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Culture supernatants of oral cancer cells induce impaired IFN-α production of pDCs partly through the down-regulation of TLR-9 expression

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ABSTRACT

Objectives: The aim of the present study was to investigate whether tumor-derived supernatants down-regulate the immune function of plasmacytoid dendritic cells (pDCs) in oral cancer and the potential molecular mechanisms of this effect.

Materials and Methods: Immunohistochemistry (IHC) and flow cytometry were used to detect tumor-infiltrating and peripheral blood pDCs. MTS and flow cytometry were employed to evaluate the immune response of $CD4^+$ T cells. Real-time PCR and ELISA assays were used to identify TLR-7 and TLR-9 expression, IFN-α production and tumor-secreted soluble cytokines.

Results: The proportion of pDCs (0.121% ± 0.043%) was significantly higher in Oral squamous cell carcinoma (OSCC) samples than in normal tissue (0.023% ± 0.016%) ($P = 0.021$). *TLR9* mRNA was significantly lower in tumor-infiltrating pDCs and positively correlated to low IFN-α production ($r = 0.956$; $P < 0.01$). The supernatant of oral cancer cells negatively regulated *TLR9* mRNA expression and the subsequent IFN-α production of pDCs, which inhibited the immune response of $CD4^+$ T cells. The neutralizing antibodies blocking assay showed that the specific inhibitory effect of pDC functionality was associated with the soluble fraction of the oral cancer environment, which is mainly mediated by IL-10 and TGF-β cooperation.

Conclusion: Tumor-derived supernatants may impair the function of tumor-infiltrating pDCs, which subsequently decreases the immune response of $CD4^+$ T cells in human oral cancer through TGF-β- and IL-10- dependent mechanisms. Careful manipulation of these impaired pDCs may help develop an important alternative immunotherapy for the treatment of oral cancer.

1. Introduction

Oral squamous cell carcinoma (OSCC) is an aggressive epithelial oral malignancy that is currently the sixth most common neoplasm in the world (Greenlee et al., 2001). Despite new therapeutic modalities being introduced for the treatment of oral cancer over the past two decades, the overall 5-year survival of OSCC patients has only improved slightly, especially in the advanced stages of the disease (Ahmedin Jemal et al., 2006). This highlights the urgent need to identify new approaches for the prevention and treatment of OSCC. Recently, much attention has been directed toward tumor immunology research. Accumulating evidence from clinical and experimental studies has shown that immune cell infiltration can significantly affect the course of malignant transformation and progression, including that of plasmacytoid dendritic cells (pDCs) (Colonna et al., 2004; Kim et al., 2007).

pDCs are one of two principal subsets of human dendritic cells (DCs). As the most important participant in the host response to viral infection, pDCs can also promote antitumor immunity through several different mechanisms, such as secretion of interferon-alpha (IFN-α) with antiviral activities, antigen capture and maturation into potent antigen presenting cells (APCs), and generation of antiviral T lymphocytes in association with LN DCs (Chengwen Liu et al., 2008; Lou et al.,

Abbreviations: pDCs, plasmacytoid dendritic cells; TLR-9, toll-like receptor 9; OSCC, oral squamous cell carcinoma; DC, dendritic cells; IFN-α, interferon-alpha; APCs, antigen presenting cells; LN, lymph node; CpG-ODN, CpG-Oligodeoxyribonucleotides; HIOEC, human immortalized oral epithelial cell lines; FCS, fetal calf serum; TGF-β, transforming growth factor beta; OCDS, oral cancer-derived supernatant

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2007; Pinto et al., 2012). However, an increasing number of studies have reported that impaired fuction of tumor-infiltrating pDCs are associated with tumor progression and poor outcome. In breast tumors, infiltration of pDCs in the primary lesion was found to be correlated with adverse outcomes (Faget et al., 2013). In ovarian cancer, the presence of pDCs not only is associated with a poor clinical outcome but also acts as a predictor of early relapse (Labidi-Galy et al., 2012). We have also demonstrated in a previous study that an increased frequency of tumor-infiltrating pDCs in primary OSCC tissues predicted a poor prognosis (Han et al., 2017). Unfortunately, the molecular mechanisms responsible for this phenomenon remain mostly unknown.

It is believed that circulating pDCs recruited to the tumor microenvironment often display a non-activated state and are associated with low IFN-α secretion. Given that IFN-α secretion of pDCs is mostly through a TLR-9-dependent pathway (Kawai et al., 2004), we wonder if there is a possible relationship between impaired function of tumor-infiltrating pDCs and TLR-9 expression in oral cancer.

To gain a more comprehensive understanding of the role that impaired function of tumor-infiltrating pDCs play in primary OSCC, this study aims to first investigate the *TLR9* mRNA expression of pDCs in cancer tissues and its relationship with IFN-α production. Second, this study determines if the supernatant of tumor cell cultures contributes to the non-activated state of tumor-infiltrating pDCs and the subsequent decrease in the T cell immune response.

