Int. J. Cancer: **77,** 695–700 (1998)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

PRESENCE AND SIGNIFICANCE OF OXYTOCIN RECEPTORS IN HUMAN NEUROBLASTOMAS AND GLIAL TUMORS

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To determine whether oxytocin (OT) could be added to the list of growth factors acting on neoplastic cells of nervous origin, we investigated the presence of oxytocin receptors (OTR) in human primary neuroblastomas and glioblastomas and related cell lines. OTR were demonstrated both at mRNA level (using a RT-PCR procedure) and at protein level (using immunocytochemical and immunofluorescence procedures). In order to clarify whether OT exerts any biological effect on these tumors through OTR, we also studied cell proliferation in 3 human neuroblastoma cell lines (SK-N-SH, SH-SY5Y, IMR-32) and one human anaplastic astrocytoma cell line (MOG-G-UVW) treated with OT 1 nM to 100 nM for 48 and 96 hr. At these doses, a dose-dependent inhibitory effect on cell proliferation was demonstrated. This inhibition was accompanied by a significant increase in the intracellular concentration of cAMP, which we have reported to be the intracellular mediator of the OT anti-proliferative effect in breast-carcinoma cell lines. Our data indicate that specific OTR are present in human neuroblastomas and glioblastomas. Through these receptors, OT could inhibit cell proliferation and modulate tumor growth. ***Int. J. Cancer*** **77:695–700, 1998.**

Like most neuropeptides, oxytocin (OT) has both peripheral and central action. Peripherally, OT participates in the cascade of events involved in parturition and lactation. These actions are mediated through specific receptors (OTR) that have been described in the uterus and in the mammary gland. In the uterus, OTR have been found both in myometrial and in endometrial cells (Takemura *et al.,* 1993). In the breast, OTR were detected in contractile myo-epithelial cells, in primary breast carcinomas and in breast-carcinoma cell lines (Bussolati *et al.,* 1996; Ito *et al.,* 1996; Cassoni *et al.,* 1994). In the latter, non-contractile, epithelial cells, OT reduces proliferative activity through the cAMP intracellular mediator (Cassoni *et al.,* 1997).

In the past 10 years, OTR have been characterized and autoradiographically localized in various areas of the central nervous system of rodents (van Leeuwen *et al.,* 1985; Tribollet *et al.,* 1988; Elands *et al.,* 1988; Freund-Mercier *et al.,* 1994). In rats, OTR have been demonstrated in the hypotalamic magnocellular nuclei, where they probably mediate the facilitatory effect of OT on its own release during the milk-ejection reflex (Freund-Mercier and Stoeckel, 1995), and in the parvocellular nuclei, where they regulate ingestive behavior (Verbalis *et al.,* 1995). OTR have been also detected in pure astrocyte primary cultures by binding procedures; however, in these cells the OTR function has not been defined (Di Scala-Guenot and Strasser, 1995).

These experimental data on OTR in normal nerve tissues induced us to investigate the presence of OTR in human neoplastic nerve tissue, such as primary neuroblastomas and glioblastomas, and in established human cell lines of neuroblastoma and anaplastic astrocytoma. In these cell lines, we also investigated the effect of OT on cell proliferation.

MATERIAL AND METHODS

Cell cultures

We grew 3 different human neuroblastoma cell lines, IMR-32, SK-N-SH and SH-SY5Y (all purchased from the ATCC (Rockville, MD) and one human anaplastic astrocytoma cell line, MOG-G-UVW (European Collection of Cell Cultures, CAMR, Salisbury, UK) as adherent monolayers in 75-ml flasks at 37°C in 5% CO_2 atmosphere in RPMI 1640 (GIBCO BRL, Gaitersburg, MD) supplemented with 10% FCS (GIBCO), penicillin (5000U/ml) (GIBCO) and streptomycin (15 mg/ml) (GIBCO). A human colon-adenocarcinoma cell line, HT29 (ATCC), was used as negative control.

