A Growth-Related Oncogene/CXC Chemokine Receptor 2 Autocrine Loop Contributes to Cellular Proliferation in Esophageal Cancer

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Abstract

Growth-related oncogene (GRO), a member of the CXC chemokine subfamily, plays a major role in inflammation and wound healing. CXC chemokines have been found to be associated with tumorigenesis, angiogenesis, and metastasis. Although elevated expression of GRO has been reported in several human cancers, the expression and role of GRO and its receptor, CXCR2, in esophageal cancer are poorly understood. This study used real-time reverse transcription-PCR (RT-PCR) and immunohistochemical approaches to show that GROα, GROβ, and CXCR2 are up-regulated in esophageal tumor tissue. Furthermore, GROα, GROβ, and CXCR2 are constitutively expressed in WHCO1, an esophageal cancer cell line that was used as a model system here. GROβ enhances transcription of EGR-1, via the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway, which can be blocked by a specific antagonist of CXCR2 (SB 225002) or specific antibody to GROβ. WHCO1 cells treated with SB 225002 exhibited a 40% reduction in cell proliferation. A stable WHCO1 GROα RNA interference (RNAi) clone displayed a 43% reduction in GROα mRNA levels as determined by real-time RT-PCR, reduced levels of GROα by fluorescence microscopy, and a 60% reduction in the levels of phosphorylated ERK1/2. A stable clone expressing GROβ RNAi displayed >95% reduction in GROβ mRNA levels, reduced levels of GROβ by fluorescence microscopy, and an 80% reduction in the levels of phosphorylated ERK1/2. Moreover, these GROα RNAi- and GROβ RNAi-expressing clones displayed a 20% and 50% decrease in cell proliferation, respectively. Our results suggest that GROα-CXCR2 and GROβ-CXCR2 signaling contributes significantly to esophageal cancer cell proliferation and that this autocrine signaling pathway may be involved in esophageal tumorigenesis.

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Introduction

A high incidence of esophageal squamous cell carcinoma has been reported in certain regions of the developing world, such as parts of Southeastern Africa, Iraq, South America, and China (1, 2). In South Africa, esophageal squamous cell carcinoma occurs with a high frequency (ASR 12.60/100,000 in males and 5.58/100,000 in females) and is one of the most common causes of cancer-related mortality in Black males (3). Despite the high mortality associated with this disease, the molecular events involved in the development of esophageal squamous cell carcinoma remain poorly understood. A better understanding of the molecular events involved in the development of esophageal squamous cell carcinoma may offer opportunities to identify diagnostic markers, therapeutic targets, or prognostic indicators for this disease. Using cDNA microarray analysis to identify genes potentially involved in the development of esophageal squamous cell carcinoma, we showed that primary esophageal tumor tissues expressed elevated levels of the chemokines growth-related oncogene (GROα) and GROβ, relative to adjacent normal tissues.2

Chemokines are a superfamily of small, cytokine-like proteins that selectively regulate the recruitment and trafficking of leukocyte subsets to inflammatory sites through chemotaxis (4). Members in this family have been divided into two major subfamilies, CXC and CC, based on the arrangement of the first two of the four conserved cysteine residues in the amino terminus of the proteins. The two cysteines are separated by a nonconserved amino acid in CXC chemokines and adjacent to each other in CC chemokines. Depending on the presence or absence of an ELR motif (Glu-Leu-Arg) immediately preceding the first cysteine, CXC chemokines are further subdivided into two groups, ELR+ CXC chemokines and ELR− CXC chemokines. ELR+ CXC chemokines, which are potent promoters of angiogenesis and bind to CXC chemokine receptor 1 or 2 (CXCR1 or CXCR2), include the GROs (GROα, GROβ, and GROγ), interleukin-8 (IL-8), epithelial-neutrophil activating protein (ENA-78), granulocyte chemotactic protein (GCP-2), neutrophil-activating protein (NAP-2), and β-thromboglobulin (5–7). ELR− CXC chemokines are potent inhibitors of the ELR+ CXC chemokine-mediated angiogenesis (6), and include monokine induced by IFN-γ (MIG), stromal cell-derived factor 1 (SDF-1), IFN-γ-inducible protein (IP-10), and platelet factor 4 (PF4; ref. 6). IP-10 and MIG bind to CXCR3 receptors, whereas SDF-1 only binds to the CXCR4 receptor (5). The biological functions of ELR+ CXC chemokines are primarily mediated via CXCR2, a 7-transmembrane G-protein coupled receptor. Signaling for this receptor is mediated via various signaling pathways, including the extracellular signal-regulated kinases (ERK; refs. 8, 9).

