the expression of caveolin-1 at gene and protein levels. Because oxytocin receptor localization within caveolin-1-enriched membrane domains is necessary for activation of the proliferative (instead of the inhibitory) response to oxytocin, its enhanced expression can be involved in the oxytocin-induced B-TEC growth as well. Altogether, these data indicate that oxytocin contributes to cell motility and growth in HMEC and B-TEC. (Mol Cancer Res 2006;4(6):351–60)

Introduction

Oxytocin receptors are classic G-protein-coupled receptors at seven-transmembrane domains (1), expressed in several normal tissues and tumors (reviewed in refs. 2, 3). Endothelial cells are among the various cell types expressing oxytocin receptor. Through oxytocin receptor, oxytocin has been reported to induce intracellular calcium increase and to promote cell proliferation in human umbilical vein endothelial cells (4). Similarly, myometrial microvascular endothelial cells express oxytocin receptor, although no specific oxytocin effects were shown at least on the gene expression in this endothelial cell subtype (5). Besides these models of "normal" endothelial cells, we showed previously that oxytocin receptors are present in "modified" endothelial cells, such as Kaposi's sarcoma KS-IMM cells (6). As shown in human umbilical vein endothelial cells, KS-IMM cells respond to oxytocin treatment by increasing proliferation and increasing the intracellular levels of calcium. Both effects can be blocked by a selective oxytocin antagonist, which inhibits cell proliferation when used alone (6).

Tumor-derived endothelial cells can be considered an additional subtype of endothelial cells and have been reported to differ from their normal counterpart because of some morphologic, functional, and structural changes (7-12). Tumor angiogenesis relies on the molecular and structural diversity of tumor-associated vasculature; therefore, such difference provides the bases for the development of targeted therapies (13-15). Specifically, at variance to normal endothelium, tumor-derived endothelial cells show various abnormalities in the basement membrane (7) as well as abnormal expression of surface molecules, such as matrix adhesion receptors (8). The expression profile of molecules related to matrix adhesion in endothelial cells possibly involved in angiogenetic processes within a neoplastic context is crucial. Recently, tumor-derived endothelial cells obtained from breast carcinomas (B-TEC) have been established and characterized (16): these cells showed an enhanced motility and the characteristics to grow and to organize in capillary like

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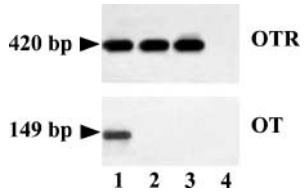

FIGURE 1. Oxytocin receptor and oxytocin mRNA detection in HMEC and B-TEC. By Southern blot, a signal correspondent to oxytocin receptor (*OTR*) was detected in both HMEC and B-TEC (*lanes 2* and *3*, respectively). On the contrary, oxytocin (*OT*) mRNA was absent in HMEC and B-TEC (*lanes 2* and *3*). T47D breast carcinoma cell line was used as a positive control for both oxytocin receptor and oxytocin (*lane 1*).

structures. They represent an interesting model to be studied to verify some of the differences between "normal" and tumor-related endothelium. Because oxytocin is known to exert either inhibiting or stimulating effects on cell proliferation in different tumors, depending on the membrane localization of oxytocin receptor (specifically in domains caveolin-rich or not; ref. 17), as well as in normal (4) and "neoplastic" (6) endothelial cells, we decided to search for a possible role of the peptide in the tumor-associated endothelial cell. Evidence of an oxytocin effect on B-TEC, which can be considered in between neoplastic cells and normal endothelium, would introduce the idea of an oxytocin role in angiogenesis-related processes.

Therefore, in the present study, we investigated (*a*) the expression of oxytocin receptor in human dermal microvascular endothelial cells (HMEC) and B-TEC, both at mRNA and protein levels, by reverse transcription-PCR and immunofluorescence; (*b*) the biological effect of oxytocin and oxytocin antagonist on their proliferation and motility by growth curves and chemotaxis experiments; (*c*) the intracellular mechanism that accompanies the activation of these functional oxytocin receptors, specifically the variations in intracellular calcium following oxytocin treatment; and (*d*) the changes in the gene profile of some matrix adhesion-related genes following oxytocin treatment by cDNA microarray.

Results

Evaluation of Oxytocin Receptor and Oxytocin mRNA in HMEC and B-TEC

Southern blot following reverse transcription-PCR procedure for oxytocin receptor mRNA detection showed a specific 391-bp intense signal in both HMEC and B-TEC (*lanes 2* and *3*) as well as in T47D breast carcinoma cell line (*lane 1*) that has been described previously to contain oxytocin receptor mRNA (ref. 4; Fig. 1).

Southern blot of oxytocin PCR products was negative for HMEC and B-TEC (*lanes 2* and *3*). Positive control T47D cells (*lane 1*; ref. 18) showed a specific oxytocin signal. Both oxytocin receptor and oxytocin signals were absent in negative controls, which included omission of reverse transferase enzyme in reverse transcription protocols (*lane 4*) and of cDNA in PCR amplifications (data not shown).

Oxytocin Receptor Detection in HMEC and B-TEC by Immunofluorescence

The reactivity of the IF3 anti-oxytocin receptor monoclonal antibody (mAb) was analyzed on HMEC and B-TEC by immunofluorescence and fluorescence-activated cell sorting. In both cell lines, immunofluorescence at confocal microscopy showed bright spots outlining the cellular membrane (Fig. 2A-C, HMEC, and Fig. 2D-F, B-TEC). By fluorescence-activated cell sorting, oxytocin receptor positivity was evident in >80% of both HMEC and B-TEC albeit with variable epitope densities (Fig. 3). No signal was detected when either the primary mAb was omitted or an unrelated mAb was used.

