Characterization of luteinizing hormone-releasing hormone receptor type I (LH-RH-I) as a potential molecular target in OCM-1 and OCM-3 human uveal melanoma cell lines

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Introduction: Uveal melanoma (UM) is the most common primary intraocular malignancy with very poor prognosis. Conventional chemotherapy only rarely prolongs the survival, therefore patients require novel treatment modalities. The discovery of specific receptors for hypothalamic hormones on cancer cells has led to the development of radiolabeled and cytotoxic hormone analogs.

Materials and methods: In the present study, our aim was to investigate the expression of mRNA for receptors of luteinizing hormone-releasing hormone type I (LH-RH-I) and LH-RH ligand in OCM-1 and OCM-3 human uveal melanoma cell lines. The presence and binding characteristics of LH-RH-I receptor protein was further studied by Western blot, immunocytochemistry and ligand competition assay. The expression of mRNA and protein for LH-RH-I receptors has been also studied using tumor samples originating from nude mice xenografted with OCM-1 or OCM-3 cells.

Results: The mRNA for LH-RH-I receptor has been detected in OCM-1 and OCM-3 cell lines and was found markedly higher in OCM-3 cells. The mRNA for LH-RH-I receptors was also observed in both UM xenograft models in vivo with higher levels in OCM-3. The presence of LH-RH-I receptor protein was found in both cell lines in vitro by immunocytochemistry and Western blot, and also in tumor tissue samples grown in nude mice by Western blot. Both human uveal melanoma models investigated showed specific high affinity receptors for LH-RH-I using ligand competition assay. The mRNA for LH-RH ligand has also been detected in OCM-1 and OCM-3 cell lines and cancer tissues.

Conclusion: The demonstration of the expression of LH-RH-I receptors in OCM-1 and OCM-3 human UM cell lines suggests that they could serve as potential molecular target for therapy. Our findings support the development of new therapeutic approaches based on cytotoxic LH-RH analogs or modern powerful antagonistic analogs of LH-RH targeting LH-RH-I receptors in UM.

Keywords: human uveal melanoma, LH-RH receptor, LH-RH ligand, targeted cancer therapy

Introduction: Uveal melanoma (UM) is an intraocular melanoma arising from melanocytes of the uveal tract, which is composed of the choroidea, ciliary body (CB), and iris. UM is the most common intraocular tumor and its prognosis depends on the size of the primary tumor, the time of diagnosis, and the presence of metastases. A number of clinical and histological risk factors have been defined over the last three decades, among others — clinicopathological factors like location, extrascleral growth, involvement of the CB, and the epitheloid cell type of the tumor. Larger tumors are associated with
a mortality rate of ~50% shortly after the diagnosis, while patients with medium-sized tumors show 50% survival rate over 15 years measured from primary tumor treatment. Nearly 50% of patients suffering from UM develop metastatic disease, that usually involves the liver and is almost inevitably fatal. When metastases develop, the median survival of the patients is only 5–7 months.

Primary tumors are treated either by brachytherapy using radioactive plaques to preserve the tissues of the eye or by enucleation. Treatment by chemotherapy only rarely prolongs survival and new treatment modalities are needed. Two distinct classes of UMs have been identified by its gene expression profile. Class I tumors exhibit low aneuploidy, and patients rarely have metastases, whereas class II tumors have a higher chance of aneuploidy and patients have a high risk to develop metastases. Monosomy 3 strongly relates to several clinical and histopathological parameters such as epithelioid cells, closed vascular patterns, large tumor diameter, and CB involvement. Several groups have already shown that there is a strong correlation between monosomy 3 and the development of metastatic diseases. Lack of chromosome 3 has been demonstrated in 5%–10% of all the patients and the remaining copy is duplicated. Monosomy 3 is present in 50%–60% of UM tumors, and is associated with isochromosome 8q and high level of 8q gain. In our previous study, we demonstrated aneuploidy of chromosome 4 in 70% of human UM specimens. Furthermore, a correlation was found between the copy number of chromosome 3 and 4 and the survival of patients. BRCA1-associated protein 1 (BAP1) is located on chromosome 3p21.1 and is thought to be a tumor suppressor gene. Inactivating somatic mutations were found in 84% of the metastasizing UMs, implicating that BAP1 mutations occur late in the UM progression. Recently, several candidate genes have been proposed in UM; LZTS1, GNAQ, DDEF1, NBS1, HDM2, BCL-2, and CCND1, among others. For most of these genes, a definite role in tumorigenesis or progression toward metastasis is still yet to be validated.

