IL-23 promotes growth and proliferation in human squamous cell carcinoma of the oral cavity

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Abstract. Interleukin (IL)-23 is a heterodimeric cytokine, comprising IL-12p40 and the cloned IL-23-specific p19 subunit, was identified as a cancer-associated cytokine in a recent study. Like IL-12, IL-23 is expressed predominantly by activated dendritic cells and phagocytic cells. These cytokines antagonistically regulate local inflammatory responses in the tumor microenvironment and infiltration by intraepithelial lymphocytes. We have previously demonstrated the expression of IL-23 and its receptors in human oral squamous cell carcinoma (HOSCC) cell lines and tissue. Hence, this study investigated whether IL-23 has a role in the growth and proliferation of oral cancer cells by examining the expression kinetics of IL-23 and NF-κB activity, *in vitro* and *in vivo*. IL-23, which constitutively expressed in oral cancer, was enhanced by TNF-α and IL-23. IL-23 promotes cell proliferation in oral cancer and enhances the transport of nuclear factor-κB (NF-κB p65, RelA) to the nucleus in HSC-3 cells. Furthermore, luciferase reporter assay showed that IL-23 strongly induces RelA activity, and confirmed this finding by knockdown of IL-23 using RNA interference. Although RelA activity was down-regulated by anti-human IL-23p19 polyclonal antibody, used to neutralize the activity of IL-23, apoptosis was not induced. Immunohistochemistry revealed a weak IL-23 immunoreactivity in the cytoplasm of inflammatory infiltrating cells and in the cancer cells derived from 14 of 40 cases (35%) of oral SCC. In contrast, strong RelA immunoreactivity was observed in 30 of 40 cases of SCC (75%), especially consistent with IL-23 positive cells in SCC tissues. These data suggest that IL-23 up-regulates the growth and cell proliferation of oral cancer by promoting the nuclear transactivation of RelA.

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Introduction

The causal relationship between chronic inflammation, innate immunity and cancer is now widely accepted, and the similarities in the regulatory mechanisms have been suggested for more than a century (1,2). Although proliferation of cells alone does not cause cancer, sustained cell proliferation in an environment rich in inflammatory cells, growth factors, activated stroma, and DNA damage-promoting agents, certainly potentiates and/or promotes neoplastic risk. Interleukin (IL)-23, a heterodimeric cytokine with many similarities to IL-12, has recently been identified as a factor linking tumor-associated inflammation and a lack of tumor immune surveillance (3). IL-23 comprises a p19 subunit that associates with the IL-12p40 subunit (4), whereas IL-12 is a combination of IL-12p35 and the same IL-12p40 subunit (5). Furthermore, IL-23p19 is a molecule structurally related to not only IL-6, but also granulocyte-colony stimulating factor (G-CSF), and the p35 subunit of IL-12. Although p19 is expressed in various tissues and cell types, it lacks biological activity and only becomes biologically active when complexed with p40, which is normally secreted by activated macrophages and dendritic cells (DCs) (4). IL-23 uses many of the same signal-transduction components as IL-12, including the IL-12 receptor (R) ß1 subunit (IL-12Rß1), Janus kinase (Jak)2, Tyk2, signal transducer and activator of transcription (Stat)1, Stat3, Stat4, and Stat5 (4,6). IL-23R, composed of the IL-12Rß1 and the IL-23R subunit, is also expressed in DCs, macrophages, and T cells (6). Consistent with the structural and biological similarities of IL-12 and IL-23, the IL-23R complex shares a subunit with that of IL-12 (IL-12Rß1); however, it does not use or detectably bind to IL-12Rß2 (4). The ability of cells to respond to either IL-12 or IL-23 is determined by expression of IL-12Rß2 or IL-23R, respectively (6). Additionally, both cytokines promote the T helper cell type 1 (Th1) costimulatory function of antigen-presenting cells (7). However, IL-23 does differ from IL-12 in the T cell subsets that it targets. IL-12 acts on naive $CD4^+$ T cells, whereas IL-23 preferentially acts on memory $CD4^+$ T cells

(7). It has been reported that IL-12 has potent antitumor activity in a variety of murine tumor models, causing regression of established tumors (8-10) and inhibiting the formation of experimental metastases (8,9) and spontaneous metastases (11,12). On the other hand, it has recently been reported that genetic deletion or antibody-mediated elimination of IL-23 in mice leads to increased infiltration of cytotoxic T cells into the transformed tissue, rendering a protective effect against chemically-induced carcinogenesis (3). So far, it has been reported that expression of IL-23 and its receptors is detectable in activated macrophages, DCs, and keratinocytes in healthy skin (13). We have also demonstrated that the human oral squamous cell carcinoma (HOSCC) cell line, HSC-3, spontaneously expresses IL-23 and its receptors mRNA and protein (14). However, the exact mechanisms by which IL-23 promotes the development of oral SCCs remain to be elucidated.

In the present study, we first examined the expression kinetics of IL-23 in oral cancer cells *in vitro* and *in vivo* in order to clarify the role of IL-23 in these cells. We demonstrated that IL-23, which constitutively expressed in oral cancer cells, is enhanced by TNF-α and IL-23. It has already been reported that TNF-α induces NF-κB activation via a common pathway based on the phosphorylation-induced degradation of IκBs (15). Nuclear factor-κB (NF-κB) has key roles in inflammation, immune response, tumorigenesis and protection against apoptosis (16-18). In most cell types, NF-κB remains bound to IκBα protein, and thereby inactive, in the cytoplasm (19,20). After stimulation by various reagents, IκBα is rapidly phosphorylated by the IκB kinase (IKK) complex and degraded by the proteasome, allowing NF-κB to translocate to the nucleus and activate its target gene (15,17,21). We also showed that IL-23 increased cell proliferation in oral cancer and up-regulated translocation of NF-κB p65, RelA into the nucleus of HSC-3 cells. We further found that IL-23 induced RelA-dependent transcriptional activity in oral cancer cells. The expression and distribution of IL-23 and RelA was also examined in oral SCC tissues.

Materials and methods

Reagents. Mouse anti-human NF-κB p65/RelA monoclonal antibody (MAb RelA) and goat anti-human IL-23p19 polyclonal antibody (PAb IL-23p19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) for immunoblot analysis and immunohistochemistry. MAb ß-actin was obtained from Sigma (St. Louis, MO, USA). PAb IL-23p19 for the neutralization of human IL-23 bioactivity was purchased from R&D Systems (Minneapolis, MN, USA). Goat anti-human IgG (H&L) antibody (R&D Systems) was used as a control for the neutralization of human IL-23 bioactivity. Recombinant human TNF-α and IL-23 (R&D Systems) were used for the stimulation of cell lines.

Cell culture. Human oral squamous cell carcinoma (HOSCC: HSC-2, HSC-3, HSC-4 and Ca9-22) cells (obtained from the American Type Culture Collection, ATCC, Manassas, VA, USA) were respectively maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin, and grown to confluency in 25 cm^2 culture flasks at 37˚C in a humidified 5% CO_2 incubator until required.