Oxytocin Effects on HMEC and B-TEC Proliferation

HMEC and B-TEC responded to oxytocin treatment with a significant increase in cell proliferation, which was evident at 48 hours and later. Oxytocin (1 μmol/L) produced a maximal (38%) increase of B-TEC number (data not shown) almost equivalent to that determined by a 100 nmol/L oxytocin concentration (36%). Oxytocin (10 and 1 nmol/L) still significantly increased cell proliferation although to a

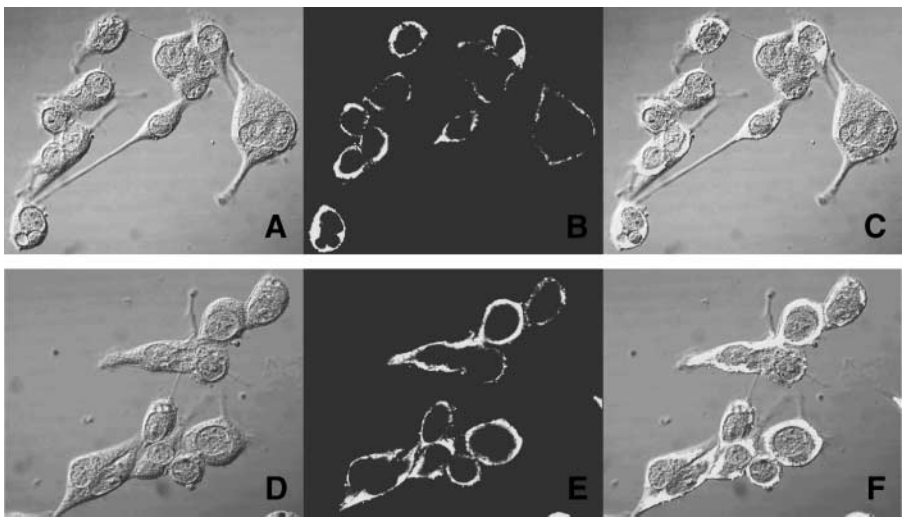

FIGURE 2. Oxytocin receptor detection in HMEC and B-TEC by immunofluorescence. By immunofluorescence, at confocal microscopy, both HMEC (**A-C**) and B-TEC (**D-F**) showed bright spots outlining the cellular membrane, deriving from immunoreactivity with the IF3 anti-oxytocin receptor monoclonal antibody and showing the presence of specific surface oxytocin-binding site.

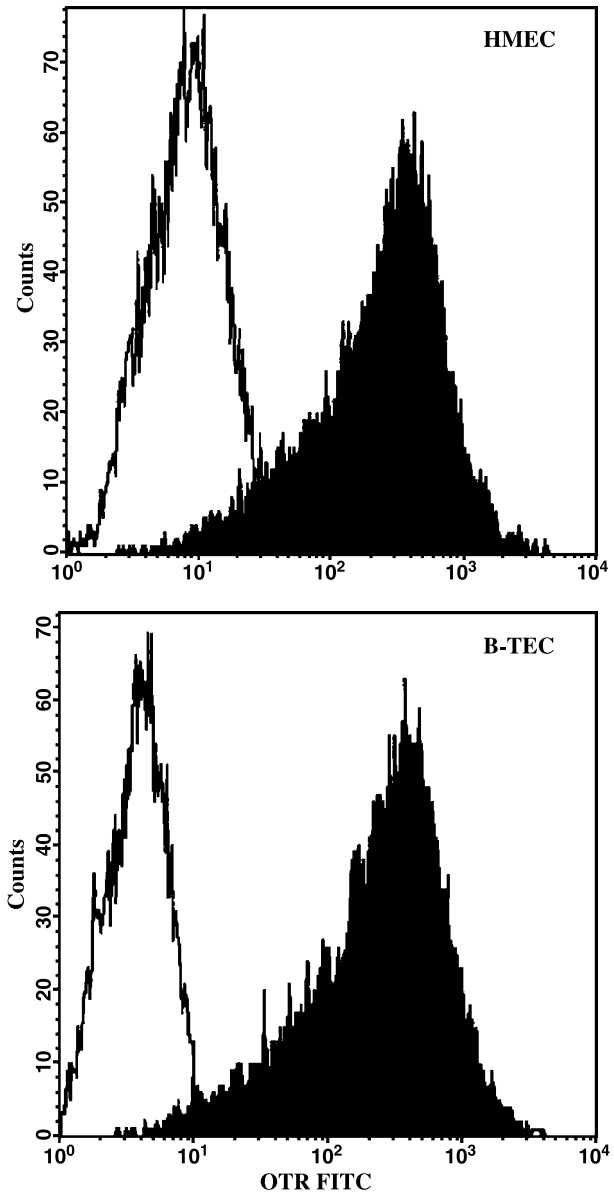

FIGURE 3. Oxytocin receptor detection in HMEC and B-TEC by flow cytometry. By flow cytometry, oxytocin receptor expression was evident in >80% of both HMEC and B-TEC. No signal was detected when an unrelated mAb was used. Each experiment was repeated at least twice, and results were reproducible. Representative experiment. Filled histogram, oxytocin receptor levels; empty histogram, unrelated antibody.

lower extent. The oxytocin effect was therefore dose dependent (1 μmol/L, 100 nmol/L, and 10 nmol/L oxytocin versus control: $P < 0.0001$ in HMEC and B-TEC; 1 nmol/L oxytocin versus control: $P < 0.0001$ in HMEC and $P < 0.001$ in B-TEC) and fully abolished by incubation with the selective oxytocin receptor antagonist, which inhibited cell proliferation when used alone (100 and 10 nmol/L oxytocin antagonist versus control: $P < 0.0001$ in HMEC and B-TEC; 1 nmol/L oxytocin antagonist versus control: $P < 0.0001$ in HMEC and $P < 0.07$ in B-TEC; Fig. 4).

In B-TEC, oxytocin effect on proliferation was also tested in serum-free conditions after 24 hours of treatment. Oxytocin (10 nmol/L) determined a 42% increase in cell proliferation ($P < 0.001$ versus control), whereas the positive controls used [10% FCS and 20 ng/mL vascular endothelial growth factor (VEGF)] determined a 50% and 58% increase in cell number, respectively ($P < 0.0001$ versus control). The effect of VEGF was significantly reduced by addition of the anti-VEGF antibody; addition of the selective oxytocin antagonist as well as addition of the calcium chelant EGTA significantly reduced the oxytocin effect (Fig. 5).

Oxytocin-Induced HMEC and B-TEC Migration

Cell motility of HMEC and B-TEC was studied by directional migration assay in Boyden chamber. Oxytocin (10 and 1 nmol/L) induced a significant chemotaxis. Preincubation with oxytocin antagonist completely inhibited the chemotactic effect of oxytocin, thus providing evidence for the receptor dependency of such effect. VEGF was used as a positive stimulus and determined a chemotactic effect that did not significantly differ from the 10 nmol/L oxytocin effect.

Table 1 shows the number of cells in the different chemotaxis experiments. Numbers are mean ± SD of a representative experiment done in triplicate; in each experiment, the cells were counted by two independent investigators. Twelve hours of 10 nmol/L oxytocin incubation determined a 15- and 25-fold increase, respectively, in the number of HMEC and B-TEC, which moved to the lower compartment, compared with control. Preincubation with the selective oxytocin antagonist (10 nmol/L) fully abolished the chemotactic effect of oxytocin, thus providing evidence for the receptor dependency of this effect. Oxytocin (1 nmol/L) still significantly promoted HMEC and B-TEC migration although to a minor extent (8- and 17-fold) than 10 nmol/L oxytocin. The positive chemotactic stimulus used (20 ng/mL VEGF) induced a 18- and 23-fold increase in the number of HMEC and B-TEC migrated cells, respectively.

