ORIGINAL ARTICLE

PRODUCTION OF GROWTH-RELATED ONCOGENE PROTEIN-α IN A HUMAN ORAL SQUAMOUS CELL CARCINOMA CELL LINE STIMULATED WITH TUMOR NECROSIS FACTOR-α: ROLE IN TUMOR ANGIogenesis AND TUMOR PROLIFERATION

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Abstract

The CXC chemokine growth-related oncogene protein-α (GRO-α) has a wide variety of biological activities including as neutrophil trafficking or migration of vascular endothelial cells. In addition, studies have shown a crosstalk between tumor cells and vascular endothelial cells; GRO-α released by endothelial cells induces invasion of tumor cells toward endothelial cells, indicating an importance of GRO-α in a tumor environment. Oral squamous cells are reported to produce GRO-α in response to cytokines such as tumor necrosis factor-α (TNF-α). However, little is known about how GRO-α is involved in oral cancer. Here, we investigated the biological role of GRO-α for both tumor growth and angiogenesis in oral squamous cell carcinoma cells. We first evaluated the effect of TNF-α on GRO-α expression in three oral cancer cells from different origins. Among the cell lines we used, KOSC-2 cells expressed the highest amount of GRO-α mRNA in response to TNF-α. TNF-α-treated condition medium from KOSC-2 cells enhanced endothelial cell chemotaxis and the chemotactic activity was partially inhibited by the addition of neutralizing anti-GRO-α antibody. In addition, GRO-α exerted tumor cell migration of KOSC-2. From these results, we conclude that GRO-α may contribute to both angiogenesis and proliferation in oral cancer.

Key words: growth-related oncogene protein-α (GRO-α); tumor necrosis factor-α (TNF-α); oral squamous cell carcinoma cells.

抄録　CXC ケモカインファミリーである GRO-α は、好中球走化性因子として知られている他、腫瘍増殖性や血管新生能を有することが明らかとなっている。これまでに口腔粘膜上皮の GRO-α 産生は報告されていたが、GRO-α の口腔癌における役割は不明である。そこで本研究では口腔癌における GRO-α を介した血管新生作用や腫瘍増殖効果について実験的に検討した。3 種類の口腔扁平上皮癌由来細胞に TNF-α 処理をしたところ、GRO-α の発現量は細胞間で大きく異なっており、TNF-α 依存的な GRO-α 産生は個々の腫瘍細胞の性質に依存することが示唆された。GRO-α を最も多く産生した KOSC-2（舌癌由来細胞株）の TNF-α 処理後の培養上清は、血管内皮細胞の走化性を亢進し、GRO-α 特異的な中和抗体の添加はその亢進を部分的に抑制した。さらに、ヒト組換え型 GRO-α は KOSC-2 の増殖を促進した。これらの結果から、口腔癌において GRO-α は腫瘍の増悪因子である可能性が示唆された。

キーワード：GRO-α；腫瘍増殖因子（TNF-α）；口腔扁平上皮癌

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**Introduction**

Tumor necrosis factor-α (TNF-α) regulates a variety of biological functions related to inflammatory reactions, cell growth and apoptosis; and the most important source of TNF-α is macrophages. TNF-α affects carcinoma cells to induce the expressions of many cytokines. Constitutive activation of nuclear factor-κB (NF-κB) is observed in many types of cancer cells, strongly suggesting a critical role in cancer development and progression. Among several carcinogens, TNF-α is thought to be the most potent activator of NF-κB. In the tumor mass, tumor-associated macrophage (TAM) should be major source of TNF-α.

Growth-related oncogene protein-α (GRO-α) / CXCL1 was first identified as a growth factor of melanoma. GRO-α belongs to the C-X-C chemokine family and has chemotactic activity for neutrophils. Some types of the C-X-C chemokine family, which contain the sequence Glu-Leu-Arg (the ELR motif) in front of the C-X-C motif, have been shown to possess a potent angiogenic property. Interleukin-8 (IL-8) / CXCL8, epithelial and neutrophil activating protein-78 (ENA-78) /CXCL5, and GRO-α are the members of this group. A variety of chemokines including GRO-α are rapidly and markedly induced by TNF-α. This indicates that GRO-α acts as the secondary mediator in response to TNF-α. TNF-α has also be reported to induce GRO-α normal oral keratinocytes; however, the role of GRO-α in oral squamous cells has not been proven by experimental analysis.