Human primary tumors

We collected 12 neuroblastomas and 4 glioblastomas which were formalin-fixed and paraffin-embedded for histology. Frozen tissues adjacent to the histological specimen were used for RNA extraction.

Immunofluorescence

For immunofluorescence, cells grown for 5 days on glass coverslips were fixed in methanol (5 min at −20°C), permeabilized in acetone (5 sec at −20°C) and air-dried. Cells were then incubated for 30 min at room temperature (RT) with the primary MAb IF3 (diluted 1:1 in PBS) directed against the NH_2 terminal sequence of OTR (Bussolati *et al.,* 1996). After being washed in PBS, cells were incubated with the appropriate secondary fluorescein-labeled anti-serum (Sera-Lab, Crawley Down, UK) for 30 min RT. Finally, cells were examined with a Leitz Orthoplan fluorescence microscope equipped with a xenon lamp and epifluorescence apparatus. Omission of the primary antibody and substitution with an unrelated antibody (Prostate Specific Antigen, PSA, Dako, Glostrup, Denmark) were used as negative controls.

Immunohistochemistry

Sections of human primary glioblastomas were collected on poly-L-lysine-coated slides and stained for OTR antigen. Endogenous phosphatase activity was blocked with 20% iced acetic acid in distilled water for 5 min RT. Endogenous peroxidase activity was blocked with 7.5% H_2O_2 for 5 min RT, with 2.28% periodic acid for 5 min and finally with 0.02% sodium borohydride for 2 min (Heyderman, 1979). Aspecific sites were blocked with 2% BSA for 20 min RT, followed by biotynilated goat anti-mouse IgM antiserum (diluted 1/200) (Vector, Burlingame, CA) for 30 min. The slides were then incubated overnight, RT, with the primary MAb IF3 directed to human OTR, diluted 1/2000 in PBS. Immunoalkaline phosphatase by the alkaline phosphatase-anti-alkaline-phosphatase (APAAP, diluted 1:50) method was used (Dako). The reaction product was developed using 4-nitroblue tetrazolium chloride (Boehringer, Mannheim, Germany) and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer). Nuclei were slightly counterstained with methyl green. Omission of the primary antibody and substitution with an unrelated antibody (PSA) were used as negative control.

Grant sponsors: Ministry of the University (Rome); Associazione Italiana per la Ricerca sul Cancro (AIRC).

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Received 29 January 1998; Revised 31 March 1998

