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TGF-β1/TβRII/Smad3 signaling pathway promotes VEGF expression in oral squamous cell carcinoma tumor-associated macrophages

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is the most common type of malignant cancer affecting the oral cavity. Tumor associated macrophages (TAMs) play a vital role in the initiation, progression and metastasis of OSCC. In this study, we investigated the correlation between macrophages and several clinical and pathological indicators, and we also explored how transforming growth factor-β1 (TGF-β1) effect on VEGF expression in TAMs. Seventy-two paraffin-embedded OSCC samples were collected. Association between macrophages density, micro vascular density (MVD) and clinical-pathological feature were explored by immunohistochemical staining. Western blot, ELISA and qRT-PCR were conducted to assess the VEGF expression in TAMs treated with or without neutralizing TGF-β1, TβRII and smad3 antibodies. Results showed that $CD68^+$ macrophages were absent in normal tissues. Macrophages density was directly correlated to low pathological differentiation, late clinical staging and poor survival rate. MVD showed positive correlation with clinical staging and macrophages density. Furthermore, OSCC-associated macrophages expressed more VEGF than macrophages in healthy lymph nodes. However, when TGF-β1 or TβRII were neutralized or the Smad3 was inhibited, VEGF expression was down regulated as well. It is concluded that TGF-β1 could promote OSCC-associated macrophages to secrete more VEGF via TβRII/Smad3 signaling pathway. This result might explain the correlation between macrophages density and worse clinical-pathological condition.

1. Introduction

Because of the high rate of both local recurrence and lymph-node metastasis, oral squamous cell carcinoma (OSCC) usually has undesirable prognosis and low 5-year survival rate that is often less than 50% [1]. Combined therapy including surgery and adjuvant chemo-/radio-therapy is the most widely adopted regime. Encouraging achievements on cancer at cellular and molecular levels contribute to the development of new anticancer drugs. However, treatment effectiveness is not as favorable as people expected. Therefore, further studies of the molecular and signaling mechanism underlying cancer initiation, progression, metastasis, and therapeutic failure are necessary.

Transforming growth factor-β1 (TGF-β1) is an important factor in tumor progression and metastasis [2]. TGF-β1 can induce biological effects through transforming growth factor β type I receptor (TGFβRI) and TGFβRII receptor, and activate Smad2 or Smad3. The phosphorylated Smad will then bind to Smad4 to express target gene [3]. Numerous studies showed that TGF-β/Smad signaling pathway was associated with tumor progression and prognosis [4,5].

Macrophages in tumor microenvironment (TME), termed as tumor associated macrophages (TAMs), are closely related to cancer growth and metastasis [6]. Recent studies have indicated that TAMs can promote the progression and metastasis of several solid carcinomas including breast, liver and ovarian cancer [7–10]. This can be partially ascribed to the fact that TAMs secrete a number of cytokines that promoting the proliferation, survival and invasion of tumor cells, such as TGF-β1, epidermal growth factor, matrix

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metalloproteinase [11–13]. TAMs also promote tumor angiogenesis by releasing tumor necrosis factor-a (TNF-a), VEGF and interleukin-8 (IL-8), which is essential for cancer growth and spread [6,12,14].

Unsurprisingly, TAMs were reported to play an indispensable role in the progression and prognosis of OSCC. Immunohistochemical analysis indicated that TAMs were significantly associated with the aggressive behavior and poor prognosis of OSCC [15]. Marcus et al. also reported that higher levels of macrophage infiltration were more likely to develop lymph node metastases in OSCC [16]. However, the signaling mechanism underlying the cross-talk between TAMs and OSCC cells remains unclear. In present study, we collected clinical materials and pathological specimens to investigate the correlation between TAMs and several OSCC clinical-pathological characteristics. Meanwhile, in vitro experiments were further conducted to explore the molecular mechanism behind this correlation.

2. Materials and methods

Our study was approved by the Ethics Committee of State Key Laboratory of Oral Diseases, Sichuan University (WCHSIRB-D-2014-095). Written informed consent was obtained from patients before surgery.

2.1. Patients' general characteristics

To explore the role of macrophages in OSCC, pathological specimens were collected from 72 patients who have been diagnosed with OSCC and underwent surgery in the Department of Head and Neck Oncology, West China Hospital of Stomatology, Sichuan University, between 2009 and 2011. The general characteristics of patients included in this study are listed in Supplementary Table 1.

2.2. Immunohistochemistry (IHC)

IHC was performed as previously described [17]. The mouse monoclonal anti-CD68 (1:150) (Dako, Denmark) and HRP labeled goat anti-mouse IgG (1:300) (Dako, Denmark) were used for IHC analysis. For immunohistochemical double staining of CD68 and CD31, the reaction was developed with DAB (ZSGB-BIO, China) for the anti-CD68 (brown staining), and with fast blue B salt (Aladdin, China) for rabbit anti-CD31 (1:100) (Santa Cruz, USA) (blue staining).