A large body of evidence indicates that CXC chemokines play a critical role in inflammatory reactions and wound healing (reviewed in ref. 10). Additional evidence suggests that certain chemokines are involved in tumorigenesis because a wide range of cancers express elevated levels of various chemokines, such as IL-8 in non–small cell lung carcinoma and gastric carcinomas (11–13), and GROα in melanoma and prostate cancer cells (14, 15). In melanoma cells, GROα acts as an autocrine/paracrine growth factor because these cells express both GROα and its receptor, CXCR2 (16, 17). IL-8 also functions as an autocrine growth factor in...
malignant melanoma cells (18) and other neoplasias, such as gastric (19), hepatic, and pancreatic cancers (20). Compared with GROα and IL-8, less is known about the role of GROβ in tumorigenesis, although reports have implicated GROβ in enhanced tumor growth of immortalized melanocytes (21).

Presently, the expression status and role of GROα and GROβ in esophageal squamous cell carcinoma is unknown. We therefore examined the expression of these two chemokines and their receptor, CXCR2, in esophageal squamous cell carcinoma tissues and normal control tissues. Our results show that tumor tissues overexpress GROα, GROβ, and CXCR2 compared with adjacent normal esophageal epithelial tissue, and, furthermore, that the esophageal squamous cell carcinoma cell lines WHCO1, WHCO5, and WHCO6 coexpress GROα, GROβ, and CXCR2. An antagonist of CXCR2 (SB 225002) reduced proliferation of WHCO1 cells, and knockdown of GROα and GROβ in WHCO1 cells using RNA interference (RNAi) reduced cell proliferation by 20% and 50%, respectively. These results implicate GRO and CXCR2 in a proliferative autocrine loop in esophageal squamous cell carcinoma cells.

**Materials and Methods**

**Northern blot analysis.** Total RNA was isolated from WHCO1 treated with 200 ng/mL anti-GROβ for the indicated times using TRIzol (Invitrogen Corporation, Carlsbad, CA) according to the instructions of the manufacturer. Northern blot analysis was done as described (22) using 5 μg total RNA. The nylon membranes containing transferred RNA were hybridized at 42°C in ULTRAhyb Ultrasensitive Hybridization Buffer (Ambion, Inc., Austin, TX) with the indicated probes (see below) labeled with [α-32P]dCTP using the Megaprimer DNA labeling system (Amersham Pharmacia Biotech UK Limited, Little Chalfont, Bucks, United Kingdom). The membrane was stripped and rehybridized with a β-actin probe to control for RNA loading. The probes included a 0.56 kb EcoRI fragment of EGR1 cDNA and a 0.63 kb EcoRI fragment of β-actin cDNA. After scanning the membranes using a phosphomager, the membranes were exposed to X-ray film.

