

Tumour-associated macrophages are recruited and differentiated in the neoplastic stroma of oral squamous cell carcinoma



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Summary

To confirm our hypothesis that macrophages recruited to fight against oral squamous cell carcinoma (SCC) invasion are functionally differentiated within neoplastic stromata, we analysed arrangements of macrophage subtypes and cancer-associated fibroblasts (CAFs) in their association with blood vasculatures in the neoplastic stroma. Surgical specimens of oral SCC were immunohistochemically examined for macrophage phenotypes (CD68, CD163, and CD204) and stromal environments (perlecan, connexin 43, and CD31). Human monocytes were co-cultured with ZK-1 cells of oral SCC origin in different culture conditions. SCC stromata were divided into two types: fascicular (fibroblast-rich) and reticular (perlecan-rich). Regardless of stromal types, CD68 positive (+)/CD163+/CD204+ macrophages were recruited when blood vessels were abundant. Connexin 43+ fibroblasts were enriched in the fascicular stroma, where blood vessels were depleted. In co-culture experiments, monocytes, in the presence of ZK-1 cells, showed $\text{TNF-}\alpha^{\text{low}}$ / $\text{IL-12}^{\text{low}}$ and $\text{IL-10}^{\text{high}}$ / $\text{VEGF}^{\text{high}}$ / $\text{MMP-9}^{\text{high}}$ with increased expression levels for fibronectin and perlecan. With direct contact with monocytes, SCC cells also expressed CD68 and CD163. SCC stromata were characterised by CD163+/CD204+ tumour-associated macrophages (TAMs) and connexin 43+ CAFs. TAMs are differentiated from monocytes by the physical contact with oral SCC cells in the perlecan-rich neoplastic stroma, which is also induced by the cross-talk between SCC cells and stromal cells including TAMs.

Key words: Tumour-associated macrophages; oral squamous cell carcinoma; cancer stroma; CD163; CD204; connexin 43.

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INTRODUCTION

The cancer microenvironment is regulated by cross-talk between parenchymal cells and stromal cells, and varieties of their interaction determine the fate and growth of cancer

parenchymal cells.¹ In terms of stroma formation, we have distinguished two types of cells that are responsible for the production of extracellular matrix (ECM): one is parenchymal cells themselves, such as those of salivary adenoid cystic carcinoma² or pleomorphic adenoma,³ and the other is stromal fibroblasts in oral squamous cell carcinoma (SCC).⁴ The stromal spaces produced by these two different cell types share a common histological architecture of poor vascularisation,^{4,5} which is distinct from granulation tissues formed against tissue damage due to cancer cell invasion. We have proposed that such granulation tissues should be distinguished from neoplastic stroma, which is generated by both cancer cells and stromal cells, as mentioned above. Since neoplastic stroma formed in salivary gland tumours lacks inflammatory cell infiltrates, it is rather easily distinguished. In the case of SCC, however, inflammatory cells often infiltrate the connective tissue space, and hence it is not always easy to distinguish true neoplastic stroma from granulation tissue. Unfortunately, to date there have been no actual trials to elucidate how inflammatory cells are recruited into neoplastic stroma, which is different from granulation tissue.

Stromal reactions represented by fibrosis and inflammatory cell infiltrations have been correlated to prognoses of individual cases of cancer.⁶ Apart from stromal types, cancer-associated fibroblasts (CAFs) have recently attracted much attention because they are now recognised as one of the important constituent cell types in neoplastic stroma, and they have been believed to regulate parenchymal cell behaviour.⁷ Lymphocytes, neutrophils, and macrophages have been recognised as major inflammatory cells in the neoplastic stroma.⁸ Although individual functions of those cancer stromal inflammatory cells have not yet been fully elucidated, it is partially disclosed that they do not always confront cancer cells but occasionally support cancer cells to grow.⁹ More recently, we have demonstrated chronologically different but cooperative functions between neutrophils and macrophages in degradation of keratin pearls of oral SCC.¹⁰

Among those inflammatory cell populations, we have paid special attention to macrophage subpopulations in the cancer stroma. This is because functions of M2 macrophages have

recently been clarified in the field of immunology, and the concept of tumour-associated macrophages (TAMs) has been established.¹¹ There are two different cell populations in macrophages: one is M1 macrophages, which release high levels of pro-inflammatory cytokines, including interleukin (IL)-1, IL-6, IL-12, and tumour necrosis factor alpha (TNF- α);¹² the other is M2 macrophages, which express such cytokines as IL-4, IL-10, and transforming growth factor (TGF)- β to support cancer cell growth, angiogenesis, ECM remodelling, and even metastasis.¹³ CD163 or CD204 have been introduced as markers for M2 macrophages,¹⁴ while CD68, a monocyte-specific glycoprotein, has been regarded as a general macrophage marker, which both M1 and M2 macrophages share.¹⁵

On the other hand, through our routine practice of histopathological diagnosis, we have come to recognise that myxoid stromal spaces are induced in the invading front of oral SCC, and that such myxoid stromal spaces lack vascularity as well as inflammatory cell infiltrates, as mentioned above.⁴ Thus, we have hypothesised that such a myxoid stroma is a true neoplastic stroma and regulates infiltration modes of inflammatory cells as well as cancer cell invasion, the latter of which we explained by our three-dimensional analysis data.¹⁶ To further confirm the hypothesis, we have designed the present study to analyse the mode of macrophage infiltration, especially that of TAMs in the neoplastic stroma. In the first part of the present study, we examine immunohistochemical modes of macrophage distribution between M2 and M1 macrophages in association with stromal types including CAF distributions. In the second part, we also examine TAM differentiation from freshly isolated monocytes when they are cultivated with oral SCC cell lines.