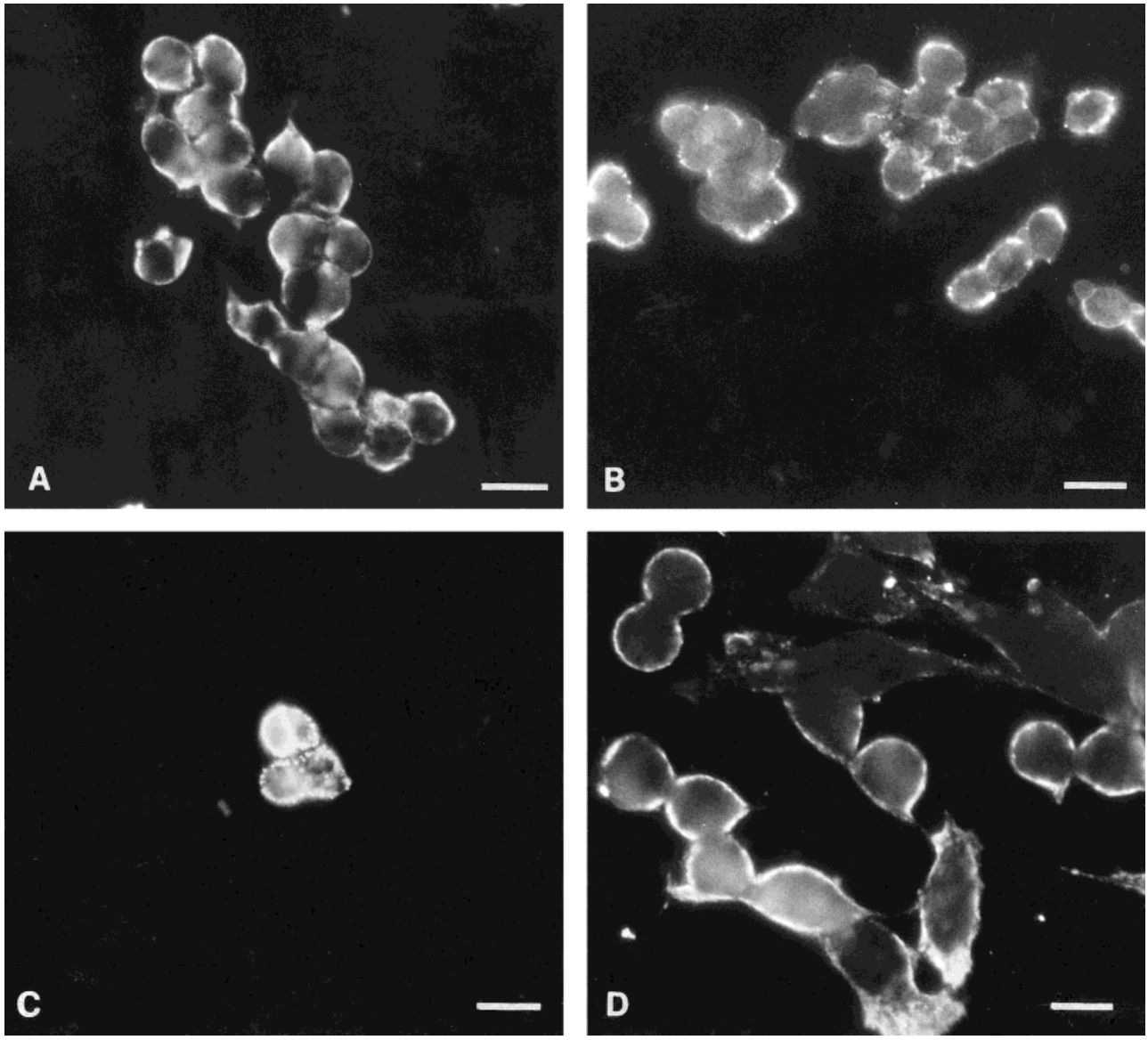

FIGURE 1 – OTR in human neuroblastoma and astrocytoma cell lines. The immunofluorescence reaction using IF3 MAb directed against the NH_2-OTR terminus reveals specific positivity (numerous fluorescent spots) along the cell membrane in all the cell lines studied. *(a)* SH-SY5Y; *(b)* SK-N-SH; *(c)* IMR32; *(d)* MOG-G-UVW) Scale bars: *a,b,c,* 7.5 µ; *d,* 5 µ.

To study the nature of OTR-positive cells in primary glioblastomas, serial sections of the tumors were stained for glial fibrillar acidic protein (GFAP, 1:100, Immunon, Pittsburgh, PA), synaptofisin (Biomeda, Foster City, CA) and neuron-specific enolase (NSE, 1:30, Biomeda).

RT-PCR

Total RNA was extracted from all cell lines and from primary neuroblastomas and glioblastomas by the guanidinium-isothiocyanate procedure (Chomczynski and Sacchi, 1987). RNA concentration was estimated by spectrophotometry; RNA degradation was monitored by agarose-gel electrophoresis.

Oligonucleotide primers were designed to amplify a 391-bp fragment of the OTR cDNA, as described by Takemura *et al.* (1993).

RNA (5 µg of each) was reverse transcribed in 20 µl reaction mixture containing 200 U of Superscript Reverse Transcriptase (GIBCO) and 50 pmols anti-sense primer for OTR. Water was used as negative control.

Each reaction product was amplified by PCR in 80 µl PCR buffer containing 1 U of Polytaq Taq DNA polymerase (Polymed, Sambuca-Firenze, Italy) and 12.5 pmols of each specific primer (OTR-F and OTR-R). The reaction was carried out for 20 cycles. Each amplified product (25 µl) was electrophoresed, stained with ethidium bromide on 1% agarose gel and transferred onto nylon membranes by Southern blotting.

