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OXYTOCIN AND OXYTOCIN-ANALOGUE F314 INHIBIT CELL PROLIFERATION AND TUMOR GROWTH OF RAT AND MOUSE MAMMARY CARCINOMAS

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The effects of oxytocin (OT) and the OT-analogue F314 were investigated on xenografts of mouse mammary and colon carcinomas (TS/A and C26 tumors) and of rat mammary carcinoma (D-R3230AC). In all cases, proliferation was previously assessed by cell counting in cultured cell lines, whereas tumor growth was checked by serial measures of tumor volume and by evaluation of tumor weight at the end of the experiment. Both cell proliferation and tumor growth were inhibited by OT and F314. These data support previous observations on the inhibitory effect of OT and F314 on the growth of MCF7, T47D and MDA-MB231 human breast cancer cell lines and open new prospects for testing the effect of this hypothalamic hormone and its analogues on the control of breast carcinoma growth.

We have observed an inhibitory effect of the neurohypophyseal peptide oxytocin (OT) and its analogue F314 on the proliferation of human breast cancer cell lines (Cassoni *et al.,* 1994). This effect was evident in both estrogen-dependent (MCF7 and T47D) and estrogen-independent (MDA-MB231) breast cancer cell lines and it was accompanied by changes in the cell phenotype. Moreover, in estrogen-dependent cells OT increased the antiproliferative effect of tamoxifen.

Our interpretation was that OT and the OT-analogue F314 may modulate breast cancer growth via specific receptors and by stimulating cell differentiation. In previous studies on developing mouse mammary gland we observed that OT inhibited the proliferation of undifferentiated stem cells whereas it increased the relative number of differentiated myo-epithelial and epithelial cells (Sapino *et al.,* 1993). These effects were quite evident when OT treatment followed estrogen and progesterone stimulation, thus suggesting that OT may act in combination with other mammotrophic hormones.

Since the inhibitory effect of OT and its analogue F314 on breast cancer cell proliferation might be of both theoretical and practical (clinical) interest, we extended previous *in vitro* studies by testing the effect of OT and F314 on the *in vivo* growth of rat and mouse mammary carcinomas.

MATERIAL AND METHODS

Animals and cell lines

Inbred BALB/c mice (female, 18–20 g in weight) and Fisher rats (female, 200 g) were obtained from Charles River (Calco, Italy). A mouse mammary carcinoma cell line (TS/A) (Nanni *et al.,* 1983), a mouse colon adenocarcinoma cell line (C26) (gift of Dr. G. Forni, Torino) and a cloned epithelial cell line (D-R3230AC) obtained in our laboratory from the rat mammary carcinoma R3230AC (Sapino *et al.,* 1992) were routinely cultured in RPMI (GIBCO, Grand Island, NY) added with 10% FCS (GIBCO). All these tumors lack estrogen and progesterone receptors.

OT and OT synthetic analogue F314 [Mpa[1], D-Tyr (Et)[2], Thr[4], Orn[8]-OT] (Melin *et al.,* 1986) were kindly supplied by Dr. P. Melin (Ferring, Malmö, Sweden) and prepared in aqueous solution to obtain the desired concentrations.

Alzet osmotic pumps (model 2002, Charles River) were used to allow continuous 14-day administration of the substances tested in the animals. To obtain the desired plasma concentration (10^{-8} and 10^{-9} M) of both peptides at steady state in the animals and to determine the correct infusion rate the following formula was used:

$$\text{plasma concentration at steady state (pmol/min)} = \frac{\text{infusion rate (pmol/min/kg)}}{\text{plasma clearance (ml/kg/min)}}.$$

Implantable OT pellets (prepared to obtain an OT plasma concentration at steady state of 10^{-8} M, calculated with the above formula) were purchased from IRA (Chicago, IL).

Tumor volumes were measured every 48 hr using a caliper. The volume was calculated using the following formula: tumor volume = 0.4 × (major axis) × (minor axis)^2.

In vitro *experiments.* Preliminary to the *in vivo* experiments we studied the growth curves of the selected cell lines (TS/A, clone D-R3230AC, C26). Cells were seeded in triplicate on 24-multiwell plates and incubated for 48 hr after plating in the presence of OT 10^{-8} M and F314 10^{-8} M. Trypsin-EDTA solution (GIBCO) was used to detach cells before counting. Cells were counted at 48 and 96 hr of culture using a hemocytometer. Each single experiment was repeated 3 times. Statistical analysis was carried out by ANOVA.