RNA extraction and real-time quantitative RT-PCR. Total RNA was extracted from monolayer HOSCC cells ($1x10^6$ cells/ml) by the acid-guanidinium-phenol-chloroform (AGPC) method reported previously (22). To confirm the expression patterns of up-regulated or down-regulated IL-23 and RelA genes in HOSCC cells, especially HSC-3 cells, derived from metastatic lymph node of tongue cancer, treated with or without TNF-α (10 ng/ml), IL-23 (10 ng/ml), or IL-23 (10 ng/ml) + PAb IL-23p19 (0.8 μg/ml), real-time quantitative RT-PCR analyses were performed using a Bio-Rad iCycler system (Bio-Rad, Tokyo, Japan) and an iScript One-Step RT-PCR kit with SYBR Green I (Bio-Rad) according to the manufacturer's instructions. Briefly, the mRNAs were reverse-transcribed into cDNAs at 50°C for 10 min and reverse transcriptase was inactivated at 95°C for 5 min. PCR amplification was performed for 45 cycles of 95°C for 10 sec and 56°C for 30 sec and was followed by detection. PCR primers were designed and synthesized by Sigma-Aldrich (Ishikari, Japan) by following special design criteria for real-time PCR primers. The following primer sequences were used in the PCR reactions: IL-23p19 forward: TGC TAG GAT CGG ATA TTT TCA CAG G; IL-23p19 reverse: GAG GCT TGG AAT CTG CTG AGT C; RelA forward: AGG CGA GAG GAG CAC AGA TAC; RelA reverse: CGG CAG TCC TTT CCT ACA AGC; GAPDH forward: CAG CCT CAA GAT CAT CAG CA; GAPDH reverse: ACA GTC TTC TGG GTG GCA GT. Each sample was tested in triplicate and for each reaction the corresponding no-RT mRNA sample was included as a negative control. The relative mRNA level of each sample for each gene was normalized to the mRNA level of GAPDH, a housekeeping gene. The results were analyzed with the Bio-Rad iCycler Software 3.0 and Microsoft Excel 97 and presented as fold induction compared with the quantity of GAPDH mRNA (set at 1). The specificity of PCR products was assessed by melting curve data and agarose gel electrophoresis to determine product size and to confirm that no by-products were formed.

Enzyme-linked immunosorbent assay (ELISA). Monolayer HSC-3 cells ($1x10^6$ cells/ml) were washed once with PBS and incubated with TNF-α (10 ng/ml) for the indicated time period in RPMI-1640 medium containing 10% FBS. The culture supernatants from HSC-3 cells were harvested and concentrated 10-fold by using an ultrafiltration kit (Millipore, Tokyo, Japan). The amounts of secreted IL-23 (heterodimeric form) protein in the supernatants were determined by a solid-phase sandwich ELISA, using ELISA kits (BioSource International, Camarillo, CA, USA) according to the manufacturer's instructions. After adding the stop solution, the optical density (OD) was determined by measuring the absorbance of the dye solution at 450 nm. This ELISA detects only the heterodimer form of IL-23, not the separate subunits. Each experiment was performed in triplicate. The minimum detectable dose of the IL-23 heterodimer is 1.5 pg/ml.

Cell proliferation assay. The assay is based on the cleavage of the tetrazolium salt WST-8 to formazan by cellular

mitochondrial dehydrogenases, the activity of which increases proportionally to the number of viable cells. The formazan dye produced by viable cells was quantified as an index of cell proliferation. Monolayer HSC-3 cells ($2x10^4$ cells/100 μl/well) were incubated for 24 h on a 96-well plate. The cells were washed once with PBS and incubated with TNF-α (10 ng/ml) or IL-23 (10 ng/ml) in the presence or absence of PAb IL-23p19 (0.8 μg/ml) for various time periods (4, 8, 12, 24 and 48 h) in RPMI-1640 medium containing 10% FBS. Ten microliters of WST-8/ECS solution (Dojindo Laboratories, Tokyo, Japan) was added to each well and incubated with the cells for 2 h at 37°C in a humidified 5% CO_2 incubator. The cells were then shaken thoroughly for 1 min on a shaker. The relative viable cell number was determined by measuring absorbance of the dye solution at 450 nm.

Morphological observation. HSC-3 cells ($1x10^4$ cells/ml) were maintained with RPMI-1640 supplemented on a 10 cm^2 glass plate (Iwaki, Chiba, Japan). The cells were washed once with PBS and incubated with 0.8 μg/ml of PAb IL-23p19 for 24 h in RPMI-1640 medium with 10% FBS. The cellular morphology was directly observed under a confocal laser microscope.

Protein extraction. To examine RelA translocation to the nucleus, we used a subcellular proteome extraction kit (S-PEK, Calbiochem, Darmstadt, Germany) according to the manufacturer's instructions to extract the cytoplasmic, cell membrane, and nuclear fractions of HSC-3 cells. Cells were treated with or without 10 ng/ml of TNF-α, 10 ng/ml of IL-23, 0.8 μg/ml of PAb IL-23p19, or IL-23 + PAb IL-23p19 for 30 min and then $5x10^6$ cells were pelleted by centrifugation, washed twice, and then resuspended in 1 ml of ice-cold Extraction I buffer containing 5 μl of protease inhibitor mixture and incubated for 10 min at 4°C with gentle agitation. The cell suspension was then centrifuged at 1,000 x g at 4°C for 10 min. The supernatant was used as the cytoplasmic fraction and the pellet was resuspended in 1 ml of ice-cold Extraction II buffer containing 5 μl of protease inhibitor mixture and incubated for 30 min at 4°C. The sample was then centrifuged at 6,000 x g at 4°C for 10 min and the supernatant was used as the cell membrane fraction. The pellet was resuspended in 500 μl of ice-cold Extraction III buffer containing 5 μl of protease inhibitor mixture and 1.5 μl of Benzonase® and incubated for 10 min at 4°C with gentle agitation. The sample was then centrifuged at 7,000 x g at 4°C for 10 min and the supernatant was used as the nuclear fraction. Each sample was subjected to immunoblot analysis.

Immunoblot analysis. For the detection of IL-23 and RelA protein by gel electrophoresis, 30-μg and 10-μg protein samples were mixed with an equal volume of SDS-PAGE sample buffer and boiled for 5 min. These amounts were loaded per lane and separated on a polyacrylamide gel of appropriate percentage, then the proteins were electroblotted onto nitrocellulose membranes. Subsequent immunoblot analysis was carried out according to the method reported previously (22).

