Analysis of local immunity in squamous cell carcinoma of the tongue and lower lip

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A R T I C L E  I N F O
Article history:
Received 1 June 2009
and in revised form 5 November 2009
Available online 26 November 2009

Keywords:
Squamous cell carcinoma
Tongue
Lower lip
Immunohistochemistry
Local immune response

A B S T R A C T
Antitumor immunity plays an important role in the development of and protection against malignancy. In general, patients with cancer are known to be immunologically compromised. The objective of this study was to evaluate local immunity in squamous cell carcinomas (SCCs) of the tongue and lower lip by immunohistochemistry, using anti-CD3, -CD4, -CD8, -CD25 and -ζ antibodies. Immunohistochemistry at the invasive front was compared considering anatomical tumor location and metastasis. The CD4/CD8 ratio was calculated for each case and associated with the variables. CD3+, CD4+, CD8+ and CD25+ cell counts were higher in SCCs of the lower lip and anti-ζ immunostaining was more evident in non-metastatic cases. CD8+ and CD25+ cell counts were also significantly correlated with tumor location (p = 0.004 and p = 0.004, respectively), with the observation of a larger number of these cells in SCCs of the lower lip. The CD4/CD8 ratio showed no significant association with metastasis or anatomical location. In conclusion, the clinical behavior of the oral SCC cases studied might be partially related to the immunohistochemical profile of the inflammatory infiltrate present at the invasive front.

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Introduction

Oral squamous cell carcinoma (OSCC), a common head and neck cancer, is characterized by an aggressive growth pattern, a high degree of local invasiveness, and cervical lymph node metastasis. Despite improved therapeutic modalities, the survival of patients with oral cancer has remained unchanged over the last three decades. The clinical practice. In many cases, these factors are inadequate and are unable to discriminate between tumors in the same clinical stage that may have distinct clinical outcomes and respond differently to the same treatment. Thus, there is a need for biological prognostic factors that better reflect the biological diversity of oral cancers and more accurately predict clinical outcomes and responses to particular types of adjuvant therapy (Shah et al., 2009).

Antitumor immunity has been suggested to play an important role in the development of and protection against malignancy. T cells serve as effector cells that are responsible for specific long-term immunity against the tumor. CD8+ cytotoxic T lymphocytes (CTLs) recognize and kill tumor cells that express peptides presented by MHC class I molecules, whereas CD4+ T helper cells are activated by the recognition of peptides presented by MHC class II molecules. The functions and interactions of these effector cells have been extensively studied and a better understanding of these mechanisms may contribute to the development of an effective immunotherapy (Chimatsu et al., 2008).

Tumor-infiltrating lymphocytes (TILs) are considered to be a manifestation of the host immune response against cancer cells (Cho et al., 2003). TILs have been studied in various types of carcinomas and are considered to be a prognostic factor. According to Naito et al. (1998), the inclusion of CD8+ cells in the TIL population seems to be important for the anticancer immune response.

Few studies have investigated the role of the host local immune response during the course of Oral SCC. Thus, the present study evaluating local immunity in OSCC may contribute to the development of new molecular markers that help identify the biological and immunological status of patients, predict disease progression, and select the appropriate treatment for individual patients. Knowledge of the events involved in the host defense is essential to improve existing treatments and to develop new therapies. We therefore investigated the local immune response in SCCs of the tongue and lower lip and compared this response between the two sites as well as between metastatic and non-metastatic cases. Local immunity was analyzed using anti-CD3 antibodies that identify T lymphocyte subpopulations; anti-CD4 antibodies specific for T helper lymphocytes; anti-CD8 antibodies that specifically identify CTLs; anti-CD25 antibodies specific for the alpha chain of the interleukin-2 receptor, and anti-ζ antibodies that identify a component of the T cell receptor (TCR) of T
lymphocytes. The model of this study is relevant and may provide relevant information contributing to the use of immunotherapy for Oral SCC.