In vivo *experiments.* 1. Forty-three BALB/c mice were inoculated s.c. in the inguinal region with 4×10^4 cells from TS/A mammary carcinoma.

In a first set of experiments, pellets prepared with placebo or OT were placed s.c., in 12 and 10 mice, respectively, 7 days after cell injections. Tumor weight was evaluated at the end of the experiment, which lasted overall 21 days (Table I).

The remaining 21 mice were divided into 3 groups and when the tumors became measurable (at least 3 mm in largest diameter), osmotic pumps releasing saline, F314 10^{-8} or 10^{-9} M were placed s.c. Tumor diameters were measured every 48 hr. The animals were sacrificed 14 days later (28–30 days after tumor implantation) and the tumor weight was recorded.

2. Ten BALB/c mice were inoculated with 6×10^4 C26 colon carcinoma cells and treated with F314 10^{-8} M or saline released by osmotic pumps placed s.c., as soon as tumors became measurable. Tumor size was measured every 48 hr. The animals were sacrificed at day 14 of treatment.

3. Tumors obtained from clone D-R3230AC mammary carcinoma cells were transplanted in 22 Fisher rats. Briefly, 3–4 mm^3 of finely minced tumor tissue were transplanted s.c. in the right dorsal region by means of a trocar. When tumors became measurable, osmotic pumps releasing saline, OT 10^{-8} M or F314 10^{-8} and 10^{-9} M were placed s.c. for 14 days. Tumor diameters were measured every 48 hr.

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TABLE I – SUMMARY OF *IN VIVO* EXPERIMENTS

Species	Animal number	Tumor xenograft	s.c. treatment[1]	Days of treatment
BALB/c	10 tests	TS/A	OT pellet	21
BALB/c	12 controls	TS/A	placebo pellet	21
BALB/c	7 tests	TS/A	10^{-8} M F314 o.p.	14
BALB/c	7 tests	TS/A	10^{-9} M F314 o.p.	14
BALB/c	7 controls	TS/A	saline o.p.	14
BALB/c	6 tests	C26	10^{-8} M F314 o.p.	14
BALB/c	4 controls	C26	saline o.p.	14
Fisher	5 tests	D-R3230AC	10^{-8} M OT o.p.	14
Fisher	5 tests	D-R3230AC	10^{-8} M F314 o.p.	14
Fisher	5 tests	D-R3230AC	10^{-9} M F314 o.p.	14
Fisher	7 controls	D-R3230AC	saline o.p.	14

[1]o.p., Alzet osmotic pumps.

At the end of the experiments all mice and rats were sacrificed and weighted. The uterus, the mammary glands and the tumor were fixed in formalin for histological evaluation. The percentages of tumor volume increase from day 1 to the end of experiment and/or tumor weight were recorded and compared in both untreated and treated animals.

Statistical analysis was carried out by *t*-test with Bonferroni's correction.

Immunofluorescence detection of OT receptor

Cells of all lines were grown for 3 days on glass coverslips and tested for indirect immunofluorescence procedure. After fixation in methanol (5 min at −20°C) and permeabilization in acetone (5 sec at −20°C), cells were air dried. Then cells were incubated for 30 min at room temperature with the IF_3 MAb (diluted 1:1 in PBS) directed against the $-NH_2$ terminal sequence of the OT receptor (Bussolati *et al.*, in press). After washing in PBS the appropriate fluorescein-labeled secondary antiserum (Sera-Lab, Crawley Down, UK) diluted 1:10 in PBS was used for 30 min at room temperature. Cells were examined with a Leitz Orthoplan fluorescence microscope equipped with a xenon lamp and epifluorescence apparatus.

RESULTS

Effect of OT and F314 on cell proliferation in vitro

OT and F314 significantly reduced proliferation in both TS/A and clone D-R3230AC breast cancer cells at each time point examined (Fig. 1*a, b*). Both substances showed a similar effect. No effect was observed on the proliferation of C26 colon carcinoma cells.

Effect of OT and F314 on tumor growth

1. Pellets releasing OT 10^{-8} M inhibited the growth of TS/A breast carcinoma in 10 BALB/c mice, as indicated by the tumor weights at the end of the experiment (Fig. 2).

The continuous release of F314 by osmotic pumps significantly inhibited growth of TS/A tumor volume in BALB/c mice from the very beginning of the experiment (growth curves, Fig. 3). Tumor weights at the end of the experiments confirmed the antiproliferative effect of the peptide (Fig. 4). The tumor weights of 10^{-9} and 10^{-8} M F314-treated mice were respectively 65% and 72% lower than in controls.