The discovery of specific receptors for hypothalamic hormones on cancer cells has led to the development of radiolabeled and cytotoxic hormone analogs. These analogs are more selective in wiping out cancer cells and less toxic than conventional chemotherapeutic agents. Hypothalamic luteinizing hormone-releasing hormone (LH-RH) is the primary regulator of gonadal function and plays a pivotal role in vertebrate reproduction. The actions of LH-RH are mediated by specific G protein-coupled receptors (GPCRs) for LH-RH present on the plasma membranes of the pituitary gonadotrophs. Those specific membrane receptors for LH-RH have been found in various animal and human cancers and can mediate direct effects of LH-RH agonists, antagonists, and cytotoxic LH-RH conjugates. Receptors for LH-RH have been demonstrated on breast, prostate, ovarian, endometrial cancers, melanomas, and renal cell and colorectal carcinomas. Previously, we have also demonstrated the expression of LH-RH ligand and LH-RH type I (LH-RH-I) receptors in human UM specimens.

In the present study, our aim was to investigate the mRNA expression of LH-RH-I receptor and LH-RH ligand in ocular choroidal melanoma (OCM)-1 and OCM-3 human UM cell lines. The presence and binding characteristics of the LH-RH-I receptor protein have been also examined by Western blot, immunocytochemistry, and ligand competition assays. In addition, we have studied the expression of mRNA and protein of LH-RH-I receptors in tumor tissue samples from nude mice xenografted with OCM-1 and OCM-3 cell lines.

Materials and methods

Cell lines and culture conditions

OCM-1 and OCM-3 human primary UM cell lines were kindly provided by the Department of Biophysics and Cell Biology, University of Debrecen and this research had approval from the Institutional Ethics Committee of the University of Debrecen. The cells were cultured in RPMI 1640 medium supplemented with L-glutamine, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin in a humidified chamber in 5% CO₂ at 37°C. Cells were subcultured every 3 days using a standard trypsinization procedure.

Animals

Athymic (nude) mice (Ncr nu/nu) were obtained from Charles River Laboratories (Germany). Mice were housed in sterile, individually ventilated cages in an air-conditioned (21°C ±2°C), humidity-controlled room (50%) with a 12/12 hour light/dark cycle. Animals were fed with autoclaved chow and water ad libitum. All experiments were conducted in accordance with the institutional guidelines for the welfare of experimental animals and regulations of the European Union. The experimental protocol was approved by the Laboratory Animal Care and Use Committee of the University of Debrecen. Six million tumor cells were subcutaneously injected into the femoral region of the mice. Four weeks after the initiation of donor animals, when tumors had developed in donor animals, tumors were aseptically dissected and mechanically minced. Approximately 3 mm³ tumor tissue was transplanted subcutaneously into nude
mice by a trocar needle. At the end of each experiment, mice were sacrificed under 3% isoflurane anesthesia using a small animal anesthetic device. Tumors were excised and weighed and necropsy was done. Tumor specimens were snap-frozen and stored at −70°C until further experiments.

RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was isolated using NucleoSpin RNA and Protein Purification Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. RNA from each sample (2,000 ng) was reverse transcribed to cDNA using a Tetro cDNA Synthesis Kit (Bioline, London, UK) in a final volume of 20 μL. In order to evaluate the expression of type I LH-RH receptors and LH-RH ligand, primer sets were designed. Gene-specific primers for LH-RH-I receptor: sense 5′-GACCTTGTCTGGAAAGATCC-3′ (EXON 1 1,844–1,863), antisense 5′-CAGGCTGATCAC CACCATCA-3′ (EXON 1 1,844–1,863), for LH-RH ligand: sense 5′-GCGTTATCTACTGACTTG-3′, antisense 5′-TCTTCTGGCCAGTTTCTCT-3′. Hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used as an internal reference gene (sense 5′-GTATTCATTATAGTCAAGGGCAT ATCC-3′, antisense 5′-AGATGGTCAAGGTCGCAAG-3′). mRNA levels of LH-RH-R-I, LH-RH, and HPRT1 have been assessed by iQ™ SYBR Green Supermix (Bio-Rad Laboratories Inc). All real-time amplifications were conducted according to the manufacturer’s protocol using MyiQ two-color real-time PCR detection system (Bio-Rad Laboratories Inc). All real-time amplifications were measured in triplicates. Results were evaluated with Bio-Rad iQ5 software (Bio-Rad Laboratories Inc) and changes in mRNA levels were calculated using the 2^(-ΔΔCt) method.

