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Original Research

Interleukin-6 secreted by oral cancer- associated fibroblast accelerated VEGF expression in tumor and stroma cells

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Abstract: Oral cancer represents the sixth most common cancer type worldwide. Patients with oral cancer express high levels of IL-6 which is associated with very poor prognosis. Previous studies illustrated that IL-6 cytokine induces angiogenesis. It has also been reported that the presence of Cancer- Associated Fibroblasts (CAFs) is essential for angiogenesis. In this study, we examined the correlation between IL-6 and CAF and the role of this correlation on VEGF production. In this study, quantitative expression level of IL-6 and VEGF in CAF and Oral Cancer Cells (OCCs) examined through Real Time PCR and ELISA and western blot analysis. In addition, maintenance and retention of IL-6 and VEGF checked out in co-culture experiment of CAF and OCC cells. These experiments demonstrated that in oral cancer, CAF cell line secretes significantly more IL-6 than OCC. Also IL-6 is a factor that causes VEGF secretion in CAF cell line. CAF is the basic and the most essential source for producing IL-6 in patients with oral cancer. Secreted IL-6 is able to induce VEGF production in both CAF and OCCs. Correlation between CAF, IL-6 and VEGF could be considered as an approach for cancer therapy.

Key words: Interleukin-6; Cancer- Associated fibroblast; VEGF; Oral cancer cells; Normal fibroblast.

Introduction

Oral cancer is the sixth most common cancer type worldwide. More than 90% of all oral malignant neoplasms are squamous cell carcinoma (SCC) (1). In spite of recent improvements in the diagnosis and treatment of OSCC, the prognosis of the patients are still poor, mainly because of the high recurrence and strong capacity for locoregional and distant metastasis (1, 2).the 5-year survival rate is 92% in OSCC patients without recurrence and 30% in cases with recurrence. The median survival is 76.8 months in patients without recurrence and 42.5 months in cases with recurrence (3). The 5-year survival rate of OSCC patients has not been significantly changed (1, 4)and additionally the incidence and prevalence of OSCC are increasing, exclusively in younger patients (4).

The recent investigations on OSCC have shown that tumor progression is not eliminated by malignant cells only, but also interactions between tumor cells and their microenvironment have great impact on the development and progression of the cancer (1, 5). The fibroblasts, increase in tumor stroma, stay permanently activated and continuously improve tumor growth, invasion and metastasis. These activated fibroblasts are identified as “Cancer-Associated Fibroblasts” (CAFs) (5). CAFs are a sub-population of cells in the cancer stroma with a myofibroblast-like phenotypes (1, 6).

Angiogenesis is a key process in development, progression, and metastasis of all cancers types particularly OSCC(7, 8). Various studies shown that in the absence of angiogenesis, tumors cannot grow beyond 1–2 mm3 in size (7)and Vascular Endothelial Growth Factor (VEGF) is introduced as a primary stimulus of angiogenesis in the vast majority of malignant tumors (7, 9). Also cytokines that secreted by malignant cells as well as stromal cells can recruit immune cells to facilitate angiogenesis process (9, 10).

Various inflammatory cells interfere with carcinogenesis which improve tumor proliferation, invasion and angiogenesis (11). Numerous cytokines, such as interleukin-6 (IL-6), known to have an important role in the pathogenesis of several cancer types (11). IL-6 is a well-known immunoregulatory cytokine that bind to receptor and triggers functions including migration, invasion, apoptosis, angiogenesis, growth and differentiation of cancer cells immune cell infiltration and stromal reaction (11, 12). Two forms of receptor IL-6 was expressed: soluble isoform has more important role in disease including cancer and Rheumatoid arthritis; another isoform is membrane- bound IL-6 receptor expressed in immune cells(13). Overexpression of IL-6

are reported in many of tumors including breast, ovary, prostate, renal cell carcinoma, multiple myelomas, leukemia, lymphomas and particularly OSCC (12, 14). Increased IL6 expression is also associated with poor prognosis in patients with several cancers (12).

This study is designed to investigate the role of IL-6 secreted by CAFs on angiogenesis in primary cell culture oral cancer.

