Differing Phenotypes between Intraepithelial and Stromal Lymphocytes in Early-Stage Tongue Cancer

Fuminori Katou,1 Haruo Ohtani,2 Yoshiko Watanabe,1 Takashi Nakayama,3 Osamu Yoshie,3 and Kenji Hashimoto4

1Department of Oral and Maxillofacial Surgery, Hamamatsu University School of Medicine, Hamamatsu, Japan; 2Department of Pathology, Mito Medical Center, National Hospital Organization, Ibarkaki, Japan; and 3Department of Microbiology, Kinki University School of Medicine, Osaka, Japan

Abstract

The significance of tumor-infiltrating lymphocytes (TIL) has attracted much attention in relation to the prognosis of patients. We herein examined the activation status of the TILs in relation to the tumor microenvironment. By using frozen sections of human early-stage tongue cancers (n = 22), the TILs in the cancer nests and those in the cancer stroma were compared for the expression of PD-1, NKG2A, NKG2D, CD69, and Ki-67. The lymphocytes in oral lichen planus, an active immune response-mediated mucosal disease, were also analyzed for comparison purposes. All of the cancer specimens were abundantly infiltrated by CD8+ T cells and CD56+ natural killer (NK) cells in the stroma, as well as in the tumor nest. The tumor nest–infiltrating (intraepithelial) CD8+ T cells frequently expressed PD-1, an inhibitory receptor, in sharp contrast to those in the stroma or in the lichen planus. Conversely, the intraepithelial CD8+ T cells only infrequently expressed NKG2D, an activating receptor, in contrast to those in the stroma or in the lichen planus. No intraepithelial CD8+ T cells expressed Ki-67, a proliferation-associated marker, whereas those in the stroma frequently expressed it. Furthermore, the intraepithelial NK cells expressed NKG2A, an inhibitory receptor, more frequently than those in the stroma or the lichen planus. Collectively, the intraepithelial CD8+ T cells and NK cells are phenotypically inactivated, whereas stromal counterparts are phenotypically just as active as those in the lichen planus. These results suggest the first-step occurrence of an immune evasion mechanism in the tumor nest of oral squamous cell carcinoma.

Introduction

The clinicopathologic significance of the tumor-infiltrating lymphocytes (TIL) in various human cancers has been an issue of great interest. CD8+ cytotoxic T cells (CTL) and natural killer (NK) cells are the most likely effector cells for an effective antitumor immunity (1). Previous studies have shown that the infiltration of lymphocytes significantly correlates with a prolonged survival time of patients, at least in certain types of cancer (2–4). However, the capacity of the TILs as effector cells may be affected by the microenvironmental milieu of the tumor tissue (5). It could therefore be important to assess the functional state of the TILs in relation to their localization in the tumor tissue.

The functional state of the TILs may be presumed by the expression of CD69, PD-1, NKG2D, and NKG2A on CD8+ T cells and NK cells for the following reasons. The CTLs express CD69 upon an acute activation with T-cell receptor engagement (6), and the NK cells also express it during the earliest activation (7, 8). PD-1 is expressed by activated T cells, and the engagement of PD-1 by PD-L1 (B7-H1), the ligand of PD-1, leads to the inhibition of the T-cell receptor-mediated T cell proliferation and cytokine secretion (9). The expression of PD-1 by CTLs thus predicts their functional exhaustion (10). NK cells are closely regulated by a balance between the signals transmitted by activating receptors (NKG2D) that recognize the ligands on tumors and by inhibitory receptors (NKG2A) that recognize HLA-E to protect HLA class I transfectants from NK cell–mediated lysis (11, 12). The engagement of NKG2A with its ligand prevents NK cell activation (13, 14). CD8+ T cells and NK cells expressing NKG2D exert cytolytic responses against tumor cells expressing MICA, a ligand for NKG2D (15, 16).

