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Detection of regulatory T cells, IFN-γ, TGF-β, and IL-10 in peripheral blood of patients with oral cancer before and after chemotherapy.

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Abstract

Objective: Oral cancer refers to malignant tumor that occurs in the mouth. Immunosuppression is frequently accompanied with different stages of oral cancer. Regulatory T cell immune function and cytokines secretion are of great significance to the oral cancer immunotherapy and recovery.
Patients and methods: In this study, monoclonal antibody technique and flow cytometry were used to analyze the characteristics of T lymphocyte immunity in patients with oral cancer. The levels of T lymphocyte subsets, Interferon (IFN)-γ, Transforming Growth Factor (TGF)-β and Interleukin (IL)-10 in peripheral blood of patients with oral cancer before and after operation or chemotherapy containing paclitaxel and cisplatin were tested.
Results: The expressions of CD3, CD4, along with the ratio of CD4/CD8 in patients with oral cancer were significantly lower than that in healthy volunteers. On the contrary, the levels of CD8 and CD56 were obviously up-regulated in comparison ($P<0.05$). The amounts of TGF-β and IL-10 were significantly elevated in patients before surgery or chemotherapy compared with that in healthy control, whereas the treatment of surgery or chemotherapy obviously inhibited those expressions ($P<0.05$). However, IFN-γ level did not present a significant change before and after surgery or chemotherapy.
Conclusion: Our data demonstrated that immunosuppression were found in patients with oral cancer and the treatment of surgery or chemotherapy can effectively alleviate the immunosuppression, which provides academic basis of immunotherapy against immunosuppression during conventional treatment of surgery or chemotherapy.

Keywords: Oral cancer, Peripheral regulatory T cells, IFN-γ, IL-10, TGF-β.

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Introduction

Oral cancer is characterized as a group of malignant tumors occurred in lip, tongue, salivary glands, gums, mouth, oropharynx, buccal mucosa, and other parts of the mouth, of which at least 80% are squamous cell carcinoma [1]. In addition to the specific pathogenic site, oral cancer severely affects the quality of life of cancer patients [2].

Currently, surgery is still the main therapy for the treatment of oral cancer. In spite of the rapid progress of medical technology, the prognosis of oral cancer is still unsatisfactory. Moreover, the surgical treatment gave rise to side-effects on swallowing, language function, and facial features.

T cells represent the main immune cells that play an important role in the anti-tumor immunity. They are divided into different subgroups according to the surface molecular markers [3].

Regulatory T (Treg) cells belong to T cell subsets and are specifically profiled with immunosuppressive function. It was found that the amount of Treg cells increased in different types of tumor, suggesting the correlation with immunosuppressive state to some extent [4]. Intensive immune tolerance creates an advantageous environment for the escape of tumor from immune attack [5,6].

In addition to direct killing effect towards tumor cells, T cells maintain the balance of immune system by secreting different types of cytokines, such as IFN-γ, TGF-β, and IL-10 [7].

Therefore, in this study, we aimed to investigate the immune function of Treg cells and cytokines on the immunotherapy and recovery of patients with oral cancer.

Materials and Methods

Main materials and reagents

FITC labelled CD3, CD4, CD8 and CD56 monoclonal antibodies were purchased from Sigma (CA, USA). Hemolysin was got from Becton Dickinson. PE labeled IFN-γ, TGF-β, and IL-10 monoclonal antibodies were bought from Sigma (CA, USA).

Main instruments

Flow cytometry FACS Calibur was provided by Becton Dickinson (San Diego, CA).

Experimental subjects

A total of 60 patients with oral squamous carcinoma diagnosed by postoperative pathology between March 2017 and February 2018 were enrolled from Xingtai People's Hospital of Hebei Medical University (Hebei, China). The patients received no anti-tumor treatment before surgery and had no systemic diseases. The pathogenic sites contained tongue, cheek, gum, and mouth bottom, and all the cases were in stage III and IV. Another 30 healthy volunteers aged from 20 to 50 y old were selected as normal control.

This study has been pre-approved by the ethical committee of Xingtai People's Hospital of Hebei Medical University (Hebei, China). All subjects have signed the consent forms before recruitment in this study.

Grouping

After signing the informed consent, the subjects were equally randomly divided into operation group and preoperative chemotherapy group. The patients in the preoperative chemotherapy group received 150 mg/m^2 taxol and 100 mg/m^2 cisplatin *via* intravenous injection on the first day.

Peripheral blood collection

A total of 1 ml peripheral blood was extracted from the healthy volunteers and treated with heparin anticoagulation. In the operation group, the blood was extracted one week before and after surgery. In the pre-operative chemotherapy group, the blood was extracted at two days before and 20 d after chemotherapy.