Effect of Oxytocin Treatment on Intracellular Free Calcium Concentration in HMEC and B-TEC

Application of 1 or 100 nmol/L oxytocin induced a slow and persistent increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) in both B-TEC and HMEC (Fig. 6A-D). The percentage of responsive cells was significantly higher in B-TEC than in HMEC [24 of 28 (85%) and 7 of 24 (29%) cells, respectively]. Moreover, the amplitude of the calcium increase in responsive cells was significantly higher in B-TEC than in HMEC ($\Delta F/F_0 = 0.3 \pm 0.08$ and 0.15 ± 0.03, respectively).

Application of oxytocin antagonist (100 nmol/L) significantly inhibited the response triggered by 100 nmol/L oxytocin in both cell types (Fig. 6B and D).

Gene Array Analysis

The expression profile of adhesion related genes was analyzed by gene array technique under basal conditions and following oxytocin 10 nmol/L treatment. Densitometric analysis was carried out in all the membranes; only transcripts

displaying at least a 2-fold overexpression or underexpression were considered to be informative. Oxytocin treatment determined a significant increase in the expression of the following genes: in HMEC, cathepsin D, integrin β_6, and matrix metalloproteinase (MMP)-17; in B-TEC, MMP-11, MMP-26, laminin B, integrin β_6, and caveolin-1. The ratios of gene expression between untreated and oxytocin-treated cells are reported in Table 2. Fig. 7 shows the pattern of intensity in untreated and oxytocin-treated cells, and some of the genes mainly modified following oxytocin-treatment are highlighted.

The increase in caveolin-1 expression in B-TEC following 10 nmol/L oxytocin treatment was also shown at protein level by flow cytometry (Fig. 8).

Discussion

In the present study, we show that (*a*) oxytocin promotes cell proliferation and migration in endothelial cells, both "normal" (immortalized, HMEC) and tumor-derived from breast carcinomas (B-TEC); (*b*) activation of oxytocin receptors is coupled to a significant increase in the intracellular levels of calcium in both HMEC and B-TEC; (*c*) the chemotactic effect of oxytocin is accompanied by the switch on of several adhesion molecule-related genes, which differ in HMEC or B-TEC; and (*d*) in B-TEC, oxytocin also induce the overexpression of caveolin-1 (gene and protein), possibly facilitating the oxytocin receptor location in caveolin-1-enriched lipid rafts, which are important in promoting the mitogenic effect of oxytocin.

Previous studies showed the presence of oxytocin receptor in endothelial cells and the effect of oxytocin in inducing cell proliferation (4). Beside these data in normal endothelial cells, a similar pattern of oxytocin receptor expression and oxytocin effect was reported by our group in "modified" endothelial cells, such as the neoplastic spindle cell counterpart of the Kaposi's sarcoma (6). In the present study, we show that in endothelial cells oxytocin induces not only proliferation but also migration. As an additional observation, we showed that the oxytocin receptor selective antagonist, which abolished the proliferative oxytocin effect, was able to decrease cell proliferation when used alone possibly through a "biased agonist" effect recently described for another oxytocin antagonist, atosiban (19). Moreover, the stimulating effects of oxytocin on cell growth and migration are not limited to endothelial cells derived from normal tissue but also involve endothelial cells derived form neoplastic tissue, specifically from breast carcinomas (B-TEC). B-TEC have been

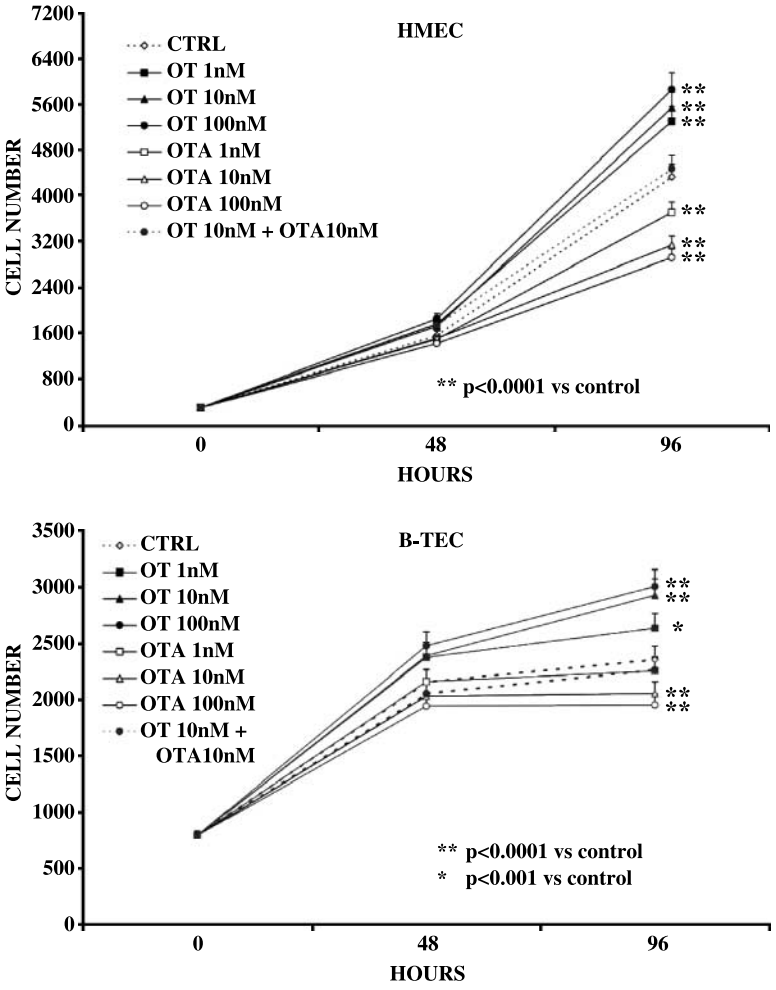

FIGURE 4. Oxytocin dose-dependent effect on HMEC and B-TEC proliferation. Oxytocin treatment induced a significant dose-dependent increase in cell proliferation since the first time point (48 hours). Oxytocin (100 nmol/L) produced a maximal (36%) increase of B-TEC number almost equivalent to the effect induced by oxytocin (1 μmol/L; 38%; data not shown). Oxytocin (10 and 1 nmol/L) still significantly increased cell proliferation although to a lower extent. The oxytocin effect was therefore dose dependent (1 μmol/L, 100 nmol/L, and 10 nmol/L oxytocin versus control: $P < 0.0001$ in HMEC and B-TEC; 1 nmol/L oxytocin versus control: $P < 0.0001$ in HMEC and $P < 0.001$ in B-TEC) and fully abolished by incubation with the selective oxytocin receptor antagonist, OTA, which inhibited cell proliferation when used alone (100 and 10 nmol/L oxytocin antagonist versus control: $P < 0.0001$ in HMEC and B-TEC; 1 nmol/L oxytocin antagonist versus control: $P < 0.0001$ in HMEC and $P < 0.07$ in B-TEC).