The membranes were hybridized at 42°C overnight with 25 pmols of digoxigenin-labelled 48-bp OTR oligoprobe (Takemura *et al.*, 1993). The membranes were washed with 2 × SSC-0.1% SDS for 10 min RT and 0.5 × SSC-0.1% SDS for 30 min at 42°C. Digoxigenin-labelled specific hybrids were visualized using an immunological detection system (Boehringer) employing anti-digoxigenin antibodies conjugated with alkaline phosphatase. Detection was performed using chemiluminescent substrate CSPD

(Boehringer), according to the manufacturer's instructions. All blots were exposed to X-ray films with intensifying screens RT for 3 hr.

Cell proliferation

For growth curves, all cell lines were plated in triplicate in 24 multiwell dishes at a density ranging from 15,000 to 35,000 cells/ml; 24 hr after plating, 1 nM, 10 nM and 100 nM OT (kind gift of Dr. P. Melin, Ferring, Malmo, Sweden) were added to the culture medium. Medium was changed every 24 hr. Trypsin-EDTA solution was used to detach cells before counting. Cells were counted after 48 and 96 hr of culture, by means of a hemocytometer, by 2 independent investigators. Each single experiment was repeated 3 times. Statistical analysis was carried out by analysis of variance (ANOVA) followed by Bonferroni correction. The value for significance is $p < 0.05$.

Measurement of intracellular cAMP

To determine whether cAMP was the intracellular mediator of the OT effect on cell proliferation, SH-SY5Y human neuroblastoma cells, after 24 hr incubation in serum-free medium, were plated in 200-ml T-flasks and treated with 100 nM OT for 10, 20, 30, 45, 60 and 90 min. The concentration of intracytoplasmic cAMP in untreated cells was used as baseline control.

The medium was removed from flasks, and the cells were washed with 2 ml of sterile PBS and mechanically detached and counted. Cells were then briefly centrifuged and re-suspended in 1 ml of ethanol-HCl and sonicated at 30 W for 3×15 sec. Extraction was accomplished overnight at −20°C. Cell extracts were then dry-evaporated and recovered with 1.0 ml of the acetate buffer provided with the RIA cAMP kit (Incstar, Stillwater, MN). Samples were diluted 1:50 and used for radioimmunoassay determination of cAMP, following the manufacturer's instructions. Values of cAMP were given as pmol/10^6 cells. The experiment was repeated 3 times. Statistical analysis was carried out by ANOVA followed by Bonferroni correction.

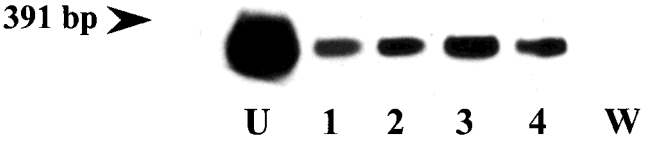

FIGURE 3 – OTR mRNA in human neuroblastomas and astrocytoma cell lines. By RT-PCR, an amplified 391-bp band corresponding to a region of the OTR cDNA is evident in all 3 human neuroblastoma (lane 1, SH-SY5Y; lane 2, SK-N-SH; lane 3; IMR32) and in the astrocytoma (lane 4, MOG-G-UVW) cell lines. Pregnant uterus was used as positive control, water (w) as negative control.

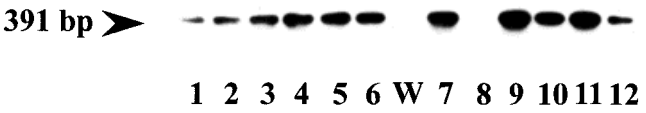

FIGURE 4 – OTR mRNA in human primary neuroblastomas. An amplified 391-bp band corresponding to a region of the OTR cDNA is evident in 11 out of 12 human primary neuroblastomas studied. Water (w) was negative control.