In the present study, we immunophenotypically compared the TILs within the cancer nests (intraepithelial TILs) and those in the stroma near the invasion front of cancer (stromal TILs) using early-stage tongue cancers, most of which are abundantly infiltrated by the TILs. We also examined infiltrating lymphocytes in tissue specimens of oral lichen planus (OLP), which is characterized by a band-like infiltration of CD4+ and CD8+ lymphocytes in the submucosa, a disruption of the basement membrane, and a degeneration of the basal keratinocytes. We considered this lesion as a good positive control of an active immune-cell attack (17, 18). We herein show that the intraepithelial CD8+ T cells and NK cells are predominantly of the immunosuppressed phenotypes, whereas their stromal counterparts displayed more activated phenotypes. We will discuss the significance of such different phenotypes in the TILs in view of the immune evasion by the tumor cells.

Materials and Methods

Tissue specimens. We collected tissue samples of the early-stage tongue cancer (T1-T2 N0) through biopsies or through surgical procedures during 2003 and 2006 at Hamamatsu University Hospital (n = 22). None of the patients received preoperative chemotherapy or irradiation therapy. The biopsy specimens of OLP were obtained from the buccal lesions in the exacerbation phase of disease (n = 7). The biopsies of OLP were performed before the initiation of topical or systemic therapy. The biopsies from the normal lingual mucosa of healthy volunteers (n = 4) were used as controls for immunohistochemistry. This study was approved by the Ethical Committee of Hamamatsu University School of Medicine. For quantitative real-time PCR, samples were snap-frozen and stored at –80°C. For enzyme-linked immunohistochemistry, the specimens were fixed in
periodeate-lysine–4% paraformaldehyde (4% PLP) overnight at 4°C. After washing in PBS-containing sucrose, the fixed specimens were embedded in OCT compound (Miles), and they were then immediately frozen. For the immunofluorescent method (we only used the double labeling method), snap-frozen specimens were used in six cases.

**Antibodies and immunohistochemistry.** We used commercially available antibodies for the immunoperoxidase staining using fixed frozen sections in all cases (for details, see Supplementary data).

**Double immunostaining.** We performed double-labeling immunofluorescent microscopy in six representative cases for a more accurate cellular identification using fresh frozen specimens and enzyme-linked double immunohistochemistry in all 22 cases using PLP-prefixed specimens for quantitative analyses as described previously (for details, see Supplementary data; refs. 19, 20).

**Quantitative real-time PCR.** Total RNA was prepared from normal tongue tissues (n = 2), early squamous cell carcinoma (n = 2), and OLP (n = 2) by using Trizol reagent (Life Technologies-Bethesda Research Laboratories). Total RNA was further purified by using RNeasy (Qiagen). Total RNA (1 μg) was reverse transcribed using an oligo(dT)18 primer and SuperScript II transcriptase (Life Technologies-Bethesda Research Laboratories). The quantitative real-time PCR was performed using the Taqman assay and 7900 HT fast real-time PCR system (Applied Biosystems). The conditions for PCR were 50°C for 2 min, 95°C for 10 min, and then 50 cycles of 95°C for 15 s (denaturation) and 60°C for 1 min (annealing extension). The primers and fluorescent probes for PD-1, NKG2A, NKG2D, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from the Taqman kit. The quantification of the gene expression was obtained using a sequence detector system software (Applied Biosystems).

**Quantification of TILs.** The cellular densities of CD8+ T cells and CD56+ NK cells in the tongue cancer and OLP were calculated manually using the immunohistochemical samples as follows; 20 microscopic areas were independently selected at a high power magnification (∗×40 objective). One field corresponded to 0.071 mm². Positive cells in the cancer nests and those in the stroma were calculated separately. The number of groups compared was four; cancer nest, cancer stroma, OLP mucosa, and OLP submucosa. The number of positive cells in 20 areas in each specimen was averaged, and the results are expressed as the number of positive cells per square millimeter.

**Morphometric analyses of PD-1+, NKG2D+, NKG2A+, CD69+, or Ki-67+ cells among CD8+ T cells and CD56+ NK cells.** We performed enzyme-linked double-labeling immunohistochemistry for these markers and CD8 or CD56 in all cases. We counted the expression of each molecule in at least 20 independently selected areas containing abundant TILs (10 cancer nests and 10 cancer stroma) per one sample by the use of a ∗×40 objective. The following quantification was done using color photograph captured with an Olympus CCD camera. The positive rates were counted thrice with an Olympus CCD camera. The positive rates were counted thrice with an Olympus CCD camera.

