Induction of Apoptosis in Pterygium Cells by Antagonists of Growth Hormone–Releasing Hormone Receptors

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PURPOSE. The aim of the study was to investigate the signaling of growth hormone–releasing hormone receptor (GHRH-R) in the pathogenesis of pterygium and determine the apoptotic effect of GHRH-R antagonist on pterygium epithelial cells (PECs).

METHODS. Fourteen samples of primary pterygium of grade T3 with size of corneal invasion ≥ 4 mm were obtained for investigation by histology, immunofluorescence, electron microscopy, explant culture, and flow cytometry.

RESULTS. We found that PECs were localized in the basal layer of the epithelium in advancing regions of the head of pterygium. These cells harbored clusters of rough endoplasmic reticulum, ribosomes, and mitochondria, which were consistent with their aggressive proliferation. Immunofluorescence studies and Western blots showed that GHRH-R and the downstream growth hormone receptor (GH-R) were intensively expressed in PECs. Their respective ligands, GHRH and GH, were also elevated in the pterygium tissues as compared to conjunctival cells. Explanted PECs were strongly immunoreactive to GHRH-R and exhibited differentiation and proliferation that led to lump formation. Treatment with GHRH-R antagonist MIA-602 induced apoptosis of PECs in a dose-dependent manner, which was accompanied by a downregulation of ERK1 and upregulation of Caspase 3 expression.

CONCLUSIONS. Our results revealed that GHRH-R signaling is involved in survival and proliferation of PECs and suggest a potential therapeutic approach for GHRH-R antagonist in the treatment of pterygium.

Keywords: pterygium, histopathology, GHRH-R signaling, GHRH-R antagonist, apoptosis

Pterygium is a common ocular-surface lesion that can cause loss of vision due to induced astigmatism or direct encroachment of the pterygium tissue onto the visual axis. Currently, the major treatment for pterygium is surgical removal, although the recurrence rate remains high. The p53 gene is expressed at high level in pterygium epithelial cells (PECs), but not in pterygium fibroblasts.2 Our recent study3 has identified that the transcriptional activity of p53 is suppressed by MDM2 in PECs. Similarly, in another ocular disease, namely, retinoblastoma, the transcriptional activity of p53 is also inhibited by MDM2.4 Despite p53 inactivity, our previous study5 has shown that antagonists of growth hormone–releasing hormone receptor (GHRH-R) can induce the expression of Caspase 3 (Casp3), which induces apoptosis in retinoblastoma cells. Therefore, we hypothesized that GHRH-R inhibitors could also induce apoptosis in PECs.

Several recent reports6,7 have shown that antagonists of GHRH-R can modulate cell proliferation and apoptosis in cancers. GHRH and GHRH-R were originally identified in the hypothalamic-pituitary axis. In addition, in our previous studies, both GHRH and its receptor are detectable in ocular tissues, including retinal ganglion cells, lens epithelium, ciliary body, and cornea in rats8 and human.9 Upon binding to GHRH-R, hypothalamic GHRH activates the synthesis and secretion of growth hormone (GH) from the pituitary, which plays a mitogenic role in stimulating cell proliferation and preventing apoptotic cell death. To study the roles of GHRH-R in these ocular tissues, we have developed several GHRH-R antagonists, including MIA-602, MIA-604, and MIA-690.7 These antagonists decrease cell proliferation and survival in cancer cell lines through pleiotropic antitumor mechanisms such as suppression of AKT/ERK signaling cascades and downregulation of PAK1-STAT3/NF-kB signaling pathway.10,11 Our previous study5 also has shown that MIA-602 can induce the expression of Casp3, which induces apoptosis in retinoblastoma cells.
Histologic Evaluation

Pterygium tissues were embedded in paraffin and sectioned for both hematoxylin and eosin (H&E) staining and immunofluorescence as described in our previous studies. In brief, three different pterygium tissues were stained with H&E and examined under a light microscope (shown in Fig. 2 and Supplementary Fig. S1). To determine the characteristics of PECs in the advancing regions, the slides were heated to induce epitope retrieval by using a Biocare Medical tissue processor (Walnut Creek, CA, USA). After blocking with 0.1% bovine serum at room temperature, rabbit polyclonal antibody to GHRH-R (1:80, ab28692; Abcam, Inc., Cambridge, MA, USA) or goat polyclonal antibody to GH-R (1:20, sc-10351; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied separately to the sections. The sections were examined under a fluorescence microscope (Diagnostic Instruments, Sterling Heights, MI, USA). Control sections were processed as above, but without primary antibody.

