Essential role of p21/waf1 in the mediation of the anti-proliferative effects of GHRH antagonist JMR-132

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Abstract

GHRH, besides its neuroendocrine action in controlling the release of GH from the pituitary, stimulates the growth of various cancers *in vivo* and *in vitro* by direct mechanism(s). However, the molecular mechanism that mediates these proliferative effects of GHRH in extrapituitary tissues remains poorly characterized. In the present study, we investigated whether the tumor suppressor *p21/waf1* is involved in the mediation of the proliferative effects of GHRH in A549 human lung cancer epithelial cells. Exposure of A549 cells to the GHRH antagonist JMR-132 caused a significant inhibition in the rate of cell proliferation. In A549 cells, GHRH suppressed while JMR-132 increased the levels of *p21* expression in a dose-dependent manner. This suggests that GHRH could regulate *p21* levels. We then evaluated whether *p21* is required in A549 cells for the regulation of cell proliferation by GHRH. To this end, we knocked-down *p21* expression in A549 cells by siRNA and assessed the effects of antagonist JMR-132 on cell proliferation. We found that the loss of *p21* expression abolished the anti-proliferative effects of JMR-132. Suppression of *p21* expression by siRNA in human HT29 colon cancer cells and non-transformed mouse osteoblasts KS483 also blocked the anti-proliferative effects of JMR-132 suggesting that the regulation of cell proliferation by GHRH is *p21* dependent. These results shed light on the molecular mechanism of action of GHRH antagonists in tumor tissues and suggest that the antineoplastic activity of GHRH antagonists could be considered for the treatment of cancers expressing *p21*.

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Introduction

The hypothalamic neuropeptide growth hormone-releasing hormone (GHRH) stimulates the production and release of GH in the pituitary (Kiaris *et al.* 2005). Besides the hypothalamus, GHRH is produced by various extrapituitary tissues as well as by cancers in which it operates as a stimulator of cell proliferation. Consequently, inhibition of action of GHRH by specific antagonistic peptide analogs may emerge as a novel anticancer strategy (Schally *et al.* 2001, Kiaris *et al.* 2003a,b, 2005, Schally *et al.* 2008). However, despite a well-documented expression of GHRH in various primary and experimental human cancers and cell lines, as well as the clearly demonstrated antineoplastic activity of GHRH antagonists *in vitro* and *in vivo*, the knowledge about the mechanism of GHRH action at the subcellular level is limited. The major receptor that mediates the effects of GHRH in the extrapituitary tissues is SV1, a splice variant of the pituitary GHRH receptor that also has ligand-independent activity (Rekasi *et al.* 2000, Kiaris *et al.* 2002, Barabutis *et al.* 2007). With regard to the effects of GHRH on

proliferation, at the level of the signaling cascades activated upon binding of GHRH to its receptor(s), a role for mitogen-activated protein kinase (MAPK), and recently Pit-1 transcription factor has been recognized in pituitary cells (Pombo *et al.* 2000, Solloso *et al.* 2008). In breast cancer cells, Ras, Raf, and MAPK have been shown to play an essential role in the mediation of the proliferative effects of GHRH (Siriwardana *et al.* 2006). It has also been shown that in MCF-7 breast cancer cells, upon binding of GHRH to SV1 receptor, the increase in cell proliferation is accompanied by elevated expression of cyclin D1, an important cell-cycle regulator (Barabutis *et al.* 2007).

In the present study, we investigated the role of *p21* in mediating the effects of GHRH in the proliferation of A549 human lung cancer epithelial cells, HT29 human colon cancer cells, and mouse non-transformed osteoblasts KS483. *p21* is a cycle-dependent kinase inhibitor that plays a major role in the regulation of cell cycle and has been recognized as a direct target of *p53* tumor suppressor gene (el-Deiry *et al.* 1993, Weiss 2003). While *p21* is rarely mutated in primary human cancers, it is frequently characterized by aberrant expression.