Results

**Tissue distribution of immune cells in squamous cell carcinoma of the tongue: intraepithelial versus stromal.** We analyzed 22 frozen specimens of the early-stage tongue cancer without any preoperative therapy. Based on observation of H&E staining, specimens containing cancer nests were thus selected and used in this study. We carefully discriminated between lymphocytes in the cancer nests (intraepithelial) and those in the cancer stroma (stromal). The cancer nest means a cluster of carcinoma cells, and the stroma means connective tissue areas around the cancer nests. The cancer nests were surrounded by abundant stromal lymphocytes in all specimens, especially richly along the tumor-host interface (Fig. 1A). The infiltrating cells in the stroma were mainly CD4+ and CD8+ T cells (Fig. 1B and C). In addition, CD83+ and/or DC-Lamp+ mature dendritic cells with a typical dendritic morphology were also observed (Fig. 2A). The stromal cells expressing CD56 were less frequent (Fig. 2B).

The cancer nests were infiltrated by numerous intraepithelial CD8+ T cells, very few CD4+ cells, abundant PD-1+ cells, and CD56+ cells (Figs. 1B–D and 2B). The CD56+ cells were judged to be NK cells because most of them expressed CD94, but not CD3, by double staining (not shown). Both NKG2A+ and NKG2D+ cells were present in the cancer nests at similar frequencies (Fig. 2C and D). In contrast, the cancer stroma was infiltrated only by NKG2D+ cells (Fig. 2D) with NKG2A+ cells being quite infrequent (not shown). The cancer nests were also infiltrated by CD1a+/Langerin+/CD83+ immature dendritic cells (not shown). This was in sharp contrast to the abundant distribution of mature dendritic cells in the stroma (Fig. 2A). These data indicated the predominance of PD-1+ cells and NKG2A+ cells in the cancer nest.

Next, we quantitatively analyzed the numbers of TILs in the early-stage tongue cancer to compare those of OLP. The number of CD8+ T cells in the cancer was similar to that of OLP (168 ± 98 cells/mm² in cancer nest versus 196 ± 70 cells/mm² in OLP mucosa; 686 ± 210 cells/mm² in cancer stroma versus 742 ± 336 cells/mm² in OLP submucosa). NK cells in the cancer nest (98 ± 58 cells/mm²) far outnumbered those in the OLP mucosa (nearly absent, [0]). In contrast, the number of NK cells in the cancer stroma (67 ± 36 cells/mm²) was roughly comparable with that of the OLP submucosa (101 ± 41 cells/mm²).

**Selective expression of PD-1, NKG2A, and NKG2D genes in clinically early-stage squamous cell carcinoma of the tongue.** To confirm the expression of the corresponding mRNA in tissue, we performed quantitative real-time PCR. As shown in Fig. 3, PD-1 and NKG2A were expressed at much high levels in cancer tissues, whereas NKG2D was expressed at comparable levels in both cancer and lichen planus. No such strong expression of these genes was seen in normal tongue tissue specimens. However, the results obtained here were the sum of intraepithelial and stromal cells in the cancer and mucosal and submucosal cells in OLP and normal tissues, respectively.

**Cellular identification of PD-1+ and NKG2A+ cells as intraepithelial CD8+ T cells and NK cells, respectively.** For the identification of PD-1+ cells and NKG2A+ cells, we performed a double immunofluorescent staining for six representative cases using fresh frozen specimens. As shown in Figs. 4 and 3A, most of PD-1+ and NKG2A+ cells in the cancer nests were double positive for CD8 and CD56, respectively. In contrast, the cancer stroma was only infrequently infiltrated by such double-positive cells (PD-1+CD8+ and NKG2A+CD56+; not shown).

