

Novel insights into GnRH receptor activity: Role in the control of human glioblastoma cell proliferation

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Abstract. Malignant glioblastoma is one of the highest proliferative and invasive tumors within the central nervous system (CNS); the therapeutical options for this disease are still very poor. Receptors for gonadotropin-releasing hormone (GnRH) have been reported to be present in glioblastoma tissues. This study aimed to determine the role of these receptors in the control of glioma growth. In two human glioblastoma cell lines, U87MG and U373, we demonstrated the expression of GnRH receptors, both at mRNA and protein levels. We also found that GnRH receptor is expressed in glioblastoma tissues from tumor patients as shown by Western blotting. In U87MG and U373 cell lines, we found the expression of mRNA for GnRH, indicating the presence of an autocrine GnRH-based system in these cell lines. Treatment of the two cell lines with a GnRH agonist resulted in a significant decrease of cell proliferation. Moreover, the GnRH agonist significantly counteracted the forskolin-induced increase of intracellular cAMP levels in these cells. These findings suggest that the GnRH receptor might represent a molecular target for an endocrine therapeutical approach against gliomas.

Introduction

Glioblastoma, the most common primary brain tumor, is also the most deadly, with median survival ~1 year after diagnosis (1). The treatment of this pathology needs a multidisciplinary approach, including surgery, radiotherapy and chemotherapy. Despite the efforts in all these fields, progress in the treatments of glioblastoma has so far been limited (1,2). A better understanding of the molecular events leading to development and progression of glioblastoma might improve diagnosis,

prognosis and therapeutic possibilities for this severe disease.

Gonadotropin hormone-releasing hormone (GnRH, also known as luteinizing hormone-releasing hormone) is the hypophysiotropic hormone that regulates gonadotropin secretion through the activation of specific GnRH receptors present in the pituitary gonadotrophs (3). GnRH-stimulated gonadotropin secretion can be blocked with antagonists or mimicked by agonists, but in the latter case sustained stimulation causes desensitization. Thus, the two treatments ultimately reduce circulating levels of gonadotropins and gonadal steroids, causing the 'medical castration' that underlies the use of GnRH analogs to treat steroid-hormone dependent neoplasms, such as those of the prostate, ovary, endometrium, or mammary (4-7). In addition to its well known physiological role in the regulation of the hypothalamus-pituitary-gonadal axis, GnRH has been shown to exert direct antiproliferative effect in many cancer types of the reproductive tissues where GnRH receptors were found (breast, ovary, endometrial and prostate) (8-10). The activation of GnRH receptors through the binding of a GnRH agonist directly to the tumor cell membrane, inhibits the proliferation of prostate cancer cells *in vitro* and *in vivo*, as well as of endometrial and ovarian cells (11,12). Agonistic activation of these GnRH receptors has been demonstrated to interact with the mitogenic signal transduction of tumor growth factor receptors (e.g. EGFR, IGF-IR), resulting in downregulation of cancer cell proliferation (3,10,13-15). Moreover, in a recent study, we demonstrated, in human melanoma cells, the presence of a GnRH-based system (GnRH and its receptor). This system participates in the mechanisms involved in cell proliferation (exerting an inhibitory activity) and in cell migration and invasion (reducing the ability of melanoma cells to invade a reconstituted basement membrane and to migrate in response to a chemotactic stimulus) (16). Van Groeninghen and coworkers reported the expression of GnRH receptors in glioblastoma and meningiomas biopsies, suggesting that these binding sites might represent a diagnostic marker and possibly a new therapeutical target for nervous system tumors (17). In the present study, we investigated whether a GnRH system (GnRH and GnRH receptors) similar to that

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previously depicted in tumors of the reproductive tract and in melanoma cells, is present in glioblastoma cells. We also explored whether this system might affect the growth of these tumor cells and through which mechanism of action.

Materials and methods

Chemicals. The GnRH agonist Zoladex [D-Ser(tBu)⁶Aza-Gly-GnRH] was kindly provided by AstraZeneca Pharmaceuticals, Divisione Farmaceutici (Milan, Italy).