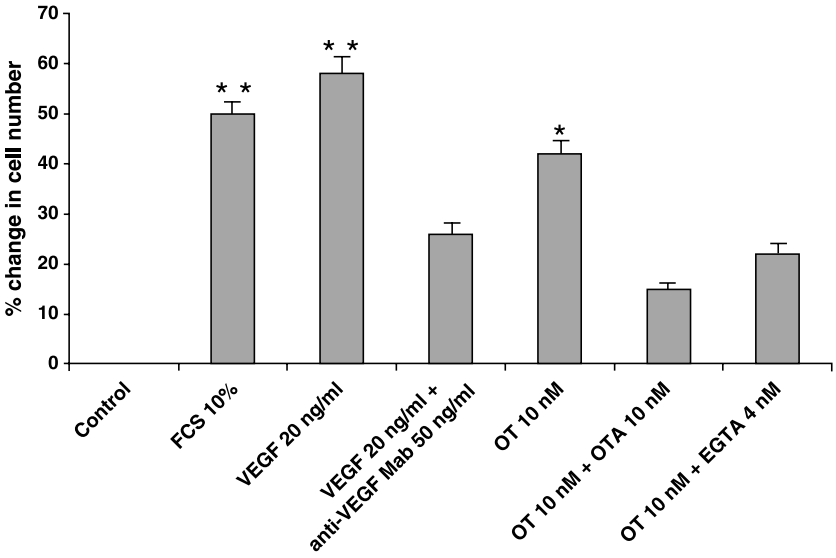

FIGURE 5. Oxytocin effect on B-TEC proliferation in serum-free medium. Twenty-four hours of 10 nmol/L oxytocin treatment in serum-free medium induced a 42% increase in B-TEC number (*, $P < 0.001$, versus control). Such effect was reverted by addition of the oxytocin receptor selective antagonist, OTA, (10 nmol/L) as well as by the calcium chelant EGTA (4 mmol/L). FCS (10%) and 20 ng/mL VEGF were used as positive controls and induced a 50% and 58% increase in the cell number, respectively (**, $P < 0.0001$, versus control). As expected, addition of the anti-VEGF antibody reduced the VEGF proliferative effect.

reported to display a proangiogenic phenotype, apoptosis resistance, and enhanced motility compared with non-tumor-associated endothelial cells (16). The effects of oxytocin are coupled to an intracellular increase in calcium levels, which is induced in both endothelial cell types, although at a significantly major extent in B-TEC. Even at low concentration (1 nmol/L), oxytocin enhances intracellular calcium levels, and the addition of a selective oxytocin antagonist (10 nmol/L) fully reverses this change, indicating that oxytocin effect is mediated by oxytocin receptor and not by other surface receptors, such as vasopressin receptors (V1aR), which could be activated by oxytocin, but only at higher concentrations (20); this observation is also in agreement with binding and PCR experiments that showed no evidence for V1aR in primary cultures from human umbilical vein endothelial cells, human aorta endothelial cells, and human pulmonary artery endothelial cells (4). As an additional evidence of the calcium involvement in the oxytocin receptor signaling, addition of a calcium chelant (EGTA) removed the mitogenic effect of oxytocin.

These findings (both induction of cell proliferation and migration) raise some questions on the possible role of oxytocin in angiogenetic processes under nonneoplastic (i.e., wounding processes) and neoplastic conditions. Interestingly, the increased cell motility is accompanied by the switch on of different adhesion molecule-related genes in HMEC and B-TEC, suggesting a certain cell type specificity in the activation of different gene patterns related to extracellular matrix remodeling, adhesion, and migration. In B-TEC, the genes of two MMP (MMP-11 and MMP-26) are 9- and 10-fold increased following oxytocin incubation, whereas MMP-17 responds to oxytocin with a 3-fold increase in HMEC. The increased expression of MMP-related genes induced by oxytocin and of cathepsin D in HMEC suggests that oxytocin may play a double role in promoting angiogenetic processes not only directly because oxytocin stimulates cell proliferation and migration, as shown here, but also indirectly through proteases involved in the breakdown of basement membranes and extracellular matrix. In B-TEC, oxytocin treatment also enhanced the expression of the laminin 1 gene, which has been reported to promote angiogenesis in endothelial cells (21). Further studies will be necessary to comprehend why oxytocin activates different gene patterns in endothelial cells as a consequence of their normal or tumor-associated status.

Table 1. Analysis of Oxytocin-Induced Migration of HMEC and B-TEC Migration Assay Was Done in the Boyden Chamber by Adding Oxytocin or VEGF in the Lower Compartments of the Chamber to Establish Positive or Absent Gradient across the Filter Barrier

Lower chamber	HMEC no. migrated cells	*P* vs vehicle	B-TEC no. migrated cells	*P* vs vehicle
Vehicle	34 ± 2		32 ± 2	
$VEGF_{121}$ (20 ng/mL)	604 ± 32	<0.001	748 ± 49	<0.001
Anti-VEGF antibody (50 ng/mL)	25 ± 7		24 ± 5	
$VEGF_{121}$ (20 ng/mL) + anti-VEGF antibody (50 ng/mL)	80 ± 12		86 ± 17	
Oxytocin (10 nmol/L)	524 ± 29	<0.001	816 ± 30	<0.001
Oxytocin (1 nmol/L)	276 ± 15	<0.005	543 ± 20	<0.001
Oxytocin antagonist (10 nmol/L; upper chamber) + oxytocin (10 nmol/L)	40 ± 2		52 ± 7	

NOTE: Cells that migrated after 12 hours of incubation to the lower surface of the filter were counted. Preincubation with a selective oxytocin antagonist or anti-VEGF antibody was done in additional experiments to further test the specificity of the used stimuli. Numbers are mean ± SD of one representative experiment done in triplicate and counted by two independent investigator.