**Quantitative analyses of PD-1, NKG2A, NKG2D, CD69, and Ki-67 expression in squamous cell carcinoma compared with those in OLP.** To clearly show the difference of activation status of TILs, we further compare the expression frequency of PD-1, NKG2A, NKG2D, CD69, and Ki-67 among CD8+ and CD56+ cells between tongue cancer and OLP, a positive control for the active...
CTL responses (summarized in Fig. 6). All these analyses were performed by enzyme-linked double immunohistochemistry in all cases using PLP-prefixed specimens as described in the Supplementary data. The intraepithelial CD8+ T cells expressed PD-1 much more frequently than did those in the cancer stroma, the OLP mucosa, or the OLP submucosa (Fig. 6A). No significant differences were observed in the CD69 expression of CD8+ T cells among these tissue groups (not shown). On the other hand, the intraepithelial CD8+ T cells expressed NKG2D less frequently than did those in the cancer stroma and the OLP submucosa (Figs. 5B and 6B). We next performed an analysis on the proliferative activity of TILs using Ki-67, one of the representative proliferation-associated markers. The intraepithelial CD8+ T cells were virtually negative for Ki-67 (Figs. 5C and 6C). In contrast, CD8+ T cells in the cancer stroma frequently expressed Ki-67 (Figs. 5D and 6C) in a similar pattern to that of NKG2D (Figs. 5B and 6B). These results suggested that the intraepithelial CD8+ T cells are mostly functionally suppressed, whereas the stromal CD8+ T cells are in a more activated state as those in the OLP tissues. The intraepithelial CD56+ cells expressed NKG2A at much higher
frequencies than did those in the cancer stroma or in the OLP tissues (Fig. 6D), suggesting that the intraepithelial NK cells are also in a certain suppressed state. No significant differences were observed in the expression of NKG2D or CD69 on CD56+ NK cells among these tissue groups (not shown). It is noteworthy that the activation status of CD8+ T cells and NK cells in the stroma closely correlated with the selective distribution of mature dendritic cells in the cancer stroma (Fig. 2A).

We finally analyzed the correlation between the expression frequencies of PD-1 and NKG2A by CD8+ T cells and NK cells, respectively, in the cancer tissues (n = 22). No statistically significant correlation was found between the two. This analysis also made it clearer that CD8+ T cells and NK cells expressed PD-1 and NKG2A, respectively, at a far higher rate in the cancer nests than in the cancer stroma (Supplementary Fig. S1).

Discussion

Our present morphologic data using early-stage tongue squamous cell carcinomas have shown the immunophenotypical heterogeneity of the TILs in relation to their microanatomic locations: the relatively suppressed immunophenotypes of the intraepithelial TILs in contrast to the activated immunophenotypes of the stromal TILs. We also examined the immunophenotypes of the lymphocytes infiltrating the OLP for comparison. OLP is an autoimmune disease caused by a CD8+ T cell–mediated immune attack against the basal epithelial cells in the oral mucosa (21). These comparative analyses confirmed that the activated phenotypes of stromal TILs are at a similar level to those in OLP. It is noteworthy that mature dendritic cells are distributed in both cancer stroma and OLP. Thus, the comparative analyses in the present study further strengthened the concept of the functional heterogeneity of the TILs in cancer, thus suggesting that the suppression of antitumor immunity could first take place in the cancer nests (22).

In head and neck cancer, Badoua et al. reported that the number of CD4+CD69+ T cells in the cancer stroma correlated with a better regional control, but they could not find any prognostic value of the stromal CD8+ T cells (23). On the other hand, Naito et al. reported that CD8+ TILs in the different microanatomic locations may have differing effects on the patients’ prognosis in colorectal cancer,
The present study has shown that the intraepithelial CD8+ TILs express PD-1 at a high rate and NKG2D at a low rate. The high expression rate of PD-1 by the intraepithelial CD8+ TILs suggests their exhausted functions (10). It has been already documented that oral squamous cell carcinoma expresses PD-L1 (24), and we also have observed that PD-1+ TILs coexist with PD-L1+ tumor cells in the cancer nests of early-stage tongue cancer.\(^5\) This in vivo close contact suggests one of the immune evasion mechanisms by the cancer cells (25, 26). In contrast, mucosal CD8+ T cells in OLP expressed PD-1 at a far lower rate, thus suggesting that OLP mucosal CD8+ T cell may survive and continue to exert their function (9). This may explain the reason of sustained damages to the epithelial cells in OLP.