Materials and Methods

Materials

Materials that were used in this study include Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco,USA), fetal bovine serum (FBS) (Invitrogen, Massachusetts, USA), and other cell culture reagents were from Sigma–Aldrich (Sigma–Aldrich, Germany). Epithelial growth factor (EGF) (Royan, Tehran, Iran), Hydrocortisone (Sigma-Aldrich, Missouri, USA), cholera toxin (Sigma-Aldrich, Missouri, USA), L-Glutamine (Invitrogen, Massachusetts, USA), Trypsin-EDTA (Invitrogen, Massachusetts, USA), The RNA isolation kit and SYBR Green PCR kit were purchased from Qiagen (Qiagen, Germany). Anti-alpha smooth muscle Actin antibody, anti VEGF and β-actin were purchased from abcam (abcam,USA). IL-6 ELISA Kit (R&D), Anti-IL-6 receptor antibody (Actemra: GENENTECH, Roche group), IL-6 human and soluble IL-6 receptor human were purchased from Gibco and Invitrogen respectively.

Methods

Sample collection and Isolation of the primary fibroblast cells using explants technique

Oral carcinoma tissue samples obtained from 60 years old male patients diagnosed with oral carcinoma with prior informed consent (Tehran University of Medical Sciences Ethical Approval Committee # IR.TUMS.REC.1394.1285) for storage and further research. Following surgical procedure, the specimens were placed into a sterile tube containing 5 ml of Dulbecco's Modified Eagle's medium (DMEM) containing Penicillin (500 U/ml)/Streptomycin (500 µg/ml) and immediately transferred to the laboratory for further process.

Samples were immersed in 70% ethanol for 20 seconds and immediately transferred to phosphate buffer saline (PBS). Samples were washed several times in PBS and transferred to DMEM medium. After removing the blood vessels and debris from the samples, they were cut into smaller parts and each sample part was divided into approximately 1 mm^3 pieces. Core sections used for isolation of cancerous fibroblasts and the tissue pieces obtained from marginal tissue used to isolate normal fibroblasts. Every two or three small tissue pieces were seeded in a 35-mm^2 tissue culture dishes covering with a sterile 22-mm^2 glass slip. DMEM:F12 (1:1) medium containing 10% FBS, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 4mM L-Glutamine was added in each culture dish. The isolated primary cells were kept in 37°C incubator with 5% CO2 for approximately three weeks to reach the 70-80% confluency. Cells were daily observed and the medium was changed every 3 or 4 days. Subsequently, cells were subcultured using 0.25% trypsin and 0.02% EDTA solution and were spared into T25 culture flasks with complete culture media at a seeding density of 4×10^4 cells/cm^2. Cells were cultured in 37°C incubator with 5% CO2.

Isolation of the primary Oral Cancer Cells (OCC) using enzymatic digestion

In order to isolate epithelial cells, enzymatic digestion procedure was applied. Initially, samples were washed completely as described previously. Samples were then cut into 1 mm3 pieces. The extracellular matrix were digested with 0.1% collagenase Type I at 37 °C, and shaken vigorously for 60 min in order to separate oral epithelial cells. The collagenase activity was neutralized by adding an equal volume of DMEM containing 10% FBS. The dissociated tissues were then passed through 100, 70 and 40 µm cell strainer to remove debris. Samples were centrifuged at 300 g for 10 min. The contaminating erythrocytes were lysed with lysis buffer (155 mM NH4Cl; 5.7 mM K2HPO4; 0.1 mM EDTA, pH 7.3) and incubated at room temperature for 10 min followed by centrifugation at 300 g for 5 min. Subsequently, DMEM:F12 (1:1) medium containing 10% FBS, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 6mM L-Glutamine, 10µg/ml Insulin, 20ng/ml EGF, 0.5µg/ml Hydrocortisone, 100 ng/ml cholera toxin and 2 mM MEM were added to cells. The isolated cells were kept in 37°C incubator with 5% CO2 for approximately two weeks to reach the 70-80% confluency. Cells were daily observed and the medium was changed every 3 or 4 days. When cells confluence reached 70-80 %, culture flasks were sub-cultured and were spared into culture flasks. The primary cells were cultured in 37°C incubator with 5% CO2 to reach the 80% confluency.

Co-culture conditions

Approximately three weeks after isolation, we established a co-culture model by the isolated fibroblasts together with OCC. The ratio of OCC:fibroblastes seeded cells was 2:1 and cells were maintained in 6-well transwell plate co-culture condition for 48 h before harvesting for latter RNA or lysate isolation.

Flow cytometry

To characterize isolated cells, immunophenotyping of oral cancer cells were performed by direct immunofluorescence staining of cell surface antigens using FITC or RPE conjugated antibodies against CD326, CD133 and appropriate isotype matched controls. Samples were analyzed on Dako flow cytometry system (brand, country) and FlowMax software.

