Immunomodulatory Effects of Peplomycin on Immunosuppressive and Cytotoxic Cells in the Lesional Skin of Cutaneous Squamous Cell Carcinoma

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Key Words
Squamous cell carcinoma · Peplomycin · Regulatory T cells · Cytotoxic T cells

Abstract
Background: Continuous intra-arterial administration of peplomycin (PEP) through a tumor-feeding artery using an intravascular indwelling catheter is one of the best treatments for cutaneous squamous cell carcinoma (SCC) on cosmetic areas. Although this reagent is useful for the treatment of SCC, its immunomodulatory effect on the tumor microenvironment is still unknown. Objective/Methods: In this study, we investigated the immunomodulatory effects of PEP on the tumor-infiltrating regulatory T cells and tumor-associated macrophages as well as CD8⁺ TIA-1⁺ cytotoxic T cells in the lesional skin of 5 patients with SCC on the lips. Results: Our data suggest that, in addition to the direct antitumor effects, PEP decreased immunosuppressive cells and increased cytotoxic T lymphocytes at the tumor sites, which might maintain antitumor immune response against SCC. © 2015 S. Karger AG, Basel
that PEP might possess immunomodulatory effects on the cancer stroma of cutaneous SCC. In this report, we evaluated the immunomodulatory effects of intra-arterial administration of PEP on cutaneous SCC using immunohistochemical staining for CD4+CD25+Foxp3+ Tregs and CD68+ TAMs, as well as CD8+TIA-1+ tumor-infiltrating lymphocytes (TILs).

Materials and Methods

Reagents

We used the following antibodies (Abs) for immunohistochemical staining: anti-human CD4 Abs (Nichirei Co., Tokyo, Japan); anti-human CD8 (Dako, Kyoto, Japan), CD25 (Vector, Burlingame, Calif., USA), CD68 (Dako), Foxp3 (Abcam, Tokyo, Japan), and anti-TIA-1 Ab (Abcam). Mouse immunoglobulin (Ig) G2a, IgG2b and IgG1 isotype controls were obtained from R&D Systems (Minneapolis, Minn., USA).

Tissue Samples and Immunohistochemical Staining

We surgically excised tissue samples of stage II or stage III SCC on the lips before and after intra-arterial administration of 5 mg PEP once a day for 7–10 days in 5 cases treated in the Department of Dermatology at Tohoku University Graduate School of Medicine. The continuous intra-arterial administration of PEP through a superficial temporal artery decreased the mass of SCC within 6 weeks in all cases. There has been no sign of local recurrence or systemic lesions for at least 12 months for all cases. The study was approved by the ethics committee of Tohoku University Graduate School of Medicine, Sendai, Japan. All patients gave informed consent. All samples were processed for staining of CD4, CD8, CD68, CD163 and TIA-1 and developed with liquid permanent red (Dako A/S, Glostrup, Denmark) or with 3,3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan) as we previously reported [9]. Double staining of Foxp3 and CD4 or CD25 was performed as described previously [10]. Briefly, formalin-fixed paraffin-embedded tissue samples were sectioned at 4 μm and deparaffinized. After autoclaving for antigen retrieval treatment, the sections were blocked with goat serum for 10 min and exposed to primary Abs at 4°C overnight. Ab binding was demonstrated via alkaline phosphatase-conjugated anti-rabbit Ig [Histofine SAB-AP(R) kit; Nichirei] for anti-Foxp3 Ab or Ig from an unimmunized rabbit, and via peroxidase-conjugated anti-mouse Ig [Histofine SAB-PO(M) kits; Nichirei] for anti-CD25 Abs, or their isotype controls. Anti-Foxp3 Ab was developed with new fuchsin (Nichirei), whereas anti-CD25 Ab was visualized with 3,3′-diaminobenzidine tetrahydrochloride.

Assessment of Immunohistochemical Staining

Staining of infiltrated lymphocytes was examined in more than 5 random, representative fields from each section. The number of immunoreactive cells was counted using an ocular grid of 1 cm² at a magnification of ×400. The percentages of each cell fraction were defined as the numbers of each cell type per total numbers of lymphocytes (CD4 + CD8 + CD68). Data are expressed as the mean ± SD for Treg fractions in each skin disorder.

Statistical Analysis

For a single comparison of two groups, Student’s t test was used. The level of significance was set at p = 0.05.

Results

Administration of PEP Enhanced Lymphocyte Infiltration within SCC on the Lip

In all 5 cases, the tumors were reduced in size by intra-arterial administration of PEP (representative figures of case 1: fig. 1). Six weeks after the administration of PEP, we excised the remaining tumors and examined them histologically to identify the tissue responses to PEP. First we immunohistochemically characterized the infiltrated cells before and after the intra-arterial administration of PEP. Substantial numbers of TILs expressed CD4 with...
Foxp3, CD25 with Foxp3 (fig. 2a) and CD68 (fig. 2c), suggesting that immunosuppressive Tregs and TAMs infiltrated into the SCC. Moreover, immunohistochemical staining for SCC after the administration of PEP (fig. 2b, d) revealed that it significantly reduced the ratio of CD25⁺Foxp3⁺ Tregs and CD68⁺ tumor-resident macrophages. The number and ratio of Tregs and CD68 are summarized in table 1 and figure 3.