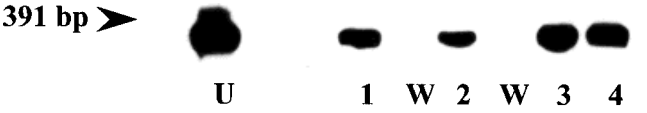

FIGURE 5 – OTR mRNA in human primary glioblastomas. An amplified 391-bp band corresponding to a region of the OTR cDNA is evident in all 4 cases of human primary glioblastomas. Water (w) was negative control, uterus (u) was positive control.

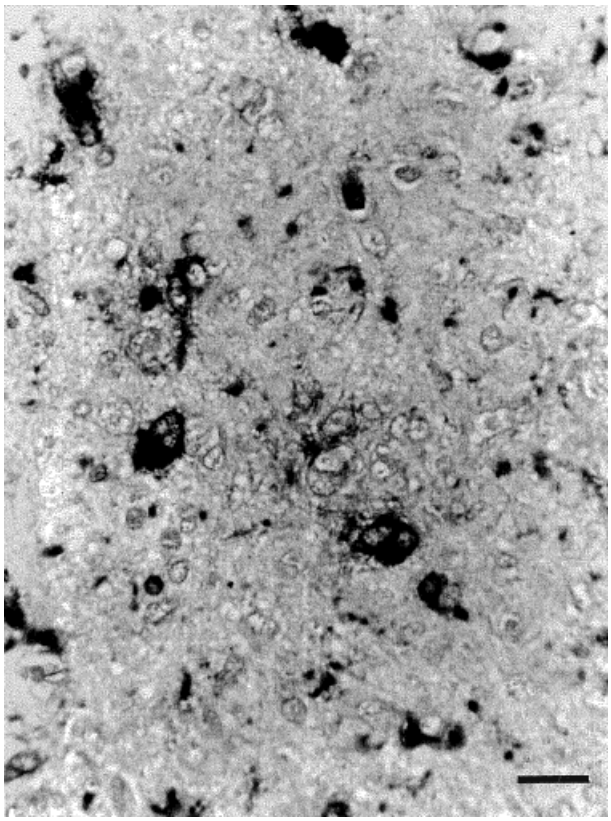

FIGURE 2 – OTR in human primary glioblastomas. The immunohistochemical reaction using the MAb for OTR shows intense positivity in neoplastic cells, mainly within the cytoplasm and along the cell projections. Scale bar, 12 µ.

RESULTS

Immunofluorescence for OTR

Anti-OTR IF3 MAb specifically stained the large majority of cells (>80%) of all neuroblastoma and astrocytoma cell lines investigated. The positive cells showed numerous immunofluorescent spots, localized mainly on the membrane of the cell body, as well as along cell projections (Fig. 1*a,b,c,d*). The omission of primary antibody and the reaction with PSA antibody gave negative results (not shown).

Immunohistochemistry for OTR

The human primary glioblastomas tested showed a large percentage of OTR-positive cells (Fig. 2). The intensity of reaction varied, but strong staining of the cytoplasm or of the cell membrane was definitely detected in neoplastic cells. The glial nature of OTR-positive cells was confirmed by intense positivity for GFAP and negativity for NSE and synaptofisin (data not shown). Unrelated PSA antibody and omission of primary antibody gave negative results.

Demonstration of OTR mRNA by RT-PCR procedure

OTR mRNA was demonstrated in primary tumors and cell lines. In fact, an amplified band of 391 bp corresponding to a region of OTR cDNA, as demonstrated (Bussolati *et al.*, 1996), was detected after RT-PCR of equal amounts of RNA extracted from the 3 different neuroblastoma cell lines and from the human anaplastic astrocytoma cell line (Fig. 3), as well as from 11 out of the 12 cases