2. OT or F314 treatment did not modify the volume and the final weight of tumors from C26 colon carcinoma cells injected in BALB/c mice (not shown).

3. Since the beginning of treatment, F314 10^{-8} M significantly inhibited the growth of D-R3230AC tumor in Fisher rats (Fig. 5). OT 10^{-8} M and F314 10^{-9} M showed a weaker, although still significant ($p = 0.05$) inhibitory effect (not shown). At day 14 of treatment, when animals were sacrificed, the tumor volume increase (compared with day 1) was 200% in controls, 52% in OT-treated, 20% in F314 10^{-8} M treated and 67% in 10^{-9} M treated animals (Fig. 6).

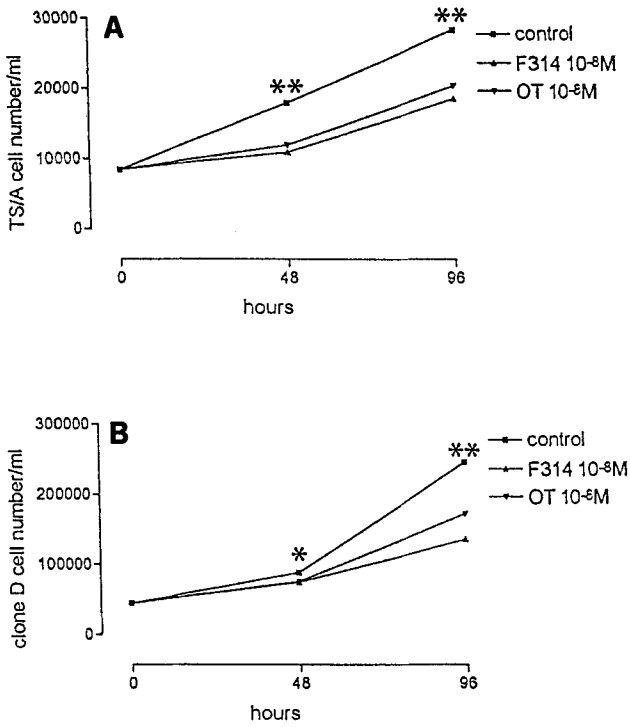

FIGURE 1 – Effect of 10^{-8} M OT and F314 on TS/A mouse mammary carcinoma *(a)* and on D-R3230AC rat mammary carcinoma *(b)* cell lines. Both peptides significantly reduce breast cancer cell growth at each time point (*, $p = 0.05$; **, $p < 0.001$). Each point is the mean of 3 different experiments. Standard deviations are below 7%. Statistical analysis carried out by ANOVA.

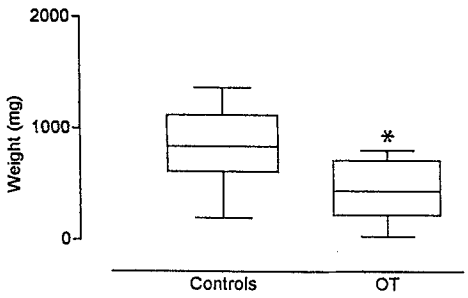

FIGURE 2 – Weights of TS/A tumor xenografts in BALB/c mice after 21 days of treatment with s.c. implanted pellets releasing OT (n = 10) or placebo (n = 12). The weight of explanted tumors is significantly different in the 2 groups (*, $p < 0.01$ treated *vs.* controls, *t*-test).

In all the *in vivo* experiments the weights of the animals, of the uterus and of the mammary glands were not modified by OT or OT-analogue F314 treatment, nor did OT and F314 treatment modify the histology of the uterus or mammary gland. Areas of necrosis in the tumors were similar in test and control animals.

Detection of OT receptor

Using an immunofluorescent technique, the clone D and the TS/A cells were specifically stained by the IF_3 MAb anti-OT

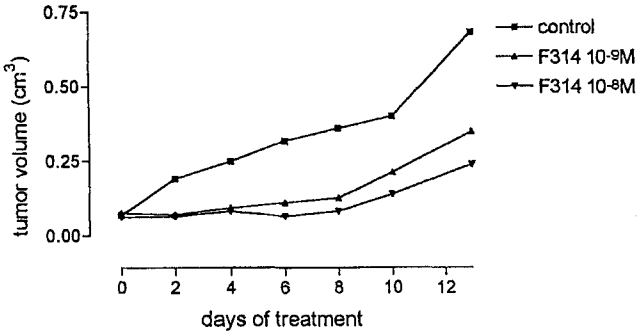

FIGURE 3 – Growth curve of TS/A tumor in 10^{-8} and 10^{-9} M F314-treated animals. Tumor growth is significantly lower in treated animals compared with controls at each time point and at both concentrations used ($p < 0.001$ tests *vs.* controls, *t*-test with Bonferroni's correction). Standard deviations are below 10%.