To study the ultrastructure of PECs, the advancing regions of pterygium were analyzed with transmission electron microscopy (TEM). Two different specimens were washed with PBS and fixed immediately in 2.5% (wt/vol) glutaraldehyde for 1 hour at room temperature, then postfixed for 1 hour in 0.5% (wt/vol) osmium tetroxide (OsO4; Sigma-Aldrich Corp., St. Louis, MO, USA). After dehydration in a series of ascending concentrations of ethanol (70% ethanol for 10 minutes, 95% ethanol for 10 minutes, 100% ethanol for 2 × 10 minutes), tissue specimens were embedded in Epon 812 (SPI Supplies, West Chester, PA, USA) and polymerized at 60°C for 24 hours. Sections at 1.5 μm were cut and stained with 0.1% toluidine blue for evaluation by light microscopy. Ultrathin sections at 50 nm were prepared, and images were acquired with a Hitachi H-7600 electron microscope (Hitachi, Tokyo, Japan). Normal bulbar conjunctiva was processed after the same procedure and served as controls.

Western Blot Analysis

Membrane proteins from three pterygium tissues were isolated by using Mem-PER Eukaryotic Membrane Protein Extraction Kit (Thermo Scientific, Waltham, MA, USA) and protein inhibitor cocktails (Complete Mini EDTA-free; Roche Diagnostics, Mannheim, Germany). Samples from normal conjunctiva were used as controls. Protein concentration was adjusted equally with protein assay kit (Bio-Rad, Hercules, CA, USA) before resuspension in 5× sample loading buffer for 5 minutes at 95°C and separated on SDS-polyacrylamide gel electrophoresis (8% for detecting GHR-H; 10% for detecting GHR-R, β-actin, and GAPDH; and 15% for detecting GHRH and GH). The procedures were performed as previously described.

Explant Culture and Treatment of Pterygium Tissues

In tissue explant culture, three fresh advancing heads of pterygium were washed with sterile PBS three times, and minced into several 1-2 mm² pieces. Minced tissues were placed into 12-well culture plates (Nunc, Roskilde, Denmark) with drops of culture medium containing DMEM (Dulbecco’s modified Eagle’s medium)/F12 (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA) and 1% penicillin/streptomycin (Life Technologies). After incubation in a humidified 5% CO₂ incubator at 37°C for 6 to 8 hours, the explants were attached to the substratum. Culture medium was added gently and changed every 2 to 3 days.
GHRH-R Signaling in Pterygium

RESULTS

External Features and Histology of Pterygium

Pterygium was characterized by a wing-shaped overgrowth onto the cornea and was grossly subdivided into head and body (Figs. 2A, 2B). Serial histologic sections (Figs. 2C, 2D, 2E) were taken from different parts of the pterygium and were indicated with lowercase c, d, and e, respectively, in Figure 2B. The typical PECs (Fig. 2C, green arrows) were the epithelial cells at the leading edge of the head that invade and disrupt the corneal epithelium (Fig. 2C, red arrows). Multiple nested aggregations of PECs (Fig. 2D, green arrows) were found adjacent to the advancing edges. In the pterygium head, mini-aggregations of PECs developed in the epithelium in the presence of a few goblet cells (black arrow) and stroma proliferation of fibroblast-vascular tissues, as well as infiltration of polymorphonuclear cells (Fig. 2E). No irregular invasion of pterygium cells or of fibroblast-vascular and inflammatory cells was detected in normal conjunctiva (Fig. 2F). The typical PECs were also noted in Supplementary Figures S1A and S1B.

Ultrastuctural Characterization of PECs

The advancing edges of pterygium were processed for TEM to identify the unique cytologic features of PECs. Toluidine blue stain showed that the PECs were clustered in the stratum basale (Fig. 3A). Under TEM, the PECs appeared as elongated cells with the basal regions anchoring to the stroma (Fig. 3B). The areas in Figure 3B indicated as c, d, and e were further elucidated at high magnification in Figures 3C, 3D, and 3E, respectively. Compared to normal conjunctival epithelial cells (Fig. 3F), PECs contained more highly dense rough endoplasmic reticulum (RER; Figs. 3C, 3D), considerable quantity of ribosomes (Fig. 3C, 3D, white arrowheads), and mitochondrion assembly (M in Fig. 3D). Notably, DNA-containing granules (Fig. 3D, white arrow) found within the mitochondrion were involved in mitochondrial reproduction. Intact RER-mitochondrion-
Expression of GHRH-R, GH-R, GHRH, and GH in Pterygium Head