Tissue samples. Glioblastoma tissue specimens (T109, T127, T625, T880 and T1145) were dissected and immediately frozen at -80°C before Western blot analysis.

Cell culture. The human glioblastoma U87MG cell line was kindly donated by Dr Gaetano Finocchiaro (Istituto Neurologico 'Besta', Milano, Italy). The human glioblastoma cell line U373 was provided by Dr Oliver Müller (Max Plank Institute, Molecular Physiology Dortmund, Germany). The two cell lines were grown in a monolayer culture in RPMI medium (Seromed, Biochrom KG, Berlin, Germany), supplemented with 10% fetal bovine serum (FBS, Life Technologies, Paisley, Scotland), glutamine (1 mM) and antibiotics (100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulphate), in a humidified atmosphere of 5% CO₂ and 95% air. The human androgen-independent DU145 prostate cancer cell line was used as a positive control, since we have previously shown that GnRH and its receptor are expressed in these cells (4,18).

RT-PCR analysis of GnRH and GnRH receptor mRNA. Total RNA from U87MG, U373, as well as from DU145 cells used as positive controls, was prepared according to a modification of the guanidinium thiocyanate/cesium chloride method. To exclude the amplification of genomic DNA, extracted RNA was treated with 10 U/mg RNase free DNase (Roche Diagnostics). RNA (2 µg) was used in a reverse transcription reaction. cDNA synthesis was performed using the Gene AMP kit (Perkin Elmer Cetus, Norwalk, CT) with an oligo (dT)₁₆ primer for reverse transcriptase. Samples containing cDNAs were then amplified in a 100 µl solution containing PCR buffer (50 mM KCl, 10 mM Tris-HCl), 2 mM MgCl₂ and 2.5 U Taq polymerase. For GnRH cDNA amplification, PCR was performed for 35 cycles (1-min denaturation at 94°C, 30-sec primer annealing at 60°C, and 2-min primer extension at 72°C) in the presence of the following primers: 5'-TGGTGCCTGGAAGGCTGCTC-3' (sense, 78/97, 20 pmol) and 5'-CTTCTTCTGCCAGTTTC CTC-3' (antisense, 285/305, 20 pmol). For the amplification of GnRH receptor cDNA, PCR was performed for 35 cycles (1-min denaturation at 94°C, 1-min primer annealing at 50°C, and 2-min primer extension at 72°C) in the presence of the following primers: 5'-GCTTGAAGCTCTGTCCTGGGA-3' (sense, -25 to -5, 30 pmol) and 5'-CCTAGGACATAG TAGGG-3' (antisense, 844-860, 30 pmol) (19). The two pairs of primers has been previously utilized in our laboratory to amplify GnRH and GnRH receptor cDNA in prostate cancer cells (6). The predicted size of the amplified cDNA fragments were 228 and 885 bp for GnRH and GnRH receptor,

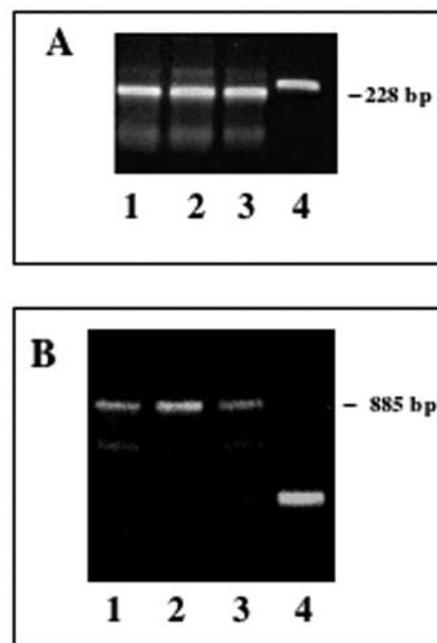


Figure 1. RT-PCR analysis of the expression of GnRH (A) and GnRH receptor (B) in U87GM and U373 glioblastoma cells. PCRs yielded products of the expected size of 228 bp for GnRH (A) and 885 bp for GnRH receptor (B). Lane 1, DU 145 cells; lane 2, U87GM cells; lane 3, U373 cells; lane 4, RT-PCR control (308 bp). One of the three experiments performed is reported.

respectively. After PCR, the amplified cDNA products were separated on a 1.5% agarose gel and stained with ethidium bromide.