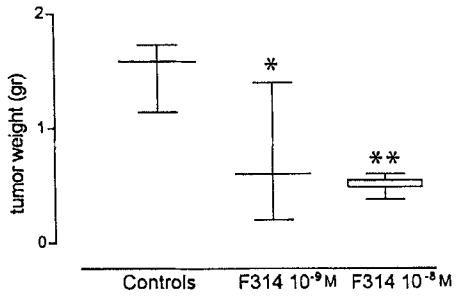

FIGURE 4 – Weights of TS/A tumor after 14 days of treatment with F314 10^{-8} and 10^{-9} M. The weight of explanted tumors is significantly lower in treated animals compared with controls (*, $p < 0.05$; **, $p < 0.001$, *t*-test with Bonferroni's correction).

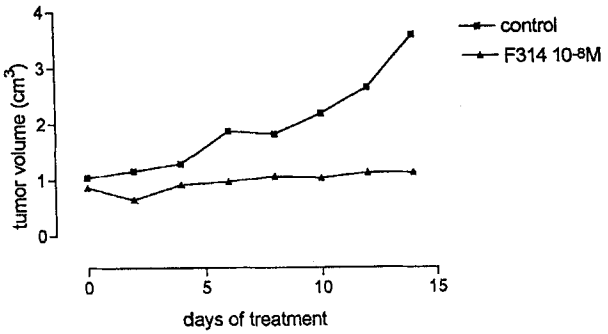

FIGURE 5 – D-R3230AC tumor growth in Fisher rats during F314 10^{-8} M treatment. The continuous release of F314 determined a significant and persistent inhibition of tumor growth after the first 48 hr ($p < 0.01$, *t*-test with Bonferroni's correction). Standard deviations are below 10%.

receptor. The pattern of positivity was similar in both cell lines and was characterized by numerous immunofluorescent spots localized on the cell membrane (Fig. 7).

The colon carcinoma C26 cells were negative.

DISCUSSION

Our present experimental data support and extend our previous observations on the *in vitro* antiproliferative effect of oxytocin in human breast cancer cell lines (Cassoni *et al.*, 1994). There are historical, clinical, biochemical and epidemiological data to support this notion (Murrell, 1995). Both OT and OT-analogue F314 inhibited the tumor growth of rat and mouse mammary carcinomas *in vitro* and *in vivo*. Differences in tumor growth between tests and controls were significant in all experimental models and at each peptide concentration used, although higher concentrations were more effective.

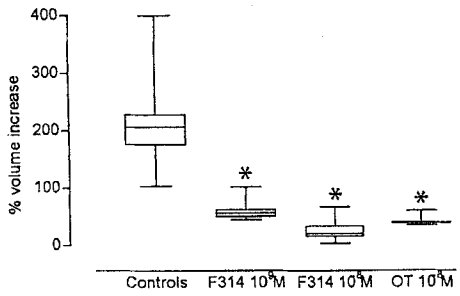

FIGURE 6 – Percentage of volume increase in D-R3230AC tumors from day 1 to day 14 of OT and F314 treatment. The inhibition of tumor growth determined by all the peptides is illustrated by the lower volume increase observed at the end of the experiment in treated animals (*, $p < 0.001$ treated *vs.* controls, *t*-test with Bonferroni's correction).

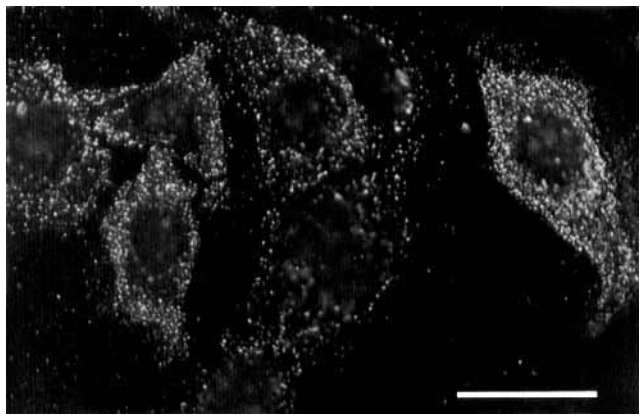

FIGURE 7 – Rat mammary carcinoma (clone D) cells. In immunofluorescence, employing IF_3 MAb against the $-NH_2$ terminal sequence of OTR, specific fluorescence is detected as distinct spots mainly over the cell surface. Scale bar = 10 μm.