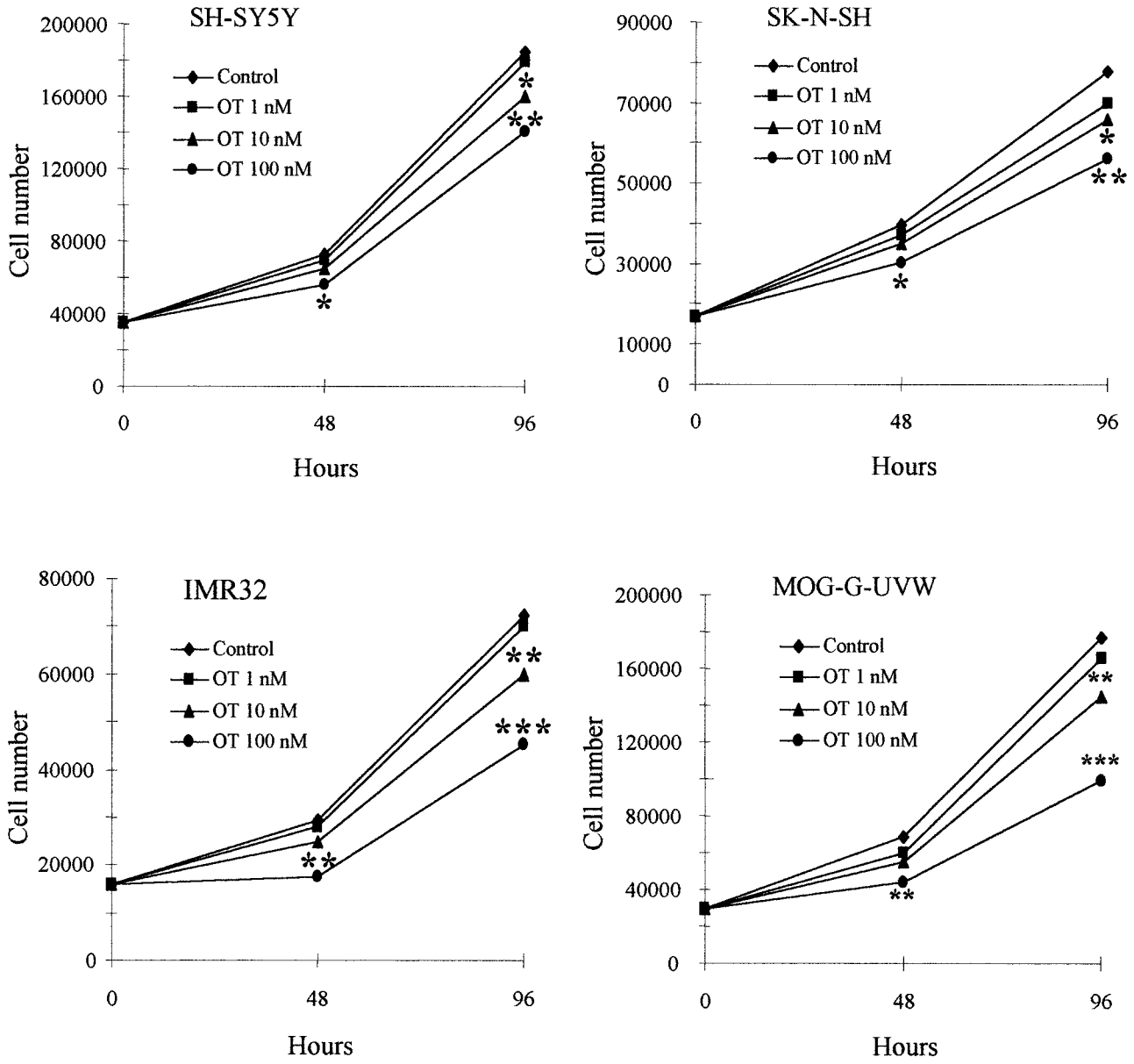

FIGURE 6 – Effect of OT on human neuroblastoma and astrocytoma cell proliferation. The addition of OT 100 nM to the culture medium significantly inhibited cell proliferation at any time point in the 4 cell lines studied (up to 43% reduction in cell number in IMR32 cells and 48% in MOG-G-UVW cells). OT at 10 nM significantly inhibited cell proliferation at the latest time point (96 hr). Data are expressed as mean of 3 different experiments. Standard deviations are below 10%. Statistical analysis was carried out by ANOVA (*$p < 0.01$; **$p < 0.001$; ***$p = 0.0001$, *vs.* control) (p value for significance: <0.05).

of neuroblastoma (Fig. 4). All the primary glioblastomas were positive for OTR-mRNA by RT-PCR (Fig. 5).