Western blot analysis of GnRH receptors in cultured U87MG and U373 cells and in glioblastoma tissues. Membrane fractions from U87MG, U373 and DU145 cells were prepared according to the protocol reported by Limonta *et al* (4). Samples were homogenized in 10 mM Tris-HCl (pH 7.6) buffer containing 1 mM dithiothreitol on ice. For tumor biopsy sample homogenization, 50 mg tissue was cut into small pieces and homogenized in 250 µl buffer H [20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1mM CaCl₂] using a Dounce glass homogeniser. The homogenates were centrifuged twice for 10 min each at 800 x g to remove cellular debris, and the resulting supernatants were centrifuged at 18,000 x g to pellet down the membrane fractions. The cell pellets, both from cell cultures and glioblastoma biopsies, were solubilized in RIPA buffer [50 mM Tris-HCl (pH 7.7), 150 mM NaCl, 0.8% Triton X-100, 0.8% sodium deoxycholate, 0.08% SDS, 10 mM ethylenediamine tetraacetate, 100 µM Na₃VO₄, 50 mM NaF, 0.3 mM phenylmethylsulfonylfluoride, and 5 mM iodoacetic acid], loading buffer was added, and samples were electrophoresed on SDS page-10% denaturing polyacrylamide gel under reducing conditions. Proteins were transferred onto a nitrocellulose filter, in 25 mM Tris-HCl (pH 8.3), 92 mM glycine and 20% methanol at 30 V overnight. Filters were probed with FIG4 mouse monoclonal antibody raised against the human pituitary GnRH receptor (clone FIG4; Lab Vision Corporation, Fremont, CA), at a concentration of 5 µg/ml,

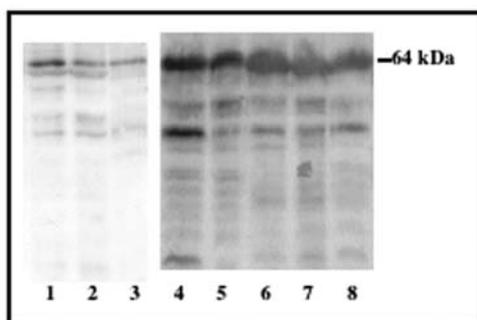


Figure 2. Western blot analysis of solubilized membrane proteins from prostate cancer cells DU145 (lane 1, used as control), glioblastoma cells U87MG (lane 2) and U373 (lane 3) and from five glioblastoma specimens (1-5) termed T109, T127, T625, T880 and T1145. The membranes were probed with the FIG4 monoclonal antibody raised against the human pituitary GnRH receptor. All glioblastomas show distinct bands at ~64 kDa.

followed by incubation with an anti-mouse IgG. The GnRH receptor expression was detected with the ECL-Western blot detection system after a 5-10 min exposure to a Hyperfilm-ECL X-ray film (Amersham, Milan, Italy), at room temperature. The specificity of FIG4 antibody for the human pituitary GnRH receptor has been previously demonstrated (4).