**Real-time reverse transcription-PCR and reverse transcription-PCR.** Total RNA was prepared from four paired normal and esophageal squamous cell carcinoma biopsies and esophageal squamous cell carcinoma cultured cells using TRIzol (Invitrogen) according to the instructions of the manufacturer and quantified using UV absorbance at 260 nm (DU60 spectrophotometer, Beckman Instrument, Inc., Fullerton, CA). cDNA was synthesized using 5 μg total RNA and oligo(dT)20 primer using the SuperScript III system for reverse transcription-PCR (RT-PCR; Invitrogen) following the instructions of the manufacturer. Target primers for amplifying GROα, GROβ, and CXCR2 were designed using Primer Designer (Scientific & Educational Software Version 2.0). GROβ forward primer: 5′-ACCTCTCTGCACGATCTT-T-3′ and reverse primer: 5′-CTCCAGAACACG- CACCCG-3′; GROβ forward primer: 5′-AGCTCTCCTCCGCAACA-3′ and reverse primer: 5′-CTCCAGAACGACCACAA-3′; CXCR2 forward primer: 5′-CTCCAAATACGACGGTAC-3′ and reverse primer: 5′-GGCTCCAGG- GAAATACA-3′. Each 50 μl reaction mixture for specific RT-PCR contained 2.5 mmol/L MgCl2, 0.5 μmol/L of each primer. Each 20 μl reaction mixture for real-time RT-PCR contained 2.5 mmol/L MgCl2, 0.5 μmol/L of each primer, and 1 μL LightCycler FastStart DNA Master SYBR Green 1 (Roche Diagnostics South Africa) as described by the manufacturer. Briefly, 1.5 × 105 cells were plated in 96-well plates in a final volume of 180 μL DMEM per well. SB 225002 (antagonist of CXCR2, 400 nmol/L, Calbiochem, Darmstadt, Germany) was added to cell and 0.001% DMSO (solvent) was added as a control. After the indicated incubation period, 18 μL of the MTT labeling reagent (final concentration 0.5 mg/mL) was added to each well and incubated for 4 hours in a humidified atmosphere. One hundred eighty microliters of the solubilization solution were added to each well and the plates were left overnight at 37°C. The spectrophotometric absorbance of samples was measured at 595 nm using a microtiter plate reader.

**Inhibition of CXCR2 activity.** CXCR2 function was inhibited with either a specific antagonist (SB 225002) or GRO antibodies. For treatment with SB 225002, WHCO1 cells were plated at 1.5 × 105 per well in 2 mL DMEM in a six-well plate. Twenty-four hours later, the medium was changed, SB 225002 was added into the medium to the final concentrations of 25, 50, 100, 200, 400, and 800 mmol/L, and 0.001% DMSO (solvent) was used as a control. Cells were incubated at 37°C for a further 48 hours. For treatment with antibodies, WHCO1 cells were plated into six-well plates at 1 × 103 per well. Three days later, GROα antibody or GROβ antibody was added to the indicated concentration and incubated for the indicated period.

**Western blot analysis.** Treated WHCO1 cells were rinsed twice with ice-cold PBS and scraped off the plate in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 25 mmol/L Tris-Cl (pH 7.5), 1% sodium deoxycholate, 20 μg/mL pepstatin, 5 μg/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride], sonicated for 5 seconds on ice with a probe sonicator (Heat System-Ultrasonics, Inc., Plainview, NY), and centrifuged for 10 minutes at 13,000 × g in a microcentrifuge. The protein concentration of the lysates was determined using the BCA Protein Assay kit (Pierce, Rockford, IL). Fifteen micrograms protein per sample was electrophoresed on 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech UK) at 4°C for 1.5 hours. Blots were incubated for 1 hour with 5% nonfat dry milk to block nonspecific binding sites and then incubated with goat polyclonal antibody against phosphorylated ERK1/2 (Santa Cruz Biotechnology) at 4°C overnight. The immunoreactivity was detected using peroxidase-conjugated antibody and visualized by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce). The bands were stripped before reprobing with antibody to ERK2 (Santa Cruz Biotechnology).