2. Materials and methods

2.1. Reagents and cell culture

Unmethylated phosphorothioate modified, human specific CpG-Oligodeoxyribonucleotides (CpG-ODN) 2006 (5-TCGTCGTTTTGTCGTTTTGTCGTT-3) was purchased from InvivoGen (San Diego, CA) and dissolved in endotoxin-free, sterile, distilled, deionized H_2O according to the manufacturer's suggestions, using the indicated concentrations. Human immortalized oral epithelial cell lines (HIOEC) and a cancerous cell line (HB) in the cellular carcinogenesis model of OSCC were used as previously described (Sdek et al., 2006; Zhong et al., 2008). Normal oral epithelial cells were obtained from surgical resections of non-cancer patients and routinely cultured. All cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Human tissue samples and patient characteristics

Tumor tissue specimens from the oral cavity were obtained during standard surgical procedures. Specimens of normal oral mucosa were obtained from patients with sleep-related breathing disorders that were undergoing

uvulopalatopharyngoplasty. Informed consent was obtained for experimentation with patients. The privacy rights of patients must always be observed.Tissue specimens were transported in sterile saline and processed immediately after excision. The characteristics of patients with oral cancer are provided in Table 1. Ethics approval was obtained from our hospital's ethical committee.

2.3. Preparation of single-cell suspensions and OSCC supernatants

Surgical tissue samples of 10 paired human primary OSCC tissues and adjacent normal epithelia were washed several times and carefully minced into small pieces in sterile, serum-free RPMI medium (RPMI1640 supplemented with 100 units/ml penicillin, 1 mM glutamine, and 100 units/ml streptomycin). Tissue was digested with collagenase type VIII (1.5 mg/ml; Sigma) and DNase type I (1.0 μg/ml) for 120 min at 37 °C with gentle agitation. The resulting cell suspensions were washed in PBS, resuspended in PBS containing trypsin/EDTA, and filtered through a 40-μm nylon cell strainer (Falcon; Becton Dickinson Labware) into cold RPMI medium containing 10% fetal calf serum (FCS). Single-cell suspensions from 10 human primary OSCC tissues were incubated at a final concentration of 1.5×10^6 cells/mL in complete RPMI in petri dishes. Culture supernatants were collected after 48 h, filtrated on 0.2 –μm and stored at −80 °C until needed for use.

Table 1
Clinical data and frequency of pDCs in patients with oral cancer.

Patient	Age(yr)	Gender	TNM	Primary site	PDC in %	
					Tumor tissue	Adjacent normal
1	45	F	$T_2N_0M_0$	Buccal	0.15	0.01
2	38	M	$T_2N_1M_0$	Tongue	0.12	0.03
3	62	M	$T_2N_0M_0$	Soft palate	0.08	0.01
4	66	F	$T_3N_0M_0$	Upper gingival	0.16	0.02
5	48	M	$T_3N_2M_0$	Tongue	0.15	0.03
6	72	M	$T_2N_0M_0$	Mouth floor	0.03	0.02
7	69	F	$T_4N_0M_0$	Hard palate	0.09	0.01
8	57	M	$T_3N_0M_0$	Buccal	0.16	0.03
9	63	F	$T_3N_1M_0$	Low gingival	0.12	0.06
10	58	M	$T_2N_0M_0$	Tongue	0.15	0.01

2.4. Isolation of pDCs from PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coats of 10 OSCC patients and 10 normal volunteer human donors using density centrifugation with Lymphoprep (Nycomed AS, Oslo, Norway). The blood donors were healthy and tested as being negative for allergies, and their ages ranged from 18 to 65 years old. Additional exclusion criteria for the blood donors included manifested infections within the previous 4 weeks, fever and medication of any kind including HIV, hepatitis B virus, and hepatitis C virus. pDCs were isolated using magnetically activated cell sorting with the BDCA-4 dendritic cell isolation kit from Miltenyi Biotec (Bergisch-Gladbach, Germany) (Evelyn Hartmann et al., 2003). pDCs were labeled with anti-BDCA-4 antibody coupled to colloidal paramagnetic microbeads and passed through a magnetic separation column, twice (LS and RS columns; Miltenyi Biotec). The purity of the isolated pDCs (lineage-negative, BDCA-2-positive, and CD123-positive cells) was>90%. Viability was> 95% as determined using the trypan blue exclusion test. pDCs were cultured at a final concentration of 5×10^5 cells/ml in 96-well plates.

2.5. Immunohistochemical staining

Immunohistochemistry was performed on 4-μm-thick paraffin sections. pDCs were detected using a mouse monoclonal anti-CD123 antibody (clone 9F5, dilution 1:400; Pharmingen, CA, USA). For CD123 staining, the streptavidin conjugate was amplified via a 3-minute incubation with tyramide (Perkin-Elmer, Wellesley, MA) followed by a second incubation with streptavidinperoxidase prior to visualization. Slides were counterstained in hematoxylin (Dako, CA), washed and dehydrated, and then permanently mounted with Clarion (Sigma-Aldrich Corp, St. Louis, MO). As a negative control, the sections were treated with PBS instead of the primary antibody. As a positive control, human tonsils were used for the detection of CD123.

2.6. Flow cytometry

The procedure was performed as previously described (Evelyn Hartmann et al., 2003). Cells were incubated at 4 °C for 30 min in PBS with 0.1% BSA and 0.01% NaN_3, in the presence of appropriate dilutions of FITC- or PE-labeled isotype controls and mouse mAbs to BDCA-2 and BDCA-4 (Miltenyi Biotec, Bergisch-Gladbach, Germany), and CD123 (Becton Dickinson, Heidelberg, Germany). The cells were then