Transfection of HOSCC cells and luciferase reporter assay. HSC-3 cells ($1x10^6$ cells/ml) were cultured for 12 h in 24-well culture plates containing RPMI-1640 supplemented with 10% FBS. pTKκB2luc, a thymidine kinase (TK) luciferase construct containing five copies of the κB motif from the CXCL10/IP-10 gene, was kindly provided by Professor Y. Ohmori (23). Cells were transiently transfected with pTKκB2luc and pRL-TK reference *Renilla* luciferase plasmid (Promega, Madison, WI, USA) by using FuGENE transfection reagents (Roche, Nutley, NJ, USA), according to the manufacturer's instructions. At 24 h after transfection, the cells were treated with TNF-α (10 ng/ml), IL-23 (10 ng/ml), TNF-α (10 ng/ml) together with PAb IL-23p19 (0.8 μg/ml), IL-23 (10 ng/ml) together with PAb IL-23p19 (0.8 μg/ml), or TNF-α (10 ng/ml) together with goat anti-human IgG (0.8 μg/ml), or IL-23 (10 ng/ml) together with goat anti-human IgG (0.8 μg/ml) for various time periods (0, 1, 4, 8 and 24 h). The activity of firefly and *Renilla* luciferase was assayed by using reagents provided by Promega, according to the manufacturer's instructions. For standardization of transfection efficiency, the luciferase activity derived from pTKκB2luc was normalized to the activity of *Renilla* luciferase. The pGL3 control luciferase plasmid was purchased from Promega.

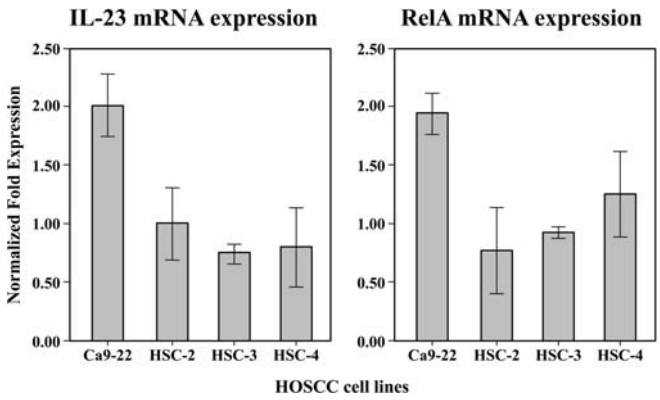

Figure 1. Detection of IL-23 and RelA mRNAs by real-time quantitative RT-PCR in HOSCC cells. IL-23 and RelA mRNAs were endogenously expressed in all HOSCC cell lines. Each column and bar represents the mean ± SEM of three independent experiments.

RNA-mediated interference. Small interfering RNAs (siRNAs) specific for human IL-23 and scrambled (control) were synthesized by Sigma-Aldrich. The sense and antisense strand sequences of the oligonucleotides were as follows. IL-23 siRNA sense, CAG CAA CCC UGA GUC CCU ATT; antisense, UAG GGA CUC AGG GUU GCU GTT; control siRNA sense, CGG AAC UCG AUA CUA CCC CTT; antisense, GGG GUA GUA UCG AGU UCC GTT. FuGENE 6 transfection reagent was mixed with 100 nM IL-23 or 100 nM control siRNA (3:3.4 μl) in serum-free medium, to a total volume of 500 μl and incubated for 30 min at room temperature. For IL-23 knockdown, HSC-3 cells ($1x10^5$ cells/ml) were rinsed with serum-free medium and transfected in 24-well plates with a RelA-dependent luciferase reporter plasmid and either an IL-23 siRNA duplex or a control siRNA using FuGENE 6 transfection reagents for 48 h at 37 °C. Cells were treated with TNF-α for 4 h, and subjected to immunoblot analysis and luciferase reporter assay.

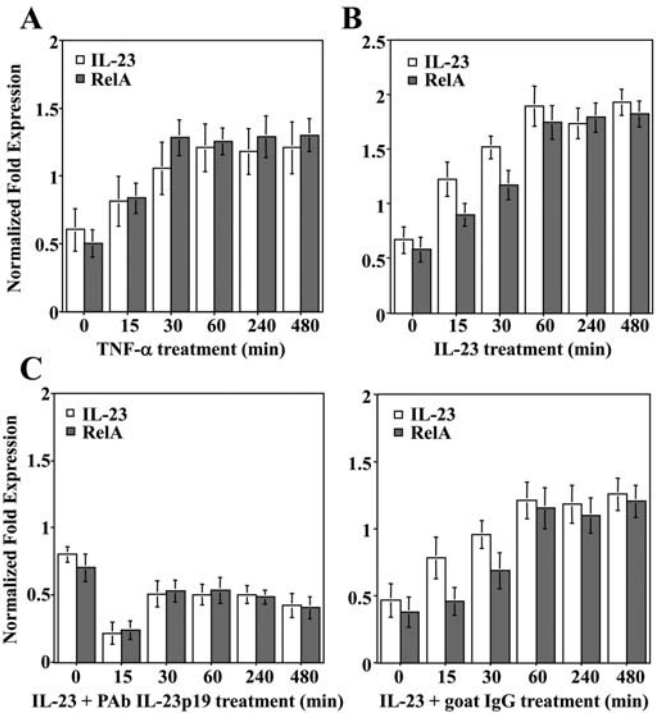

Figure 2. Effects of TNF-α or IL-23 on IL-23 and RelA mRNA expression. TNF-α and IL-23 induced a rapid increase in the levels of both IL-23 and RelA mRNA. TNF-α stimulation led to a more rapid peak of IL-23 and RelA expression at 30 min (A), after which mRNA levels reached a plateau, whereas IL-23 treatment led to a peak of expression of both genes at 60 min and subsequent plateau (B). The relative quantity of IL-23 and RelA mRNA is suppressed in HSC-3 cells by the neutralization of IL-23 (C). The goat IgG was used as a control antibody for the effect of PAb IL-23p19. Each column and bar represents the mean ± SEM of three independent experiments.

Primary tumor samples. Formalin-fixed, paraffin-embedded specimens were obtained from 40 patients with SCC treated in the Department of Oral and Maxillofacial Surgery, Meikai University Hospital, Japan. The pathological diagnosis of oral lesions was based on histological examination of hematoxylin and eosin-stained slides and made according to the WHO classification (24). The postsurgical TNM stage was determined according to the pTNM pathological classification of the UICC (International Union Against Cancer) (25). All specimens were obtained from surgical biopsies. None of the patients had undergone preoperative chemotherapy or radiotherapy. The labeling index was defined as the percentage of tumor cells displaying immunoreactivity, and calculated by counting the number of IL-23p19 and NF-κB-positive tumor cells among 1,000 tumor cells in each section. Tissue sections with <5% reactive cells were defined as negative (-), and those with ≥5% positive reactive cells were defined as positive (+).

Immunohistochemical examination. The sections were immersed in absolute methanol containing 0.3% H_2O_2 for 20 min at room temperature to block endogenous peroxidase activity. Immunohistochemistry was carried out with each antibody according to the method reported previously (14).