**RNA interference.** To create the small interfering RNA (siRNA) plasmid constructs, complementary strands of oligonucleotides specifically targeting GROα and GROβ were synthesized. For GROα siRNA, oligo1: 5′-CTGTTTAGTGAATGCTTCCAGCATTTACGTTTATTTTTTT-3′, oligo2: 5′-AATTAAAAACGTCTTTGAGAATGTGTATCT-3′, and 5′-GACGCTTCTGACCCACATTTACCAGCAGTTTTTT-3′, for GROβ siRNA,
nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich South Africa Ltd., Aston Manor, South Africa). Fluorescence was observed by using a ×40 objective lens on an inverted microscope (Zeiss Axiosvert 200 M, Jena, Germany). The specificity of the antibodies was tested using specific blocking peptides for either anti-GROα or anti-GROβ using immunofluorescence staining.

Statistical analysis. The Student’s t test was used for statistical significance of differences in the expression of GROα, GROβ, and CXCR2 between groups. P < 0.05 was considered to be significant.

Results

Overexpression of GROα, GROβ, and CXCR2 in esophageal squamous cell carcinoma. An initial study using cDNA microarray analysis indicated that both GROα and GROβ were overexpressed by 1.7-fold in esophageal squamous cell carcinoma biopsies compared with adjacent normal tissue from the same individual (data not shown). Because the cDNA microarray was done with pooled RNA from three patients, using reciprocal labeling, further analysis was carried out to verify the difference in GROα and GROβ expression between normal and tumor esophageal tissue. Specific primers were designed to quantitate GROα and GROβ mRNA levels in tissue, and the PCR products generated (423 and 409 bp for GROα and GROβ, respectively; Fig. 1A) were sequenced to verify their identity. Real-time RT-PCR using total RNA isolated from esophageal squamous cell carcinoma and adjacent normal tissues confirmed that GROα was indeed overexpressed (by 2-fold to 5-fold, P < 0.05) in all four of the esophageal tumor tissues analyzed (Fig. 1B). Similarly, GROβ mRNA levels were also overexpressed (by 1.5-8 fold, P < 0.05) in three of the four esophageal tumor tissues examined, relative to adjacent normal tissue (Fig. 1C). To establish whether GROα and GROβ could potentially function as autocrine signaling molecules in esophageal squamous cell carcinomas, we determined the expression of CXCR2 (a receptor for GROα and GROβ) in the same tissues. CXCR2 mRNA levels were also overexpressed (by 1.6-20 fold, P < 0.05) in the tumor tissues examined (Fig. 1D). To verify the expression of these two chemokines and their receptor, CXCR2, immunohistochemical analysis was done using esophageal tumor and matched normal tissue sections obtained from esophageal cancer patients that had undergone esophagectomies. The immunohistochemical analysis showed that GROα was overexpressed in 7 of 10 tumor sections analyzed, and that GROβ was overexpressed in 11 of 14 tumor sections examined (Fig. 2A-D). These results confirmed that both GROα and GROβ were overexpressed in esophageal tumor tissue, and, furthermore, that these two chemokines were primarily located in the cytoplasm (Fig. 2A-D). CXCR2 levels were elevated in 88% of tumor sections examined (n = 65), whereas it was either absent or was present at very low levels in matched normal tissue

Figure 1. Expression of GROα, GROβ, and CXCR2 in esophageal normal and tumor tissues. Total RNA was extracted from biopsies (normal and tumor tissue) obtained from patients diagnosed with esophageal squamous cell carcinoma. The total RNA was subjected to RT-PCR, and the PCR products were analyzed by agarose gel electrophoresis (A, lane 1, DNA molecular weight marker VIII; lane 2, GROα RT-PCR product; lane 3, GROα RT-PCR product; lane 4, CXCR2 RT-PCR product). Real-time RT-PCR was also carried out with GROα-primer, GROβ-primer, and CXCR2-specific primers (B-D). Columns, ratio of GROα, GROβ, and CXCR2 mRNA relative to GAPDH in esophageal squamous cell carcinoma tumor samples and normal esophageal samples; bars, SD of each sample measured in triplicate.


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Our results also showed that the CXCR2 receptor was localized in the plasma membrane and cytoplasm of the cells examined. These findings demonstrating that esophageal squamous cell carcinomas coexpress GROα, GROβ, and CXCR2, and suggest that these chemokines could play an autocrine role in this poorly understood cancer.