Immunofluorescence

In brief, CAFs and NF were fixed in 10 % paraformaldehyde for 30 minutes at room temperature. Cells washed by PBS three times and blocked in 2% bovine serum albumin (BSA) at room temperature. Next cells were incubated overnight by α-SMA antibody at 4°C. After washing, Cells were incubated in the secondary antibodies (Anti-C3c antibody (FITC), Abcam) for 2 hours at room temperature, in dark. Next, also, incubation was done in the DAPI for 10 minutes in the dark.

ELISA assays for measurement of IL-6

The expression levels of IL-6 by each cells assessed

with ELISA. Fibroblasts and OCC were seeded into a 6-well plate at a density of 1×10^5 /ml and were incubated overnight the cell lines were then exposed to each of the conditions described in this study, and incubated in DMEM:F12 containing 10% FBS. After 48 h, culture supernatants were collected, centrifuged, and measured using the IL-6 ELISA Kit (R&D Systems Inc., Minneapolis, MN, USA). The procedure was followed according to the manufacturer's instructions.

Real-time quantitative PCR

For quantitative analysis of gene expression, treated cells were harvested and total RNA was extracted using RNeasy RNA extraction kit (brand, country) according to the manufacturer's instructions. 1 µg of isolated RNA was reverse-transcribed by Qiagene RT-PCR kit (brand, country). Evaluation of gene expression was performed using Quanti Fast SYBR Green mastermix (takara, Japan) using ABI StepOne instrument (Corbett Research, Australia). VEGF and IL-6 expression levels were assessed by QuantiTect primers. The data was normalized to the expression levels of Actin transcript level as the internal control.

Western blot analysis

OCC and fibroblasts cell lysates was prepared by homogenization in modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 1 % Triton X-100, 0.2 % sodium deoxycholate, 0.2 % SDS, 1 mM Na-EDTA, 1 mM PMSF) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). 20 µg of total protein was fractionated by SDS-PAGE and immunoblotted with anti-VEGF or Actin. Immunoblotted protein bands were visualized with enhanced chemiluminescence and protein bands were quantified using Scion Image software. Each experiment was performed at least three times.

Statistical analysis

All statistical analyses were performed using SPSS 13.0. (SPSS, Chicago, IL). Comparisons among all groups were performed with the one-way analysis of variance (ANOVA). If statistical significance was found, the Tukey post hoc test was performed. Values of $p<0.05$ were considered statistically significant. Results are expressed as mean ± SD of three independent experiments.

Results

Isolation primary OCCs, CAFs and NFs

Primary Oral Cancer Cells (OCC)

Epithelial cell adhesion molecule (EpCAM) (CD326) is generally expressed on the basolateral surface of epithelial and carcinoma cells and it is a Ca^{2+} independent adhesion molecule which is expressed in OCC. In order to, to investigate this issue, the characterization of OCC cell lines were examined by flow cytometry and the results revealed high levels of CD326 marker expression.

The CD133 (prominin-1) is presently considered as a useful biomarker for prognosis and detection of cancer stem cell in a variety of human cancer types, including oral cancer. In this study the cells were examined by flow cytometry and the results showed that cells were positive for CD133 (Figure 1).

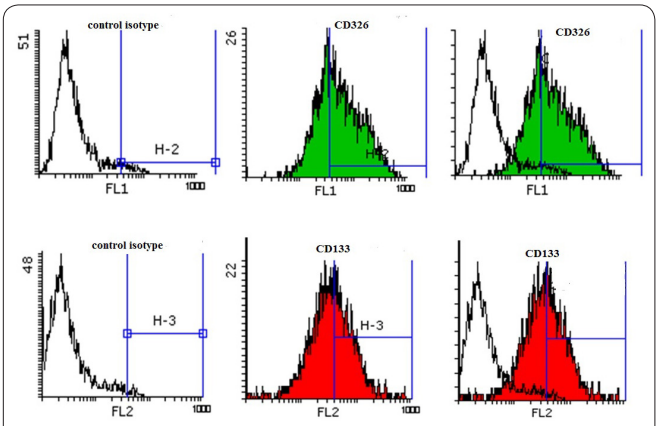

Figure 1. Flow cytometry analysis shown that primary OCC cells were positive expression for CD326 (green histogram) and CD133 markers (red histogram).

Cancer-associated fibroblast and normal fibroblast

To prove that isolated cells are fibroblast, CAFs and NF evaluated by vimentin immunebloting. Result confirms that CAF and NF are fibroblasts (Figure 2). To differentiate between CAF and NF α-SMA, staining immunofluorescence was performed. Data showed α-SMA expression in CAFs is more than NF (Figure 3).