Ethical considerations. The study was approved by the Research Ethics Committee of the Meikai University School of Dentistry, Saitama, Japan (reference number: A0801).

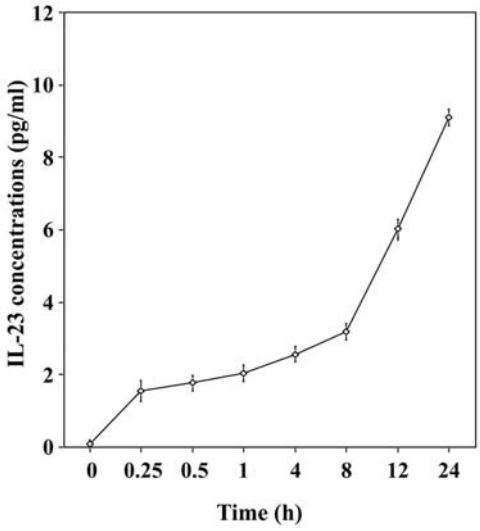

Figure 3. ELISA. HSC-3 cells were treated with TNF-α (10 ng/ml) for the indicated times before harvest of culture supernatants. After concentration of the supernatants by means of an ultrafiltration kit, the levels of IL-23 protein were measured by ELISA. The maximum amount of TNF-α-induced heterodimeric IL-23 was observed after 24 h and this was an increase of over 8-fold compared with cells not treated with TNF-α. Each column and bar represents the mean ± SEM of three independent experiments.

Results

Detection of IL-23 and RelA gene expression in HOSCC cells stimulated with TNF-α or IL-23. To investigate the regulation of expression of IL-23 and RelA mRNA in HOSCC cells by TNF-α or IL-23 treatment, real-time quantitative RT-PCR analysis was carried out using specifically designed primer pairs. Firstly, the relative level of IL-23 and RelA mRNA was determined in HSC-2, HSC-3, HSC-4, and Ca9-22 cell lines without TNF-α or IL-23 treatment (Fig. 1). IL-23 and RelA mRNAs were endogenously expressed in all HOSCC cell lines. We then determined the effect of TNF-α and IL-23 on the expression levels of IL-23 and RelA mRNA by treating HSC-3 cells with 10 ng/ml TNF-α or IL-23 over a time course (Fig. 2A and B). TNF-α and IL-23 induced a rapid increase in the levels of both IL-23 and RelA mRNA. TNF-α stimulation led to a more rapid peak of IL-23 and RelA expression at 30 min, after which mRNA levels reached a plateau, whereas IL-23 stimulation led to a peak of expression of both genes at 60 min and subsequent plateau. Moreover, to confirm whether IL-23 affected specifically in HSC-3 cells, inhibition assay for IL-23 was performed by stimulating cells with IL-23 in the presence of PAb IL-23p19, a neutralizing polyclonal antibody against IL-23, and analyzing the relative quantities of IL-23 and RelA mRNA by quantitative RT-PCR (Fig. 2C). Treatment of HSC-3 cells with IL-23 together with PAb IL-23p19 suppressed expression of IL-23 and RelA mRNA, whereas control treatment with goat IgG had no effect on expression of these genes. Taken together, these results suggest that TNF-α and IL-23 induce the expression of IL-23 and RelA and that IL-23 may play a critical role in the proliferative activity of HOSCC cells.

Detection of heterodimeric IL-23 in the culture supernatant of HSC-3 cells. We used a specific ELISA to confirm whether

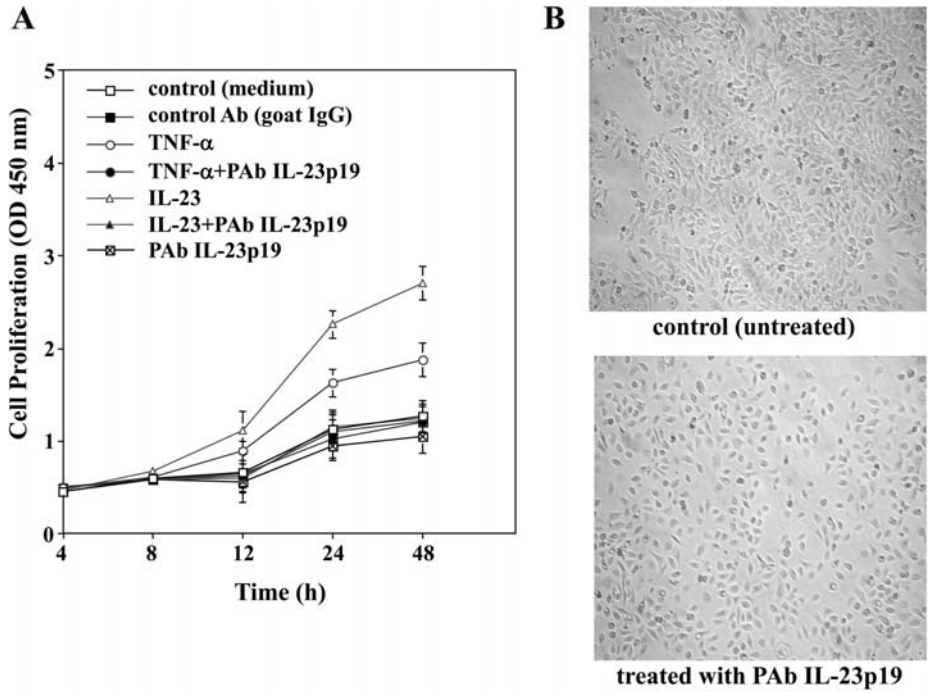

Figure 4. Cell proliferation assay and confocal laser microscopy. HSC-3 cells were incubated with TNF-α (10 ng/ml) or IL-23 (10 ng/ml) in the presence or absence of PAb IL-23p19 (0.8 μg/ml) for various time periods (4, 8, 12, 24 and 48 h). HSC-3 cell numbers increased in a time-dependent manner after treatment with 10 ng/ml of IL-23, and after 2 days culture, it reached by approximately 5-fold in comparison with the data of 4 h treatment (A). At 48 h, the number of cells treated with IL-23 had increased by approximately 2-fold compared with the untreated control. The increase in cell number was lower after treatment with TNF-α than with IL-23. The number of HSC-3 cells treated with IL-23 or TNF-α in the presence of PAb IL-23p19 was lower than that caused by the treatment of cells with TNF-α or IL-23 with the cell number ultimately being reduced to the control level. Cell numbers were slightly lower than that of controls 48 h after treatment with PAb IL-23p19 alone. We used goat anti-human IgG (H&L) antibody as a control for the effect of PAb IL-23p19. No significant morphological changes were observed by confocal laser microscopy in the cell structure of HSC-3 treated with PAb IL-23p19 (B).

heterodimeric IL-23 is secreted into the culture supernatant of HOSCC cell lines. The level of heterodimeric IL-23 was below the detectable limit (1.5 pg/ml) in the supernatant of untreated HSC-3 cells, but the level increased markedly after treatment by TNF-α in a time-dependent manner (Fig. 3). The maximum amount of TNF-α-induced heterodimeric IL-23 was observed after 24 h and this was an increase of over 8-fold compared with cells not treated with TNF-α. This finding suggests that HSC-3 cells secrete a low level of IL-23 that is markedly increased by TNF-α.

