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Interleukin-22 promotes the migration and invasion of oral squamous cell carcinoma cells

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ABSTRACT

The roles of interleukin-22 (IL-22) in carcinogenesis have been proposed in various neoplasms. Increased expression of IL-22 has been observed in oral squamous cell carcinoma (OSCC) lesions as well as in other cancers. OSCC is still associated with poor prognosis and a high mortality rate because of its invasiveness and frequent lymph node metastasis. In the present study, we investigated the effects of IL-22 on OSCC cells. The human OSCC cell lines Ca9-22 and SAS were stimulated with IL-22 (1–10 ng/mL), and their migration abilities were examined using a cell scratch assay. A Matrigel invasion assay was performed to evaluate the invasion abilities of OSCC cells. Signal transducer and activator of transcription 3 (STAT3) phosphorylation, matrix metalloproteinase (MMP) and epithelial-mesenchymal transition (EMT)-related genes and proteins were also examined. IL-22 treatment promoted the migration and invasion abilities of OSCC cells without increasing their viability. IL-22 stimulation also induced STAT3 phosphorylation, MMP-9 activity and EMT-related genes and proteins. Our findings suggest that IL-22 has possible roles in the development of OSCC.

KEYWORDS

Interleukin-22 (IL-22); oral squamous cell carcinoma (OSCC); prognosis; invasion

1. Introduction

Interleukin 22 (IL-22) is a member of the IL-10 cytokine superfamily produced by activated helper T cells and innate lymphocytes. The IL-22 receptor consists of IL-22 receptor 1 (IL-22R1) and IL-10 receptor 2 (IL-10R2). While IL-10R2 is widely expressed in various cells, IL-22R1 expression is restricted to epithelial cells. Therefore, IL-22 is considered to mediate the crosstalk between immune cells and tissue epithelial cells [1]. For instance, IL-22 induces the secretion of antimicrobial peptides from epithelial cells to protect mucosal surfaces from pathogens [2]. In human keratinocytes, the retardation of epidermal differentiation and the induction of proinflammatory-associated gene expression are caused by IL-22 [3,4]. Moreover, IL-22 promotes the migration of keratinocytes and induces hyperplasia of the reconstituted human epidermis.

Recent studies have been reported the possible roles of IL-22 in the pathogenesis and prognosis of various human cancers, including oral squamous cell carcinoma (OSCC) [5–8]. Increased IL-22 and IL-22R1 expression levels have been observed in OSCC lesions [5] as well as in other cancers. Another study reported that increased expression of IL-22 seemed to correlate with cancer progression and metastasis [9]. In vitro studies have shown that stimulation with IL-22 promotes the migration and proliferation of lung cancer cells [8] as well as the migration and invasion of gastric cancer cells [7]. Thus, IL-22 is considered to have an important role in carcinogenesis.

OSCC is the most common malignant disease affecting the oral cavity [10]. OSCC is still associated with poor prognosis and a high mortality rate because of its invasiveness and frequent lymph node metastasis [11]. Various etiological factors, such as ethnicity, smoking, sexual behaviors and subsequent viral infections, occupational activity, external agent exposure, dietary micronutrient deficiency, and alcohol consumption have been reported to affect the progression of OSCC [11–14]. These factors could induce chronic inflammation in the oral mucosa. Infiltration of inflammatory cells into the submucosa could then occur to repair the inflamed tissues. A previous study by others using various OSCC cell lines revealed that IL-22 stimulation upregulated the expression of antiapoptotic and mitogenic genes through signal transducer and activator of transcription 3 (STAT3)-dependent and STAT3-independent pathways [5]. We hypothesized the involvement of IL-22 in OSCC migration and invasion because it was reported that activation of...
STAT3 was associated with the cellular invasion ability [15]. To demonstrate this hypothesis, we investigated the effect of IL-22 on the migration and invasion of OSCC cell lines.