Expression of GROα, GROβ, and CXCR2 in cultured esophageal cancer cell lines. Three cultured esophageal cancer lines (WHCO1, WHCO5, and WHCO6) were tested to identify a model system in which the role of CXCR2 and its ligands (GROα and GROβ) could be explored in esophageal squamous cell carcinoma. RT-PCR analysis using total RNA isolated from the three cell lines revealed that GROα, GROβ, and CXCR2 were expressed in all three cell lines examined (Fig. 3). WHCO1 was selected as a model system to further explore the role of CXCR2 and its ligands in esophageal cancer because this cell line constitutively expresses GROα, GROβ, and CXCR2.

Transduction of GROβ signal through the ERK1/2 pathway. Our next objective was to determine whether the observed expression of GROα, GROβ, and CXCR2 in WHCO1 cells constituted a functional autocrine loop in these cultured cells. Because recent evidence indicated that GROα could signal via the ERK1/2 pathway (24), we treated WHCO1 cells with SB 225002 (a specific inhibitor of CXCR2; ref. 25, 26), and measured levels of phosphorylated ERK1/2 in treated cells by Western blot analysis. Increasing concentrations of SB 225002 substantially reduced the levels of phosphorylated ERK1/2 in WHCO1 cells, leaving unphosphorylated ERK2 levels unaffected (Fig. 4A). Densitometric analysis of the bands indicated that 100 nmol/L SB 225002 reduced p-ERK1/2 levels by 30%, similar to the IC50 value of 88 nmol/L reported by Catusse et al. (26). Although the results in Fig. 4A strongly suggest that the CXCR2 receptors are activated in WHCO1 cells (leading to phosphorylation of ERK1/2), the identity of the ligand/s responsible for activating CXCR2 is less clear. To address this question, WHCO1 cells were incubated with either anti-GROα or anti-GROβ for the indicated times, followed by measurement of phosphorylated ERK1/2 levels. Treatment of WHCO1 cells with anti-GROβ substantially reduced the amount of phosphorylated ERK1/2 in treated cells (Fig. 4B). In contrast, treatment of WHCO1 cells with anti-GROα had no effect on the levels of phosphorylated ERK1/2 measured in the cell lysates, regardless of the time of exposure to the antibody (Fig. 4C). As another marker of the functionality of the GROβ/CXCR2 autocrine loop in WHCO1 cells, we also measured the effect of anti-GROβ treatment on the expression of EGR-1 mRNA because previous reports have identified EGR-1 as a downstream target of ERK1/2 signaling (27–29). Depletion of GROβ in the medium of WHCO1 cells (using anti-GROβ) resulted in a time-dependent reduction of EGR-1 mRNA levels in treated cells (Fig. 5). These results strongly support the hypothesis that GROβ can signal via the ERK1/2 pathway. The mechanisms underlying this process are currently under investigation.
suggested that GROβ signals via the ERK1/2 pathway and constitutes an autocrine loop with CXCR2 in cultured WHCO1 cells.

**Signaling through CXCR2 promotes proliferation of WHCO1 cells.** Because it is known that GROα stimulates cell proliferation in melanoma cells through an autocrine loop (14, 16), we explored the possible contribution of the GROα/CXCR2 and GROβ/CXCR2 loops to proliferation of WHCO1 cells. Blocking CXCR2 signaling in WHCO1 cells with 400 nm SB 225002 significantly decreased cell proliferation by ~40% to 50% (Fig. 6), suggesting that CXCR2-mediated signaling contributed substantially to proliferation of WHCO1 cells in culture.