IL-23 promotes proliferation of HSC-3 cells. We investigated whether IL-23 promotes the proliferation of HSC-3 cells by carrying out a cell proliferation assay (Fig. 4A). The cell proliferation assay showed that HSC-3 cell numbers increased in a time-dependent manner after treatment with 10 ng/ml of IL-23, and after 2 days culture, it reached by approximately 5-fold in comparison with the data of 4 h treatment. At 48 h after treatment with 10 ng/ml IL-23, the cell number had increased by approximately 2-fold compared with the untreated control. The increase in cell number was lower after treatment with 10 ng/ml TNF-α than with 10 ng/ml IL-23. We then treated HSC-3 cells with IL-23 or TNF-α in the presence of PAb IL-23p19, a neutralizing antibody specific for IL-23. PAb IL-23p19 abolished the induction of cell proliferation by TNF-α or IL-23 with the HSC-3 cell number ultimately reducing to the control level, cell numbers were slightly higher than that of controls 48 h after treatment with PAb IL-23p19 alone though. This result indicates that promotion of cell proliferation is a specific effect of IL-23, and further that IL-23 has a stronger cell proliferative effect than TNF-α in oral SCC. We then investigated whether the inhibition of cell proliferation by PAb IL-23p19 is the result of apoptosis or a specific effect against cell proliferation via the neutralization of IL-23. Since the apoptosis rapidly induces dramatic effects on cell structure, the effect of PAb IL-23p19 in HSC-3 cells was examined by confocal laser microscopy (Fig. 4B). We found no significant morphological changes in the cell structure of HSC-3. This result suggests that the inhibition of cell proliferation by PAb IL-23p19 is due to a specific effect for cell proliferation via the neutralization of IL-23, but not the result of apoptosis.

IL-23 induces RelA nuclear translocation in HSC-3 cells. To examine how RelA expression is regulated in HSC-3 cells upon stimulation with TNF-α or IL-23, immunoblot analysis was carried out followed by densitometric analysis. RelA protein was localized to the cytoplasm of HSC-3 cells and was transported to the nucleus upon stimulation with TNF-α or IL-23, an effect that reached a peak after 30 min (Fig. 5). It was also found that IL-23 induced the nuclear transactivation of RelA slightly stronger than that of TNF-α. Analysis of the time course of RelA protein expression up to 24 h revealed a decrease in the rate of nuclear translocation of RelA (data not shown).

Regulation of RelA expression in HSC-3 cells after treatment with IL-23 in the presence of PAb IL-23p19 was also analyzed. RelA was primarily localized in the cytoplasm

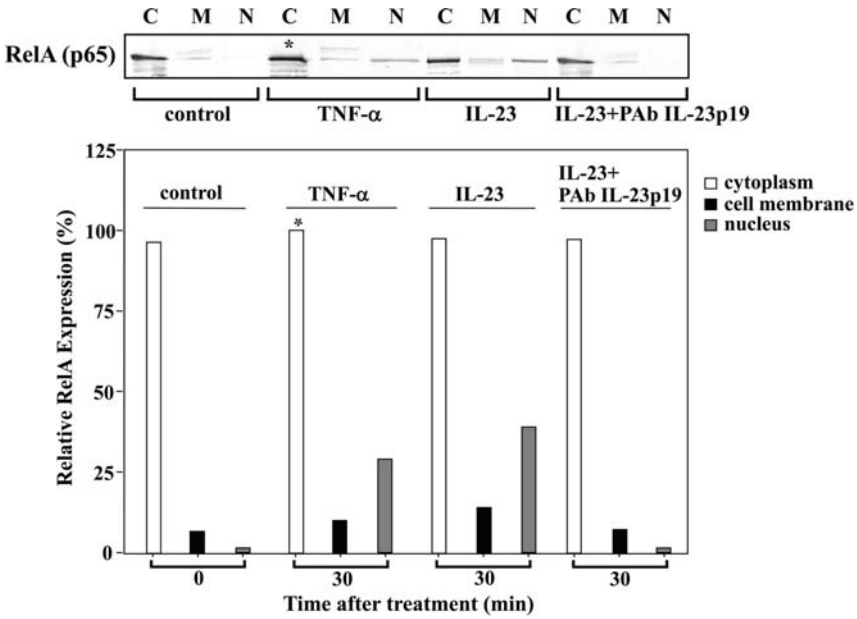

Figure 5. Nuclear transactivation of RelA. HSC-3 cells were treated with or without TNF-α (10 ng/ml), IL-23 (10 ng/ml), or IL-23 (10 ng/ml) + PAb IL-23p19 (0.8 μg/ml) for 30 min. RelA protein was localized to the cytoplasm of HSC-3 cells and was transported to the nucleus upon stimulation with TNF-α or IL-23, an effect that reached a peak after 30 min. TNF-α and IL-23 induced RelA transportation to the nucleus and that PAb IL-23p19 complete inhibited the nuclear translocation of RelA protein. Immunoblot analysis was also evaluated by densitometric analysis. Filters were scanned and computer-generated images were analyzed with the National Institutes of Health IMAGE program to obtain densitometric values. For each series of samples (cytoplasm, cell membrane and nucleus), the relative density of each image was calculated and expressed as a percentage of the value (arbitrarily set at 100) indicated by an asterisk. C, cytoplasm; M, cell membrane; N, nucleus.

with small quantities also detected in the cell membrane; however, RelA transport to the nucleus was almost nothing in HSC-3 cells treated with 10 ng/ml IL-23 and 0.8 μg/ml PAb IL-23p19 for 30 min (Fig. 5). Hence, we found that IL-23 promotes nuclear translocation of RelA in oral SCC. As shown in Fig. 2C, a decrease in the level of IL-23 mRNA in HSC-3 cells treated with PAb IL-23p19 was observed by real-time quantitative RT-PCR, and that RelA mRNA was also markedly down-regulated almost immediately after treatment. Taken together, these results show that IL-23 induces RelA transportation to the nucleus and that PAb IL-23p19 completely inhibits the nuclear translocation of RelA protein, even though the relative quantity of RelA mRNA is observed. Thus, the down-regulation of IL-23 by PAb IL-23p19 suppresses RelA activity in HSC-3 cells. This result is suggestive of the existence of an autocrine mechanism, in which tumor growth is promoted by the IL-23 produced by autologous cancer cells. Furthermore, this effect may play a critical role in proliferative activity of HSC-3 cells.