2. Materials and methods

2.1. Cell culture

The human OSCC cell lines Ca9-22 and SAS were obtained from the JCRB cell bank. Ca9-22 cells were incubated in MEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 100 U ml\(^{-1}\) penicillin, and 100 U ml\(^{-1}\) streptomycin. SAS cells were incubated in DMEM/F12 (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum, 100 U ml\(^{-1}\) penicillin, and 100 U ml\(^{-1}\) streptomycin. We used these two cell lines for different purposes according to the different characteristics of the two cell lines, i.e., Ca9-22 is a noninvasive cancer cell line, and SAS is an invasive cancer cell line [16].

2.2. Cell viability assay

To examine the effects of IL-22 on cell viability, we used a formazan formation assay using the Cell Counting Kit-8 (Dojindo, Tokyo, Japan). Briefly, Ca9-22 and SAS cells were plated on a 96-well plate at 1\times 10^4 cells per well. The cells were grown for 48 h before treatment. Thereafter various concentrations of IL-22 (1–10 ng/mL) were added. Following treatment for 24 h or 48 h, the cells were incubated with substrate for 3 h at 37°C. The cell viability was determined by measuring the OD absorbance at 450 nm. The cell viability is presented as the percentage relative to the untreated control.

2.3. Migration assay

The migration assay was performed as previously described [17]. Ca9-22 cells were grown to confluence in 24-well microplates (Corning, Corning, NY, USA). A linear scratch was performed using a 2-mm-wide Cell Scratcher (Iwaki Glass, Chiba, Japan), followed by washing once with phosphate-buffered saline (PBS). Thereafter complete medium containing various concentrations of IL-22 (1–10 ng/mL) were added. Following treatment for 24 h or 48 h, the cells were incubated with substrate for 3 h at 37°C. The cell viability was determined by measuring the OD absorbance at 450 nm. The cell viability is presented as the percentage relative to the untreated control.

2.4. Invasion assay

Invasion ability of OSCC cells was measured by Matrigel invasion assay as previously described [18,19]. Briefly, BioCoat Matrigel Invasion Chambers (Corning) were used. A total of 5 \times 10^4 SAS cells were plated in 500 μL of culture medium on the Matrigel chambers with or without IL-22 (5 ng/mL or 10 ng/mL) for 48 h. At the end of the incubation period, the Matrigel was removed, and we cleaned the upper side of the membrane with a cotton swab to remove cellular debris. The cell membranes were fixed and stained with Diff-Quik stain (Sysmex, Hyogo, Japan). The bottom side cells were counted under a stereomicroscope (Carl Zeiss). The invasive activity is expressed as the fold-change relative to the unstimulated control.

2.5. Western blotting

SAS cells were homogenized in cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) after IL-22 (10 ng/mL) stimulation. Protein concentrations were measured using an RC DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). The protein samples (20 μg/lane) were separated by 10% SDS-PAGE (Thermo Fisher Scientific) and were transferred onto PVDF membranes (iBlot Transfer Stack, PVDF, Thermo Fisher Scientific). After blocking with Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) or TBS containing 5% skim milk, the blotting membranes were incubated with an anti-E-cadherin antibody (1:500; Abcam, Cambridge, UK), anti-vimentin antibody (1:500; Abcam), anti-phospho-STAT3 (Tyr705) antibody (1:1000; Cell Signaling Technology), anti-STAT3 antibody (1:1000; Cell Signaling Technology), or β-actin antibody (1:1000; Cell Signaling Technology) at 4°C overnight. Then, the membranes were incubated with an anti-rabbit IgG, HRP-conjugated secondary antibody (Cell Signaling Technology) and were visualized using ECL (GE Healthcare, Fairfield, CT, USA).

2.6. Gelatin zymography

The enzyme activities of secreted matrix metalloproteinase-2 (MMP-2) and MMP-9 were determined by gelatin zymography [18]. Following IL-22 stimulation for 24 h, SAS cell culture media were collected, and protein concentrations were analyzed using an RC DC Protein Assay Kit (Bio-Rad). Equal amounts of proteins were used for electrophoresis in zymogram gels (Thermo Fisher Scientific). After electrophoresis, the gel was incubated with renaturing buffer (Thermo Fisher Scientific) for 30 min and then with developing buffer (Thermo Fisher Scientific) at 37°C overnight. Next, the gel was stained using
SimplyBlue SafeStain (Thermo Fisher Scientific) to visualize the specific bands. We used the LL-MMP mixed enzyme marker (Life Laboratory Co., Yamagata, Japan) as a positive control. The results were analyzed using ImageJ software (NIH), and the data are expressed as the fold-change relative to the unstimulated control.