Materials and methods

Peptides

Human GHRH (hGHRH) (1-29)-NH₂ was obtained from Dr Parlow (National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, USA). The synthesis of the GHRH antagonist JMR-132 [PhAc⁰,D-Arg²,Phe(4-I)⁶,Ala⁸,Har⁹,Tyr(Me)¹⁰,His¹¹,Abu¹⁵,His²⁰,Nle²⁷,D-Arg²⁸,Har²⁹] hGHRH(1-29)-NH₂, where Abu is α -aminobutyric acid, Har is homoarginine, Nle is norleucine, PhAc is phenylacetyl, and Tyr(Me) is *O*-methyltyrosine was reported previously (Buchholz *et al.* 2007).

Cell culture

A549 human lung alveolar and HT29 human colon cancer cells were obtained from American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM containing 10% fetal bovine serum (FBS) and antibiotics/antimycotics. KS483 cells (provided by Dr Karperien, The Netherlands) were grown in a-MEM Phenol Red free medium supplemented with 10% FBS DCC treated. Tissue culture reagents were obtained from Invitrogen. The rate of cell proliferation was assessed after exposure of cells for 48 h to GHRH or JMR-132 at concentrations indicated and the cells being counted under inverted microscope after standard trypan blue exclusion assay. Experiments have been performed in triplicates and average values are shown.

siRNA and western blot analysis

Suppression of *p21* in A549 and HT29 cells, or KS483 cells, was achieved by transfecting cells with siRNA specific for human or mouse *p21* (Ambion, Austin, TX, USA) respectively according to the manufacturer's instruction using Lipofectamine 2000 reagent. Forty eight hours post-transfection cells were lysed by using RIPA reagent and total protein was subjected to western blot analysis. Antibodies for *p21* and actin were obtained from Sigma. All experiments have been performed at least three times independently and similar results were obtained.

Statistical analysis

Statistical analysis was performed with the Student's *t*-test and results were considered significant when $P < 0.05$.

Results and discussion

Initially, we evaluated the effects of GHRH and JMR-132 on the proliferation of A549 cells. As depicted in Fig. 1, the exposure of A549 cells to JMR-132 at 10^{-6} M for

Figure 1 Proliferation of A549 cells in the presence of GHRH or JMR-132 at 10^{-6} M concentration. All experiments were performed in triplicates and average values are shown. JMR 132 inhibited the proliferation of A549 cells by about 35% ($P < 0.05$).

48 h resulted in 35% ($P < 0.05$) decrease, while GHRH caused about 10%, yet insignificant, stimulation on the rate of cell proliferation suggesting that these cells are sensitive to JMR-132 treatment. Consistent with previous findings on the effects of GHRH antagonists on cell proliferation, no evidence of cytotoxicity was noted in the cells exposed to JMR-132.

Subsequently, we evaluated the effects of these peptides on the expression of the tumor suppressor gene *p21/waf1*. A549 cells were exposed to GHRH or JMR-132 at 10^{-10} M, 10^{-8} M and 10^{-6} M and the expression of *p21* was assessed by western blot analysis. As shown in Fig. 2, increasing concentrations of GHRH caused a dose-dependent reduction while JMR-132 produced an elevation in the expression of *p21*. This is consistent with the effects of these peptides on the proliferation of A549 cells. It is noted, however, that whereas GHRH caused a considerable reduction in *p21* levels, its effects on cell proliferation were relatively small when compared with those of JMR-132, implying that *p21* levels might not be the limiting factor for the increase in cell proliferation elicited by GHRH. Furthermore, JMR-132 stimulated *p21* expression at 10^{-10} to 10^{-6} M while it inhibited cell proliferation only at 10^{-6} M. Probably this is due to the fact that *p21* induction is necessary but not sufficient for cell proliferation inhibition, or that a certain threshold in *p21* expression levels should be achieved in order to cause inhibition of cell proliferation.

To determine whether these findings are causatively associated with the anti-proliferative effects of JMR-132 or reflect the reduced proliferation of A549 cells, we knocked down *p21* expression by siRNA and then evaluated if JMR-132 was capable of suppressing A549 proliferation. As shown in Fig. 3, in the absence of *p21*, JMR-132 at 10^{-6} M lost its ability to inhibit A549 cell proliferation, indicating that this cell-cycle regulator is required for the suppression of cell proliferation by antagonistic analogs of GHRH. For this experiment, we used siRNA for *p21* at 5 nM, because at higher concentrations siRNA for *p21* exerted toxic effects (Fig. 3a and unpublished observations).