We next investigated the effect of TNF-α or IL-23 on RelA-dependent transcriptional activity in HSC-3 cells by using a luciferase reporter assay. Both TNF-α and IL-23 caused strong induction of luciferase activity (Fig. 6A and B). In HSC-3 cells stimulated with TNF-α, RelA-dependent transcription was gradually increased in a time-dependent manner. Maximum RelA-dependent transcription was observed after 24 h, with a 6.5-fold increase in luciferase activity, compared with cells not exposed to TNF-α. Similarly, in HSC-3 cells stimulated with IL-23, maximum RelA-dependent transcription was also observed after 24 h, with a 9-fold increase in luciferase activity compared with cells not exposed to IL-23. Thus, IL-23 induced a stronger RelA-dependent transcriptional activity than TNF-α. To examine whether constitutive RelA activity was observed in HSC-3 cells, a luciferase reporter assay was performed in the absence of TNF-α or IL-23 for the indicated time periods. We found that there was no constitutive RelA activity in HSC-3 cells (Fig. 6C). Furthermore, the blocking of constitutive IL-23 expression by PAb IL-23p19 has no effect on constitutive RelA activity. These findings suggest that constitutive IL-23 expression is not associated with RelA activation in HSC-3 cells. In addition, the increase in luciferase activity was completely dependent on the presence of κB sites, since the control plasmid lacking the κB elements did not respond to TNF-α or IL-23 (Fig. 6D). We then attempted a luciferase reporter assay to test whether PAb IL-23p19, used for neutralization of IL-23, can suppress induction of luciferase activity by TNF-α or IL-23. PAb IL-23p19 down-regulated luciferase activity in HSC-3 cells with half-maximal inhibition of luciferase activity detected 4 h after PAb IL-23p19 treatment (Fig. 6A and B). Taken together, these data indicate that production of IL-23 is induced by TNF-α or IL-23, which in turn increases RelA-dependent transcriptional activity in HSC-3 cells.

IL-23 up-regulates RelA activation in HSC-3 cells. To determine whether endogenous IL-23 functions as an up-regulator of RelA activation by TNF-α in HSC-3 cells, we used an siRNA approach to reduce the expression of IL-23 and determined the effects on the basal and TNF-α-induced activity of RelA. As expected, the level of the IL-23 protein was markedly reduced by IL-23 siRNA (Fig. 7A). We then assessed the effect of IL-23 siRNA on RelA-dependent transcriptional activity by TNF-α over 4 h. As shown in Fig. 7B, IL-23 knockdown

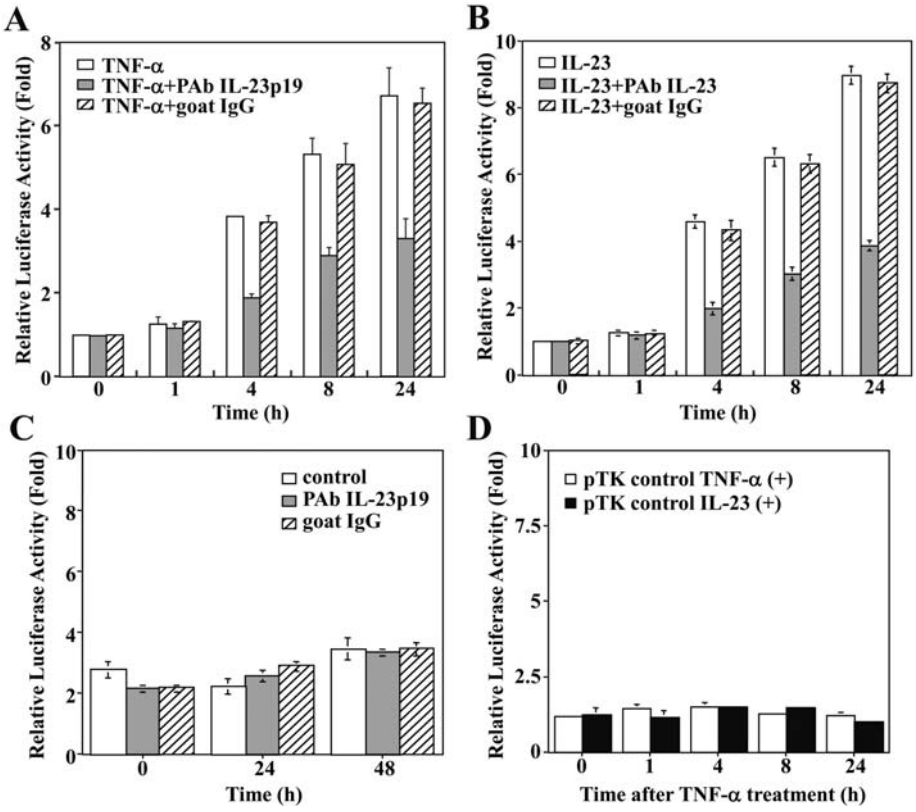

Figure 6. Luciferase reporter assay. The cells were transfected with the plasmids and 24 h later the cells were treated with TNF-α (10 ng/ml), or IL-23 (10 ng/ml), TNF-α (10 ng/ml) together with PAb IL-23p19 (0.8 μg/ml), or IL-23 (10 ng/ml) together with PAb IL-23p19 (0.8 μg/ml), or TNF-α (10 ng/ml) together with goat anti-human IgG (0.8 μg/ml), or IL-23 (10 ng/ml) together with goat anti-human IgG (0.8 μg/ml) for various time periods (0, 1, 4, 8 and 24 h). We used goat anti-human IgG (H&L) antibody as a control for the effect of PAb IL-23p19. A luciferase reporter assay indicated the effect of TNF-α and PAb IL-23p19 on RelA-dependent transcriptional activity in HSC-3 cells (A). A luciferase reporter assay indicated the effect of IL-23 and PAb IL-23p19 on RelA-dependent transcriptional activity in HSC-3 cells (B). A luciferase reporter assay was performed with PAb IL-23p19 or goat anti-human IgG in the absence of TNF-α and IL-23 for the indicated times (0, 24 and 48 h) (C). Constitutive RelA activity was not observed in HSC-3 cells. Furthermore, the blocking of constitutive IL-23 expression by PAb IL-23p19 had no effect on constitutive RelA activity. The increase in luciferase activity was completely dependent on the presence of κB sites, since the control plasmid lacking the κB elements did not respond to TNF-α or IL-23 (D). Relative luciferase activities are shown as fold induction compared with the activity of untreated sample (A) or pTK control IL-23 (+) sample at 24 h (D). Each column and bar represents the mean ± SEM of three independent experiments.