2.7. Quantitative reverse transcription PCR (RT-qPCR)

RT-qPCR was performed to determine the mRNA expression levels of Snail and Slug in SAS cells 24 h after IL-22 (5 or 10 ng/ml) stimulation. Total RNA was extracted from SAS cells using the ReliaPrep RNA Cell Miniprep system (Promega, Madison, WI, USA) according to the manufacturer’s protocol. For cDNA synthesis, reverse transcription was performed using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA, USA). RT-qPCR analysis was conducted using iQ SYBR Green Supermix (Bio-Rad). Peptidylprolyl isomerase A (PPIA) was used as an endogenous gene for normalization. The primers used to amplify cDNA were as follows: Snail, forward (5'-TGCAGGACTCTAATCCAAGTTTACC-3') and reverse (5'-GTGGGATGGCTGCCAGC-3'); Slug, forward (5'-TTTCCAGACCTTGTTGCTTC-3') and reverse (5'-CTCAGATTTGACCTGTCTGCAATG-3'); PPIA, forward (5'-ATGCTGGACCCCAA CACAAAAT-3') and reverse (5'-TCTTTTCACCTTGGCCAAAACCACC-3'). All reactions were performed using a QuantStudio3 real-time PCR system (Thermo Fisher Scientific). The data are expressed as the fold-change relative to the unstimulated control.

2.8. Statistical analysis

All data were obtained from three to five independent experiments. The data are expressed as the means ± standard deviations (SD) and were analyzed by the Tukey-Kramer test using Statcel 3 software (OMS, Tokorozawa, Japan). A probability level of 5% (p < .05) was considered statistically significant.

3. Results

3.1. IL-22 did not affect the viability of OSCC cells

First, we examined the effect of IL-22 on OSCC cell viability because IL-22 has been reported to enhance the cell proliferation of several cancer cells, such as non-small-cell lung cancer cells [8] or colon cancer cells [9]. We used Ca9-22 cells as a noninvasive OSCC cell line and SAS cells as an invasive OSCC cell line [16]. In this study, Ca9-22 cells and SAS cells were treated with 1–10 ng/mL of IL-22 for 24 h or 48 h. Compared to the control cell viability, the Ca9-22 (Figure 1(A)) and SAS (Figure 1(B)) cell viability did not significantly change after cells were treated with 1–10 ng/mL IL-22 (Figure 1).

3.2. The migration and invasion abilities of OSCC cells were enhanced by IL-22

To evaluate the effects of IL-22 on OSCC migration and invasion activities, we performed scratch and Matrigel assays. Ca9-22 cells were scratched by a 2-mm-wide Cell Scratcher and then treated with different dosages of IL-22 (1 ng/mL or 10 ng/mL). The migration assay showed that 10 ng/mL of IL-22
significantly enhanced cellular migration at 24 h after the scratch (Figure 2(A)). Next, to examine the effect of IL-22 on the invasion ability of OSCC cells, we used SAS cells as an invasive OSCC cell line. SAS cells were plated on Matrigel chambers with or without IL-22 (5 ng/mL or 10 ng/mL) for 48 h. As shown in Figure 2(B), IL-22 treatment promoted the Matrigel invasive activity of SAS cells (Figure 2(B)).

3.3. IL-22 stimulated STAT3 signaling in OSCC cell lines

IL-22 signals through the JAK/STAT pathway induce phosphorylation of STAT3, STAT1 and STAT5. In particular, activation of STAT3 signaling has been reported to be associated with IL-22-regulated cancer progression, including OSCC [5,8,9,15,20]. We observed that IL-22 induced STAT3 phosphorylation in SAS cells (Figure 3), similar to the effects in other OSCC cell lines [5].