Materials and methods

Patients

The study was approved by the Ethics Committee of the Federal University of Rio Grande do Norte (Natal-RN, Brazil). Thirty cases of SCC of the tongue and 20 cases of SCC of the lower lip, embedded in paraffin and obtained from surgical biopsies of patients submitted to tumor resection without previous chemotherapy and/or radiotherapy, were selected for this study.

Patients who had been followed up clinically for at least 5 years were preferentially selected to determine the presence or absence of cervical lymph node metastases at the time of diagnosis. Patients who developed cervical or distant lymph node metastases during treatment or follow-up were excluded. The criterion for inclusion in this study was the presence or absence of cervical metastases at the time of diagnosis and before the beginning of treatment. Metastases were demonstrated by imaging exams such as computed tomography or magnetic resonance. Additional inclusion criteria were surgical treatment performed according to standard procedures and consisting of resection of the primary tumor, complete clinicopathological data, and availability of sufficient paraffin-embedded tumor material.

Sections (5 μm) were cut from paraffin-embedded tumor specimens and stained with hematoxylin and eosin for the grading of histological malignancy as proposed by Bryne (1998). The following four parameters of the tumor invasion front were analyzed: degree of keratinization, cellular pleomorphism, invasion pattern, and intensity of the inflammatory infiltrate. A score of 0 to 4 was attributed to each parameter and cases with a total score of 8 or lower were classified as low-grade malignancy and those scoring higher than 8 were classified as high-grade malignancy.

Immunohistochemistry

The paraffin-embedded material was cut into 3-μm thick sections and the specimens were mounted on previously cleaned glass slides with organosilane (3-aminopropyltriethoxy-silane; Sigma Chemical Co., St. Louis, MO, USA) as adhesive and submitted to immunohistochemistry using the streptavidin–biotin method as follows: deparaffinization, hydration in a decreasing ethanol series, removal of formalin pigment with 10% ammonium hydroxide in 95° ethanol, and the streptavidin steps. Next, the slides were incubated with the secondary antibody chemistry using the streptavidin with organosilane (3-aminopropyltriethoxy-silane; Sigma Chemical and the specimens were mounted on previously cleaned glass slides. The reaction was developed with diaminobenzidine. The slides were counterstained with Mayer’s hematoxylin and coverslipped with Permount.

For determination of the effectiveness of the technique, non-metastatic cervical lymph node specimens obtained by prophylactic cervical node resection were used as positive control. Specimens in which the primary antibody was omitted served as negative control.

Immunohistochemical and statistical analysis

A single examiner blinded to the patient diagnosis determined the number of immunostained cells twice at different times. Cells were counted under an Olympus light microscope in 10 random histological fields at 400× magnification as described by Reichert et al. (2002). The median value of 10 histological fields was established for each case using the Windows Excel program. As suggested by Reichert et al. (2002), the intensity of anti-ζ antibody staining was classified as weak or strong in the case of mild or intense positivity, respectively.

Statistical analysis was performed to compare anti-CD3, anti-CD4, anti-CD8, anti-CD25 and anti-ζ antibody immunostaining according to anatomical location (tongue and lower lip) and the presence or absence of metastases. The Student’s t-test and Mann–Whitney test were used according to the nature of the data. The CD4/CD8 ratio was calculated for each case by dividing the number of CD4+ cells by the number of CD8+ cells. The Mann–Whitney test was used to determine the association between this ratio and the independent variables mentioned above. Fisher’s exact test was used to analyze anti-ζ staining classified as strong or weak. The level of significance was set at 5% for all tests.

Results

Thirty patients with SCC of the tongue (19 males, mean age: 58.79 years, and 11 females, mean age: 63.63 years) and 20 patients with SCC of the lower lip (15 males, mean age: 65 years, and 5 females, mean age: 77 years) were studied.