Effect of OT treatment on cell proliferation

OT reduced the proliferation activity in all neuroblastoma cell lines and in the astrocytoma cell line in a dose-dependent way. In the neuroblastoma cells, OT 1 nM did not significantly reduced proliferation, whereas OT 100 nM inhibited cell proliferation after the first 48 hr up to 43% in IMR32 cells. OT 10 nM inhibited cell proliferation at the second time point, after 96 hr of treatment. The proliferation rate of the anaplastic-astrocytoma MOG-G-UVW cells was significantly inhibited by OT 10 and 100 nM (up to 48% at 96 hr of treatment with OT 100 nM) (Fig. 6). The proliferation activity of HT29 colon-carcinoma cells was not affected by OT (not shown).

Intracellular cAMP determination

Intracellular cAMP levels were increased after 20 min of treatment with OT 100 nM. The difference between untreated and treated cells became significant at 45 and 60 min of treatment ($p < 0.01$ and $p < 0.001$ respectively *vs.* control) (Fig. 7).

DISCUSSION

In this study we found evidence of specific OTR in human neoplastic nerve tissues, and we demonstrated that activation of

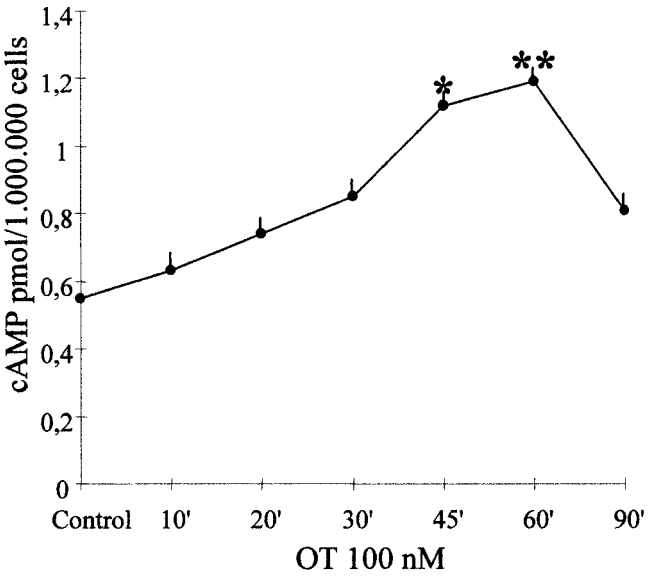

FIGURE 7 – Intracellular cAMP determination. Intracellular cAMP levels were increased after 20 min of treatment with OT 100 nM. The difference between untreated and treated cells became significant at 45 and 60 min of treatment. Data are expressed as mean ± SD of 3 different experiments. Statistical analysis was carried out by ANOVA (*$p < 0.01$; **$p < 0.001$, *vs.* control).

these receptors by OT reduces cell proliferation of neoplastic nerve and glial cells *in vitro.*

OT binding sites, as well as OTR mRNA, have been found in various areas of the brain, and in astrocyte and neurone cultures (Tribollet *et al.,* 1988; Yoshimura *et al.,* 1993; Di Scala-Guenot and Strosser, 1995). In addition, OTR have been visualized within cell bodies and dendrites of neurons in supra-optic, paraventricular and accessory magnocellular hypothalamic nuclei of lactating rats (Freund-Mercier and Stoeckel, 1995).

All these studies demonstrate the presence of OTR in normal nerve tissues of rodents. In our experimental model, we demonstrate that OTR could also be expressed in human neoplastic tissues of neuronal and glial origin. Indeed, by RT-PCR, we detected the presence of OTR in normal human brain of autoptic origin as well (data not shown).