3.4. IL-22 upregulated MMP-9 activity in OSCC cell line

MMPs are a family of degradative enzymes. Among MMPs, MMP-2 and MMP-9 are associated with cellular invasion [21]. Therefore, we investigated the MMP-2 and MMP-9 activities in the supernatant of SAS cells cultured with or without IL-22 (5 ng/mL or 10 ng/mL) using gelatin zymography (Figure 4(A)). While IL-22 tended to upregulate the MMP-2 (72 kDa) levels in the supernatant, the upregulation was not significant (Figure 4(B)). In contrast, the MMP-9 levels in the supernatant were significantly increased by IL-22 (Figure 4(C)).

3.5. IL-22 induced epithelial-mesenchymal transition (EMT)-related proteins in the OSCC cell line

EMT is an essential process for tumor prognosis and metastasis because cancer cells partially obtain migratory and invasive abilities through EMT. Thus, we investigated the expression of EMT-related proteins and genes in IL-22-treated SAS cells. Among the various molecules involved in EMT, we examined E-cadherin as an indicator of epithelial features and vimentin as an indicator of mesenchymal features. We observed that vimentin expression was significantly increased by IL-22 treatment (Figure 5(B)). Although E-cadherin expression tended to decrease after IL-22 treatment, the change was not significant (Figure 5(A)). We also examined two EMT-related genes, Snail and Slug, and as shown in Figure 5(D,E), IL-22 treatment significantly upregulated their expressions in SAS cells.
4. Discussion

In the present study, we investigated the involvement of IL-22 in OSCC migration and invasion. IL-22 is well known for its pathophysiological roles in inflammatory processes. As easily predicted from its actions in tissue inflammation and subsequent cancer progression, IL-22 can be a new prognostic marker of malignancies. The association of increased levels of IL-22 and the prognosis of various human malignancies, including gastric cancer [6,7,20], lung cancer [8], colon cancer [9], and OSCC [5] were recently reported. OSCC is an extremely aggressive cancer because of its high invasive ability. Multiple mechanisms are associated with the invasion of OSCC cells, such as aberrant expression of cell adhesion molecules (e.g., E-cadherin, N-cadherin, desmoglein, and claudin-1), upregulation of tumor microenvironment molecules (TME) (e.g., MMP and VEGF) and cell signaling pathways (e.g., STAT3, EGFR and EMT-related signaling pathways) [21]. In this study, we disclosed the possible roles of IL-22 in modulating the biological features of OSCC.
by increasing migration and invasion associated with the modulation of several invasion-related molecules and cell signaling.

IL-22 enhanced cell proliferation of non-small-cell lung cancer cells [8] or colon cancer cells [9]. However, in the present study, IL-22 showed no effect on the viability of the OSCC cell lines. This result is consistent with those of a previous report on IL-22 and OSCC cell viability [5]. These findings suggest multiple mechanisms of IL-22 on carcinogenesis and cancer progression in various neoplasms.

The binding of IL-22 to IL-22R activates receptor-associated Janus kinase (JAK) 1 and tyrosine kinase (JAK) 2, leading to the activation of signal transducers and activators of transcription (STAT) signaling. This results in the upregulation of various genes involved in the promotion of cell proliferation, survival, and migration, including the expression of E-cadherin and vimentin.

Figure 5. The effect of IL-22 on E-cadherin and vimentin expression in the OSCC cell line. (A) Representative Western blotting experiments. Expression of the E-cadherin (B) and vimentin (C) levels following stimulation with IL-22 for 24 h represents the relative quantities in the treated cells compared to that in the unstimulated cells. Gene expression changes of Snail (D) and Slug (E) following stimulation with IL-22 for 24 h, showing the relative quantities in the treated cells compared to that observed in the unstimulated cells. Data represent three independent experiments. Error bars represent the SD. *p < .05; **p < .01; Tukey-Kramer test.
kinase 2, which subsequently activate STAT3, STAT1 and/or STAT5 [22]. Naher et al. [5] reported that IL-22 induced transient tyrosine phosphorylation of STAT3 (pY705-STAT3) in the OSCC cell line. STAT3 has been shown to be involved in cancer progression, as constitutive STAT3 activation has been reported to induce tumor cell proliferation, survival, and invasion [23]. Recent clinical findings have indicated that activation of STAT3 signaling is associated with cancer metastasis in oral carcinoma [15, 24]. Interestingly, Yu et al. [25] observed that IL-22 mediated oral mucosal wound healing via STAT3. In the present study, IL-22 induced STAT3 phosphorylation in SAS cells. STAT3 activation is caused by various cytokines including IL-6, IL-10, IL-22, IFN-γ, and TNF-α and growth factors such as G-CSF and VEGF. We suggest that STAT3 phosphorylation by IL-22 is, at least in part, essential to enhance the invasion ability of OSCC cell lines.