In accordance with the high expression of PD-1, the intraepithelial CD8+ TILs express a much lower expression of NKG2D than do the stromal CD8+ TILs. NKG2D is one of the most potent activating receptors for the effector functions of both CD8+ T cells and NK cells in peripheral tissues (15, 16, 27–29). Our data, thus far, indicate that the intraepithelial CD8+ T cells are phenotypically inactivated. Indeed, at early stage of tumor development, NKG2D can effectively reject the tumor cells (15). However, sustained surface expression and proteolytic shedding of MICA, a NKG2D ligand, by tumor cells impairs the immune response, thus promoting tumor immune evasion (27–29). The intraepithelial CD8+ TILs express Ki-67 only at a very low rate, whereas both the stromal CD8+ TILs and the submucosal CD8+ T cells in OLP show high rates of expression of Ki-67. The increased proliferative activity of the stromal CD8+ T cells is probably related to the abundant distribution of mature dendritic cells in the cancer stroma and the submucosa of OLP, thus suggesting the presence of a certain level of ongoing immune responses. The loss of the proliferative activity in the intraepithelial CD8+ T cells is also consistent with their high rates of expression of PD-1.

Our results are in contrast to the highly suppressed state of the lymphocytes, both in the circulating blood and in the cancer stroma of oral cancer patients with the advanced stages (mostly stage III or stage IV; ref. 30). This discrepancy may be ascribed to the different stages of disease. The previous analyses of the immune cells in colorectal cancer may support this notion. The number of intraepithelial CD8+ T cells negatively correlates with the cancer stages; they are the highest in stage I and show decreases in the advanced stages (31).

We also analyzed the functional phenotypes of NK cell, another possible effector cell. NKG2A was more prominently expressed on the intraepithelial NK cells than those in the cancer stroma or in the OLP suggesting the suppressed function of the intraepithelial NK cells, quite similar to the high PD-1 expression on the intraepithelial CD8+ T cells (13, 32). Therefore, the intraepithelial NK cells might have partially lost a tumor-specific triggering capacity (8).

Taken together, the intraepithelial CD8+ TILs, as well as the intraepithelial NK cells, in our clinically early-stage tongue cancer are in a certain state of an inactivated phenotype and are poor in the proliferation activity in sharp contrast to the more activated phenotype of their stromal counterparts. Given the systemically immunosuppressed status in patients with an advanced stage cancer, we have possibly visualized in humans the ongoing immune evasion process that initially starts in the cancer nest (33–35).

Finally, we need to pay attention to the possible differences among various cancers. For example, the intraepithelial CD8+ T cells in a colorectal cancer express Ki-67 at levels higher than do the stromal CD8+ T cells (31). In renal cell carcinoma, a higher labeling index of Ki-67 in the intraepithelial CD8+ T cells

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\(^5\) F. Katou, H. Ohtani, Y. Watanabe, unpublished data.
significantly correlates to a longer survival of patients (36). These are adenocarcinomas. Our present data are based on squamous cell carcinoma of the tongue. Therefore, it must be kept in mind that the immune responses could vary with the different histologic types of cancer.

To conclude, we herein showed the importance of the analyses of the TIL properties in relation to their in situ localization. The further accumulation of knowledge on the TILs in different stages of various human cancers may help to develop a more effective design for immunostimulatory therapy.

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Figure 6. Expression frequencies of PD-1, NKG2D, Ki-67, and NKG2A among CD8+ T cells or CD56+ NK cells in early-stage tongue cancer and OLP, as measured by an enzyme-linked double immunostaining (Fig. 4B–D). The data were derived from all cases examined. Note that the intraepithelial CD8+ T cells express PD-1 most frequently (A) and NKG2D at a relatively low rate (B), and they are virtually negative for Ki-67 (C). The stromal CD8+ T cells express NKG2D and Ki-67 at a similar rate to those in the OLP submucosa (B and C). Expression frequencies of NKG2A among the CD56+ NK cells in early-stage tongue cancer and OLP (D). Note that the intraepithelial NK cells express NKG2A most frequently. The data are presented as the median ± 75% quartiles in box plots due to their nonparametric distribution. The whiskers show the 5th and 95th percentiles. Statistical significance was evaluated by the Kruskal-Wallis test followed by the Mann-Whitney U test between two particular groups. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

References

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