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Forkhead box-P3$^+$ regulatory T cells and toll-like receptor 2 co-expression in oral squamous cell carcinoma

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ABSTRACT

Background: The function of forkhead box-P3 (FoxP3) regulatory T cells (Treg) and toll-like receptor (TLR)2 protein in the oral cancer microenvironment is not fully understood, but evidence from other malignancies suggests it is likely they are involved with tumour development and progression. The aim of this study was to investigate the distribution of FoxP3$^+$cells, TLR2$^+$ cells and double-labelled FoxP3$^+$TLR2$^+$ immune cells in oral squamous cell carcinoma (OSCC), using immunohistochemistry (IHC) and immunofluorescence (IF).

Methods: 25 archival cases of OSCC were immunostained with anti-FoxP3 and anti-TLR2 antibodies. Inflamed hyperplastic oral mucosal tissues were used as controls. The proportion of single-labelled, double-labelled and negative cells was determined.

Results: A higher frequency of double-labelled FoxP3$^+$TLR2$^+$ Tregs was observed within the immune cells of OSCC compared to inflamed controls using IHC ($p < 0.05$). Cell-to-cell contact between single-stained TLR2$^+$ cells and FoxP3$^+$ cells was noted. Double IF studies validated demonstration of co-expression of FoxP3$^+$/TLR2$^+$ immune cells in OSCC.

Conclusion: The presence of FoxP3$^+$TLR2$^+$ cells within the OSCC microenvironment may represent a dendritic cell-dependent pathway capable of inhibiting Treg suppressive activity, potentially enhancing the anti-tumour response. Modulation of TLR2-Treg interactions should be further explored to determine if they have a role in the therapeutic management of OSCC.

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1. Introduction

Oral and oropharyngeal cancer is the sixth most common cancer in the world (Warnakulasuriya 2009). Oral squamous cell carcinoma (OSCC) is the most common form of oral and oropharyngeal cancer, with two-thirds of cases occurring in developing countries (Parkin et al., 2005).

The stroma of OSCC is almost always infiltrated by variable numbers of mononuclear immune cells, mostly lymphocytes, known as tumour infiltrating lymphocytes (TILs) (Rollins, 2006; Uppaluri et al., 2008). This phenomenon was initially thought to be evidence of an anti-tumour response (Yu and Fu, 2006), however functional analysis showed that the infiltrate was richly populated with CD4$^+$ cells, with few NK cells (Snyderman et al., 1991). This population of CD4$^+$ cells was later identified as forkhead box-P3 (FoxP3)$^+$ regulatory T cells (Treg), which in normal circumstances, are important in maintaining immune homeostasis and preventing autoimmune diseases (Schwarz et al., 2008). Studies have shown expansion of this CD4$^+$FoxP3$^+$ Treg population in the tumour microenvironment (TME) of a range of cancers, including in OSCC, possibly induced by the local environment which is rich in interleukin (IL)10 and tumour growth factor (TGF)β (Bergmann et al., 2008; Watanabe et al., 2010; Lim et al., 2014). Malignant oral keratinocytes secrete TGFβ (Gasparoto et al., 2010), strongly indicating that OSCC cells are able to modulate the function of Tregs to their advantage.

For tumour antigens to induce an immunogenic effect, they have to be presented to antigen presenting cells (APCs). The antigen presentation mechanism is commonly chaperoned by a group of molecules belonging to the heat shock protein (HSP) family that interacts with transmembrane proteins such as the toll-like receptor (TLR) family. TLR2 recognises a wide variety of pathogen-associated molecular patterns (PAMPs) from exogenous pathogens, as well as endogenous damage-associated molecular patterns (DAMPs) including HSPs and high-mobility group box 1 protein (Sato et al., 2009). TLR2 expression on immune cells protected mice from developing colorectal cancer (Lowe et al., 2010) and mature dendritic cells (DC) injected with bacterial DNA were able to overcome immune tolerance in colon carcinoma (Heckelsmiller

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et al., 2002) using the TLR pathway. More keratinocytes in OSCC expressed TLR2 than keratinocytes in control epithelium (Ng et al., 2011). Whether or not this finding denotes the stimulation of an anti-tumour response or survival enhancement in OSCC is yet to be proven.

The expansion of the Treg population in OSCC is increasingly thought to be responsible for suppression of an anti-tumour response, which, in turn, facilitates cancer progression and leads to a poorer prognosis. TLRs, particularly TLR2, play a role in Treg expansion and facilitate their suppressive capacity (Sutmuller et al., 2006). On the other hand, TLR2 can induce loss of Treg function in a murine model with a shift to a pro-inflammatory (Th1) cytokine profile (Sutmuller et al., 2006). More recently it has been shown that TLR2 agonists induce tumour regression by modulating Treg and cytotoxic T cell (Tc) function (Zhang et al., 2011).

The ability of TLRs to modulate Treg and/or the TME has initiated interest in their potential role in cancer immunotherapy. It is not known whether activation of TLR2 receptors on Treg facilitates reversal of suppression of the anti-tumour response.