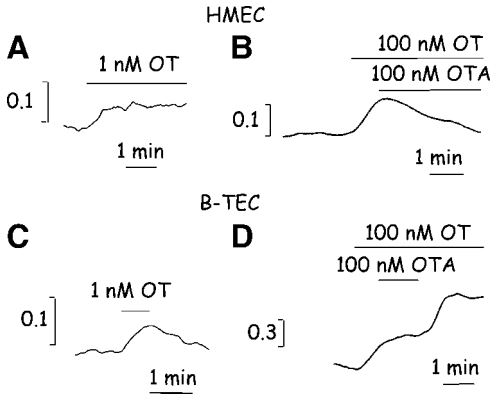

FIGURE 6. Effect of oxytocin treatment on $[Ca^{2+}]_i$ in HMEC and B-TEC. Application of 1 or 100 nmol/L oxytocin induced a slow and persistent increase in $[Ca^{2+}]_i$ in both B-TEC and HMEC (Fig. 5A-D). Application of oxytocin antagonist (100 nmol/L) significantly inhibited the response triggered by 100 nmol/L oxytocin in both cell types (Fig. 5B and D), thus confirming its specificity through oxytocin receptor activation. The percentage of responsive cells was significantly higher in B-TEC (85%) than in HMEC (29%) as well as the amplitude of the calcium increase itself ($\Delta F/F_0$ = 0.3 ± 0.08 and 0.15 ± 0.03, respectively).

In a previous work, Guzzi et al. showed that the localization of oxytocin receptor in caveolin-1-enriched domains turns the receptor-mediated inhibition of cell growth into a proliferative response (17). Interestingly, in the present study, we observed that in B-TEC oxytocin treatment determines a 3-fold increase in the expression of caveolin-1 gene, which is accompanied by an enhanced expression at protein level. This increase in caveolin-1 synthesis could participate in the effect of oxytocin in cell proliferation of tumor-associated endothelium and its angiogenic meaning. Because this increment in the caveolin-1 expression following oxytocin treatment is specific for B-TEC but absent in HMEC, we are tempted to imagine that the oxytocin effect in endothelial cells associated with tumor involves different pathways and possibly modifies the expression of those components of the cell membrane. Another factor that can be involved in the mitogenic effect of oxytocin in both HMEC and B-TEC is integrin β_6. In fact, we observed here that oxytocin switches on this integrin subunit gene (with a 3-fold increase in HMEC and a 8-fold increase in B-TEC) in both endothelial phenotypes. Integrin β_6 is known to direct bind to extracellular signal-regulated kinase 2 (22); on the other hand, different temporal patterns in extracellular signal-regulated kinase 2 phosphorylation have been reported to be activated in oxytocin receptor-expressing cells in relation to their localization within caveolin-1-enriched domains (23). Therefore, a challenging hypothesis of an activated oxytocin receptor/caveolin-1/integrin β_6/extracellular signal-regulated kinase 2 loop in B-TEC can be envisaged and worth further studies. In addition, in neoplastic cells, the overexpression of the integrin β_6 subunit greatly amplifies activation of extracellular signal-regulated kinase by serum-derived growth factor (22). In B-TEC, we can therefore suppose an alternative/additional indirect mechanism through which oxytocin may induce cell proliferation, enhancing extracellular signal-regulated kinase sensitivity to activating factors.

In conclusion, in the present study, we show that oxytocin participates in endothelial cell growth and migration in both normal and neoformed tumor-derived cells and that many different molecular mechanisms may be involved in such effects. Because oxytocin has been reported to be synthesized and locally released by breast carcinoma cells (18), the existence of an intratumoral source of peptide in breast cancers could be effective in regulating the neoformed vessels even at those low concentrations that previously resulted biologically ineffective on the neoplastic epithelial cells themselves (24-26).

Materials and Methods

Breast Tumor-Derived Endothelial Cells

Tumor-derived endothelial cells were obtained from breast carcinomas as reported previously (16). Briefly, specimens were minced finely with scissors and then digested by incubation for 1 hour at 37°C in DMEM containing collagenase IV (Sigma-Aldrich, Mannheim, Germany). After washings in medium plus 10% FCS, the cell suspension was forced through a graded series of meshes to separate the cell components from stroma and aggregates. Endothelial cells were isolated from cell suspension, using anti-CD105 antibody coupled to magnetic beads, by magnetic cell sorting using the MACS system (Miltenyi Biotech, Auburn, CA) and grown in complete EBM (Cambrex Bioscience, East Rutherford, NJ) supplemented with 10% FCS, 50 units/mL penicillin, and 50 μg/mL streptomycin as described previously (16, 27). HMEC were obtained from derma using anti-CD31 antibody coupled to magnetic beads, by magnetic cell sorting using the MACS system, immortalized by infection of primary cultures with a replication-defective adeno-5/SV40 virus, and grown as described previously (28).

Detection of Oxytocin Receptor and Oxytocin mRNA by Reverse Transcription-PCR in HMEC and B-TEC

Total RNA was extracted from HMEC and B-TEC using the TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. The concentration of RNA was estimated by spectrophotometry, and RNA degradation was assessed by 1% agarose gel electrophoresis. Total RNA (1 μg) was first digested, with 10 units RNase-free DNase (Boehringer Mannheim, Mannheim, Germany) in a 10 μL solution containing 2 mmol/L $MgCl_2$, to avoid DNA contamination. The solution was kept at room temperature for 10 minutes and then heated for 5 minutes at 70°C to inactivate DNases, 40 pmol/L oligodeoxythymidine primer [$oligo(dT)_{16}$] was added,

Table 2. Fold Increase in Gene Expression following 24-Hour 10 nmol/L Oxytocin Treatment in HMEC and B-TEC

Adhesion molecule genes	Fold increase in oxytocin-treated B-TEC	Fold increase in oxytocin-treated HMEC
Integrin β_6	×8	×3
Cathepsin D		×3
MMP-11	×9	
MMP-17		×3
MMP-26	×10	
Caveolin-1	×3	
Laminin B	×5	

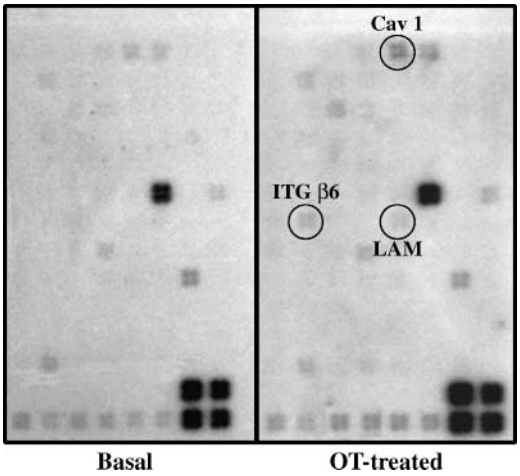

FIGURE 7. Adhesion molecule gene array analysis following 10 nmol/L oxytocin treatment in B-TEC. The expression profile of adhesion-related genes was analyzed by gene array technique under basal conditions and following 24-hour 10 nmol/L oxytocin treatment. In B-TEC, oxytocin treatment determined a significant increase in the expression of the following genes: MMP-11 and MMP-26, laminin B (*LAM*), integrin β_6 (*ITG* β_6), and caveolin-1 (*Cav 1*). The pattern of intensity in untreated and oxytocin-treated cells, and some of the genes mainly modified following oxytocin treatment are highlighted.

and the solution was heated at 70°C for 10 minutes and then chilled on ice to allow primer hybridization. The final solution was reverse transcribed using 200 units SuperScript II reverse transcriptase (Invitrogen) following the manufacturer's instructions in the presence of 40 units/μL recombinant RNasin RNase inhibitor (Promega, Madison, WI). Negative control samples for further PCR amplification included omission of the reverse transcriptase enzyme.