Immunostaining of GHRH-R and GH-R in pterygium head demonstrated strong immunoreactivity of the PECs in the basal layers (Figs. 4C, 4D), whereas only a basal level of immunoreactivity of GHRH-R was found in the normal conjunctival epithelium (Fig. 4B). No detectable staining was observed in the pterygium head processed without the primary antibody (Fig. 4A). To verify the involvement of GHRH-R signaling in pathogenesis of pterygium, cells from the pterygium head were isolated and analyzed by Western blot. GHRH-R, GH-R, and their respective ligands (GHRH and GH) were predominantly expressed in pterygium heads but barely detected in normal conjunctiva (Fig. 4E). Of note, GH-R and GH presented as two isoforms in both pterygium and conjunctiva. The immunoreactive bands of GHRH-R, GH-R, GHR, and GH were normalized to the band intensity of their corresponding internal controls GAPDH and β-actin. Except for GH, the protein levels of GHRH-R, GH-R, and GHRH were significantly upregulated in the pterygium head when compared with normal conjunctiva (Fig. 4F).

GHRH-R Expression in Explant Culture of PECs

Explant cultures of the advancing pterygium head (Figs. 5A–C) showed that the PECs were actively migrating out from the explant in 24 hours. The cells proliferated rapidly in the following days (up to day 9) and formed lumps of cell aggregates (Figs. 5B, 5C; arrows). Before treatment with GHRH-R antagonist, the cells were processed for immunostaining of GHRH-R on day 5 in culture. All cells in the lump were intensely immunoreactive to GHRH-R antibody (Fig. 5D).

GHRH-R Antagonist Induced Apoptosis Through Suppression of ERK1 Expression and Induced Casp3 Expression in PECs

Explanted PECs were treated with 5 μM or 10 μM GHRH-R antagonist MIA-602 for 48 hours. Cells were then stained with propidium iodide, and the sub-G1 fractions of cells were quantified by using flow cytometry. Treatment with MIA-602 significantly induced apoptosis in the three samples examined (Figs. 6A, 6B), and the effects were dependent on the dose of the antagonist used. These findings support strongly that blocking of GHRH-R activity is effective in causing cell death in pterygium cells. To investigate the molecular pathways regulated by the GHRH-R antagonist in apoptosis, mRNA was extracted from MIA-602–treated cells and subjected to reverse transcription coupled with quantitative real-time PCR (qRT-PCR). In our previous study, we have found that GHRH-R antagonist suppresses ERK1 expression and induces Casp3 expression in retinoblastoma cells. In pterygium cells, we observed that MIA-602 could also significantly suppress ERK1 expression and induce Casp3 expression in a dose-dependent manner (Fig. 6C), suggesting that GHRH-R antagonist suppresses the cell proliferation pathway and induces the apoptosis pathway.

DISCUSSION

This study investigated the contribution of GHRH-R signaling to the pathogenesis of pterygium. Our major findings were as follows: (1) PECs are predominately localized in the advancing regions of pterygium during aggressive invasion; (2) GHRH-R and GH-R are intensely expressed in the PECs; (3) explanted PECs are strongly immunoreactive to GHRH-R with differentiation and proliferation followed by lump formation, which is highly consistent with the aggressive growth of pterygium; and (4) blocking GHRH-R activity-specific antagonist induces...
apoptosis of PECs in a dose-dependent manner, indicating a novel function of GHRH-R signaling in pterygium pathogenesis.

We have shown in a previous study that PECs exhibit stem cell–like properties and might act as a proliferation battery for the overgrowth of pterygium.15 These cells have been found to express high levels of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), which are less expressed in the pterygium fibroblasts and absent in normal conjunctival, limbal, or corneal cells.16 Through the secretion of MMPs and TIMPs in the advancing regions, PECs dissolve the Bowman’s layer of cornea for invasion and induce activation of the fibroblasts by Wnt/β-catenin pathway or microRNA-200a for proliferation.17,18 Investigation of PECs in the advancing region of pterygium showed that these cells display an aggressive behavior characterized by a multiple nested pattern of proliferation and gross invasion on the superficial cornea. Loss of Bowman’s layer is the first stage of PEC migration and invasion, and PECs have a high proliferative potency for continuous invasion. This is consistent with our findings that the cytoplasm of these cells accumulated several types of organelles, including RER, ribosomes, and mitochondria, which contribute to protein synthesis and energy production. In particular, desmosomal cadherin is known to promote cell growth, migration, and invasion through regulation of the signaling of cyclin-dependent kinase 2 (CDK-2), activator protein 1 (AP-1), and protein kinase C (PKC).19,20 The expression of E-cadherin is limited to the epithelial cells of pterygium head and not detected in the normal cornea and conjunctiva.21 Thus, our findings indicate that desmosomes between PECs not only provide hyperadhesive intercellular junctions, but may also facilitate the signal transduction mediating aggregation-dependent cell survival. Also, the RER-mitochondria contact would allow regulation of calcium signaling, metabolism, and cell survival, which may be involved in the pathogenesis of pterygium.22 These intracellular phenomena lead to continuous overgrowth of pterygium cells