Cell proliferation studies. U87MG cells were plated at a density of 1400 cells/cm² in 10-mm dishes, U373 cells were plated at a density of 880 cells/cm² in 10-mm dishes in standard culture medium. Cells were allowed to attach and start growing for 3 days; the seeding media were then changed to experimental media. Cells were treated, for 7 days, with Zoladex (10⁻¹⁰-10⁻⁶ M); medium was changed every two days. At the end of the treatment, cells were collected and counted by hemocytometer.

cAMP accumulation in U87MG cells. U87MG cells were plated at a density of 2,800 cells/cm² in 24 multiwell/plates. After 2 days, cells were washed with 1 ml of serum-free medium, pretreated with 3 isobutyl-1-methylxanthine, 0.5 mM, (IBMX, a phosphodiesterase inhibitor) (Sigma Chemicon Co.) for 15 min at 37°C and then treated with forskolin (FSK, 5 μM), either alone or in the presence of Zoladex (1 μM) for 15 min at 37°C. At the end of the treatment, cells were extracted with ethanol 65% at 4°C for 5 min, 2 times, and centrifuged for 3 min (10,000 × g). The supernatants were collected, lyophilized in a vacuum concentrator, and stored at -20°C. cAMP content in each samples was determined by the ³H-cAMP assay according to the manufacturer's instructions (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK).

Statistical analysis. The data from experiments involving cell proliferation and cAMP determination were analyzed according to Dunnett's test (20) after one-way ANOVA.

Results

Expression of GnRH and GnRH receptors in cultured glioblastoma cells and in glioma tissues. RT-PCR revealed

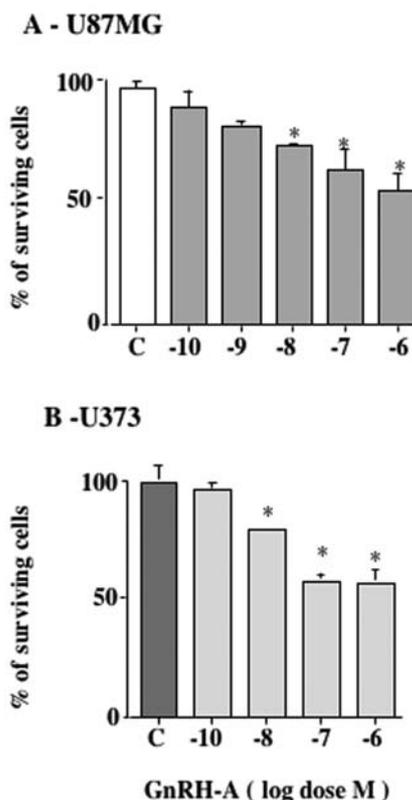


Figure 3. Effect of GnRH-A on U87MG (A) and U373 (B) glioblastoma cell growth. Both cell lines were treated, every days, with different doses of GnRH-A (10⁻¹⁰ M-10⁻⁶ M) for a period of 7 days. Cell growth was determined by hemocytometer count and the results were calculated as the relative percentage of cells vs. control cells. Values are means ± SE. *P<0.05 vs. control.

expression of GnRH (Fig. 1, lanes 2 and 3) and GnRH receptor mRNA (Fig. 1B, lanes 2 and 3) in U87MG cells and U373 cells. The size of the amplified cDNA corresponded to that found in human prostate cancer cells (Fig. 1A and B, lane 1) utilized as positive controls. The presence of GnRH receptors in glioblastoma cells was then demonstrated at the protein level. By Western blotting, a band of ~64 kDa was identified in U87MG and U373 cell membrane preparations (Fig. 2A, lanes 1 and 2). A band of the same size was also detected in membrane preparations from DU145 human prostate cancer cells, used as a positive control (Fig. 2, lane 3). The molecular size of these bands corresponded to that reported for the human pituitary GnRH receptor (21).

Western blotting revealed expression of the GnRH receptor in all five glioblastoma tissues analyzed (T109, T127, T625, T880, T1145) (Fig. 2). The membrane fractions of the tumors showed distinct bands at ~64 kDa.