There are no reports describing the association of Tregs and TLR2 in the OSCC TME. The aim of this study was to investigate the presence and distribution of Tregs and TLR2$^+$ cells in OSCC, as well as to correlate their distribution with the depth of tumour invasion.

2. Methodology

2.1. Sample selection

The experiment was approved by University of Otago Institutional Ethics Committee (09/111). The database of the Medlab Dental Oral Pathology Diagnostic Service, Faculty of Dentistry, University of Otago was searched for specimens reported as primary OSCC. The original haematoxylin and eosin (H&E) slides were obtained to verify the diagnosis and confirm the suitability of the specimens using the following selection criteria: i) each section must contain a minimum of three regions with more than 50 tumour infiltrating lymphocytes (TILs) associated with the invasive tumour front and ii) the histological material was accessioned during the five year interval January 2006–December 2010.

Twenty-five formalin-fixed paraffin-embedded (FFPE) specimens were selected from the archives using these inclusion criteria. For controls, 12 samples of inflammatory hyperplastic tissue were selected using the same criteria. It was considered important that inflamed tissue rather than normal tissue was used for controls to determine whether there was a difference between inflammation in the TME by comparison with non-specific inflammation. Human tonsil tissue was used as a positive control for immunohistochemistry (IHC) and immunofluorescence (IF).

2.2. Sectioning of tissues

Sections from all specimens were cut at 4 μm thickness. For IF, six samples of primary OSCC from the same pool were randomly selected. An additional serial 4 μm section was cut for each sample to be used as a negative control. All sections were embedded on adhesive positively charged Histobond® microscope slides (Marienfield Laboratory Glassware, Germany).

2.3. Immunohistochemistry

The primary antibodies and isotype-matched controls used for IHC, together with optimised working conditions, are presented in Table 1. The IHC staining for all sections was performed using the BenchMark XT (Ventana® Medical Systems Inc., USA) automated slide stainer. Immunohistochemical double-labelling with anti-FoxP3 (320002, Clone: 150D isotype IgG, Biolegend, USA) and anti-TLR2 (309702, Clone TL2.1 isotype IgG, Biolegend, USA) was performed by manual titration of the anti-FoxP3 antibody and detected with ultraView Universal DAB Detection kit (Ventana® Medical Systems Inc., USA). This was followed by a 4 min cell conditioning denaturation cycle at 70C. The anti-TLR2 primary antibody was applied and detected with Enhanced Alkaline Phosphatase Red Detection kit (Ventana® Medical Systems Inc., USA). For negative control sections, anti-mouse/anti-rabbit IgG was used as isotype control replacing both primary antibodies at the same concentration.

2.4. Immunofluorescence

Double-labelling IF was performed using the same anti-FoxP3 and anti-TLR2 antibodies to validate the IHC results. To prevent cross-reaction(s) between individual detection methods, antibodies from different species were used (mouse-rabbit monoclonal combination). The optimised working conditions and secondary antibodies for IF are presented in Table 1.

2.5. Data analysis

2.5.1. Immunohistochemistry analysis

The IHC staining pattern, intensity and distribution between the test and control samples was assessed using light microscopy with various magnifications from 40× to 100×. Immunopositivity for FoxP3 was defined as distinct and clear brownish nuclear staining and for TLR2 was defined as red cell membrane and/or cytoplasmic staining (Ng et al., 2011). A double-labelled cell was defined as a cell with distinct brown nuclear staining with a red ring around the cell membrane. The staining pattern and expression of both antigens in the OSCC sections were compared with the hyperplastic/inflammatory tissue control group. For quantitative analysis, tissue sections of all samples were scanned at 40× magnification to identify areas with the most inflammatory cells (more than 50 TILs). Using an ocular grid three representative TIL-rich regions scattered along the invasive tumour front were selected from each stained SCC section at 400× magnification. Similarly, three representative inflamed regions scattered along the superficial lamina propria were selected for the inflamed/hyperplastic control tissues. A threshold of 1000 cells was counted for each section (0.12 mm^2 area). The results were expressed as the percentage of positive mononuclear cells per total number of immune cells present in the area.

Tumour depth was measured for every section according to defined criteria (Darragh et al., 2012) where tumour depth of less than 3 mm was defined as superficially invasive OSCC and tumour depth greater than 3 mm was considered a deeply invasive OSCC.

2.5.2. Immunofluorescence analysis

All IF sections were viewed (×40 magnification) under a fluorescence microscope. The area of interest was observed and captured under different wavelengths. Images were superimposed and screened to disclose positive cells (fluorescing cell membrane rings and/or nuclear and/or cytoplasmic fluorescence) using image J (Image Processing and Analysis in Java, ImageJ version 1.49, National Institute of Health, USA). A cell was identified as co-expressing two targeted protein markers when the superimposed and screened images showed both green (Alexa Fluor 488®) and red fluorescence (Alexa Fluor 595®) on the cell membrane and/or cytoplasm, depending on the type of fluorochrome used (Table 2).

2.5.3. Statistical analysis

Data entry and descriptive analysis was performed using SPSS Version 17.0 (SPSS Inc., Chicago, IL, USA). This software was used to analyse any difference in expression between the test group and

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