The neuroblastoma cell lines here used were established from highly malignant tumors and some of them (SK-N-SH and its sub-clone SH-SY5Y, for example) can switch between a neuronal and a surface-adherent epithelioid phenotype (Ross *et al.,* 1983). This is interesting, since the different cell phenotype can influence the answer to different stimuli. In fact, it has been demonstrated that some growth factors such as insulin, IGF-I and IGF-II are mitogens only on undifferentiated neuroblastoma cells, whereas the same growth factors do not affect cell proliferation of more differentiated phenotypes (Pahlman *et al.,* 1995). Similarly, it has been reported that 2 other growth factors, bFGF and EGF, may either inhibit or stimulate cell proliferation, even in closely related neuroblastoma cell lines (Janet *et al.,* 1995). Specific receptors for all these and other growth factors have been reported in neuroblastoma cells (Janet *et al.,* 1995), and our results indicate that OTR can be added to the list.

In addition, the evidence of OTR mRNA in tissues from primary human neuroblastomas indicates that expression of OTR is not related to the *in vitro* system but is tumor-specific. Our data on the inhibitory effect of OT on cell proliferation suggest that OT can be included in the number of factors involved in the regulation of neuroblastoma growth. Further investigations would clarify whether this effect is accompanied by a differentiating process, and whether it varies in different culture conditions, as reported for other growth factors. At present, we have found an anti-proliferative answer to the peptide treatment in all the neuroblastoma cell lines studied.

Moreover, in our study the OTR were identified in human neoplastic cells of glial origin. The presence of OTR in the anaplastic-astrocytoma cell line (MOG-G-UVW) and in human primary glioblastomas is in agreement with earlier demonstrations of the receptor, by binding procedures, in normal glial cells (astrocytes) from rat hypothalami in culture (Di Scala-Guenot and Strosser, 1992, 1995*a*,*b*). *In vitro,* neuron cells were shown to modulate expression of OTR in rat astrocyte cells and it has been suggested that, *in vivo,* OTR expression in astrocytes could be a feature of a reactive gliosis following neuronal injury (Di Scala-Guenot and Strosser, 1995*b*). Thus, it is possible that in some way OTR regulate the proliferation activity of such cells. In fact, in our *in vitro* experiments, OT inhibits the proliferation of MOG-G-UVW cells.

We have demonstrated that, in breast-carcinoma cell lines, OT participate in the modulation of cell proliferation, both *in vitro* and *in vivo,* through specific OTR (Cassoni *et al.,* 1994, 1996). Interest in the proliferation-regulating activity of OT within the nervous system, as compared with the breast, is amplified by the fact that OT is normally produced by nerve cells in some areas of the CNS (Sawchenko and Swanson, 1983; Richard *et al.,* 1991), suggesting a possible role of the peptide as autocrine regulator.

In breast-carcinoma cell lines, we have demonstrated that intracellular cAMP participated in the OT anti-proliferative effect: in fact, cAMP was increased 4-fold after 60 min of OT 100 nM treatment (Cassoni *et al.,* 1997). In the neuroblastoma SH-SY5Y cell line, we observed a similar effect, with 100% increase of intracellular cAMP after 60 min of OT 100-nM treatment. In nerve tumors, intracellular cAMP concentrations have been reported to be increased during differentiation processes and to be inversely related to cell proliferation (Gaetano *et al.,* 1995; Tsai *et al.,* 1995).

In conclusion, we have demonstrated the presence of OTR in neoplastic nerve cells and tissues. Through these receptors, OT increases intracellular cAMP concentrations and inhibits cell proliferation. All these data suggest that a differentiation process is possibly involved in the inhibiting effect exerted by OT on cell proliferation of neoplastic cells of neuronal and glial origin.

ACKNOWLEDGEMENTS

We thank Mr. N. Ferraro and Mr. A. Rua' for photographic assistance and Miss C. Ravizza for technical assistance.

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