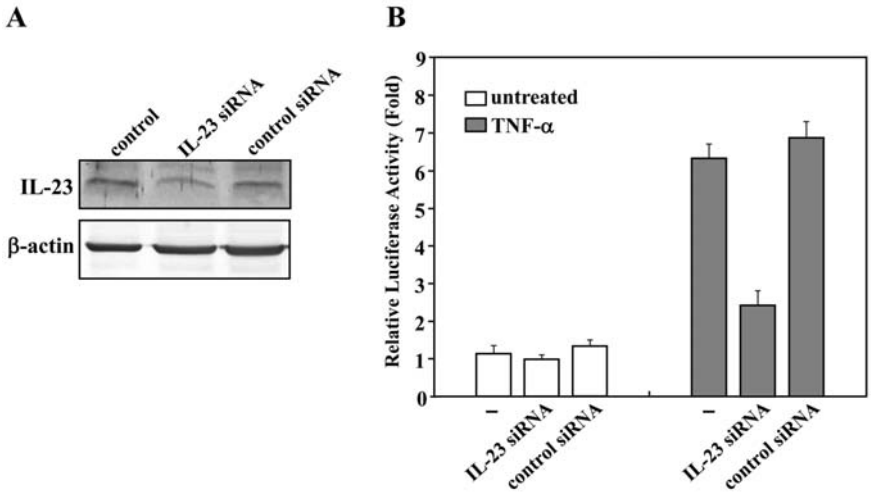

Figure 7. IL-23 siRNA-mediated interference. HSC-3 cells ($1x10^5$ cells/ml) were transfected with a RelA-dependent luciferase reporter plasmid and either an IL-23 siRNA duplex or a control siRNA for 48 h. Cells were treated with TNF-α for 4 h, and subjected to immunoblot analysis and luciferase reporter assay analysis. The endogenous level of the IL-23 protein was markedly reduced by IL-23 siRNA (A). A luciferase reporter assay indicated that IL-23 knockdown by IL-23 siRNA greatly reduced RelA activation by TNF-α in HSC-3 cells compared with the scrambled siRNA control (B). However, IL-23 siRNA had no effect on constitutive RelA activity in HSC-3 cells. The relative luciferase activities are shown as fold induction compared with the activity of untreated samples. Each column and bar represents the mean ± SEM of three independent experiments.

Table I. Correlation between the expression of IL-23, NF-κB and clinicopathological variables in 40 cases of oral squamous cell carcinomas.

No.	Age	Gender	Location	Differentiation	pTNM	Stage	Immunohistochemistry	
							IL-23	NF-κB
1	87	M	Oral floor	Well	T2N2bM0	IVA	+	+
2	48	F	Gingiva	Well	T4N0M0	IVA	-	+
3	56	F	Buccal mucosa	Well	T2N0M0	II	-	+
4	75	M	Tongue	Well	T4N2cM0	IVA	+	+
5	55	M	Oral floor	Well	T2N0M0	II	+	+
6	54	M	Tongue	Well	T4N2bM0	IVA	-	+
7	54	M	Oral floor	Well	T2N2aM0	IVA	-	+
8	70	M	Tongue	Well	T2N0M0	II	-	+
9	67	M	Maxillary gingiva	Well	T4N3M0	IVB	-	+
10	92	M	Soft palate	Well	T2N0M0	II	-	-
11	66	M	Tongue	Well	T2N0M0	II	-	+
12	87	M	Tongue	Well	T2N0M0	II	-	-
13	48	F	Tongue	Well	T1N0M0	I	-	-
14	85	M	Tongue	Well	T2N1M0	III	+	-
15	56	F	Tongue	Well	T1N2bM0	IVA	-	+
16	67	M	Mandibular gingiva	Well	T1N2bM0	IVA	+	+
17	55	M	Tongue	Well	T1N0M0	I	+	+
18	85	M	Buccal mucosa	Well	T1N0M0	I	+	-
19	50	M	Mandibular gingiva	Well	T3N1M0	III	+	+
20	67	M	Mandibular gingiva	Well	T4N0M0	IVA	+	+
21	79	M	Buccal mucosa	Well	T2N0M0	II	+	+
22	54	M	Buccal mucosa	Well	T1N0M0	I	-	+
23	54	M	Mandibular gingiva	Well	T4N1M0	IVA	-	-
24	62	M	Mandibular gingiva	Well	T1N0M0	I	+	+
25	60	M	Maxillary gingiva	Well	T1N0M0	I	+	-
26	79	M	Mandibular gingiva	Moderately	T4N2cM0	IVA	-	+
27	85	F	Tongue	Moderately	T2N1M0	III	-	+
28	54	M	Tongue	Moderately	T1N0M0	I	-	+
29	54	M	Tongue	Moderately	T4N2bM0	IVA	-	+
30	75	M	Mandibular gingiva	Moderately	T1N0M0	I	-	-
31	66	F	Mandibular gingiva	Moderately	T1N0M0	I	-	-
32	65	M	Mandibular gingiva	Moderately	T2N0M0	II	-	-
33	88	M	Mandibular gingiva	Moderately	T2N0M0	II	-	+
34	79	M	Mandibular gingiva	Moderately	T2N0M0	II	-	+
35	54	M	Buccal mucosa	Moderately	T2N0M0	II	+	+
36	64	M	Buccal mucosa	Poorly	T2N0M0	II	-	+
37	62	M	Tongue	Poorly	T2N1M0	III	-	+
38	60	M	Tongue	Poorly	T2N1M0	III	-	+
39	60	M	Tongue	Poorly	T1N0M0	I	-	+
40	68	M	Maxillary gingiva	Poorly	T3N0M0	III	+	+

by IL-23 siRNA greatly reduced RelA activation by TNF-α in HSC-3 cells compared with the scrambled siRNA control. However, IL-23 siRNA had no effect on constitutive RelA activity in HSC-3 cells. These data indicate that endogenous IL-23 functions as an up-regulator of RelA activation induced by TNF-α in HSC-3 cells, further suggest that IL-23 produced by autologous cancer cells promotes tumor growth.

Immunohistochemical detection of IL-23 and RelA and clinicopathological variables in SCC tissues. The correlations between IL-23, RelA expression, and clinicopathological variables in SCC tissues are summarized in Table I. Immunohistochemical detection of IL-23 and RelA was carried out in 40 cases of SCC at various stages. A weak positive reaction to PAb IL-23p19 was observed in the cytoplasm of infiltrated

