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Galanin Receptor 1 Is Anti-proliferative

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human oropharyngeal SCC cell lines (20) were grown to 60–70% confluence in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml l-glutamine as described previously (21). An HPV16-immortalized human oral keratinocyte cell line (HOK16B, a gift from Dr. No-Hee Park, University of California, Los Angeles) was maintained in low-Ca” keratinocyte growth medium (KGM, Biowhittaker) (22).

**Galanin ELISA**—Conditioned medium was collected from HOK16B, UM-SCC17B, and UM-SCC22B, and a panel of well characterized oropharyngeal UM-SCC cell lines (UM-SCC-11A, -11B, -14A, -14B, -17B, -74A, -81B, -22A, and OSCC3) were collected at 24 h and assayed for GAL secretion by a competitive ELISA. 

**Western Blot Analysis**—An N-terminal peptide from GALR1 was selected based on its location in the first extracellular domain. Membranes were incubated with the primary antibody to the GAL substrate. Finally, plates were read at 480 nm with a microplate reader. The principle of this assay was that GALR1 in the conditioned medium would competitively inhibit the binding of GAL antibody to the GAL substrate.

**Proliferation Assay**—Rabbit anti-GAL polyclonal antibody or rabbit anti-GALR1 polyclonal antibody was used to investigate the role of GAL and GALR1 respectively, on cell proliferation. HOK16B, UM-SCC22A, and UM-SCC17B cells (1.5–3.0 × 10⁴) were cultured overnight in a 24-well plate in KGM or DMEM. The following day, these cells were treated with medium containing anti-GAL or anti-GALR1 antibodies or vehicle control for 24 h. Each antibody dilution was normalized to 1:50 rabbit serum concentration. Each treatment was performed in triplicate. Cells in three wells were counted prior to the incubation. After 24 h, total cell number was determined by trypan blue enumeration assay. GALR1, respectively, on cell proliferation. HOK16B, UM-SCC-22A, and UM-SCC-17B cells (1.5–3.0 × 10⁴) were cultured overnight in a 24-well plate in KGM or DMEM. The following day, these cells were treated with medium containing anti-GAL or anti-GALR1 antibodies or vehicle control for 24 h. Each antibody dilution was normalized to 1:50 rabbit serum concentration. Each treatment was performed in triplicate. Cells in three wells were counted prior to the incubation. After 24 h, total cell number was determined by trypan blue enumeration assay.
rabbit anti-GALR2 polyclonal antibody (1:3000, Alpha Diagnostics); rabbit anti-GALR3 antibody (1:1000, Alpha Diagnostics); mouse anti-glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (GAPDH, 1:5000, Chemicon International); anti-active MAPK (ERK1/2) (1:2000, Cell Signaling Technology, MA); and anti-ERK (Cell Signaling Technology). Membranes were washed in Tris-buffered saline containing 0.1% Tween 20 (Bio-Rad). Affinity-purified horseradish peroxidase-linked donkey anti-rabbit or goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) as a secondary antibody was used to detect primary antibodies. Visualization of the immunoreactive proteins was accomplished by the chemiluminescence system (Pierce, Rockford, IL) and exposure to film.

MAPK activation was evaluated following incubation of immortalized keratinocytes (HOK16B cells) and UM-SCC-11A cells in serum-free medium with 1:50 concentration of GALR1 polyclonal antibody or with 1:50 normal rabbit serum. For MAPK inhibition experiments, cells were preincubated in serum-free medium for 6 h prior to treatment with 10 nM U0126 for 24 h (Sigma). For experiments involving GAL stimulation, cells were stimulated with 150 nM GAL for 10 min following a 6-h preincubation in serum-free medium. Following standard Western blotting techniques as described above, the activation of the MAPK pathway was evaluated using anti-active MAPK (ERK1/2) (1:2000, Promega).
**Total RNA Isolation**—RNA was isolated using the TRIzol reagent according to the manufacturer’s instructions. cDNA was synthesized from 1/8 H9262 g of RNA using the reverse transcription system kit (Promega).