RNA quality was assessed by amplification of β_2-microglobulin mRNA. Oxytocin receptor primers were designed according to Takemura et al. (29). Primer sequences for oxytocin receptor and oxytocin are listed in Table 3. PCR experiments were carried out in a final volume of 20 μL containing 1 μL cDNA, 1 μmol/L sense and antisense primer, 200 μmol/L deoxynucleotide triphosphate, 1.5 mmol/L $MgCl_2$, 1× PCR buffer, and 0.5 units Taq polymerase (Applied Biosystems, Foster City, CA). Each reaction consisted of 40 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C (β_2-microglobulin and oxytocin receptor) and 61°C (oxytocin) for 1 minute, and extension at 72°C for 1 minute. PCR products were then visualized under UV light in 1% agarose gels containing ethidium bromide. MCF7 human breast carcinoma cells and normal human hypothalamus (obtained from autopsy) were used as positive controls for oxytocin receptor and oxytocin, respectively. Negative control samples included omission of cDNA in the PCR mixture.

To further test reverse transcription-PCR product specificity, Southern blot analysis was done. Probe sequences are reported in Table 3. Membranes were hybridized at 42°C overnight with 25 pmol digoxigenin-labeled oxytocin receptor and oxytocin oligonucleotide probe. The membranes were then washed with 2× SSC-0.1% SDS for 10 minutes at 42°C and 0.5× SSC-0.1% SDS for 30 minutes at room temperature. Digoxigenin-labeled specific hybridization was visualized using an immunologic detection system (Boehringer Mannheim) employing anti-digoxigenin antibodies conjugated with alkaline phosphatase. Detection was done using the chemiluminescent substrate disodium 3-(4-methoxyspiro;1,2-dioxetane-3,2-(5-chloro)tricyclo(3.3.1.^{3,7})decan;-4-yl) phenylphosphate CSPD (Boehringer Mannheim) according to the manufacturer's instructions. All blots were exposed to X-ray films at room temperature for 3 or 4 hours.

Immunofluorescence and Flow Cytometry for Detection of Oxytocin Receptor and Caveolin-1 in HMEC and B-TEC

For standard immunofluorescence procedure, HMEC and B-TEC were grown on glass coverslips for 5 days. After washing in PBS, cells were fixed in 5% paraformaldehyde with 2% sucrose (pH 7.6) for 5 minutes (or in methanol for 10 minutes and acetone for 5 seconds at −20°C) and then incubated at room temperature for 30 minutes with anti-human oxytocin receptor IF3 mAb (30) diluted 1:2 in PBS. Cells were then rinsed in PBS and finally incubated for 30 minutes at room temperature with the appropriate fluorescein-labeled secondary antiserum (Sera-Lab Ltd., Sussex, United Kingdom) diluted 1:2 in PBS.

Samples were observed by differential interference contrast and fluorescence confocal microscopy. Cells were imaged at 20°C using a confocal scanning laser microscope (FV300) mounted on an IX71 inverted microscope (both from Olympus, Hamburg, Germany) with a PlanApo ×60 oil, 1.4 numerical aperture, objective lens. Images were analyzed and processed using FluoView 300 and Adobe Photoshop CS software. An

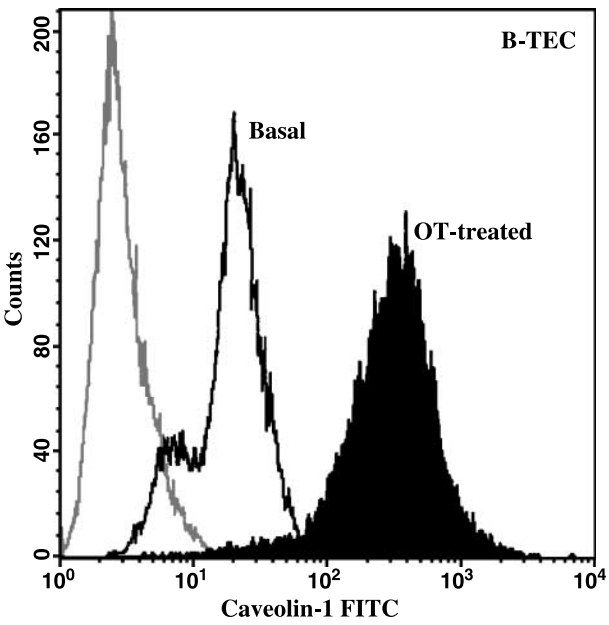

FIGURE 8. Caveolin-1 expression following 10 nmol/L oxytocin treatment in B-TEC. Following 10 nmol/L oxytocin treatment, B-TEC showed a 10-fold increase in caveolin-1 expression as evidenced by flow cytometry. X axis, fluorescence intensity/cells; Y axis, number of cells registered/channel. Each experiment was repeated at least twice, and results were reproducible. Representative experiment. Gray histogram, unrelated antibody.

Table 3. Oxytocin and Oxytocin Receptor Primer and Probe Sequences Used for Reverse Transcription-PCR and Southern Blot Analysis

	Sense primer	Antisense primer	Probe
Oxytocin	5′-ACCATTTCTGGGGTGGCTAT-3′	5′-GGTCTTGGGCCTCTGCTG-3′	5′-GTGTTCGGAGCCATCAAGTT-3′
Oxytocin receptor	5′-CCTTCATCGTGTGCTGGACG-3′	5′-CTAGGAGCAGAGCACTTATG-3′	5′-ACGGGCCACCTCTTCCACGAACTCG TGCAGCGCTTCCTGTGCTGCTCC-3′

unrelated primary mAb (Common Leucocytic Antigen, DAKO, Glostrup, Denmark) and omission of primary antibody were used as negative controls.