Metastases were observed in 12 (40%) of the 30 patients with tongue SCC, but in only one (5%) of the 20 cases of lower lip SCC. The presence of metastases was significantly associated with SCC of the tongue (p = 0.008). Thirteen (43.33%) tongue SCC cases and 13 (65%) lower lip SCC cases were classified as low-grade malignancy, whereas 17 (56.66%) tongue SCC cases and 7 (35%) lower lip SCC cases were classified as high-grade malignancy.

With respect to the immunohistochemistry results, all cases were positive for the antibodies studied, but presented differences in the number of immunostained cells. A significant difference (p = 0.004) in anti-CD8 and anti-CD25 immunostaining was observed between the two anatomical locations of SCC (Table 2). CD8+ and CD25+ cell counts were higher in SCCs of the lower lip when compared to tongue SCCs (Figs. 1, 2, 3 and 4).

Analysis by the Student’s t-test showed no significant difference in the presence of anti-CD3, anti-CD4 or anti-ζ staining between anatomical locations (p>0.05) (Table 3), but slightly higher median CD3+ and CD4+ cell counts were observed in lower lip SCCs when compared to tongue SCCs. CD4+ cells were less frequent than CD8+ cells in all specimens (Fig. 5).

Table 1

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Dilution</th>
<th>Laboratory</th>
<th>Antigen retrieval</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>1:10</td>
<td>Dako, CA, USA</td>
<td>Citrate, pH 6.0, 30 min, Steamer</td>
<td>60 min</td>
</tr>
<tr>
<td>CD4</td>
<td>1:20</td>
<td>Newcasta Laboratories LTD, UK</td>
<td>Citrate, pH 6.0, 30 min, Steamer</td>
<td>120 min</td>
</tr>
<tr>
<td>CD8</td>
<td>1:20</td>
<td>Dako</td>
<td>Citrate, pH 6.0, 30 min, Steamer</td>
<td>60 min</td>
</tr>
<tr>
<td>CD25</td>
<td>1:40</td>
<td>Dako</td>
<td>Citrate, pH 6.0, 30 min, Steamer</td>
<td>60 min</td>
</tr>
<tr>
<td>ζ</td>
<td>1:400</td>
<td>Santa Cruz Biotechnology, CA, USA</td>
<td>No treatment</td>
<td>120 min</td>
</tr>
</tbody>
</table>

Table 2

Parameters used for the calculation of the Mann-Whitney (U) test for the evaluation of the immunopositive cells for anti-CD3 and anti-CD25 antibodies according to the anatomic location.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Location</th>
<th>n</th>
<th>Median</th>
<th>Q25–Q75</th>
<th>Mean of the ranks</th>
<th>p</th>
</tr>
</thead>
<tbody>
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<td>Anti-CD8</td>
<td>Tongue</td>
<td>30</td>
<td>12.75</td>
<td>7.12–18.12</td>
<td>20.63</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Lip</td>
<td>20</td>
<td>21.25</td>
<td>13.75–25.75</td>
<td>32.80</td>
<td></td>
</tr>
<tr>
<td>Anti-CD25</td>
<td>Tongue</td>
<td>30</td>
<td>8.00</td>
<td>5.50–15.87</td>
<td>20.72</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Lip</td>
<td>20</td>
<td>17.75</td>
<td>13.5–25.12</td>
<td>32.68</td>
<td></td>
</tr>
</tbody>
</table>
No significant difference in immunostaining was observed between metastatic and non-metastatic cases for any of the antibodies studied ($p > 0.05$) (Tables 4 and 5), but, CD4+, CD3+, CD8+, CD25+ and $\zeta$ cell counts were slightly higher even in node-negative patients. No significant difference in the CD4/CD8 ratio was observed between node-negative and node-positive patients ($p = 0.371$). This ratio was 1:2.27 for metastatic cases and 1:3.70 for non-metastatic cases. There was no significant association between the CD4/CD8 ratio and anatomical tumor location, with a ratio of 1:3.44 for tongue SCCs and 1:3.70 for lower lip SCCs ($p = 0.332$).