In general, IL-6 was released from CAFs

Since inflammatory factors, like IL-6, are involved in growth and development of tumor cells, the release rate of IL-6is analyzed through ELIZA method in order to reveal if IL-6 is released from cancer cells or fibroblasts. To investigate source of IL-6, the gene expression and release rate of IL-6 is analyzed by Real-time and ELISA method in order to reveal if IL-6 is released from

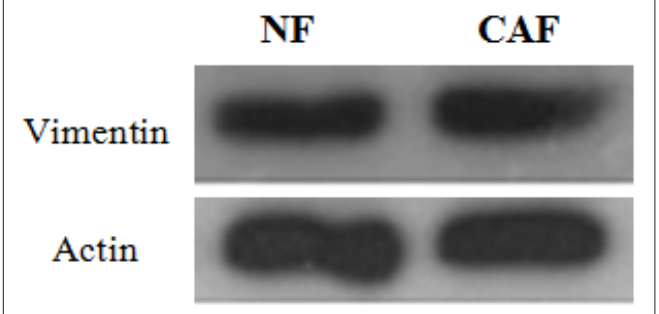

Figure 2. Western bloting of vimentin expression in fibroblasts. CAF and NF cells were expressed vimentin and confirm that as fibroblast cells in the tumor tissue.

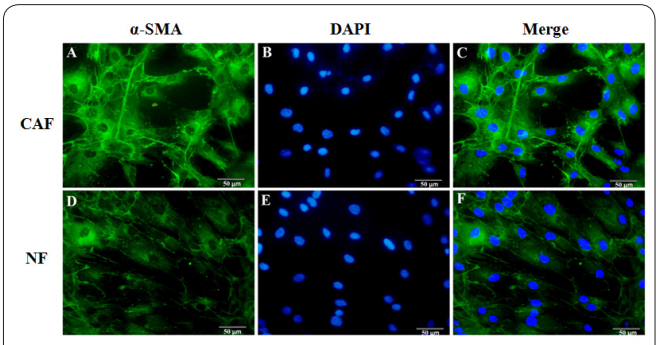

Figure 3. Immunofluorescent stained with α-SMA antibody and FITC. Immunofluorescent staining for α-SMA (A, D) showed NF reduced expression compared to CAF. (B and E) Nucleus of cells stained with DAPI. (C and F) is merged of α-SMA and DAPI. The nucleus can be observed as blue and α-SMA as green.

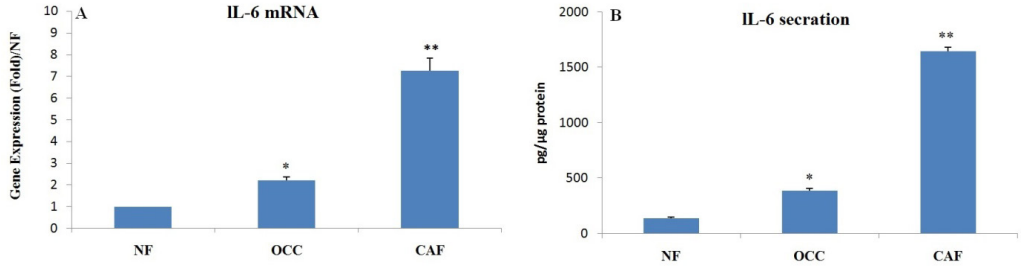

Figure 4. IL-6 mRNA and protein levels were assessed in primary OCC and fibroblasts. (A) Real-time PCR analysis showed that IL-6 expression level in the CAF is 7.2 and 4.3 times more than NF and OCC respectively. (B) ELISA analysis confirms mRNA expression data. P<0.01 versus NF and OCC cells.

cancer cells or fibroblasts. Real-time display that Il-6 expression level is 3and 7.2 times toward OCC and NF respectively (Figure 4A). The amount of IL-6 release in CAF was 4.3 and 11.8 times higher than OCC and NF respectively (Figure 4B). This finding shows that IL-6 mainly secretion from CAFs and not from OCC.

LPS or OCC induced IL-6 expression in NF

Now, since tumor cells and their stroma environment (including fibroblasts, white cells, etc.) are related to one another and according to above result, this indicated some factor passable induced IL-6 secretion from CAF. To investigated whether IL-6 expression can increase to level was observed in NF was exposed to OCC (co-cultural); and it seen that the rate of IL-6 gene expression and release was 6 and 9.2 times higher than control cells, respectively (Figure 5A-B). In order to more specifically analyze the NF cells, OCC was treated with LPS. The results showed that the amount of IL-6 gene expression and release was 4 and 6.8 times higher than control cells respectively, but low change observed in IL-6 release from OCC cells (Figure 5C-D). In sum, above mentioned-results showed that the main origin of IL-6 is fibroblast cells, and it is produced by the effects of tumor cells on fibroblast cells.