However, this approach failed to differentiate between CXCR2 ligands secreted by WHCO1 cells and tissue culture serum–derived CXCR2 ligands. Furthermore, we could not exclude nonspecific effects of the antagonist (SB 225002) used at this concentration on cell proliferation. To address this problem, we used RNAi vectors to inhibit GROα and GROβ expression in stably transfected WHCO1 cells. Using fluorescent immunocytochemistry, we showed that stably transfected WHCO1 cells containing siRNA against GROβ expressed significantly lower levels of GROβ than cells transfected with vector only (Fig. 7A, top). Using commercially available competing ligands for GROα and GROβ, we confirmed that >90% of the fluorescence signal observed for these antibodies was specific (data not shown). Real-time RT-PCR analysis confirmed >95% (P < 0.05) reduction in GROβ mRNA levels in the GROβ RNAi clone relative to the vector control (Fig. 7B, top). Furthermore, the stably transfected clone expressing siRNA against GROβ displayed an 80% reduction in phosphorylated ERK1/2 levels relative to control cells (Fig. 7C), suggesting that secreted GROα contributed to the activation of this mitogen-activated protein kinase signaling pathway. Although WHCO1 cells stably transfected with pcDNA3.1-U6/GROαRNAi expressed lower levels of GROα (Fig. 7A, bottom) than control cells, the reduction in GROα expression was less substantial than the knockdown observed for GROβ (Fig. 7A, top). This was confirmed by real-time RT-PCR analysis that indicated that GROα mRNA levels had been knocked down by 50% (P < 0.05) in the GROα RNAi clone (relative to the vector control; Fig. 7B, bottom). The GROα RNAi clone only displayed a 60% reduction in the level of phosphorylated ERK1/2 relative to control cells (Fig. 7C). Reducing GROβ expression levels in WHCO1 cells with GROβ RNAi reduced the proliferation rate of these cells by ~50% relative to wild-type or the vector control WHCO1 cells (Fig. 8). WHCO1 cells stably transfected with GROα RNAi, however, displayed a 20% reduction in cell proliferation relative to vector control cells (Fig. 8). Cell cycle analysis by flow cytometry suggested that the reduced proliferation of GROα-RNAi and GROβ-RNAi-expressing stable clones of WHCO1 was not due to severe disruption of the cell cycle or cell cycle block because GROα-RNAi and GROβ-RNAi-expressing clones displayed cell cycle profiles similar to wild-type and vector control cells (data not shown).

**Discussion**

Hanahann and Weinberg (30) identified six biological characteristics that cells acquire en route in their transformation to cancer. One of these highlights the ability of cancer cells to lose their dependence on surrounding cells for proliferative signals, and, instead, to resort to alternative proliferative signals that facilitate unregulated growth. The results of this study suggest that esophageal...
squamous cell carcinoma cells express the functional autocrine loop involving GROα, GROβ, and their receptor, CXCR2, which contributes substantially to the proliferation of esophageal cancer cells in culture. The immunohistochemical evidence presented here suggests that the GROα/CXCR2 and GROβ/CXCR2 chemokine loop probably contributes to proliferation in most primary esophageal squamous cell carcinoma tissues in situ. Recent studies have shown an increasingly important role of chemokines in the biology of a wide array of cell types, including cancer cells, in addition to their role in regulating directional migration of leukocytes during inflammatory responses (31). Although this is the first description of a proliferative chemokine autocrine loop operating in esophageal cancer, other studies have implicated GROα and IL-8 in the progression of malignant melanoma, hepatic cancer, and pancreatic cancer via an autocrine loop involving CXCR2 (14, 16–18, 20).

Although the GROβ/CXCR2 chemokine loop clearly contributes to proliferation in WHCO1 cells, the role of GROα in these cells is less obvious, despite our demonstration that esophageal cancer cells express both GROα and GROβ (by immunohistochemistry and RT-PCR) and other studies showing that CXCR2 binds both GROα and GROβ (9).