Surface expression of oxytocin receptor on B-TEC and HMEC was also investigated by flow cytometry analysis (FACSort, Becton Dickinson, Milan, Italy). Briefly, 2×10^5 cells were incubated with 1 μg of the IF3 mAb, whereas the secondary reagent was a FITC-conjugated $F(ab')_2$ fragment of a goat anti-mouse immunoglobulin antibody (Caltag, Burlingame, CA). Intensity of fluorescence was recorded on a logarithmic scale by scoring at least 10,000 cells per sample; background fluorescence intensity was obtained by incubating the cells with the goat anti-mouse immunoglobulin reagent alone.

Oxytocin Effects on HMEC and B-TEC Proliferation

HMEC and B-TEC were seeded in triplicate in 96-multiwell plates at a density ranging from 500 to 800 per well. To evaluate the effect on cell proliferation, 24 hours after plating, oxytocin, the specific oxytocin receptor antagonist $d(CH2)_5[Tyr(Me)^2,Thr^4,Tyr\text{-}NH_2^9]OVT$ (oxytocin antagonist, kindly provided by Dr. M. Manning, Medical University of Ohio, Toledo, OH, as oxytocin; ref. 31), and both peptides together were added to culture medium at concentrations ranging from 1 nmol/L to 1 μmol/L. The medium was changed every 48 hours. At 48 and 96 hours of culture, cells were fixed in 2.5% glutaraldehyde, stained with 0.1% crystal violet in 20% methanol, and solubilized in 10% acetic acid. Cell growth was evaluated by measuring absorbance at 590 nm in a microplate reader (Multiskan Bichromatic, Thermo-Labsystems Oy, Helsinki, Finland). A calibration curve was set up with known numbers of cells and a linear correlation between absorbance and cell counts was established up to 1×10^5 cells. Each experiment was repeated thrice with cells seeded in quadruplicate. The HT29 (oxytocin receptor–negative) human colon carcinoma cells were used as a negative control.

An additional study on the oxytocin effect on cell proliferation was done in serum-free medium: 24 hours after seeding, cells were serum-starved and incubated in presence of 10 nmol/L oxytocin, 10 nmol/L oxytocin antagonist, and 10 nmol/L oxytocin plus oxytocin antagonist for 24 hours. In this set of experiments, 20 ng/mL recombinant VEGF (Strathmann Biotech GmbH, Hanover, Germany) was used as a positive control and the neutralizing monoclonal anti-VEGF antibody (50 ng/mL; Genentech, San Francisco, CA) was used as a negative control. Cell growth was evaluated as reported above. Each experiment was repeated twice with cells seeded in quadruplicate.

Statistical analysis was carried out using ANOVA followed by Bonferroni's correction. Cutoff for significance was 0.05.

Oxytocin Effects on HMEC and B-TEC Migration

Cell motility in response to oxytocin was investigated as chemotaxis in Boyden chambers. Chemotaxis across a polycarbonate filter (8-μm pore size) was done as follows. RPMI or EBM (for HMEC and B-TEC, respectively) containing 0.25% bovine serum albumin and the stimulus (10 and 1 nmol/L oxytocin or 20 ng/mL VEGF) or the vehicle alone was placed in the lower compartment of the chamber. Cells (2×10^5) suspended in the same medium were seeded in the upper compartment of the Boyden chamber. In selected experiments, cells treated with oxytocin or VEGF, respectively, were preincubated for 30 minutes at 37°C with the oxytocin antagonist (10 nmol/L) or with the neutralizing monoclonal anti-VEGF antibody (50 ng/mL). After 12 hours of incubation at 37°C, the upper surface of the filter was scraped with a rubber policeman. The filters were then fixed and stained with DiffQuick (Harleco, Gibbstone, NJ) and 10 fields at ×200 magnification were counted by two independent investigators.

Calcium Measurements

Confocal fluorimetric measurements were done using an Olympus Fluoview 200 laser scanning confocal system (Olympus America, Inc., Melville, NY) mounted on an inverted IX70 Olympus microscope equipped with a ×60 oil immersion objective (numerical aperture, 0.17).

Cells were seeded on glass coverslips at a density of 5,000 per cm^2. For calcium measurements, the cells were loaded with Fluo-3 AM (2 μmol/L, 30 minutes, 37°C) and excited by a 488 nm, 20 mW argon-ion laser. Emission signals were filtered by a 510- to 540-nm bandpass filter and detected with the scanning head.

During experiments, cells were continuously perfused with a standard Tyrode solution of the following composition: 154 mmol/L NaCl, 4 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 5 mmol/L HEPES, 5.5 mmol/L glucose, and NaOH (pH 7.35). Stimulations were applied using a microperfusion system (inner pipette diameter, 200 μm). *X-Y* plane images (resolution 800 × 600 pixels) were acquired every 1.6 seconds, stored in multiTIFF file format, and subsequently analyzed with ImageJ, a public domain Java image processing software tool.[3] For each image sequence, regions of interest corresponding to single cells were selected and fluorescence intensity of each region of

[3] W. Rasband. ImageJ [imaging software]. Version 1.32. NIH; 2004.

interest was computed. Changes in intracellular calcium concentration, $[Ca]_c$, were represented as $\Delta F/F_0$ to normalize the traces.

Gene Array

The human extracellular matrix and adhesion molecules gene array, GEArray kit (SuperArray, Inc., Bethesda, MD), was used to characterize the gene expression profiles associated with different experimental conditions, specifically basal growth condition or following 24-hour 10 nmol/L oxytocin treatment. The kit included duplicate spots of 96 genes, including cell adhesion molecules, extracellular matrix proteins, proteases and proteases inhibitors, and four housekeeping genes (β-actin, glyceraldehyde-3-phosphate dehydrogenase, cyclophilin A, and ribosomal protein L13a). Total RNA was extracted from HMEC and tumor-derived endothelial cells using the TRIzol reagent following the manufacturer's recommendations and used as a template for biotinylated probe synthesis. For the probe synthesis, each RNA sample (5-10 μg) combined with a specific primer mix was added to a prewarmed (42°C for 2 minutes) labeling mix containing 200 units Moloney murine leukemia virus SuperScript II reverse transcriptase, 40 units recombinant RNasin RNase inhibitor, and a deoxynucleotide triphosphate mix with 10 nmol biotin-16-dUTP (Roche Molecular Biochemicals, Mannheim, Germany) and then incubated for 120 minutes at 42°C. The arrays were prehybridized at 68°C for 1 hour with GEAhyb hybridization solution containing 100 μg/mL heat-denatured salmon sperm DNA (Amersham Pharmacia Biotech, Piscataway, NJ) to block nonspecific hybridization. The filters were then hybridized overnight at 68°C with denatured biotinylated cDNA probes in 5 mL hybridization solution with continuous agitation. The filters were then extensively washed at low- and high-stringency conditions. After membrane incubation with alkaline phosphatase–conjugated streptavidin (diluted 1:5,000), gene expression was detected by chemiluminescence signal using the alkaline phosphatase substrate and images were acquired with Lumi-Imager (Roche Molecular Biochemicals); exposure times ranged from 2 to 40 minutes. Each GEArray membrane was spotted with a negative control of pUC18 DNA and two positive control genes, β-actin and glyceraldehyde-3-phosphate dehydrogenase. Densitometric analysis was done with Lumi Analyst Image Analysis Software version 3.1 (Roche Molecular Biochemicals).