IL-6 induces expression and release of VEGF in CAFs and NF

IL-6 is one of the factors inducing the angiogenesis that affects the target cells and leads to increase in their expression and release of VEGF. To investigate whether IL-6 induced angiogenesis, primary oral cancer cell and fibroblasts were treated by IL-6 1ηg ml^{-1} /soluble IL-6 receptor human 40ηg ml^{-1} (ratio is 1/40) and Anti-IL-6 receptor antibody (Actemra) 10µg ml^{-1} for 48 h. VEGF gene expression and protein was evaluated by Real-time PCR and western blot respectively (Figure 6A-C). CAFs and NF treated by IL-6 and VEGF mRNA increase 2.3 and 3 times respectively. To confirm this data, IL-6 was suppressed by anti-IL-6 receptor antibody. In CAFs and NF, VEGF mRNA was at same rate as control group. To more investigate, VEGF western blot in CAFs and NF, showed that increase in present of IL-6 and was at the same rate as control when suppressed by anti-IL-6 receptor anti body. In addition to, VEGF mRNA and protein were assessed in OCC. IL-6 increase VEGF mRNA and protein in OCC but not same level as seen in CAFs or NF (Figure 7). In sum, this data showed that CAFs play a more signification role in angiogenesis than OCC in oral cancer.

Discussion

Increasing progress in research and clinical trial demonstrated that there is a strong relation between inflammation and cancer. It was well proven that inflammatory molecules increase the risk of tumor growth. These inflammatory molecules are secreted from immune cells or other cells like cancer cells (15-17). One

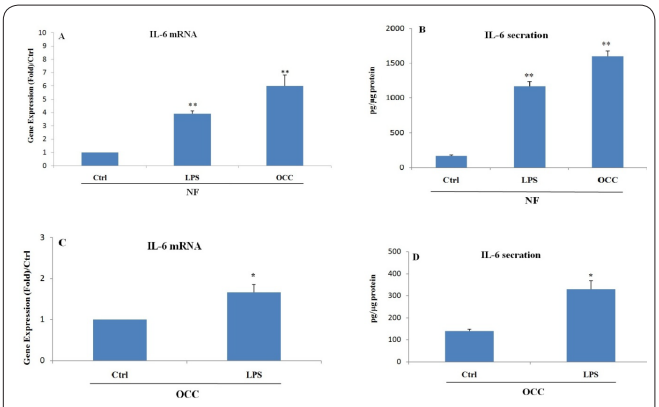

Figure 5. IL-6 release in response to LPS and calculator with OCCs. (A-C) Co-culture of NF with OCCs or LPS increased mRNA expression of IL-6 more than the control (**P<0.01). Low rise IL-6 mRNA rate were seen in OCCs treated by LPS (*P<0.05). (B–D) ELISA analysis of IL-6 levels in the supernatants, IL-6 protein rate release from NF cocultured with OCCs or LPS was much higher relative to control (**P<0.01). Low rise IL-6 protein rate were seen in OCCs (*P<0.05).

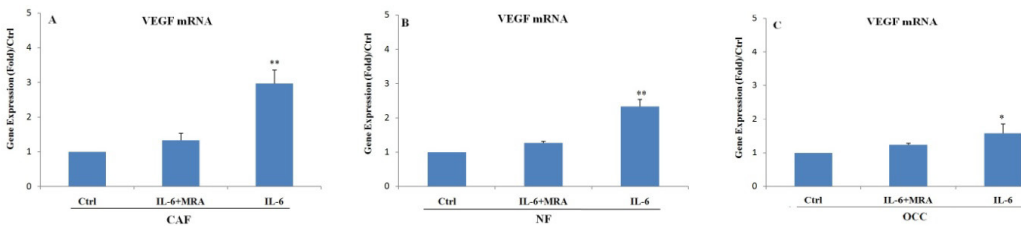

Figure 6. Analysis of VEGF mRAN expression and protein in fibroblasts and OCCs treated by IL-6 and Actemra. (A) Real-time PCR showed that VEGF mRNA rate was increased in CAFs, NF and OCCs teated with IL-6 and decrease in present of Actemra (**P<0.01). (B) IL-6 positively regulates VEGF.