Immunocytochemistry

Immunoperoxidase staining

To detect LH-RH-I receptors, OCM-1 and OCM-3 cells were fixed in ice-cold methanol (10 minutes). Endogenous peroxidase activity was blocked in 3% hydrogen peroxide (10 minutes). Samples were permeabilized with 0.1% Triton X-100 and blocked with 1% bovine serum albumin (BSA) – 1% FBS solution in 0.1% Triton X-100 (room temperature, 1 hour). Samples were incubated with primary anti-LH-RH-R antibody (sc-13944 rabbit polyclonal; Santa Cruz Biotechnology Inc; 1:50) (overnight, 4°C) and anti-rabbit fluorescein isothiocyanate secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA; 1:1,000). Samples were rinsed and mounted with ProLong® Diamond Antifade Mountant with DAPI (Molecular Probes). Staining was evaluated using an Olympus FV-1000 confocal microscope (Olympus Corporation, Tokyo, Japan).

Western blot

Total protein was isolated using NucleoSpin RNA and Protein Purification Kit (Macherey-Nagel) according to the manufacturer’s instructions. Total protein amount of the supernatant was determined by a Nanodrop ND-1000 UV-Vis Spectrophotometer (ThermoFisher Scientific). Equal amounts of proteins (20 μg) were separated in 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred to polyvinylidene fluoride membrane using standard procedures. Upon blocking with 5% BSA, membranes were incubated with primary antibodies (overnight, 4°C): anti-LH-RH-R, 1:200 dilution (sc-13944 rabbit polyclonal; Santa Cruz Biotechnology Inc), anti-GAPDH, 1:1,000 dilution (D16H11 rabbit monoclonal; Cell Signaling Technology, Danvers, MA, USA). Proteins were detected with anti-rabbit horseradish peroxidase conjugated antibody (mouse sc-2357; Santa Cruz Biotechnology Inc) and Luminata Forte Western substrate kit (Agilent Technologies). Samples were rinsed with tap water, dehydrated through a graded series of alcohol, and mounted with ProLong® Diamond Antifade Mountant (Molecular Probes, Eugene, OR, USA).

Expression of LH-RH-I receptor and LH-RH ligand

Immunofluorescent labeling

To investigate LH-RH-I receptors, OCM-1 and OCM-3 cells were fixed in 4% paraformaldehyde at room temperature for 10 minutes, permeabilized with 0.1% Triton X-100 at room temperature for 1 hour, and blocked with 5% BSA in 0.1% Triton X-100 solution at room temperature for 1 hour. Samples were incubated with primary anti-LH-RH-R antibody (sc-13944 rabbit polyclonal; Santa Cruz Biotechnology Inc; 1:50) (overnight, 4°C) and anti-rabbit fluorescein isothiocyanate secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA; 1:1,000). Samples were rinsed and mounted with ProLong® Diamond Antifade Mountant with DAPI (Molecular Probes). Staining was evaluated using an Olympus FV-1000 confocal microscope (Olympus Corporation, Tokyo, Japan).

Preparation of membranes and radioligand binding studies

Preparation of membranes for receptor studies was performed as described previously. Receptor binding was characterized using sensitive in vitro ligand competition assay based on binding of [125I][D-Trp9]-LH-RH as radioligand to membrane homogenates. The binding characteristics of receptors for LH-RH-I were determined

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in the membrane fraction of OCM-1 and OCM-3 human UM cell lines (1.8–2.4 × 10^8 cells each) and in OCM-1 and OCM-3 tumors grown in nude mice. Radioiodinated derivatives of [D-Trp^6]LH-RH were prepared by the chloramine-T method and purified by reverse-phase high-performance liquid chromatography as described. The radioligand has been well-characterized previously and showed high affinity binding to LH-RH-I receptors expressed in human and rat pituitaries and human breast, prostate, and other cancers. In brief, membrane homogenates containing 50–160 µg protein were incubated in duplicate or triplicate with 60–80,000 cpm [^125I][D-Trp^6]LH-RH and increasing concentrations (10^{-12}–10^{-6} M) of nonradioactive peptides as competitors in a total volume of 150 µL of binding buffer. At the end of incubation, 125 µL aliquots of the suspension were transferred onto the top of 1 mL of ice-cold binding buffer containing 1.5% BSA in siliconized polypropylene microcentrifuge tubes (Sigma-Aldrich Co, St Louis, MO, USA). The tubes were centrifuged at 12,000 × g for 3 minutes at 4°C. Supernatants were aspirated and the bottoms of the tubes containing the pellet were cut off and counted in a gamma counter. Protein concentration was determined by the method of Bradford using a protein assay kit (Bio-Rad Laboratories Inc). The LIGAND-PC computerized curve-fitting program of Munson and Rodbard was used to determine the type of receptor binding, dissociation constant (K_d), and the maximal binding capacity of the receptors (B_max).