References

1. Kimura T, Tanizawa O, Mori K, Brownstein MJ, Okayama H. Structure and expression of a human oxytocin receptor. Nature 1992;356:526–9.

2. Gimpl G, Fahrenholz F. The oxytocin receptor system: structure, function, and regulation. Physiol Rev 2001;8:629–83.

3. Bussolati G, Cassoni P. Editorial: the oxytocin/oxytocin receptor system-expect the unexpected. Endocrinology 2001;142:1377–9.

4. Thibonnier M, Conarty DM, Preston JA, Plesnicher CL, Dweik RA, Erzurum SC. Human vascular endothelial cells express oxytocin receptors. Endocrinology 1999;140:1301–9.

5. Weston GC, Cann L, Rogers PA. Myometrial microvascular endothelial cells express oxytocin receptor. BJOG 2003;110:149–56.

6. Cassoni P, Sapino A, Deaglio S, et al. Oxytocin is a growth factor for Kaposi's sarcoma cells: evidence of endocrine-immunological cross-talk. Cancer Res 2002; 62:2406–13.

7. Baluk P, Morikawa S, Haskell A, Mancuso M, McDonald DM. Abnormalities of basement membrane on blood vessels and endothelial sprouts in tumors. Am J Pathol 2003;163:1801–15.

8. Garlanda C, Dejana E. Heterogeneity of endothelial cells. Specific markers. Arterioscler Thromb Vasc Biol 1997;17:1193–202.

9. Jain RK. Molecular regulation of vessel maturation. Nat Med 2003;9:685–93.

10. Mollica F, Jain RK, Netti PA. A model for temporal heterogeneities of tumor blood flow. Microvasc Res 2003;65:56–60.

11. McDonald DM, Baluk P. Significance of blood vessel leakiness in cancer. Cancer Res 2002;62:5381–5.

12. Morikawa S, Baluk P, Kaidoh T, Haskell A, Jain RK, McDonald DM. Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. Am J Pathol 2002;160:985–1000.

13. Pasqualini R. *In vivo* phage display and vascular heterogeneity: implication for targeted medicine. Curr Opin Chem Biol 2002;6:399–404.

14. Nathwani AC. Antiangiogenetic gene therapy for cancer treatment. Curr Hematol Rep 2004;3:267–73.

15. Miller KD. Redefining the target: chemotherapeutics as antiangiogenics. J Clin Oncol 2001;19:1195–206.

16. Grange C, Bussolati B, Bruno S, Sapino A, Camussi G. Isolation and characterization of breast tumor-derived endothelial cells. Oncol Rep 2006;15: 381–6.

17. Guzzi F, Zanchetta D, Cassoni P, et al. Localization of the human oxytocin receptor in caveolin-1 enriched domains turns the receptor-mediated inhibition of cell growth into a proliferative response. Oncogene 2002;21:1658–67.

18. Cassoni P, Marrocco T, Sapino A, Allia E, Bussolati G. Oxytocin synthesis within the normal and neoplastic breast: first evidence of a local peptide source. Int J Oncol 2006;28:1263–8.

19. Reversi A, Rimoldi V, Marrocco T, et al. The oxytocin receptor antagonist atosiban inhibits cell growth via a "biased agonist" mechanism. J Biol Chem 2005;22:16311–8.

20. Chini B, Mouillac B, Balestre MN, et al. Two aromatic residues regulate the response of the human oxytocin receptor to the partial agonist arginine vasopressin. FEBS Lett 1996;397:201–6.

21. Dixelius J, Jakobsson L, Genersch E, Bohman S, Ekblom P, Claesson-Welsh L. Laminin-1 promotes angiogenesis in synergy with fibroblast growth factor by distinct regulation of the gene and protein expression profile in endothelial cells. J Biol Chem 2004;279:23766–72.

22. Ahmed N, Niu J, Dorahy DJ, et al. Direct integrin $\alpha_v\beta_6$-ERK binding: implications for tumour growth. Oncogene 2002;221:1370–80.

23. Rimoldi V, Reversi A, Taverna E, et al. Oxytocin receptor elicits different EGFR/MAPK activation patterns depending on its localization in caveolin-1 enriched domains. Oncogene 2003;22:6054–60.

24. Cassoni P, Sapino A, Negro F, Bussolati G. Oxytocin inhibits proliferation of human breast cancer cell lines. Virchows Arch 1994;425:467–72.

25. Cassoni P, Sapino A, Papotti M, Bussolati G. Oxytocin and oxytocin-analogue F314 inhibit cell proliferation and tumor growth of rat and mouse mammary carcinomas. Int J Cancer 1996;66:817–20.

26. Sapino A, Cassoni P, Stella A, Bussolati G. Oxytocin receptor within the breast: biological function and distribution. Anticancer Res 1998;18:2181–6.

27. Bussolati B, Deambrosis I, Russo S, Deregibus MC, Camussi G. Altered angiogenesis and survival in endothelial cells derived from renal carcinoma. FASEB J 2003;17:1159–61.

28. Conaldi PG, Serra C, Mossa A, et al. Persistent infection of human vascular endothelial cells by group B coxsackieviruses. J Infect Dis 1997;175:693–6.

29. Takemura M, Nomura S, Kimura T, et al. Expression and localization of oxytocin receptor gene in human uterine endometrium in relation to the menstrual cycle. Endocrinology 1993;132:1830–5.

30. Bussolati G, Cassoni P, Ghisolfi GP, Negro F, Sapino A. Immunolocalization and gene expression of oxytocin receptors in carcinomas and non-neoplastic tissues of the breast. Am J Pathol 1996;148:1895–903.

31. Elands J, Barberis C, Jard S, et al. [^{125}I-] labeled d(CH_2)$_5$[Tyr(Me)^2,Thr$_4$, Tyr-NH_2^9]OVT: a selective oxytocin receptor ligand. Eur J Pharmacol 1988; 147:197–207.

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