In healthy oral mucosa, IL-8 and monocyte chemotactic protein-1 (MCP-1) /CCL2 mRNA are constitutively expressed whereas mRNA expression of GRO-γ /CXCL3, a member of GRO family chemokine, is significant lower. In contrast, high level of GRO-α expression is shown to be correlated with both tumor angiogenesis and lymph node metastasis in oral cancer. Furthermore, microarray analysis revealed that GRO-α is more markedly expressed in oral cancer cells than in normal oral epithelial cells. These suggest the essential role of GRO-α in oral cancer cells.

Endothelial cells express NF-κB-dependent GRO-α, mostly in response to TNF-α. CXC chemokines including GRO-α and IL-8 secreted by endothelial cells have been shown to induce tumor cell invasion. On the other hand, the role of GRO-α, which is produced from oral squamous carcinoma, is incompletely understood.

We have been studying the effect of TNF-α on human oral squamous cell carcinoma, and here we report the expression of GRO-α is cell line-specific, even in response to TNF-α. We also studied the effect of GRO-α on tumor growth and endothelial cell chemotaxis.

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**Materials and Methods**

**Reagents**

Cell culture medium Humedia EB-2 and its supplements were purchased from Kurabo (Osaka, Japan). Primer oligo(dT), M-Mulv reverse transcriptase were from GIBCO-BRL (Gaithersburg, MD, USA). Digoxigenen (DIG)-labeling and detection systems were obtained from Boehringer Mannheim (Mannheim, Germany) and a GRO-α enzyme-linked immunosorbent assay (ELISA) kit from R&D Systems (Minneapolis, MN, USA). An RNeasy total RNA isolation kit and Taq DNA polymerase were from Qiagen (Hilden, Germany). A Northern Max kit and a Lig’nScribe kit were from Ambion (Austin, TX, USA).

**Cell culture**

A cell line of human oral squamous cell carcinoma, KOSC-2, was a generous gift from...
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The other human oral squamous cell carcinoma cell lines, HSC-3 and Ca9-22 were purchased from JCRB Cell Bank (Osaka, Japan). The cells were cultured using RPMI-1640 (KOSC-2) or DMEM (HSC-3 and Ca9-22) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. The cells were subjected to the stimulation with TNF-α when they reached about 80% confluence.

Human umbilical vein endothelial cells (HUVECs) were purchased from KURABO (Tokyo, Japan). The cells were cultured in Humedia EB-2 supplemented with 2% FBS, 10 ng/mL recombinant human (r(h)) epidermal growth factor, 5 ng/mL r(h) basic fibroblast growth factor, 1 μg/mL hydrocortisone and 10 μg/mL heparin. CD45+ cells were found in the cultures.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the cells using an RNeasy total RNA isolation kit. Single-strand cDNA was synthesized from 1 μg of total RNA using primer oligo(dT)12,18 and M-Mulv reverse transcriptase. A CFX96 Real-Time PCR System (Bio-Rad) was used for quantitative analyses of GRO-α and 18S rRNA expression. The sequences of the primers were:

GRO-α-F (5’-ATGGCCCGCGTGCTCTCC-3’),
GRO-α-R (5’-GTTGGATTCTCAGTTCCAG-3’),
18S rRNA-F: 5’-ACTCAACACGGGAAACCTCA-3’,
and rRNA-R: 5’-AACCAGACAAATCGCTCAGTTCAG-3’.