### Results

#### Expression of type I LH-RH receptors in human UM cell lines in vitro

mRNA expression for LH-RH-I receptors was analyzed in OCM-1 and OCM-3 cell lines by RT-qPCR. LH-RH-I receptors were detected in both these human UM cell lines, with a slightly higher expression of LH-RH-I receptor observed in OCM-3 cells (Figure 1A). Western blot analysis confirmed the presence of LH-RH-I receptors in OCM-1 and OCM-3 cells and revealed a signal corresponding to a protein of ~68 kDa, which is the molecular mass of the LH-RH-I receptor reported earlier (Figure 2). In accordance with the receptor mRNA data, a slightly higher protein expression of LH-RH-I receptors was observed in OCM-3 cells by Western blot and immunocytochemical analysis (Figure 3).

#### Expression of type I LH-RH receptors in vivo in tumor xenograft models

The mRNA expression for receptors for LH-RH-I in OCM-1 and OCM-3 tumors grown in nude mice was analyzed by RT-qPCR. mRNA for LH-RH-I receptors could be detected in all tumor xenografts (Figure 1A). In accordance with our in vitro results, the level of the LH-RH-I receptor transcript in our OCM-3 model was considerably higher than in the OCM-1 tumor samples in vivo. Western blot analysis also confirmed the presence of LH-RH-I receptor protein in OCM-1 and OCM-3 tumor tissues. Similar to our in vitro finding, higher expression level of LH-RH-I receptor was found in OCM-3 tumor xenografts, also detected by Western blots (Figure 2).

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**Figure 1** RT-qPCR analysis of the expression of mRNA for LH-RH-I receptor (A) and LH-RH ligand (B) in human uveal melanoma cell lines: 1) OCM-1, 2) OCM-3, 3) xenografted OCM-1, 4) xenografted OCM-3, 5) positive control, human pituitary. Y-axis represents fold change in gene expression, normalized to HPRT1 gene. Data represent mean values ± SE (n=3).

**Abbreviations:** LH-RH-I, luteinizing hormone-releasing hormone type I; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; OCM, ocular choroidal melanoma; SE, standard error.
Radioligand binding studies
The presence of specific LH-RH binding sites and characteristics of binding of $[^{125}\text{I}][\text{D-Trp}^6]\text{LH-RH}$ to membrane receptors on OCM-1 and OCM-3 human UM models were determined using ligand competition assays. Analyses of the typical displacement of radiolabeled $[\text{D-Trp}^6]\text{LH-RH}$ by the same unlabeled peptide revealed that the one-site model provided the best fit, indicating the presence of one class of high affinity LH-RH-I receptors in crude membranes derived from human UM cells. In cell membranes of OCM-1 and OCM-3 human UM cell lines, ligand competition studies also revealed a single class of high affinity binding sites for LH-RH-I with mean dissociation constants ($K_d$) of 4.11±0.3 and 4.26±0.6 nM, respectively (Table 1). The concentration of LH-RH-I receptors was 233.6±21.7 fmol/mg membrane protein in OCM-1 cells while OCM-3 cells showed a markedly higher receptor level (1.029±168.5 fmol/mg membrane protein) (Table 1). Receptors for LH-RH-I were also found in membranes of OCM-1 and OCM-3 tumor tissue samples. Radiolabeled $[\text{D-Trp}^6]\text{LH-RH}$ was bound to a single class of specific, high affinity binding sites in both human UM models investigated. Mean $K_d$ values were 5.85±0.7 nM for OCM-1 tumors and 6.18±0.8 nM for OCM-3 tumors (Table 2). Mean $B_{\text{max}}$ values were 267.3±38.5 fmol/mg membrane protein in OCM-1 tumors and about 2.7 times higher (713.0±29.4 fmol/mg membrane protein) for OCM-3 xenografts (Table 2). Biochemical parameters essential to establish the identity of specific binding sites were also determined. Thus, the binding of $[^{125}\text{I}][\text{D-Trp}^6]\text{LH-RH}$ was found to be reversible, time- and temperature-dependent, and linear with protein concentration in human UM samples. The specificity of LH-RH binding was demonstrated by competitive binding experiments using several peptides structurally related or unrelated to LH-RH. The binding of radiolabeled $[\text{D-Trp}^6]\text{LH-RH}$ was completely displaced by increasing concentrations ($10^{-12}$–$10^{-6}$ M) of LH-RH agonist buserelin and LH-RH antagonist cetrorelix (data not shown).