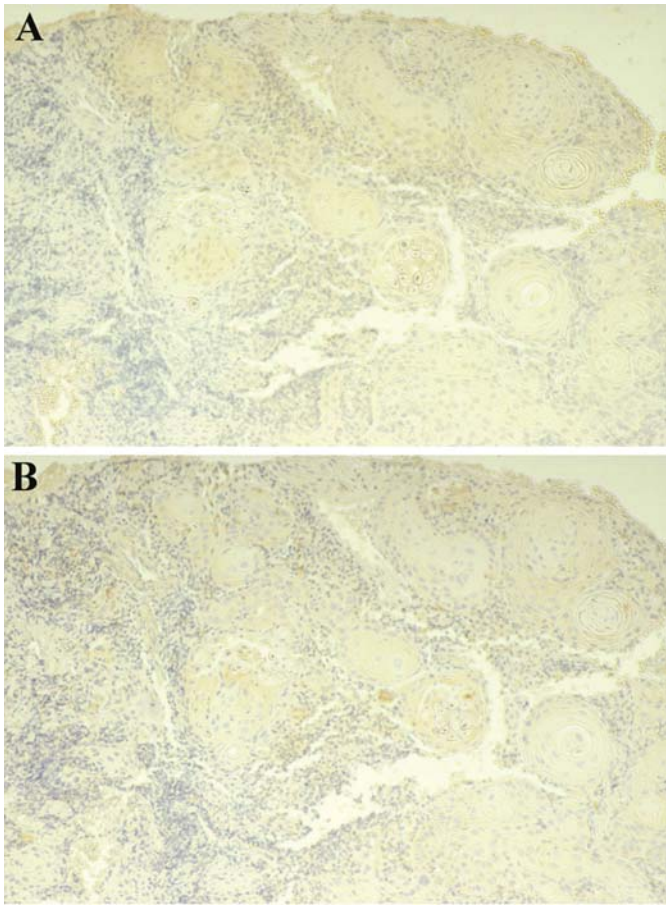

Figure 8. Immunohistochemistry. Immunohistochemical detection of IL-23 and RelA was carried out for 40 cases of SCC at various stages. A weak positive reaction was observed for PAb IL-23p19 in the cytoplasm of cancer cells and infiltrated inflammatory cells (x66) (A). A strong positive reaction for MAb RelA was observed in the cytoplasm of cancer cells, especially consistent with the observation of IL-23-positive cells in SCC tissues (x66) (B).

inflammatory cells and cancer cells in 14 of 40 cases (35%) of SCC (Fig. 8A). Whereas, strong RelA immunoreactivity was observed in 30 of 40 cases of SCC (75%), especially consistent with the observation of IL-23-positive cells in SCC tissues (Fig. 8B).

Discussion

The epidemiologic relationship between cancer and inflammation has been well established. Many cancers arise at the site of chronic inflammation and inflammatory mediators are often produced in tumors (2,26). The frequent use of anti-inflammatory drugs reduces the incidence of a variety of human tumors (27). Although blockading some of these mediators has been shown to be efficacious in experimental settings, it is still unclear whether the inflammatory reaction at the tumor site promotes tumor growth or simply implies the failed attempt of the immune system to eliminate the rising malignancy. However, transgenic mice with chronic inflammatory bowel disease caused by the absence of IL-10 develop colon cancer (28), and the adoptive transfer of inflammation-suppressing regulatory T cells can inhibit colon polyposis in transgenic mice carrying a mutated adenomatosis polyposis coli (APC) gene (29). In recent years, accumulating evidence has shown that although human tumors are less immunogenic than foreign pathogens, a surprisingly wide range of tumor specific epitopes are recognized by the adaptive immune system (30). Tumor antigen-specific T cells (31), which are cytotoxic to autologous tumor cells *in vitro*, are found in the circulation and within tumors, and can be expanded therapeutically. Despite the identification of tumor-associated antigens and their application for immunization of patients with cancer, the majority of clinical trials show disappointing results that contrast with the effectiveness of immunotherapy in experimental tumors (32). The basis for this poor clinical outcome may be the ability of cancer cells to escape from destruction by adoptive and innate immunity effector mechanisms. We also observed in our previous study that tumor cells possess the potential to escape immune surveillance by killing host T lymphocytes through cytokines such as RCAS1 (33).

Cytokines comprise a large family of secreted proteins that bind to and signal through defined cell surface receptors on a wide variety of target cells and play a pivotal role in the establishment and maintenance of homeostasis. Many cytokines share structural features and functions during development, immune response, or inflammation. In a previous study, IL-23 was identified as a cancer-associated cytokine (3). The expression of IL-23, but not that of its close relative IL-12, was significantly increased in the vast majority of human tumors from various organ types when compared with normal adjacent tissue from the same individual (3). The role of IL-23 in tumorigenesis is clearly demonstrated in mice lacking IL-23p19; these mice are almost completely resistant to endogenous tumor formation when challenged in a chemical carcinogenesis protocol (3). By comparison, a sizeable number of reports have described tumor-inhibitory effects of IL-23 in fast growing, immune-sensitive mouse tumor models, genetically engineered to overexpress an IL-23-like product with linked p40 and p19 subunits. Following implantation in immune-naive hosts, IL-23-overexpressing tumors show reduced growth and metastasis consistent with the stimulation of a $CD8^+$ memory T cell response (34-38). As described, the precise role of IL-23 and its function at the cancer invasive front is controversial.

In this study, the results of real-time quantitative RT-PCR indicated that IL-23 induced NF-κB activation, and the IL-23 knockdown analysis suggested that IL-23 regulates NF-κB activation induced by TNF-α in HSC-3 cells. It has already been reported that TNF-α induces NF-κB activation via a common pathway based on the phosphorylation-induced degradation of IκBs (15). In the present study, TNF-α was also used to activate NF-κB in HSC-3 cells to mimic the local inflammatory response in the metastasized region, and some tumor cells were reported to produce TNF-α (15). We further found that blocking the function of IL-23 inhibits the proliferative activity of tumor cells without inducing apoptosis. As follows, two separate lines of evidence allowed us to conclude that IL-23 is a potent and specific promoter of NF-κB activation in HSC-3 cells: i) IL-23 promoted nuclear transactivation of NF-κB, and ii) IL-23 increased NF-κB-dependent transcriptional activity. Indeed, NF-κB controls

genes that code for anti-apoptotic proteins, some acting at the mitochondrial level (17) or directly blocking caspase activation (39). Up-regulation of NF-κB could therefore result in an increase in crucial anti-apoptotic influences both at the mitochondrial and the membrane death receptor levels. Additionally, we found that although both IL-23 and TNF-α have effects such as increased cell proliferation, promoted nuclear transactivation of NF-κB and increased NF-κB-dependent transcriptional activity, IL-23 is stronger in each effect than TNF-α. Furthermore, our *in vivo* studies showed strong RelA-specific immunoreactivity in 75% of SCC cases, especially consistent with the observation of IL-23-positive cells in SCC tissues. These data also suggested that IL-23 might promote NF-κB activity, alternatively IL-23 function might be activated by NF-κB in SCC tissues. Finally, we noted that IL-23 was secreted not only by DCs and macrophages, as shown in previous studies (4), but also by autologous cancer cells. Consequently, we consider the existence of an autocrine mechanism, in which tumor growth is promoted by IL-23 produced by autologous cancer cells. From these combined data, we believe that IL-23 plays a significant role in the growth and proliferation of oral cancer. Thus, IL-23 could be used as a predictor of poor prognosis in patients with oral cancer, and its antibody might be able to use as an inhibitor of oral cancer progression. Identification of the signaling pathways underlying these events might provide the key to elucidating the mechanism of development of oral cancer. Further investigations into the role of IL-23 will be required to fully understand IL-23-mediated tumor proliferation and to establish an IL-23-based oral cancer therapeutic strategy.

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