Amplifications were performed using iQ SYBR Green Supermix (Bio-Rad), according to the manufacturer’s specifications. Cycling conditions were as follows: 50°C, 2 min; 95°C, 3 min; 40 cycles of 95°C (15s) + 58°C (30 s) + 72°C (30 s). A melting curve was generated by acquiring fluorescence measurements while slowly heating to 95°C at a rate of 0.1°C per second. Melting curves and quantitative analysis of the data were performed using a CFX manager, as previously reported.

ELISA for GRO-α

After the treatment with TNF-α, the KOSC-2 cells were washed twice with RPMI-1640 and incubated for 2 h in RPMI-1640 containing 0.5% human serum albumin (RPMI-HSA). The medium was collected and subjected to ELISA for GRO-α.

Endothelial cell chemotaxis

Endothelial cell chemotaxis was examined using a 24-well chemotaxis chamber as described previously. Briefly KOSC-2 cells were grown to confluence and stimulated for 4 h with 10 ng/mL TNF-α. Then the medium was replaced with Medium 199 containing 0.5% HSA (M199-HSA), and the cells were conditioned for 2 h. Aliquots (100 μL) of the conditioned medium, M199-HSA containing 1 ng/mL r(h) GRO-α, 10 pg/mL vascular endothelial growth factor (VEGF), or control medium were placed in lower chambers and upper chambers filled with 100 μL of HUVEC suspension (1x10⁵ cells/mL M199-HSA). When indicated, an anti-GRO-α neutralizing antibody was added to the medium. After incubating for 4 h at 37°C, the membrane from each chamber was fixed with methanol and stained with Giemsa solution. Transmigrated cells in random four low-power fields were counted under a microscope.

Wound assay

Confluent monolayers of KOSC-2 cells were wounded using a scalpel and a rubber policeman as described. Then the cultures were washed with 20 mM phosphate-buffered saline, pH 7.4 (PBS), and further incubated in the conditioned medium of the cells stimulated for 4 h with 10 ng/mL TNF-α. The cells were washed with PBS, fixed with 10% formaldehyde, and photographed under a microscope. Control
medium and the medium containing 1 ng/mL r(GRO-α) were also tested in parallel.

**Statistics**

For chemotaxis assay (Fig. 3.), data were analyzed using one-way analysis of variance (ANOVA) to compare the treatment effects. Tukey’s post-hoc analyses were applied for multiple comparisons, with the statistical significance set at P<0.05.

**Results**

Expression of GRO-α in oral squamous cell lines stimulated with TNF-α

We first asked whether most of the oral squamous cancer cells can induce GRO-α in response to TNF-α. In this study, we used three oral cancer cells from different donors to observe GRO-α expression in response to TNF-α. TNF-α (10 ng/mL) transiently expressed
GRO-α in KOSC-2 and HSC-3 cells. In both cells, GRO-α mRNA reached the maximal level 4 h after the stimulation with TNF-α (Fig 1A). The induced levels of mRNA levels of GRO-α in KOSC-2 were markedly higher than that in HSC-3 cells. In contrast, no such increase of GRO-α was observed in Ca9-22 (Fig 1A). These observations suggested that the induction of GRO-α in response to TNF-α varies depending on the cell type. The time course of GRO-α protein secretion corresponded with that of the mRNA expression (Fig. 1B).

Fig. 2 Concentration-dependent induction of GRO-α by TNF-α. KOSC-2 cells were incubated with 0.01-100 ng/mL TNF-α for 4 h. (A) The expression of mRNA for GRO-α or GAPDH was analyzed by RT-PCR. (B) The conditioned medium of KOSC-2 cells was collected and subjected to ELISA for GRO-α. Means (± SD) of three experiments are shown.

TNF-α enhanced GRO-α mRNA expression of KOSC-2 cells in a concentration-dependent manner (Fig. 2A). The expression of GRO-α was observed from the treatment with 0.1 ng/mL TNF-α. TNF-α also stimulated the secretion of GRO-α protein and the maximal effect was observed at 10 ng/mL (Fig. 2B).