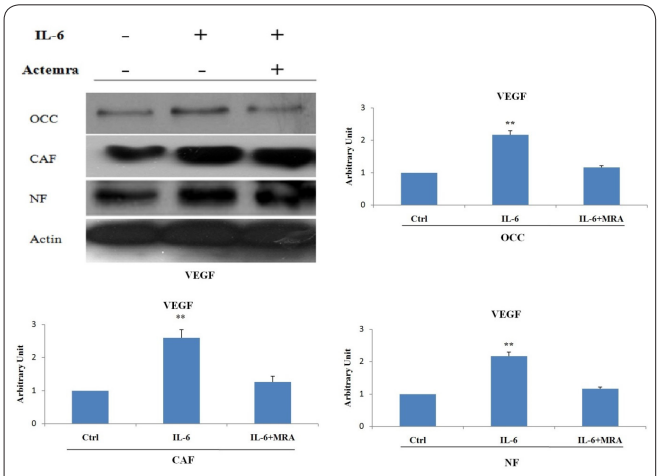

Figure 7. Western blot analyses of protein extracts from CAFs, NF and OCCs cells treated with IL-6. The results for the detection of VEG. Data represent are expressed as mean ± SD three independent experiments (**P<0.01).

of the most important inflammatory molecules is IL-6, which plays a significant role in growth and differentiation of tumor cells. Several studies showed that quantity of IL-6 is extraordinary in patients with cancer (breast, clone and prostates); Moreover, high rate of IL-6 secreted in serum represented direct relation with invasion, metastases and resistance to medicine. Moreover, in comparison with those who have lower rates of IL-6, high rates of IL-6 have weak prognosis. (18, 19).

In the current study, IL-6 was inhibited by anti-human IL-6 receptor. Results showed that this inhabitation in NF and CAF reduced VEGF expression, in comparison to the samples with the absence of inhabitation. In addition, although OCC has lower VEGF expression level than fibroblasts, but in the presence of IL-6 inhibitor the expression level of VEGF will be decreased in fibroblasts too.

Several studies showed that inflammatory factors like IL-6 are potential treatment of some cancer types (20-22). Most of these studies indicated and confirmed the relation between cancer cells and inflammation. In present study, we have investigated the relationship between stroma and IL-6. Generally, cancer medicines prevent cancer cell proliferation. Recently, the Stroma of tumor tissue has received a significant attention in treatment (15-17). This Stroma environment has had a significant effect on angiogenesis in a way that any changes in tumor Struma environment will lead to changes in invasion, angiogenesis and metastasis of tumor cells. According to this study, it was cleared that in addition to VEGF inhibitor medicines, inhibiting the inflammation especially IL-6 factor is crucial for inhabiting the angiogenesis,the reason is that inflammatory factors like IL-6 will induce VEGF in tumor Stroma cells, especially fibroblasts.

There are several researches which expressed IL-6 has important role in tumor growth. The secretion sources of IL-6 are different. Some research clarified that immune cells in cancer Stroma secreted IL-6 (16, 17, 23). Also, other studies showed that IL-6 secreted from cancer-associated fibroblast and epithelial cancer cells (24-27). Nagasaki et al. (2013) reported that IL-6 secreted by CAF but not epithelial cancer cells. In addition, Nilsson et al. (2005) showed that IL-6 released from ovarian epithelial cancer cells (28). Although, our data clarified that in oral cancer, IL-6 release from CAF. According to stroma condition, it was shown that OCC can induced IL-6 produce from NF same as level reported in CAF. This finding are aligned with some other reports (26, 29).

In the present study, we showed that in compression with OCC primary cells, the rate of IL-6 released in CAF cells is much more. Moreover, the results of this study demonstrated that in the present of OCC or LPS, the release rate of IL-6 through NF is high; while the amount of IL-6 released in OCC cells has low increase in response to LPS. It seems that OCC cause CAF and NF to trigger an inflammatory response and induce IL-6 expression (30). in addition, some studies showed that OCC cells are able to generate Il-6, but in this study we found that CAF is the major origin of IL-6 production (31, 32). Furthermore, in the presence of LPS, the amount of IL-6 was increased very low in OCC. Hwang et al. (2012) showed that cancer cells release IL-6, but our data represent that the expression level of IL-6 by CAFs is significantly higher than OCC (31). These results confirm that in oral cancer often IL-6 produces by CAF. Also, OCC and LPS can upregulate IL-6 production by NFs to the same level produced by CAFs.

Angiogenesis is one of the hallmarks of cancer(33). For further growth, tumor needs angiogenesis. One of the major factors of angiogenesis is VEGF, which is released from tumor cells and induces the angiogenesis in tumor tissues (34).

Present study showed that the rate of VEGF expression in CAF and NF will be increased in the presence of OCC or LPS. According to the above-mentioned results, it seems that increase in VEGF has direct relationship with IL-6 (26). For proving this statement, it was showed that CAF and NF cells cause increasing levels of VEGF expression in response to IL-6; while IL-6 has fewer effects on VEGF rates of OCC cells. Therefore, it seems that the effects of IL-6 are through para-crine and autocrine on fibroblastic cell (26).