MATERIALS AND METHODS

Samples

We microscopically reviewed haematoxylin and eosin (H&E) stained surgical specimens of oral squamous cell carcinoma (SCC) from the surgical pathology files of the Division of Oral Pathology, Niigata University Graduate School of Medical and Dental Sciences during the three years from 2009 to 2011, and we selected 100 cases of SCC, which simultaneously contained normal mucosal parts and/or carcinoma *in situ* (CIS) foci within their tissue sections. Sections of main SCC foci were selected from those that contained enough stromal spaces for histological evaluation. The surgical samples had been fixed in 10% formalin and routinely processed and embedded in paraffin. Serial sections cut at 5 μ m from paraffin blocks were used for H&E and immunohistochemical stainings. The experimental protocol for analysing surgical materials was reviewed and approved by the Ethical Board of the Niigata University Graduate School of Medical and Dental Sciences (Oral Life Science).

Antibodies

Mouse monoclonal antibodies against human CD68 (clone PG-M1, isotype IgG3), CD31 (JC70A, IgG1), and α -smooth muscle actin (α SMA) (clone 1A4, isotype IgG2a) were purchased from Dako (Denmark). A mouse monoclonal antibody against human CD163 (10D6, IgG1) was purchased from Novocastra (UK). A mouse monoclonal antibody against human CD204 (SRA-E5, IgG1) was purchased from Trans Genic (Japan). Rabbit polyclonal antibodies against connexin 43 (Cnx43) (GJA1) were obtained from Abcam (UK). Antibodies against the mouse perlecan core protein were raised in rabbits, as described elsewhere.¹⁶

Immunohistochemistry

Immunohistochemical stainings were performed using ChemMate Envision system (Dako) in serial sections, as described elsewhere.¹⁷ For CD68, CD163, CD204, and Cnx43, sections were autoclaved in citrate buffer (pH 6.0) for

10 min at 121°C. For α SMA, sections were autoclaved in Tris-EDTA (pH 9.0) for 10 min at 121°C. For CD31, sections were pretreated with 0.2% trypsin (type II; Sigma-Aldrich, USA) in 0.01 M Tris-HCl (pH 7.6) containing 0.1% CaCl₂ for 30 min at 37°C. For perlecan, they were pretreated with 3% hyaluronidase (bovine testicular origin, type I-S; Sigma) in PBS for 30 min at 37°C. Reaction products were visualised with 0.02% 3,3'-diaminobenzidine in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.005% hydrogen peroxide. Finally, the sections were counterstained with haematoxylin. For control studies on antibodies, the primary antibodies were replaced with pre-immune mouse or rabbit IgGs.

Cells cultured in chamber slides were fixed with 4% formaldehyde for 30 min on ice and were added with 0.2% Triton X-100 in PBS for permeabilisation.¹⁸ After removal of plastic chamber frames, the slides were subjected to immunoperoxidase staining, as mentioned above, but without enzymatic or autoclave pretreatments.

Evaluation of immunohistochemical results

After tissue sections were screened at lower magnification, three fields from different SCC stromal types were randomly selected at higher magnification using a 40 \times objective lens. Those representative areas for every category were photographed on a Nikon Eclipse microscope equipped with a Nikon DXM1200C digital camera (Nikon, Japan). Numbers of mononucleated macrophages that were positive for CD68, CD163, and CD204 were separately counted manually in a unit field of 0.25 \times 0.25 mm on serial sections. Positive-stained cells that were smaller in size than circulating monocytes (10 μ m) were excluded from counting.¹⁹

Cancer-associated fibroblasts (CAFs)

In addition to perlecan, stromal spaces were also characterised by CAFs, which have been widely identified by immunohistochemistry for α SMA. In the present study, we introduced immunohistochemistry for Cnx43, one of the gap junction molecules, in addition to that for α SMA positivity, to determine if both α SMA and Cnx43 were simultaneously expressed in the same stromal fibroblasts. α SMA+ and Cnx43+ fibroblasts were manually counted using a 20 \times objective lens in the same unit field of the stromal space (0.25 \times 0.25 mm) in the same manner as that used for macrophage counting, as mentioned above.

Microvessel density (MVD)

The entire sections were scanned at low magnification in order to identify the most highly vascularised areas (hotspots), and three hotspot areas were photographed at high magnification using a 20 \times objective lens. Microvascular numbers, which were identified with CD31+ endothelial cells or with their clusters with or without vascular lumina separating each other from adjacent vessels, were counted manually in a unit field of 0.25 \times 0.25 mm as MVD.²⁰

Cells

ZK-1, a SCC cell system established from a SCC of the human tongue, was maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS; Gibco, Thermo Fisher Scientific, USA), 50 mg/mL streptomycin, and 50 IU/mL penicillin (Gibco).¹⁸ Monocytes from human peripheral blood were collected from healthy volunteers after obtaining informed consent. Heparinised fresh human blood samples (7 mL each) were mixed with an equal volume of PBS(-) (free from calcium and magnesium) and were subjected to Ficoll-Paque density gradient centrifugation at 2000 rpm for 20 min to recover peripheral blood mononucleated cells (PBMCs). The PBMCs thus obtained were allowed to adhere to 60 mm plastic dishes in FCS-free RPMI 1640 (Gibco) at 37°C for 2 h, and adherent cells were collected as monocytes by a plastic cell scraper. The purity of monocytes in the samples was determined to be 60–80% by flow cytometry (forward scattering/side scattering plots) using a BD FACS Aria II Cell Sorter (BD Biosciences, USA). The viability and shape of isolated monocytes were confirmed by trypan blue and Giemsa stains, respectively.

Cell culture conditions

Cell culture experiments were performed under four different conditions, as described below.

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