MMP-9 has been reported to play a crucial role in the invasion of OSCC cells [16] and other cancer cells [26]. Ji et al. [7] reported that IL-22 stimulation promoted the invasion of gastric cancer cells by upregulating MMP-9 activity. Porphyromonas gingivalis, a periodontal pathogen, caused activation of MMP-9 in OSCC and subsequent OSCC invasion [16]. Periodontitis is considered to be one of the independent risk factors of OSCC [27], and increased IL-22 production was observed at the periodontitis lesion in clinical human samples [28] and animal periodontitis models [29]. In the present study, IL-22 stimulation enhanced MMP-9 activity. Taken together, IL-22-stimulated upregulation of MMP-9 in OSCC might play an essential role in the progression of OSCC.

We also observed that IL-22 is associated with expression of EMT-related molecules in SAS cells. EMT also contributes to cancer invasion. Abe et al. [30] reported that the vimentin and N-cadherin expression levels were significantly correlated with the invasion depth of cancer cells and lymphovascular invasion by immunohistochemical analysis. IL-22 induces mesenchymal gene and protein expression and reduces epithelial gene and protein expression in primary healthy salivary gland epithelial cells [31]. In the present study, IL-22 upregulated vimentin, Snail, and Slug expression in SAS cells. Although E-cadherin expression showed a decreasing tendency with IL-22 treatment, the change was not significant. Further studies are required to elucidate the IL-22-induced mechanisms of EMT using primary tumor samples, because the response to IL-22 varies in different OSCC cell lines [5].

Recent reports have suggested the involvement of oral microbiota in the progression of OSCC [32, 33]. Fusobacterium was reported to be highly associated with OSCC staging [32, 33]. Yang et al. [33] reported that the abundance of Fusobacterium increased significantly with the progression of OSCC in the healthy control to OSCC stages 1 through 4. Interestingly, Fusobacterium-infected mice showed high inflammatory cytokine levels, including IL-22 [34]. In our study, we observed that IL-22 induced the migration and invasion of OSCC cells. Thus, Fusobacterium may promote OSCC progression by inducing IL-22 secretion.

It is well known that smoking is one of the most important independent risk factors for OSCC carcinogenesis [13]. Interestingly, studies have shown that smoking is associated with IL-22 production from Th17 cells [35, 36]. The proportion of Th17 cells in the peripheral blood of smoking patients with psoriasis has been reported to increase compared with that in the peripheral blood of nonsmoking patients. Weng et al. [36] reported that cigarette smoke directly alters Th17 cell function by affecting the ROCK2-IRF4 axis and then increases the synthesis of IL-22. In addition, some specific viruses, such as Epstein-Barr virus (EBV) and human papilloma virus (HPV), have been known to associate the development of OCSS [12]. While high levels of IL-22 expression were observed in uterine cervical cancer patients [37], there are few reports showing the association between IL-22 and EBV or HPV infection in the oral cavity.

This study has potential limitations. We studied the effects of IL-22 on OSCC using an in vitro cell line model in this study. Further clinical studies are needed to elucidate the pathophysiological significance of IL-22 in OSCC patients.

In conclusion, we revealed increased migration and invasion of OSCC cell lines by IL-22 treatment in vitro. IL-22 induced STAT3 phosphorylation, MMP-9 activation and EMT-related protein production. As the aggressiveness of OSCC is at least partially promoted by IL-22 and IL-22R1, these compounds might be effective therapeutic targets to prevent OSCC progression.

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Disclosure statement

The authors declare no conflicts of interest.

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