Effect of a GnRH agonist on glioblastoma cell proliferation. The observation that GnRH receptors are expressed in U87MG and U373 cells, both at mRNA and protein levels, prompted us to investigate the role of these receptors in the regulation of glioblastoma cell proliferation. Treatment of both cell lines with a potent GnRH agonist (Zoladex) resulted in a significant decrease of the proliferation rate, Zoladex being significantly effective at doses ranging from 10⁻⁸ to

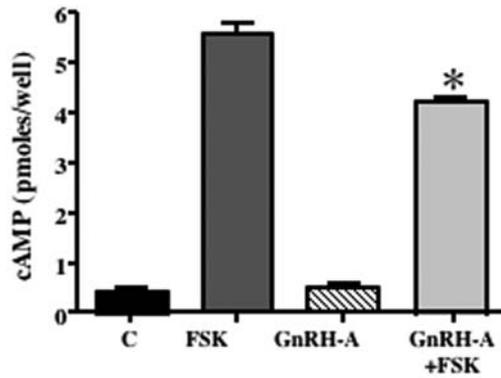


Figure 4. Effect of GnRH-A (1 μ M) on FSK (5 μ M)-induced cAMP accumulation in U87MG cells. Each experimental group consisted of six replicates. Each experiment was repeated 3 times, with identical results. Results are expressed as the mean cAMP pmoles per well \pm SE. * $P < 0.05$ vs. FSK.

10^{-6} M in both cell lines, as shown in Fig. 3; a Zoladex concentration of 10^{-8} M resulted in a 24.8% inhibition vs. controls, whereas a Zoladex concentration of 10^{-6} M resulted in a 42.5% inhibition vs. controls.

Effect of a GnRH agonist on cAMP accumulation. To investigate if, in glioblastoma cells, GnRH receptors might be coupled to the $G_{\alpha i}$ protein, we studied the effects of Zoladex on FSK-induced cAMP accumulation, in U87MG cells (Fig. 4). Zoladex, when given alone, did not affect cAMP levels. FSK, as expected, substantially stimulated cAMP accumulation. Zoladex, significantly counteracted the increase in cAMP levels induced by FSK. Similar results were obtained in U373 cells (data not shown).

Discussion

Glioblastoma represents the most aggressive form of malignant gliomas and despite the efforts in surgery, radiotherapy, chemotherapy, the dismal outcome has not improved over the past half century. The identification and validation of new anticancer molecules could lead to successful treatment strategies for neuro-oncology patients. In this study, we demonstrate the presence of GnRH and its receptors in the two human glioblastoma cell lines studied (U87MG and U373) and in glioblastoma tumor specimens. The activation of these receptors by means of the potent GnRH agonist Zoladex induces a dose-dependent inhibition of cell proliferation. Moreover, the accumulation of the second messenger cAMP, whose production depends on the activity of the membrane bound enzyme adenylyl cyclase, is reduced by the treatment with the GnRH agonist. GnRH and its receptor have previously been investigated (by us and by other laboratories) in several tumor types affected by sex steroid hormones (prostate, uterine, breast, ovarian tumors) (8,10,18,22) but scarce data on their occurrence in glioblastoma are available. In 1998 van Groeninghen and coworkers demonstrated the expression of GnRH receptors in glioblastoma biopsies, suggesting that these receptors might represent a diagnostic marker and a new therapeutic target for nervous system tumors (17). To our knowledge, this study represents