**Reverse Transcriptase-PCR**—The cDNA was used as template in PCR reactions to amplify GAL, GALR1, GALR2, and GALR3. Specific 5' and 3' -primers spanning the intron-exon boundaries in the region were GAL (5'-GCGCACAATCATTGAGTTTC-3' and 3'-TGCATAAATTGGCCGAAGAT-5'), GALR1 (5'-AAGAAGGCTACGTGGTGTG-3' and 3'-TGGATGCTTCAGACTTCTTTGA-5'), GALR2 (5'-CCACCATCTAGTACCCCTGGAC-3' and 3'-ACTGGCGGTAGTAGCTCAGG-5'), GALR3 (5'-CATGTACGCCAGCAGCTTTA-3' and 3'-ACGGTGCCGTAAGCTGAG-5'), and GAPDH (5'-GAGAAGGCTGGGGCTCATTTGACAG-3' and 3'-CCATCCACAGTCTTCTGGGTGCA-5').

**Human brain cDNA, used as a positive control, was a gift from Dr. A. Swaroop (University of Michigan).**

**Statistical Analysis**—All of the statistical comparisons were performed using the Student’s t test, and experiments were done in triplicate. A P value of ≤0.05 was used as a measure of significance.

**RESULTS**

**Galanin Is Secreted by Human Oropharyngeal Keratinocytes**—Although the growth effects of GAL have been studied in neuronal and non-neuronal cells (12–14), the role of GAL and its receptors in epithelial cell proliferation has not been explored. Hence, in this study, initial experiments investigated the expression and secretion of GAL by normal and malignant oral epithelial cells. Using reverse transcriptase-PCR, GAL mRNA was identified in immortalized oral keratinocytes (HOK16B) and oropharyngeal SCC cell lines including UM-SCC-17B, UM-SCC-11A, UM-SCC-22B, and UM-SCC-14A (Fig. 1A). As expected, the human brain, the positive control, showed a signal of similar molecular mass. The water control was appropriately negative. In order for GAL to stimulate the GALRs, it must be secreted. Secretion of the 3-kDa GAL neuropeptide from immortalized and malignant keratinocytes was investigated using a competitive ELISA (Fig. 1B). HOK16B and all of the SCC cell lines secreted GAL. Secretion was statistically greater in five SCC cell lines (17B and 14A (p ≤ 0.05) and 11A, 14B, and 74A (p ≤ 0.01)) when compared with the non-malignant HOK16B cells. In conditioned medium collected at 24 h, GAL secretion in UM-SCC-11A, UM-SCC-11B, UM-SCC-14A, UM-SCC-14B, UM-SCC-17B, UM-SCC-74A, and UM-SCC-81B consisted of a minimum of 175 ng/ml/million cells, whereas HOK16B, UM-SCC-22A, UM-SCC-22B, and OSCC3 secreted less than 50 ng/ml (Fig. 1B). Thus, GAL is transcribed in and secreted by non-malignant and malignant keratinocytes.

**Galanin Receptor 1 Is Anti-proliferative**—Several studies have shown that GAL has an autocrine mitogenic effect on neuronal cells and modulates regeneration and survival in these cells (12–14, 27). Hence, we hypothesized that if GAL has an autocrine mitogenic effect in oral epithelial cells, an anti-GAL antibody should...
compete for secreted GAL, thereby inhibiting proliferation. To test this hypothesis, HOK16B and two cancer cell lines, UM-SCC-22A and UM-SCC-17B, were incubated with varying concentrations of anti-GAL antibody for 24 h. Cell proliferation was determined by the trypan blue enumeration assay. These cell lines were selected, because they represent low and intermediate levels of GAL secretion, \( \frac{1}{H_{11021}} \) 50 ng/ml and \( \frac{1}{H_{11022}} \) 175 ng/ml GAL, respectively (Fig. 1B). All three cell lines exhibited a reduction in cell number with anti-GAL antibody; however, compared with control-untreated cells, HOK16B proliferation at 24 h was not significantly inhibited at any of the antibody concentrations tested (Fig. 2). In contrast, both of the cancer cell lines exhibited a dose-dependent inhibition of proliferation that was also significantly different from control at the highest antibody concentration (1:50,000). At this concentration, UM-SCC-22A exhibited a 38% inhibition \( \left( \frac{1}{H_{11006}} 3.9 \right) \), whereas UM-SCC-17B, which secretes more GAL, exhibited a 63% \( \left( \frac{1}{H_{11006}} 3.0 \right) \) inhibition. Thus, we conclude that GAL up-regulates proliferation in malignant keratinocytes.