Regulatory T cell surface marker detection

Heparinized blood was initially diluted with RPMI 1640, 2% Fetal Bovine Serum (FBS) anti-biotic, anti-mycotic, glutamine supplemented media, within 2 h of collection. PBMCs were isolated from diluted blood at 400 g for 30 min. The PBMCs were washed in RPMI-1640 at 300 g for 5 min) and viable cells counted using trypan blue. One million PBMC were then for flow cytometry analysis per condition. Staining with cell surface antibodies of anti-CD3, anti-CD4, anti-CD8, anti-CD56, and anti-CD25 was performed in PBS/0.5% BSA/0.02% sodium azide (PBA) buffer for 60 min at 4°C in the dark, respectively. Flow cytometry data were acquired using FACS calibur flow cytometer (BD, Franklin Lakes, New Jersey, USA). Acquisition was stopped after 20,000 CD4 prevents were collected within the lymphocyte gate. Analysis was performed using FlowJo software (V.8.8.6, Tree Star, Ashland, Oregon, USA).

Cytokines detection in T lymphocytes

Heparinized blood was treated as described in regulatory T cell surface marker detection. In brief, one million PBMC per condition were fixed and permeabilized with the BD Pharmingen transcription factor buffer set and then intracellularly stained with fluorescent antibodies specific for IFN-γ, IL-10 and TGF-β. Flow cytometry data were collected and analyzed as stated above.

Statistical analysis

All data analyses were performed on SPSS 16.0 software. The measurement data were presented as mean ± standard deviation ($\chi \pm s$) and compared by t-test or one way ANOVA. $P<0.05$ was considered as statistical significance.

Results

The typing changes of peripheral T lymphocytes

We applied flow cytometry to test the proportion of each type of peripheral T lymphocytes (Table 1). Of note, the amounts of CD3 and CD4 decreased, and CD8 level elevated, along with the decreasing ratio of CD4/CD8 in the oral cancer patients on one week before operation ($P<0.05$). However, statistical elevation of CD56 expression in oral cancer patients was observed compared with that in healthy volunteers ($P<0.05$), suggesting that the patients were in immunosuppressive state before operation. The proportion of CD3+ T cells slightly increased at one week after surgery compared with pre-operation but was statistically lower than that in the healthy volunteers ($P<0.05$). Similar results were found on the level of CD4. The ratio of CD4/CD8 also exhibited similar trend with statistical significance. It indicated that the immune level was improved in oral cancer patients after surgery but the value still cannot reach to the level of healthy people.

Table 1. The typing changes of peripheral T lymphocytes.

Group	n	CD3 (%)	CD4 (%)	CD8 (%)	CD56 (%)	CD4/CD8 (%)
Healthy volunteers	30	76.83 ± 3.88	53.41 ± 12.33	24.16 ± 6.14	11.46 ± 5.46	2.56 ± 1.33
Before operation	30	58.38 ± 11.20*	30.59 ± 6.39*	30.84 ± 8.79*	23.59 ± 8.12*	1.25 ± 0.43*
After operation	30	61.88 ± 9.80*	44.32 ± 7.49*	24.55 ± 4.96#	14.65 ± 7.46*#	1.60 ± 0.48*#
Before chemotherapy	30	56.89 ± 9.27*	32.15 ± 6.78*	32.54 ± 6.88*	24.15 ± 6.48*	1.26 ± 0.45*

After chemotherapy	30	70.89 ± 5.88	50.46 ± 4.87*#	29.66 ±5 .87*#	18.62 ± 6.21*#	1.54 ± 0.52*#

*p<0.05, compared with healthy volunteers; #p<0.05, compared with pre-operation.

The proportion of CD3+ T cell significantly was declined in patients receiving pre-operative chemotherapy compared with healthy volunteers and the proportion obviously increased after chemotherapy. The proportion of CD4+ T cell was markedly decreased, whereas the CD8+ T cell ratio was apparently up-regulated in oral cancer patients compared with control. Chemotherapy significantly elevated CD4+ T cell proportion and CD4/CD8 ratio. Similarly, the change of CD56 was consistent with that of CD8+ T cell. To sum up, immunosuppression was alleviated after chemotherapy or operation compared with patients receiving no treatment.

TGF-β level changes in oral cancer patients before and after operation or chemotherapy

TGF-β is a multi-functional protein that can be secreted by multiple cells. It contributed to a regulatory role on the growth and differentiation of a variety of cells under physiological condition [7]. Previous study showed that TGF-β overexpression is associated with progression and metastasis of oral cancer. It is thought to be an important inhibitory factor affecting immune function in patients with oral cancer [8,9]. In this study, we examined TGF-β expression levels before and after surgery or chemotherapy. As shown in Table 2, TGF-β was significantly increased before and after operation or chemotherapy compared with healthy volunteers (P<0.05). Its level obviously declined after surgery or chemotherapy compared with pre-operation or before chemotherapy (P<0.05). It suggested that chemotherapy and surgery can improve the immunosuppressive state and convert that to the state of normal healthy volunteers.

Table 2. *TGF-β level changes in oral cancer patients before and after operation or chemotherapy.*

Group	n	TGF-β+ (%)
Healthy volunteers	30	4.69 ± 1.75
Before operation	30	5.52 ± 1.20*
After operation	30	4.86 ± 0.80#
Before chemotherapy	30	5.55 ± 1.27*
After chemotherapy	30	4.94 ± 0.88

*p<0.05, compared with healthy volunteers; #p<0.05, compared with pre-operation.