the first report of an inhibitory activity of GnRH agonists on the proliferation of glioblastoma cells expressing GnRH receptors. In the authors' laboratory, the presence of GnRH receptor has been demonstrated in prostate cancer cells, either androgen-dependent (LNCaP) or androgen-independent (DU145) (18,24). Similar findings have been obtained by other authors in breast, ovarian and endometrial cancer cells and tissues (25,26). We have also shown that the activation of these receptors by means of potent GnRH agonists leads to a decrease of proliferation of prostate cancer cells, both androgen-dependent and androgen-independent. We further confirmed the antiproliferative effect observed in *in vitro*, and *in vivo* experiments on androgen-independent prostate cancer cells xenografted into nude mice (11). Further, an autocrine growth regulatory system that reduces cancer growth through a direct effect in the ovary and endometrium, *in vitro* and *in vivo* has been demonstrated based on GnRH production and its receptor expression. Recently, we showed that both GnRH and GnRH receptors are expressed in human melanoma cells (BLM and Me15392). This system seems to participate in the mechanisms regulating cell proliferation exerting an inhibitory activity since GnRH receptor activation by an exogenous GnRH agonist brings about a significant decrease of tumor cell proliferation. Therefore, tumors expressing GnRH receptors respond to GnRH agonists treatments with a decrease of cell proliferation. It is interesting that in U87MG we found that Zoladex completely counteracts the forskolin-induced increase of intracellular cAMP levels; this indicates that in these cells the locally expressed GnRH receptor may be linked to a $G_{\alpha i}$ protein which, through inhibition of cAMP accumulation, may mediate the antiproliferative activity of autocrine GnRH. These data confirm that human GnRH receptors, expressed in tumor cells, are coupled to a $G_{\alpha i}$ -cAMP intracellular signal pathway. In the authors' laboratory it has previously been observed that in prostate cancer cells, GnRH receptors are coupled to a $G_{\alpha i}$ protein which, through the inhibition of cAMP accumulation, may mediate the antiproliferative effects of GnRH analogs (27). Similar observations are reported in ovarian and endometrial cancer cells in which GnRH agonists reduce the pertussis toxin-induced ADP ribosylation of $G_{\alpha i}$. Other lines of evidence have proposed a role for the ERK pathway in mediating the antiproliferative activity of GnRH analogs in ovarian and endometrial cancer (28-30).

In reproductive system tumor cells, GnRH receptors are coupled to signal transduction systems that differ to that observed for pituitary GnRH receptors; pituitary GnRH receptors are known to be coupled to a phospholipase C signaling system, thus mediating LH synthesis and secretion (30). This difference might be responsible for the different actions of GnRH in tumor cells and in anterior pituitary. Several studies have reported the presence of a second form of GnRH (GnRH-II; His⁵, Trp⁷, Tyr⁸-GnRH-I) in humans (31). In certain mammals, this decapeptide is involved in sexual function and reproduction, but its role in humans is less clear (31). GnRH-II is expressed in the human central nervous system, but also widely in peripheral tissues. It has been shown that GnRH-II exerts anti-tumorigenic effects in steroid hormone-dependent malignancies (9,32,33). Chen and

coworkers demonstrated that both GnRH isoforms, GnRH-I and GnRH-II, are expressed and secreted in human neuronal cell lines TE-671 (medulloblastoma) and LAN-1 (neuroblastoma) (34). These data suggest that GnRH-II might play a role in cerebral pathology in addition to GnRH-I.

Our present findings suggest that GnRH agonists may have potential as a treatment for primary tumors such as malignant glioblastomas. However, to confirm this effect *in vivo* it is crucial to better clarify the capacity of GnRH agonists to cross the blood-brain barrier. A recent study demonstrates that GnRH agonists have significant effects on memory, but it has been suggested that the changes in brain functions observed by the authors might be attributed to the indirect effects of reduced LH, FSH and gonadal hormones in the brain (35). However, a role of GnRH in Alzheimer's disease has been recently underlined, as GnRH agonist leuprolide acetate decreases the levels of A β protein (the marker of Alzheimer's disease neuropathology) affecting the activity of hippocampal neurons (36). An interesting study on the capacity to cross the blood-brain barrier by peptide hormones, was recently performed by Schally and coworkers by using antagonists of another hypothalamic peptide hormone (growth hormone-releasing hormone, GHRH). The authors found that GHRH antagonists (MZ-5-156 and JV-1-36) reduce the tumorigenicity and growth of U87MG human glioblastoma cells transplanted onto mouse flanks and found that the peripheral administration of the iodinated GHRH antagonist JV-1-42 rapidly crosses the blood-brain barrier (37). In order to obtain effective intratumoral concentrations of GnRH agonists, another chance is to develop different routes of administration which can circumvent the blood-brain barrier (gene therapy, nanoparticles) (38). Based on the observation that GnRH agonists have significant anti-glioblastoma activity in cell cultures, further evaluation of their potential preventive or therapeutic utility *in vivo* is at present ongoing in our laboratory.

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