Analysis of strong and weak anti-$\zeta$ staining intensity showed no significant association with metastasis or anatomical location ($p > 0.05$) (Tables 3 and 5).

**Discussion**

In the present study, we showed that large numbers of TILs at the invasive front are related to the clinical behavior of Oral SCCs. According to O'Regan et al. (2006), SCCs account for approximately 90% of all oral cancers. The most important factor affecting the outcome of this tumor is the clinical stage of the disease at first diagnosis. However, the presence of clinically positive lymph nodes is the single most important predictor of survival. Once regional metastases have occurred, the 5-year survival rate of patients with OSCC decreases by one half that of patients with early-stage disease (Bell et al., 2007).

Although SCCs with identical morphological characteristics correspond to 90% of all oral cancers, these tumors may show a wide variation in biological behavior. According to Bryne (1998), these tumors consist of heterogeneous cell populations, with more aggressive cells being present in deeper areas when compared to central or superficial areas of the tumor. Based on this fact, Bryne (1998) proposed a histological malignancy grading system of the tumor invasive front. This system evaluates tumor factors such as the degree of keratinization and cellular pleomorphism, and host-related factors such as the inflammatory infiltrate and the pattern of neoplastic invasion. In the present study, we used the histological malignancy grading system proposed by Bryne (1998) since we agree with the author that the tumor–host relationship is important.

We evaluated the quality of the inflammatory infiltrate at the invasive front in a series of SCCs of the tongue and lower lip. According to Gabriel et al. (1999), quantitative and qualitative analysis of TILs is important to determine possible changes in the invasive front of the tumor.

According to Kacani et al. (2003), CD8+ CTLs and CD4+ helper T cells are present in OSCCs. The latter can be type h1 or h2, which...
express IFN-γ and IL-4, respectively. Both lymphocyte populations were detected in the present study using anti-CD3, anti-CD4 and anti-CD8 monoclonal antibodies.

CD8+ T cells can lyse tumor cells directly and destroy large tumor masses (Cho et al., 2003; Russ et al., 2005). In the present series, most lymphocytes were CTLs, in agreement with Wakabayashi et al. (2003). According to these authors, most non-hematopoietic tumors express more MHC I than MHC II molecules, which are restrictive elements of CTL and CD4+ T helper cell recognition, respectively. This fact also explains the smaller number of CD4+ cells found here.

Although no significant association was observed between CD8+ T cell count and metastasis, the number of CD8+ T cells was significantly associated with tumor location, a finding that may partially explain the higher aggressiveness of SCCs of the tongue. The reduced number of CTLs at this site may facilitate the dissemination of neoplastic cells and the development of metastases. According to these authors, most non-hematopoietic tumors express CD4+CD25+FOFP3+ and have immunosuppressive properties due to their capacity to inhibit T cell activation by the release of immunosuppressive cytokines.

In the present study, anti-CD25 antibody expression was found to be significantly associated with the anatomical location of the tumor (p = 0.004). This antibody was more frequently detected in carcinomas of the lower lip and was slightly more prevalent in non-metastatic cases. Our results regarding anti-CD25 are in accordance with the immune response to T cell activation since, once activated, these cells express IL-2 receptors that can be identified by immunohistochemistry using the anti-CD25 antibody (Sheu et al., 1999). However, according to Satoh et al. (2005), CD25 is also expressed on the surface of regulatory T cells (Treg or suppressor), which can be classified as CD4+CD25+FOFP3+ and have immunosuppressive properties due to their capacity to inhibit T cell activation by the release of immunosuppressive cytokines.