The modest reduction (20%) in cell proliferation observed in a GROα knockdown clone (Fig. 8) probably reflect an incomplete knockdown of GROα mRNA levels because fluorescent microscopy revealed a larger knockdown effect on GROβ than GROα. The real-time RT-PCR results (Fig. 7B) also confirm that GROα was incompletely knocked down. Although only a 20% reduction of cell proliferation was observed in the GROα knockdown clone, our results still suggest that GROα signaling is mediated through ERK1/2, because a 60% reduction in phosphorylated ERK1/2 was shown in this clone. The apparent inability of anti-GROα treatment to reduce phosphorylated ERK1/2 levels in WHCO1 cells (Fig. 4C) could reflect the inability of the GROα antibody to remove secreted GROα from the culture medium.

Using cultured esophageal cancer cells as a model system, our results clearly suggest that GROβ signaling is also mediated through ERK1/2, and that blocking GROβ (either with an antibody, CXCR2 antagonist or RNAi) substantially reduced signaling through ERK1/2. This is consistent with previous evidence indicating that CXCR2 can signal through ERK1/2 in addition to other pathways (24). We also showed that GROβ makes a major contribution to the elevated transcription of EGR-1 in cultured esophageal cancer cells. Preliminary immunohistochemical results obtained in our laboratory

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**Figure 7.** RNAi mediated GROα and GROβ knockdown in WHCO1 cells. To selectively knock down GROα and GROβ, WHCO1 cells were stably transfected with either pcDNA3.1-U6/GROαRNAi or pcDNA3.1-U6/GROβRNAi and selected with G418. Cells were grown on coverslips and the immunofluorescence staining of GROα in the clone expressing GROα RNAi (A, top) and GROβ in the clone expressing GROβ RNAi (A, bottom) was done as described in Materials and Methods. Nuclei were visualized using DAPI. Magnification, x400. cDNA synthesized from RNA extracted from these clones was subjected to real-time RT-PCR using GROα-specific and GROβ-specific primers (B), whereas the levels of phosphorylated ERK1/2 were detected by Western blot using a specific antibody to phosphorylated ERK1/2 (C).

**Figure 8.** Proliferation of WHCO1 cells transfected with either pcDNA3.1-U6/ GROαRNAi or pcDNA3.1-U6/GROβRNAi. To determine the effect of GROα/RNAi and GROβ/RNAi silencing on the proliferation of WHCO1 cells, the MTT assay was done as described in Materials and Methods and the absorbance measured at 595 nm using a microtiter plate reader.
show that EGR-1 is significantly overexpressed in esophageal squamous cell carcinoma tissue compared with normal esophageal squamous epithelial tissue. The relationship between GROβ and EGR-1 expression in esophageal cancer is presently being explored further in our laboratory. The implications of the relationship between GROβ and EGR-1 are very significant because EGR-1 is known to regulate the expression of many genes involved in tumorigenesis, including insulin-like growth factor-II (32, 33) and vascular endothelial growth factor (34).

Because ELR-containing chemokines, such as GROα, GROβ, and IL-8, are potent angiogenic factors (8), our observation that esophageal squamous cell carcinoma cells secrete GRO may facilitate the gain in these cells of yet another important phenotypic hallmark of cancer (30). In this context, the GROα/CXCR2 and GROβ/CXCR2 loops may play an important role in the development and maintenance of squamous esophageal cancer, and consequently this chemokine system represents a significant therapeutic target that should be considered in the future treatment of esophageal cancer. Approaches targeting chemokine systems in other cancers have already shown considerable promise. A small molecular inhibitor of CXCR4 (AMD3100) reduced proliferation and induced apoptosis of intracranial xenograft brain tumors in mouse model studies (35). Progress made in the development of orally bioavailable, potent antagonists of CXCR2 (36) suggest that clinicians may soon have at their disposal a range of small molecule antagonists of CXCR2. The results of the present study (showing reduced proliferation of cultured cancer cells in response to CXCR2 antagonist or RNAi treatment) have highlighted the feasibility of disrupting the GROα/CXCR2 loop as a treatment strategy in squamous esophageal cancer. Studies showing that mouse gene knockouts of CXCR2 display minimal pathology (37) suggest that this treatment route warrants further investigation, despite the pleiotropic effects of chemokine systems.

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