Figure 4. Expression of GHRH-R, GHR, GHRH, and GH in pterygium heads (PTHs). (A–D) Immunofluorescence studies of GHRH-R and GHR expression: negative control in PTH without primary antibody (A), with basal level of GHRH-R detected in the normal conjunctival epithelium (red in [B]); of note, GHRH-R (red in [C]) and GHR (green in [D]) stained strongly on the PECs. (E) The expression of GHRH-R and GHR, and their ligands GHRH and GH, was further evaluated by using Western blot, showing that they were predominantly expressed in PTHs. Two isoforms of GHR and GH were observed in both conjunctiva and PTH. (F) The normalized intensities were obtained from the intensity values of each band over their internal control (GAPDH and β-actin, respectively); the expression levels of GHRH-R, GHR, and GHRH were significantly upregulated in PTHs compared to those of normal conjunctiva. bl, basal layer; Con, conjunctiva; PTH1-3, pterygium head obtained from three patients; se, superficial epithelium. Data are shown as mean ± SD; Mann-Whitney test; compared with the value in the conjunctiva, respectively; *P < 0.05; #, no significant difference; n = 3 in each group. Scale bar: 80 μm.

Figure 5. Explant cultures of advancing pterygium head. (A–C) The PECs (arrows) left the explanted tissues and proliferated in a 9-day culture. PECs showed multiple proliferation and lump formation (arrows in [B, C]). (D) On day 5, the cells were processed with immunofluorescence using GHRH-R antibody, showing strong immunostaining in PECs (red). T, tissue. Scale bar: 50 μm.
centripetal to the cornea, and the cells then proliferate rapidly followed by lump formation in the explanted condition. Our work showed an abundant level of GHRH-R and GH-R, and their ligands GHRH and GH in the pterygium tissues. Expression of the receptors was restricted to the PECs that were localized at the basal regions of the epithelium. There was no detectable level of GHRH-R and GH-R in the superior regions and in the fibroblasts (data not shown). Our data suggest that these PECs are hyperactive cells that may play a pivotal role in the pathogenesis of pterygium. Since they express GHRH-R, these cells respond to the autocrine and paracrine growth factor GHRH and augment the local synthesis of GH, which binds to its receptor GH-R for production of downstream molecules and exerts extrapituitary functions.8 GHRH/GHRH-R signaling regulates the survival and proliferation of pancreatic islet beta cells and endometrial cells, and promotes cardioprotection and wound healing. These activities are linked to the GHRH-related activation of survival kinase pathways, including MAPKs, ERK 1/2, and STAT3,23 which enhance survival of PECs.

The expression of GHRH-R was also found on the PECs emerging from the tissue of the pterygium head. With intensive GHRH-R expression, PECs grew rapidly and formed a lump after 5 days in culture. However, blocking GHRH-R activity with a potent antagonist, MIA-602, induced substantial cell death in these PECs in a dose-dependent manner. This finding is consistent with our previous report showing that three times higher incidence of apoptosis is observed after MIA-602 intervention in the retinoblastoma cells.5 The synthetic GHRH-R antagonists, therefore, possess potent and effective properties against cell proliferation through direct silencing of the GHRH-GH axis. There may be subsequent suppression of MAP kinases ERK-1/2, AKT/ERK, PAK1/STAT3, and P53 signaling pathways.6 Moreover, GHRH-R antagonists might also alter activities of cadherin, cyclin D1, CD44, PI3K/AKT, NF-kB, MMP-2, and MMP-9 to inhibit the epithelial-to-mesenchymal transition in the experimental benign prostate hyperplasia and prostate cancer cells.24,25 We found that GHRH-R antagonist suppresses the expression of ERK1 but induces Casp3 expression in the pterygium tissue, suggesting that MIA-602 induces apoptosis, as well as suppressing proliferation of PECs, which is consistent with previous findings showing that a polyphenol antioxidant, rosmarinic acid, inhibits the viability of PECs through upregulation of caspase 9 and caspase 3.26

In conclusion, PECs are actively proliferating cells supporting the growth and invasion of pterygium. Here we reported for the first time that GHRH-R signaling is present in these cells and plays a role in their survival and proliferation. The blockade of GHRH-R, using the synthetic GHRH-R antagonist, was effective in inhibiting the proliferation of PECs. Our findings demonstrated a novel action of GHRH-R signaling in pterygium and suggest a potential therapeutic use of GHRH-R antagonists for pterygium treatment.

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GHRH-R Signaling in Pterygium

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