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Some studies suggest that alterations in the expression of TCR-associated transduction molecules, such as components of protein CD3 and protein ζ (zeta), are responsible for immune system deficiencies in various types of cancer (Reichert et al., 2002; Whiteside, 2004). In the present study, ζ+ cell counts were not significantly associated with anatomical location or metastasis. Nevertheless, a slightly larger number of these cells were observed in non-metastatic cases. With respect to the staining intensity for this domain, the percentage of

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Location</th>
<th>n</th>
<th>æ ± dp (95%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD3</td>
<td>Tongue</td>
<td>30</td>
<td>21.91-11.12</td>
<td>0.234</td>
</tr>
<tr>
<td></td>
<td>Lip</td>
<td>20</td>
<td>26.12-13.45</td>
<td>0.324</td>
</tr>
<tr>
<td>Anti-CD4</td>
<td>Tongue</td>
<td>30</td>
<td>4.26-2.70</td>
<td>0.487</td>
</tr>
<tr>
<td></td>
<td>Lip</td>
<td>20</td>
<td>4.77-2.37</td>
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</tr>
<tr>
<td>Anti-ζ</td>
<td>Tongue</td>
<td>30</td>
<td>20.25-10.89</td>
<td>0.795</td>
</tr>
<tr>
<td></td>
<td>Lip</td>
<td>20</td>
<td>19.57-7.40</td>
<td>0.234</td>
</tr>
</tbody>
</table>

CD4+ helper T cells were observed at this site, as well as reduced anti-CD3 antibody staining, a marker specific for both lymphocyte subpopulations. These findings suggest that the smaller number of CD8+ cells found in these situations may also be the result of an insufficient number of CD4+ helper T cells, which are necessary to recruit and maintain CTLs.

Calculation of the CD4/CD8 ratio for each case showed no significant association of this parameter with metastasis or anatomical location (p > 0.05). These findings disagree with Sheu et al (1999), who suggested alterations in the CD4/CD8 ratio to be a prognostic factor of cervical carcinoma progression and to be indicative of a poor antitumor response. This discordance might be due to differences in the sample size and in the methods used in the two studies.

The release of cytokines such as IL-2 is known to be necessary for the proliferation of lymphocytes and their differentiation into effector cells (Kacani et al., 2003). Once lymphocytes become active, they start to express IL-2 receptors that can be identified by immunohistochemistry using the anti-CD25 antibody (Sheu et al., 1999). However, according to Satoh et al. (2005), CD25 is also expressed on the surface of regulatory T cells (Treg or suppressor), which can be classified as CD4+CD25+FOFP3+ and have immunosuppressive properties due to their capacity to inhibit T cell activation by the release of immunosuppressive cytokines.

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<table>
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<th>Antibody</th>
<th>Metastasis</th>
<th>n</th>
<th>æ ± dp (95%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD8</td>
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</tr>
<tr>
<td></td>
<td>Absent</td>
<td>37</td>
<td>18.50-24.50</td>
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</table>

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Metastasis</th>
<th>n</th>
<th>æ ± dp (95%)</th>
<th>p</th>
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<tbody>
<tr>
<td>Anti-CD4</td>
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<td>14.43-8.56</td>
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<tr>
<td>Anti-ζ</td>
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<td>0.757</td>
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<tr>
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<td>Absent</td>
<td>37</td>
<td>20.28-7.21</td>
<td>0.234</td>
</tr>
</tbody>
</table>

Fig. 5. A few amount CD4+ cells CD4+ cells in tongue SCCs case (SABC, 200×).
cases showing weak or strong expression was similar for SCCs with and without metastasis.

In conclusion, the present results indicate that the inflammatory infiltrate at the invasive front of SCCs of the tongue and lower lip may influence tumor aggressiveness. Further studies investigating the antitumor response of patients with OSCCs may contribute to improve cancer therapies. The profile of cytokines secreted by these patients and the integrity of TCR components of lymphocytes should be investigated, since most studies show lymphocyte count deficiencies in aggressive SCCs. Thus, the results of the present study suggest that reinforcement of the immune system may improve the response of these patients to treatment.

References