Several studies have showed that CAF and IL-6 illustrated potential therapeutic targets in cancers(35, 36). In sum, according to what we discussed above, when a high level of metastasis has been observed in oral cancer, decrease of inflammatory factors like IL-6 or signaling pathways of IL-6 lead to decrease in angiogenesis and decrease of metastasis consequently.

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Conflict of interest
The authors declare no conflict of interest.

Author' contribution
Study conception and design by Ahmad Nasimian and Pouyan Aminishakib; acquisition of data by Masoume Mirkeshavarz, Abbas Karimi and Fatemeh Kamali; analysis and interpretation of data by Mohamad Javad Kharazifard, Meysam Ganjibakhsh, Parvaneh Farzaneh and Neda sadat Gohari; drafting of manuscript by Nazanin Mahdavi and Faezeh Vakhshiteh; critical revision by Seyed Abolhassan Shahzadeh Fazeli, Pouyan Ami-

nishakib and Ahmad Nasimian.

References

1. Li H, Zhang J, Chen S-W et al. Cancer-associated fibroblasts provide a suitable microenvironment for tumor development and progression in oral tongue squamous cancer. Journal of translational medicine. 2015; 13(1): 198.
2. Hernández Guerrero JC, Jacinto Alemán LF, Jiménez Farfán Farfán MD, Macario Hernández A, Hernández Flores F, Alcántara Vázquez A. Prevalence trends of oral squamous cell carcinoma. Mexico City's General Hospital experience. 2013.
3. Wang B, Zhang S, Yue K, Wang X-D. The recurrence and survival of oral squamous cell carcinoma: a report of 275 cases. Chin J Cancer. 2013; 32(11): 614-618.
4. Feller L, Lemmer J. Oral squamous cell carcinoma: Epidemiology, clinical presentation and treatment. 2012.
5. Li J, Jia Z, Kong J et al. Carcinoma-associated fibroblasts lead the invasion of salivary gland adenoid cystic carcinoma cells by creating an invasive track. PloS one. 2016; 11(3): e0150247.
6. Tang D, Gao J, Wang S et al. Cancer-associated fibroblasts promote angiogenesis in gastric cancer through galectin-1 expression. Tumor Biology. 2016; 37(2): 1889-1899.
7. Liang Y, Brekken RA, Hyder SM. Vascular endothelial growth factor induces proliferation of breast cancer cells and inhibits the anti-proliferative activity of anti-hormones. Endocrine-related cancer. 2006; 13(3): 905-919.
8. Mărgăritescu C, Pirici D, Stîngă A et al. VEGF expression and angiogenesis in oral squamous cell carcinoma: an immunohistochemical and morphometric study. Clinical and experimental medicine. 2010; 10(4): 209-214.
9. Aggarwal S, Devaraja K, Sharma SC, Das SN. Expression of vascular endothelial growth factor (VEGF) in patients with oral squamous cell carcinoma and its clinical significance. Clinica Chimica Acta. 2014; 436: 35-40.
10. Gopinathan G, Milagre C, Pearce OM et al. Interleukin-6 stimulates defective angiogenesis. Cancer research. 2015; 75(15): 3098-3107.
11. Zhao ZF, Li JX, Ye R, Wu X, Gao LL, Niu BL. Interleukin-6 as a potential molecular target in esophageal squamous cell carcinoma. Oncology letters. 2016; 11(2): 925-932.
12. Chen C-J, Sung W-W, Lin Y-M et al. Gender difference in the prognostic role of interleukin 6 in oral squamous cell carcinoma. PLoS One. 2012; 7(11): e50104.
13. Mihara M, Hashizume M, Yoshida H, Suzuki M, Shiina M. IL-6/IL-6 receptor system and its role in physiological and pathological conditions. Clinical science. 2012; 122(4): 143-159.
14. Nikakhlagh S, Ranjbari N, Khorami E, Saki N. Association between Serum Levels of Interleukin-6 and Stage of Laryngeal Cancer. Iranian journal of otorhinolaryngology. 2015; 27(80): 199.
15. Sansone P, Storci G, Tavolari S et al. IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. The Journal of clinical investigation. 2007; 117(12): 3988-4002.
16. Wan S, Zhao E, Kryczek I et al. Tumor-associated macrophages produce interleukin 6 and signal via STAT3 to promote expansion of human hepatocellular carcinoma stem cells. Gastroenterology. 2014; 147(6): 1393-1404.