**GALR1, GALR2, and GALR3 Are Expressed in Immortalized and Malignant Keratinocytes**—Three galanin receptor isoforms, GALR1, GALR2, and GALR3, have been identified in neuronal cells. The pro-proliferative effects of GAL on human keratinocytes suggested that these cells express at least one of the three receptors. To verify this hypothesis, the expression of these three receptors in immortalized and malignant oral keratinocytes was investigated by reverse transcriptase-PCR and immunoblot analysis. GALR1, GALR2, and GALR3 mRNAs were identified in non-malignant (HOK16B) and malignant (UM-SCC-17B, -11A, -22B, and -14A) oral keratinocyte cell lines (Fig. 3A, top, middle, and bottom panels, respectively). Signals of corresponding molecular mass were detected in the human brain, which was used as a positive control. GAPDH served as an internal control. Protein expression was confirmed using isofrom-specific galanin receptor antibodies (Fig. 3, B–D).

**An Antibody to the N Terminus of GALR1 Acts as a Competitive Inhibitor of Receptor Activation**—The GALR1 gene has been mapped to the chromosome 18q23 region, which has been implicated in the progression of head and neck SCC. Thus, to investigate GALR1 function and signaling in immortalized and malignant oral keratinocytes, an antibody was generated to the

![Fig. 6. Proliferation and MAPK activation are increased in UM-SCC-11A cells at 24 h following treatment with anti-GALR1 antibody. A, UM-SCC-11A cells were incubated in the presence of anti-GALR1 antibody, and proliferation was determined as described under “Experimental Procedures.” rbt, rabbit; ctrl, control. B, anti-GALR1-treated UM-SCC-11A cells were lysed, and MAPK activation was evaluated by immunoblotting with anti-active ERK monoclonal antibodies (Ab).](image1)

![Fig. 7. Proliferation of CHO cells, which do not express GALR1, is unaffected following anti-GALR1 antibody treatment. A, whole cell lysate of CHO cells (30 µg each) was electrophoresed, transferred, and blotted with antibodies to GALR1 or GAPDH. B, CHO cells were incubated in the presence of anti-GALR1 antibody (Ab), and proliferation was determined as described under “Experimental Procedures.” rbt, rabbit; ctrl, control.](image2)
N terminus, the ligand binding domain of GALR1, to serve as a competitive inhibitor of ligand binding. This strategy was adopted, because GALR1-specific agonists or inhibitors were unavailable. A peptide representing the ligand binding domain of the extracellular N-terminal region was synthesized and inoculated into rabbits to generate a GALR1-specific polyclonal antibody. Antibody specificity was verified by immunoblot analysis of whole cell lysates of 293T cells transfected with full-length GALR1 cDNA and HOK16B cells (Fig. 4). As expected, in 293T cells, the antibody detected a peptide in cells transfected with increasing concentrations of GALR1 cDNA, whereas no signal was detected in the vector control (pcDNA). This signal is specific for GALR1 and was not detected by antibodies to GALR2 or GALR3 (Fig. 4A). In HOK16B cells, the N-terminal antibody detected a single band at 70 kDa (Fig. 4B, left panel), which was not detected when the antibody was preincubated with increasing concentrations of the immunogenic peptide prior to Western blotting (Fig. 4B, middle and right panels).

Antibodies to receptors may have a stimulatory or inhibitory effect on receptor activation (28). To investigate whether the N-terminal GALR1 antibody inhibited receptor signaling, 293T cells were transfected with GALR1 and stimulated with GAL in the presence or absence of GALR1 antibody. Because stimulation of GALR1 facilitates MAPK activation (29–32), we investigated whether the GAL-induced MAPK effects in 293T cells were blocked by preincubation with the N-terminal GALR1 antibody (Fig. 4C). In 293T cells transfected with GALR1 as a model for GALR1 signaling, GAL stimulated ERK activation (Fig. 4C), whereas in empty vector (pcDNA) transfected cells, GAL did not induce ERK activation. Furthermore, MAPK activation was blocked when GALR1 cells were preincubated with the N-terminal GALR1 antibody prior to GAL treatment (Fig. 4C). Thus, the N-terminal GALR1 antibody specifically detects GALR1 and inhibits receptor activation.