IL-10 level changes in oral cancer patients before and after operation or chemotherapy

IL-10 is also an important cytokine participated in immunosuppression [10]. We examined IL-10 expression levels before and after surgery or chemotherapy. As shown in Table 3, IL-10 was significantly increased before and after operation or chemotherapy compared with healthy volunteers (P<0.05). Its level obviously declined after surgery or chemotherapy compared with pre-operation or before chemotherapy (P<0.05), indicating the immunosuppressive state was inhibited by chemotherapy and surgery, though the level was not as similar as that of normal healthy volunteers.

Table 3. *IL-10 level changes in oral cancer patients before and after operation or chemotherapy.*

Group	n	IL-10+ (%)
Healthy volunteers	30	4.67 ± 0.45
Before operation	30	5.39 ± 0.88*
After operation	30	5.00 ± 0.51#
Before chemotherapy	30	5.22 ± 1.27*
After chemotherapy	30	4.92 ± 0.64#

*p<0.05, compared with healthy volunteers; #p<0.05, compared with pre-operation.

IFN-γ level changes in oral cancer patients before and after operation or chemotherapy

IFN-γ is a kind of cytokine secreted by Th1 that plays a crucial role in anti-tumor immunity [11]. We examined CD4+IFN-γ+ cell proportion before and after surgery or chemotherapy, aiming to explore the T cell immunity in oral cancer patients before and after surgery or chemotherapy. As shown in Table 4, the proportion of CD4+IFN-γ+ cell showed no significant difference before and after operation or chemotherapy.

Table 4. *IFN-γ level changes in oral cancer patients before and after operation or chemotherapy.*

Group	n	IFN-γ+ (%)
Healthy volunteers	30	5.78 ± 0.88
Before operation	30	5.52 ± 1.20
After operation	30	5.89 ± 1.80
Before chemotherapy	30	6.22 ± 1.27
After chemotherapy	30	5.89 ± 0.88

Discussion

Malignant tumor is one of the important reasons that affect human survival and quality of life. Millions of people die each year from cancer in the world. Oral cancer accounts for the sixth among various malignant tumors [12]. Anti-tumor immune system consists of the immune surveillance and killing through immune cells and cytokines [13]. However, its specific mechanism in the process of killing malignant tumors has not

been elucidated [14,15]. Immune cells derive from the bone marrow and enter the peripheral blood after thymus activation [16,17]. Therefore, this study selected peripheral blood samples to test T cell typing and cytokine changes, aiming to understand the changes in cellular immune function in patients with oral cancer.

Surgery or chemotherapy has certain effect on the improvement to immune state. In this study, our results also validated the partial function of surgery or chemotherapy on the immune status of patients with oral cancer cannot. Additionally, active or passive immunotherapy is needed to improve the efficacy of oral cancer surgery and chemotherapy. The observation of the changes of factors secreted by T cell and T lymphocyte immunophenotype is thus of great significance during the immunotherapy.

Lymphocytes are one of the most important immune cells in the body that can be divided into three subgroups (cytotoxic T cells, helper T cells, regulatory T cells) [18]. At present, it was revealed that the anti-tumor immunity is mainly based on cellular immunity. Distribution of different subtype T lymphocytes in the peripheral blood can reflect the state of anti-tumor immunity.

Previous study demonstrated that compared with lymphocytes in adjacent tissues, a significant decrease in CD4/CD8, CD4-positive T cells, NK cells, and NKT cells was found in hepatocellular infiltrated lymphocytes [19]. Of note, our data showed that CD8+ cells proportion and CD56+ cell proportion presented same reducing trend after surgery/chemotherapy group in comparison with that before surgery/chemotherapy, indicating the immunosuppressive state was inhibited by the treatment.

It was also observed that detection of peripheral blood T cell subsets has important clinical significance in evaluating the clinical efficacy and prognosis in patients with gastric cancer, colorectal cancer, lung cancer, breast cancer, and liver cancer [20-22].

In this study, we also detected immunosuppression related cytokines IFN-γ, TGF-β, and IL-10, among which, TGF-β and IL-10 were significantly upregulated in oral cancer patients. Either surgery or chemotherapy can reduce their levels, but cannot restore to normal. IFN-γ level showed no significant change in oral cancer patients before and after surgery or chemotherapy.

Earlier studies reported that TGF-β expression is significantly elevated and is not associated with age, sex, and histological differentiation in patients with oral cancer [8,9]. However, it was related to the clinical stage and lymph node metastasis, which was similar to our results. Similar results were obtained on IFN-γ and IL-10 expression levels [23,24].

In general, the immunosuppressive state is not the first factor of tumor. However, regulation of immune function has an important role in the occurrence, treatment, and prognosis of oral cancer [25,26]. Detection of immune indicators is of great significance to monitor the disease and provides parametric basis for medication.

Conclusion

Our data demonstrated that immunosuppression was alleviated after chemotherapy or operation compared with patients before receiving treatment, indicating the great significance of immune therapy regulating immunosuppression combined with conventional treatment for oral cancer.

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Disclosure of Conflict of Interest

None.

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