We also detected numerous cells that expressed TIA-1⁺ and CD8⁺, suggesting the activation of cytotoxic T lymphocytes (CTLs) infiltrating into the SCC (fig. 4a, c). In contrast to immunosuppressive cells such as Tregs...
and TAMs, the administration of PEP significantly increased the ratio of TIA-1+ and CD8+ cells within the tumor (fig. 4b, d). This therapy did not have an effect on the ratio of CD4+ cells. The number and ratio of CD4, CD8 and TIA-1 are summarized in table 1 and figure 3.

Discussion

In this report, we demonstrated that continuous intrarterial administration of PEP through a facial artery using an intravascular indwelling catheter significantly decreased the ratio of CD4+/CD25+Foxp3+ Tregs and CD68+...
TAMs in the tumor, and significantly increased the ratio of CD8\(^+\)TIA-1\(^+\) CTLs. These findings suggest that the effect of continuous intra-arterial administration of PEP is not only the direct cytotoxic effect on SCC, but might also induce an antitumor immune response by the decrease in Tregs and TAMs and increase in the effector T cells.

The induction of effector T cells, such as CTLs, has been a long-standing goal in cancer immunology and medical oncology. In this context, we previously reported that the intratumoral injection of an antitumor reagent, such as cationic liposome-encapsulated polyinosinic-polycytidylic acid and IFN-β, significantly increased the CTLs in melanoma [11, 12]. These data suggest that these reagents induce an antitumor effect against melanoma not only by killing the tumor but also by modulating the host immune systems at the tumor site. However, though we successfully induced CTLs by these reagents, there was no effect on the long-term survival of the melanoma-bearing host [11, 12]. This discrepancy may be explained by the recruitment of immunosuppressive cells, such as Tregs and TAMs, in the tumor microenvironment. Indeed, Mahnke et al. [13] reported that the depletion of Tregs in melanoma patients in vivo resulted in enhanced immune functions and the substantial development of antigen-specific CD8\(^+\) T cells in vaccinated individuals. More recently, Telang et al. [14] assessed the efficacy of depleting Tregs in stage IV melanoma patients and concluded that the depletion of Tregs had a significant clinical effect in patients with unresectable stage IV melanoma. These reports suggested that depletion of Tregs could be an optimal supportive therapy for human skin tumor.

Recently, it was reported that Tregs in tumors not only suppressed effector T cells directly, but modified the phenotype of tumor-infiltrating macrophages to express inhibitory B7-H molecules and to produce IL-10 in both human and mouse models [15, 16]. We previously reported that depletion of Tregs significantly down-regulated the expression of immunosuppressive molecules, such as B7-H1, B7-H3 and B7-H4, on myeloid-derived suppressor cells (MDSCs) and reduced the tumor growth, indicating the concerted immunosuppressive activity of Treg and MDSCs [15]. MDSCs are a heterogeneous population of cells that promote an immunosuppressive environment in tumor-bearing hosts [17, 18]. In humans, MDSCs are a less defined and phenotypically heterogeneous group of cells that have only immunosuppressive activities in common [17, 18]. Interestingly, Tiemessen et al. [16] reported that CD4\(^+\)CD25\(^+\)Foxp3\(^+\) Tregs produce IL-10, IL-4 and IL-13 and are able to steer monocyte differentiation toward alternative activated M2 macrophages. In addition, several reports also suggested the therapeutic effect of a selective reduction of MDSCs by chemotherapeutic drugs, such as gemcitabine or 5-fluorouracil [19, 20]. In aggregate, together with Tregs, immunosuppressive macrophages, such as MDSCs and TAMs, contribute to establishing the tumor microenvironment in skin cancer [17, 18, 21, 22], and the decrease in Tregs, MDSC or TAM could be an optimal supportive therapy for human skin tumor.

Concerning human SCC, recently several clinical reports suggested the contribution of tumor-infiltrating Foxp3\(^+\) Tregs on the establishment and progression of the tumor [23–25]. In addition, Tabachnyk et al. [25] recently reported the immunomodulatory effect of neoadjuvant radiochemotherapy (i.v. administration of cisplatin and 5-fluorouracil with 50.4-Gy radiation) for oral SCC and suggested that the induction of cytotoxic T cells and a decrease in Foxp3\(^+\) Tregs correlated with a better disease-free survival. They concluded that concurrent radiochemotherapy for oral SCC drives the composition of inflammatory cells in a prognostically favorable direction [25].

In addition to 2 previous cases of SCC on the ear successfully treated with intra-arterial administration of PEP [3], in this report, we described 5 cases of SCC on the lips successfully treated with intra-arterial administration of PEP through a superficial temporal artery. Notably, all of these 7 cases have achieved clinically complete remission. Moreover, we described the immunomodulatory effect of PEP on the TILs in cutaneous SCC. Since we did not directly assess the suppressive or effector function of these infiltrating cells in this study, further analysis of the mechanisms underlying this phenomenon could offer fundamental insights into the mechanisms of TILs in SCC. Such clarifications will need to be addressed in future investigations.

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**Disclosure Statement**

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References