**GALR1 Inhibits Proliferation in HOK16B and UM-SCC11A—**
Oropharyngeal SCC cells secrete GAL, which binds to GALR1, GALR2, and GALR3, all of which are variously expressed in SCC cell lines (Fig. 3). To identify the GALR1-specific effects, the antibody generated to the N-terminal ligand binding domain of GALR1 (Fig. 4) was used as a competitive inhibitor to investigate the functional role of this receptor in human keratinocytes. Because GALR1 has a proliferative role in neuronal cells (12–14), subsequent studies focused on a possible proliferative function for this receptor in SCC cells. The treatment of HOK16B with increasing concentrations of GALR1 rabbit polyclonal antisera showed a dose-dependent increase in total cell number when compared with the preimmune rabbit serum control (Fig. 5A). This increase was significantly different from the control at a 1:50 antibody dilution. Because varying serum concentrations in the different treatment groups can contribute to pro-mitogenic effects, serum content in all of the treatment groups was normalized with preimmune serum. Preincubation of the antibody with the immunogenic peptide abrogated the increase in the cell number observed in cells treated with GALR1 antibody alone (Fig. 5B). In cells treated with non-immune rabbit serum and peptide, the GALR1 N-terminal peptide also induced a slight but statistically insignificant increase in cell number. We postulate that the peptide may exert some effect by competitively binding endogenously secreted GAL. The MAPK pathway is a significant mitogenic pathway in oropharyngeal keratinocytes (33). Hence, we investigated whether the pro-proliferative effects of GALR1 antibodies correlated with MAPK activation. HOK16B were treated as above with GALR1 antibody (1:50), and whole cell lysates were prepared. As shown in Fig. 5C, in the presence of anti-GALR1 antibody, there was an increase in active phospho-

**ERK when compared with the rabbit serum control. In contrast, total ERK and GAPDH, which were used as loading controls, were unchanged. It is unlikely that ERK activation was due to a disparity in serum concentration, because the serum concentration was equivalent in both treatment groups. Thus, GALR1 inhibits proliferation by inhibition of MAPK activation.**

Incubation of UM-SCC-11A, a cell line with very high GAL secretion but low GALR1 expression, behaved similarly. It exhibited a dose-dependent increase in cell number with increasing concentrations of GALR1 antibody (Fig. 6A) and ERK activation (Fig. 6B) when compared with the serum control. This effect plateaued at a 1:50 antibody dilution. Thus, in both HOK16B (Fig. 5B) and UM-SCC11A cells (Fig. 6B), the MAPK pathway was activated following inhibition of GALR1, consistent with the anti-mitogenic function for GALR1 observed in human oral keratinocytes (Figs. 5A and 6A).

**Chinese Hamster Ovary (CHO) Cells Are Unaffected by GALR1 Inhibition—**
Previous studies have shown that CHO cells do not express GALR1 (34). These findings were confirmed using the anti-GALR1 antibody, which detected no signal in CHO cell lysates (Fig. 7A). To verify the specificity of the inhibitory effects of the N-terminal antibody on proliferation, CHO cells were treated using the same protocol described above for HOK16B and UM-SCC11A. As expected, in cells that do not express GALR1, the anti-GALR1 antibody did not affect proliferation (Fig. 7B). Thus, the N-terminal GALR1 antibody specifically inhibits the GALR1 receptor in GALR1-expressing cells.