Figure 2 Modulation of *p21* expression by GHRH and JMR-132 in A549 cells. Western blot analysis shows that GHRH causes dose-dependent suppression (upper panel), whereas JMR-132 causes dose-dependent induction in the levels of *p21* (lower panel).

In order to test whether our findings represent a more general mechanism of GHRH action or they are limited to A549 lung cancer cells we evaluated the consequences of *p21* knock-down in cells of diverse origin, such as human colon cancer cells and mouse non-transformed osteoblasts KS483. In HT29 cells, we found that JMR-132 stimulated *p21* expression at 10^{-6} M– 10^{-10} M, notwithstanding the fact that stimulation was slightly more potent at 10^{-8} M (Fig. 4). Subsequently, we knocked-down *p21* by siRNA and tested the effects on cell proliferation, of JMR-132 at 10^{-8} M alone or in combination with *p21* siRNA at 10 nM. As shown in Fig. 4, suppression of *p21* expression significantly inhibited the anti-proliferative effects of JMR-132 ($P < 0.01$). Similar results were obtained with KS483 with the exception that in these cells, while treatment with JMR-132 10^{-6} M had no considerable effect on *p21* expression (not shown), it effectively blocked the suppression of *p21* caused by estradiol at 10^{-7} M. Furthermore, siRNA for *p21* at 5 nM

Figure 3 Requirement of *p21* for the anti-proliferative effects of JMR-132 in A549 cells. (a) Transfection of siRNA for *p21* reduced considerably *p21* levels as revealed by western blot analysis. The *p21* suppression was more efficient at 5 nM siRNA, while at higher concentrations siRNA treatment was toxic. (b) In the presence of 5 nM of siRNA for *p21*, JMR-132 was incapable of suppressing the proliferation of A549 cells ($P < 0.005$).

significantly inhibited the anti-proliferative effects of JMR-132 in the presence of 10^{-7} M estradiol (Fig. 5). Therefore, we conclude that modulation of *p21* expression by GHRH antagonists likely represents a general mechanism for the action of these agents in cell growth.

Figure 4 Effects of JMR-132 in *p21* expression and proliferation of HT-29 human colon cancer cells. (a) Effective stimulation of *p21* expression by JMR-132 at 10^{-6} M– 10^{-10} M. (b) siRNA for *p21* at 10 nM potently inhibited *p21* expression. (c) JMR-132 at 10^{-8} M inhibited HT-29 cell proliferation and this effect was abolished in cells transfected by siRNA for *p21* at 10 nM. * $P < 0.01$ versus controls.

Figure 5 Effects of JMR-132 in *p21* expression and proliferation of KS483 mouse non-transformed osteoblasts. (a) While treatment with JMR-132 10^{-6} M alone had no considerable effects in *p21* expression (not shown), effectively blocked the suppression of *p21* caused by estradiol at 10^{-7} M. (b) siRNA for *p21* at 5 nM potentially inhibited *p21* expression. (c) In the presence of 10^{-7} M estradiol, JMR-132 at 10^{-6} M inhibited KS483 cell proliferation and this effect was abolished in cells transfected by siRNA for *p21* at 5 nM. * $P < 0.01$ versus controls.

In none of the cells used above, siRNA for *p21* alone did not affect cell proliferation considerably, and scrambled siRNA also did not affect *p21* expression (data not shown).

Thus, considering previous findings on the mechanism of action of GHRH analogs in extrapituitary tissues, the interaction of GHRH with its membrane receptors, may initiate a signaling cascade that involves Ras, Raf, and MAPK and which eventually results in the modulation of *p21* expression in the nucleus. This mechanism of modulation of *p21* expression appears to play a major role in the mediation of the effects of GHRH analogs on cell proliferation.

These results shed light on the mechanism of action of GHRH antagonists by linking directly GHRH to the cell-cycle machinery, indicating that *p21* is a critical target of GHRH analogs. Our results also imply that the GHRH antagonists should be considered particularly for the therapy of cancers that express *p21* activity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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