17. Wang L, Miyahira AK, Simons DL et al. IL6 Signaling in Peripheral Blood T Cells Predicts Clinical Outcome in Breast Cancer. Cancer Research. 2017; 77(5): 1119-1126.
18. Salgado R, Junius S, Benoy I et al. Circulating interleukin-6 predicts survival in patients with metastatic breast cancer. International journal of cancer. 2003; 103(5): 642-646.
19. Chang CH, Hsiao CF, Yeh YM et al. Circulating interleukin-6 level is a prognostic marker for survival in advanced nonsmall cell lung cancer patients treated with chemotherapy. International journal of cancer. 2013; 132(9): 1977-1985.
20. Tawara K, Oxford JT, Jorcyk CL. Clinical significance of interleukin (IL)-6 in cancer metastasis to bone: potential of anti-IL-6 therapies. Cancer management and research. 2011; 3: 177.
21. Casneuf T, Axel AE, King P et al. Interleukin-6 is a potential therapeutic target in interleukin-6 dependent, estrogen receptor-α-positive breast cancer. Breast Cancer: Targets and Therapy. 2016; 8: 13.
22. Smolen JS, Maini RN. Interleukin-6: a new therapeutic target. Arthritis research & therapy. 2006; 8(2): S5.
23. Sousa S, Brion R, Lintunen M et al. Human breast cancer cells educate macrophages toward the M2 activation status. Breast Cancer Research. 2015; 17(1): 101.
24. Woods KV, Adler-Storthz K, Clayman GL, Francis GM, Grimm EA. Interleukin-1 regulates interleukin-6 secretion in human oral squamous cell carcinoma in vitro: possible influence of p53 but not human papillomavirus E6/E7. Cancer research. 1998; 58(14): 3142-3149.
25. Tang C-H, Chuang J-Y, Fong Y-C, Maa M-C, Way T-D, Hung C-H. Bone-derived SDF-1 stimulates IL-6 release via CXCR4, ERK and NF-κB pathways and promotes osteoclastogenesis in human oral cancer cells. Carcinogenesis. 2008; 29(8): 1483-1492.
26. Nagasaki T, Hara M, Nakanishi H, Takahashi H, Sato M, Takeyama H. Interleukin-6 released by colon cancer-associated fibroblasts is critical for tumour angiogenesis: anti-interleukin-6 receptor antibody suppressed angiogenesis and inhibited tumour–stroma interaction. British journal of cancer. 2014; 110(2): 469-478.
27. Liu S, Ginestier C, Ou SJ et al. Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks. Cancer research. 2011; 71(2): 614-624.
28. Nilsson MB, Langley RR, Fidler IJ. Interleukin-6, secreted by human ovarian carcinoma cells, is a potent proangiogenic cytokine. Cancer research. 2005; 65(23): 10794-10800.
29. Shintani Y, Fujiwara A, Kimura T et al. IL-6 Secreted from Cancer-Associated Fibroblasts Mediates Chemoresistance in NSCLC by Increasing Epithelial-Mesenchymal Transition Signaling. Journal of Thoracic Oncology. 2016; 11(9): 1482-1492.
30. Hugo HJ, Lebret S, Tomaskovic-Crook E et al. Contribution of fibroblast and mast cell (afferent) and tumor (efferent) IL-6 effects within the tumor microenvironment. Cancer microenvironment. 2012; 5(1): 83-93.
31. Hwang YS, Lee SK, Park K-K, Chung W-Y. Secretion of IL-6 and IL-8 from lysophosphatidic acid-stimulated oral squamous cell carcinoma promotes osteoclastogenesis and bone resorption. Oral oncology. 2012; 48(1): 40-48.
32. Bernabé DG, Tamae AC, Biasoli ÉR, Oliveira SH. Stress hormones increase cell proliferation and regulates interleukin-6 secretion in human oral squamous cell carcinoma cells. Brain, behavior, and immunity. 2011; 25(3): 574-583.
33. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. cell. 2011; 144(5): 646-674.
34. Sakurai T, Kudo M. Signaling pathways governing tumor angiogenesis. Oncology. 2011; 81(Suppl. 1): 24-29.
35. Coward J, Kulbe H, Chakravarty P et al. Interleukin-6 as a therapeutic target in human ovarian cancer. Clinical Cancer Research. 2011; 17(18): 6083-6096.
36. Hsu CP, Chen YL, Huang CC et al. Anti-interleukin-6 receptor antibody inhibits the progression in human colon carcinoma cells. European journal of clinical investigation. 2011; 41(3): 277-284.