**GALR1 Inhibits Keratinocyte Proliferation via the MAPK Pathway—**We observed that oral keratinocyte cell proliferation is stimulated by GALR1 inhibition and that this correlates with the activation of the MAPK pathway in these same cells. To directly investigate whether GALR1 inhibits MAPK activation in SCC, UM-SCC-11A cells were transfected with GALR1 or...
pcDNA control vector. Transfected cells were subsequently treated with GAL in the presence or absence of anti-GALR1 antibody. ERK activation was determined by immunoblot analysis (Fig. 8A) and quantified by densitometry. ERK activation was significantly decreased in untreated or GAL-treated GALR1-transfected cells (Fig. 8, A and B). As predicted based on the results in Fig. 6, when GALR1 cells were preincubated with the anti-GALR1 antibody prior to GAL treatment, ERK was significantly activated compared with vector controls (Fig. 8, A and B). These findings are consistent with an inhibitory effect of GALR1 on MAPK activation (Fig. 8A and B). To verify the role of the MAPK pathway in GALR1 signaling and regulation of cell growth in these tumor cells, a specific MAPK inhibitor, U0126, was used in anti-GALR1 antibody-treated HOK16B and UM-SCC-11A cells. Proliferation and MAPK activation for both cell lines were investigated. Following a 24-h anti-GALR1 antibody incubation, HOK16B and UM-SCC11A cell numbers were increased (Fig. 9, A and C, respectively). However, preincubation with U0126 abrogated this up-regulation of proliferation. ERK activation was increased in HOK16B cells and UM-SCC-11A cells as described above; however, when U0126 was added, ERK activation (Fig. 9, B and D, respectively) returned to control levels.

**DISCUSSION**

In this study, we show that GAL is secreted by oropharyngeal keratinocytes and inhibition of GAL binding in both non-malignant and malignant (SCC) keratinocytes decreases proliferation. This is the first report showing GAL and galanin receptor expression and function in epithelial cells of the oropharyngeal region and one of a very few reports characterizing the effects exerted by these important proteins at the cellular level. GAL distribution has been fairly well characterized in the central nervous system (10). High concentrations of GAL (1–5 pmol/mg protein) have been detected in the hippocampus, amygdala, nucleus accumbens, and hypothalamus (10, 35). GAL has also been shown in a number of peripheral organs, such as the pituitary, pancreas, respiratory tract, and gastrointestinal tract (10, 35). Variable GAL secretion by individual SCC cells may play a role in the regulation of proliferation in these cells. Consistent with this hypothesis, we observed that anti-GAL antibody treatment resulted in the inhibition of proliferation in GAL-secreting head and neck SCC cells. In the gastrointestinal tract, GAL is secreted by enteric nerves where it exerts a paracrine regulation of intestinal smooth muscle contraction and relaxation as well as regulation of pancreatic secretions (19, 36, 37). Because keratinocytes secrete GAL, it is likely that normal cells utilize GAL in an autocrine loop to regulate cell proliferation. This regulation may also be mediated by GAL secretion from neuronal tissue that lies in proximity to the oral epithelium. Further characterization of GAL secretion and the functional impact of varying endogenous levels may help us to better understand disregulated proliferation in malignant keratinocytes.

Growth regulation may be disrupted in SCC cells as a result of differential galanin receptor expression. A number of reports have implicated 18q loss of heterozygosity (LOH) in the progression of head and neck SCC (3, 4). Tumor progression following a
chromosomal loss would be consistent with a tumor suppressor hypothesis where mutation of one allele and a loss of another may lead to the loss of function of a potential tumor suppressor gene. This loss of function could have a profound impact on disregulated proliferation in head and neck SCC. Takebayashi et al. (38) identified the important 18q loci lost in head and neck cancer and speculated that tumor suppressor gene(s) were located in this region. Therefore, the identification of this gene(s) would help elucidate the mechanism by which head and neck SCC development and progression occur. GALR1 was identified as a gene within the minimally lost region with the highest LOH frequency in head and neck SCC (38). Because GALR1 regulates neuronal growth and development, it is an excellent candidate for growth regulation in oropharyngeal epithelial cells.

Our results in non-malignant and malignant keratinocytes consistently point to a growth suppressive function for GALR1. In UM-SCC-11A, a cell line that does not exhibit 18q LOH, and consistently point to a growth suppressive function for GALR1. In normal or reduced GALR1 expression may lead to unchecked cell proliferation (Fig. 10). Alternatively, normal GALR1 function and a loss of GALR1 function could generate the same effect (Fig. 10).

Additionally, disregulation of signaling proteins downstream from individual receptors may also affect this regulation. Consistent with this speculation, Wittau et al. (39) showed that activation of GALR2 stimulates G12/13 to induce MAPK activation and clonal growth of small cell lung cancer cells. Thus, GAL effects may be regulated at the level of the receptor or by differential intracellular signaling mechanisms (29).

Future studies will focus on the outcomes of differential galanin receptor activation as well as the initiation of downstream signaling molecules following binding of GAL to GALR1 and GALR2.

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