Expression of LH-RH mRNA in human UM cell lines and tumor xenografts
In addition to LH-RH receptor studies, the expression of LH-RH ligand in OCM-1 and OCM-3 models was also...
Correlation between type I LH-RH receptor and LH-RH ligand

According to our statistical analysis, there is a significant correlation between the expression of LH-RH-I receptor and LH-RH ligand in OCM-1 cell line and in OCM-1 tumor xenografts in vivo (Pearson $r=0.8380$; $p=0.0373$, CI =0.95%). Moreover, we also observed a significant correlation between the expressions of LH-RH-R-I and LH-RH ligand in OCM-3 cells and OCM-3 tumors grown in nude mice (Pearson $r=0.9878$; $p=0.0002$, CI =0.95%) (Figure 4).

Discussion

UM is the most common primary intraocular cancer of the eye. Risk factors for the development of UM are, among others, Caucasian ethnicity, light eye color, ocular melanocytosis and dysplastic naevus syndrome. Therefore, a better understanding of the molecular background of UM and the development of new therapeutic approaches are urgently needed.

Table 2 Binding characteristics of LH-RH-I receptors in OCM-1 and OCM-3 human uveal melanoma xenografted into nude mice

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$K_D$ (nM)</th>
<th>$B_{max}$ (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCM-1</td>
<td>5.85±0.7</td>
<td>1,029.1±294.4</td>
</tr>
<tr>
<td>OCM-3</td>
<td>6.18±0.8</td>
<td>713.0±40.3</td>
</tr>
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Notes: Binding characteristics were obtained from ligand competition assays (each done in triplicate). Based on the binding of radiolabeled [D-Trp$^5$]LH-RH to membrane homogenates. All values represent mean ± SE.

Abbreviations: LH-RH-I, luteinizing hormone-releasing hormone type I; OCM, ocular choroidal melanoma; SE, standard error; $K_D$, dissociation constant; $B_{max}$, maximal binding capacity.
inflict permanent damage to pituitary function and does not induce any cardiotoxicity.\textsuperscript{49,51,52} AN-152 has been tested in Phase I/II trials in castration-resistant prostate cancer and in Phase II and III clinical trials in endometrial and ovarian cancers.\textsuperscript{47,49,53,54} Modern LH-RH antagonists such as degarelix could be also tried.

In this study, we provide evidence for the existence of LH-RH-I receptors in two human UM cell lines and demonstrate that OCM-3 cells express LH-RH-I receptors at a higher level than OCM-1 cells. The same expression pattern has been observed in our in vivo models. Moreover, a remarkable expression of mRNA for LH-RH ligand was detected in both cell lines and cancer tissues grown in nude mice. Significant correlation was found between the LH-RH-I receptor and LH-RH ligand expression in OCM-1 and OCM-3 cell lines. The presence of LH-RH-I receptor protein was confirmed in both cell lines cultured in vitro and tissue samples from nude mice using Western blot. In addition, using ligand competition assay we examined the binding of \([^{125}I][D-Trp^6]LH-RH\) to membrane preparations of OCM-1 and OCM-3 human UM models. In both human UM models investigated, specific high affinity LH-RH-I receptors were found. The expression of LH-RH ligand and co-expression of LH-RH-I receptors support the idea that they might play a role in an autocrine and/or paracrine regulatory system in human UM; however, further studies are required to confirm this.

Our results provide further support to the hypothesis that locally produced LH-RH may participate in the regulation of tumor growth.\textsuperscript{19,20,25,50,55,56}

\[ \begin{align*}
\text{Conclusion} & \\
\text{In this study we demonstrated the expression of LH-RH-I receptor as a potential therapeutic target and LH-RH ligand in two human UM cell lines and tumor xenografts grown in nude mice. Our findings support the development of new therapeutic approaches based on LH-RH antagonists or cytotoxic analogs of LH-RH targeting LH-RH receptors in UM.} \\
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\text{Acknowledgments} & \\
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\text{Disclosure} & \\
\text{The authors report no conflicts of interests in this work.} \\
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References