2.3. Macrophages-counting and Micro vascular density (MVD)

Macrophage counting was performed with an improved method from previous report [18]. Four areas with most condensed macrophages were randomly selected at 100× magnification. The cell counting was performed at 200× magnification. Macrophages with a brown-yellow staining in each area were counted twice and took the average. If the number of macrophages in each microscopic field was less than 15 or more than 30, it was considered as low or high expression, respectively. Numbers between 15 and 30 were considered as intermediate expression. To evaluate MVD, endothelial cells showing blue staining that well defined with surrounding vessel, connective tissue and tumor cells were recorded as one vessel.

2.4. Immunofluorescence staining

Fresh cancer tissue was embedded in OCT (Sakura Finetek, Japan) and cut into 10 mm sections using a cryostat (Leica CM1950, Germy). Sections were exposed to FITC conjugated CD68 (1:100) (Dako, Denmark), CD206 (1:100) (Biolegend, USA) and iNOS (1:100) (Abcam, USA) separately with rabbit *anti*-VEGF (Sant Cruz, USA) overnight at 4 °C. Slices were incubated with DAPI (Beyotime, China) and then mounted with Antifade Mounting Medium (Beyotime, China).

2.5. Cell culture

Eight to ten weeks male C57BL/6J mice (20–25 g) were purchased from the animal center of Sichuan University. Bone marrow cells were isolated as previously reported [18] and treated with 40 ng/ml recombinant mouse macrophage colony stimulating factor (M-CSF) to induce macrophages differentiation. Macrophages of the third passage were stimulated by concentrated recombinant human TGF-β1 (rhTGF-β1) (Dapcel Inc, USA) or Cal27 CM containing TGF-β1 neutralizing antibody (5 ng/ml) or anti-Tor vehicle (nIgG) (antibodies were treated for 24 h before the stimuli) for 24 h.

Cal27 cell line provided by the State Key Laboratory of Oral Disease, Sichuan University were cultured in DMEM/F12 (Gibco, USA) with 10% fetal bovine serum (FBS, Gibco, USA) and incubated at 37 °C in a humidified atmosphere with 5% CO_2. DMEM/F12 without FBS was added 24 h before Cal27 cells culture medium collection and it was considered as conditional medium (CM).

2.6. Flow cytometry

Fresh cancer tissue and healthy lymph nodes were digested with collagenase (Sigma, USA) and then centrifuged twice (1500 rpm, 3 min each). Abandon the supernatants and exposed the samples to FITC conjugated anti-human CD68 (1:100) (Dako, Denmark) or PE conjugated anti-mouse CD68 (1:100) (Biolegend, USA) to separate or identify $CD68^{+}$cells.

2.7. VEGF ELISA

ELISA was carried out to test the concentration of VEGF secreted by TAMs. Supernatants of primary cultured macrophages stimulated by Cal27 CM, normal medium (F12) or rhTGF-β1 for 24 h was collected. For some cell samples, 10 ng/ml TβRII neutralizing antibody (R&D, USA) or Smad3 inhibitor (Sigma, USA) was applied 1 h before the above stimulus was applied. The procedure was performed according to the manufacturer's instructions of R&D ELISA kit (R&D, Minneapolis, USA). The absorbance at 410 nm was measured using a microplate reader (Multiskan MK3, USA) and VEGF levels were expressed in pg/mL.

2.8. Quantitative real-time PCR (qRT-PCR)

$CD68^{+}$ macrophages from flow cytometry were used for qRT-PCR. RNA was extracted using PureLink RNA Mini Kit (Invitrogen, USA) and reversely transcribed using thermal cycler (SimliAmp™, USA). Primers sequences included VEGF (F-CTCACCGGAAAGACCGATTA; R-CGGAATATCTCGGAAAACTGC) and GAPDH (F-TGCTGAGTATGTCGTGGAGTCTA; R-AGTGGGAGTTGCTGTTGAAATC). All primers used above were synthesized by Life technologies, USA. Then, qRT-PCR was performed using the SYBR Premix Ex TaqTM II kit (Takara, Japan) and ABI 7300 Real-time PCR system (Applied Biosystems, USA). The same protocol was performed to detect VEGF mRNA from cultured macrophages that were stimulated by 1, 2, 5 and 10 ng/ml TGF-β1 or treated with Cal27 CM with or without neutralizing antibody for TGF-β1 (5 μg/ml, R&D, USA).

2.9. Western blot

Macrophages treated with rhTGF-β1, Cal27 CM or F12 were cultured in 6-well plates ($>5 \times 10^5$). The total protein was extracted by RIPA buffer (Beyotime, China). Mouse *anti*-GAPDH (Millipore,

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