The antiproliferative effect described here cannot be related to non-specific toxicity of the peptides, since it was observed *in vitro* and *in vivo* only on the mammary carcinomas and not on the C26 mouse colon carcinoma cell line. Moreover, the areas of necrosis were similar in test and control tumors, and the weights of the animals and of the uterus were not modified by OT or F314 treatment.

In vitro, the addition of either OT or F314 at a concentration of 10^{-8} M significantly inhibited the proliferation of D-R3230AC and TS/A cells, similar to our previous observations on human breast cancer cell lines (Cassoni *et al.*, 1994).

In vivo, the effect of both OT and F314 is even more striking than *in vitro* and appears from the first 48 hr of treatment. We cannot exclude that the *in vivo* effect of OT on mammary tumors might be enhanced by the OT-mediated activation of other endocrine systems (Arey and Freeman, 1992).

We presume that the effects of the OT and OT-analogue F314 observed in these rodent mammary carcinoma cells are likely due to the activation of specific OT receptor (OTR), as we had already demonstrated in human breast carcinoma cells. Besides breast cancer cells, OTRs have been observed in other

non-contractile epithelial cells, such as those of endometrial glands (Takemura *et al.,* 1993). However, an RT-PCR technique successfully used to demonstrate the presence of OTR mRNA in human tissues and breast cancer cell lines (Cassoni *et al.,* 1994) gave negative results in the tumors investigated here as well as in other tissues (mammary gland and uterus) of rodent origin (data not shown). This might be due to differences in the sequence of the investigated fragments of mouse and human OTR (Rozen *et al.,* 1995; Kimura *et al.,* 1992). However, we were able to confirm the presence of OTR on the rodent mammary cell lines using the IF_3 MAb against the OTR, generated in our laboratory (Bussolati *et al.,* 1996, in press).

The presence of OTR subtypes has been suggested in pregnant rat myometrium and decidua (Chan *et al.,* 1993; Chen *et al.,* 1994). We cannot exclude the possibility that in epithelial cells OT binds to a receptor subtype different from the OTR expressed in contractile cells, such as myometrial cells. This could determine a different biological response to OT of epithelial and myometrial cells (inhibition of proliferation or contraction). We can also suppose that the different receptor subtypes could activate different intracellular pathways of transduction. In breast carcinoma cells we did not observe the Ca^{++} increase that is known to mediate the OT signal in myometrial cells (Arnaudeau *et al.,* 1994), whereas a significant intracellular amount of cAMP increase became evident 30 min after OT treatment (data not shown).

Whatever the mechanism involved, the effect described *in vitro* appears reproducible *in vivo* using both OT or the OT-analogue F314.

The OT-analogue F314 used in this study acts as an OT antagonist, preventing uterine contraction by binding to OTRs. However, our data suggest an agonistic effect of F314 on breast carcinoma cells. Actually, many OT antagonists are "partial antagonists" and can exhibit an agonistic activity as well, mainly as a function of dose (Chan *et al.,* 1993). Concentrations of F314 up to 10^{-8} M indeed determine tumor growth inhibition almost equivalent to that induced by OT both *in vitro* and *in vivo.* As already hypothesized for OT, this agonistic effect of F314 in mammary tumors could depend on either a different OT-receptor subtype or a different mechanism of transduction in epithelial cells compared with myometrial cells. In clinical trials, administration of F314 to block preterm labor does not induce any toxic effects even at high concentrations (Akerlund *et al.,* 1987; Andersen *et al.,* 1989; Goodwin *et al.,* 1994). Our present observations confirm the lack of toxicity in long-lasting (14 and 21 days) treatment of OT and of F314.

Taken together, these results might open new therapeutic prospects, since the presence of OTRs has been described as being relatively common in primary carcinomas of the breast (Bussolati *et al.,* in press).

In conclusion, the inhibitory effect previously described on human breast cancer cells appears to be reproducible both *in vivo* and *in vitro* in experimental mammary carcinomas. These observations provide a possible rationale for further clinical trials aimed at testing the response of carcinomas of the breast to OT and OT-analogues.

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