GRO-α has chemotactic activity for endothelial cells

The results of endothelial cell chemotaxis are summarized in Fig. 3. VEGF is known as
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A potent angiogenic factor, and thus we used VEGF as a positive control for this migration assay for endothelial cells. As we expected, only small amount of VEGF could induce chemotaxis in HUVECs. The conditioned medium from TNF-α-treated KOSC-2 cells significantly enhanced the transmigration of endothelial cells, and r(h) GRO-α was also found to be active in this assay. To evaluate the possible role for GRO-α in the TNF-α-treated conditioned medium, we added anti-neutralizing antibody against GRO-α in the conditioned medium, and
found partial, but significant ($P<0.05$) inhibition by GRO-$\alpha$ neutralization. These data suggest a positive role of GRO-$\alpha$ as a secondary mediator. In agreement with this result, $r(h)$ GRO-$\alpha$ induces chemotaxis of HUVECs.

GRO-$\alpha$ promotes migration of KOSC-2 cells

The results of KOSC-2 cell migration in wound assay are shown in Fig. 4. Twenty hours after wound assay, control KOSC-2 cells grew into the wounded area. In contrast, the growth was faster in the cells incubated with the medium conditioned by TNF-$\alpha$-treated cell. $r(h)$ GRO-$\alpha$ also showed a migration promoting activity on KOSC-2 cells.

Discussion

TNF-$\alpha$ was first identified as a factor that induces necrosis of tumor cells; however, various functions of this cytokine have been demonstrated thereafter$^{22, 23}$. In some case, it serves as a "tumor growth factor"$^{24}$. TNF-$\alpha$ activates transcriptional factors such as AP-1 and NF-$\kappa$B, and subsequently induces the expression of various chemokines$^{25, 26}$.

In the present study, we initially found that TNF-$\alpha$ induces expression of GRO-$\alpha$ in KOSC-2 cells. GRO-$\alpha$ is known to be expressed in various types of cells including endothelial cells, bronchial epithelium, macrophages and polymorphonuclear neutrophils$^{27-30}$. Previous report has shown the expression of GRO-$\alpha$ by TNF-$\alpha$ in oral keratinocytes$^{12}$. However, Ca9-22 derived from an oral squamous cell carcinoma did not express GRO-$\alpha$ in response to TNF-$\alpha$. Moreover, super-induction of GRO-$\alpha$ was observed in TNF-$\alpha$-treated KOSC-2 cells. These results suggested that the level of GRO-$\alpha$ is dependent on individual oral squamous cell carcinoma. GRO-$\alpha$ has a neutrophil chemotactic activity and plays an important role in inflammatory responses, but the ubiquitous nature of its expression suggests that GRO-$\alpha$ is involved in biological events other than leukocyte chemotaxis$^9, 10$. In fact some members of C-X-C chemokines that contain ELR motif are demonstrated to act as an angiogenic factor, while the members that lack ELR motif serve as an angiostatic factor$^{10}$. In the present study, we found that the conditioned medium from TNF-$\alpha$-treated KOSC-2 cells contained a substantial amount of GRO-$\alpha$ protein and enhanced endothelial cell transmigration. $r(h)$ GRO-$\alpha$ was also found to enhance endothelial migration. Although the medium conditioned by the TNF-$\alpha$-treated KOSC-2 cells contains many endothelial chemotactic factors, such as IL-8, ENA-78, or VEGF (data not shown), GRO-$\alpha$ may partly account for the activity in the conditioned medium.

GRO-$\alpha$ was originally found as a factor that promotes the growth of melanoma cells, and a subsequent report demonstrated the growth-enhancing effect on other malignant tumors$^{7, 8, 31}$. We demonstrated, in the wound assay, that GRO-$\alpha$ enhances the growth of KOSC-2 cells; and TNF-$\alpha$ may control the autocrine regulation mechanism of the growth of KOSC-2 cells.

In summary, TNF-$\alpha$ stimulates the secretion of GRO-$\alpha$ by KOSC-2 cells and may control the tumor spread through angiogenesis and growth of the tumor cells.

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References


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