Hypoxia and CD11b+ Cell Influx Are Strongly Associated With Lymph Node Metastasis of Oral Cancer

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Abstract. Background/Aim: Treatment failure in oral cancer is mainly caused by uncontrolled cervical lymph node (LN) metastasis. We previously reported that CD11b+ cells are recruited into tumor hypoxic areas following radiation, leading to re-vascularization and relapse. Since lymphatic vessel formation has similarities with vascular formation, we examined whether surgery induces hypoxia and stimulates lymphangiogenesis. Materials and Methods: The recruitment of CD11b+ cells and the formation of lymphatic vessels were examined using orthotopic tongue cancer mouse models with glossectomy. Results: Surgery on OSC-19 tumor induced LN metastases and hypoxia, followed by CD11b+ cell influx. These phenomena were not observed in the no tumor or SAT tumor models. Stimulation of lymphangiogenesis was observed in the CD11b+ cell influx area, as the tumor grew. The localization of CD11b+ cells was changed from the lymph nodules to the medullary sinuses. Conclusion: Surgery-induced hypoxia in oral tumors leads to CD11b+ cell infiltration, lymphangiogenesis, and LN metastasis.

As of 2018, head and neck cancers were the seventh most common cancers worldwide, and there were approximately 890,000 newly diagnosed cases and 450,000 deaths yearly (1). Oral cancer accounts for approximately 40% of them, and the majority (90%) of oral cancers are squamous cell carcinomas (OSCCs) arising from the oral mucosa epithelium. Although the 5-year-survival rate of tongue cancer is approximately 60-70%, the prognosis of advanced tongue cancer with lymph node (LN) metastasis is poor (2). Despite the fact that metastasis to cervical LNs is the most reliable predictor for failure of OSCC treatment, the cellular and molecular mechanisms are poorly understood. Treatment outcomes become worse when extracapsular spread or multiple node metastases occur. Therefore, early diagnosis and eradication of metastatic LNs are clinically critical issues.

During LN metastasis, cancer cells spread through lymphatic vessels and colonize draining LNs. In general, LN metastasis is thought to occur via lymphangiogenesis, which is composed of the expansion of the lymphatic capillary vessel network; it is a novel prognostic parameter in some cancers (3, 4). However, the molecular and cellular mechanisms of lymphangiogenesis are largely unknown, even if they are driven by the incorporation of progenitor cells or local lymphatic endothelial cell proliferation. Recent reports have revealed that lymphatic vessels are strongly involved in immune cell and stroma cell trafficking and inflammation. One of the most reliable molecular mechanisms related to these phenomena is the vascular endothelial growth factor C (VEGF-C)/VEGFR3 pathway. Both blood vessels and lymphatic vasculatures are similarly stimulated by the VEGF family. Hypoxia is a major condition, which favors both the induction of angiogenesis/vasculogenesis and lymphangiogenesis. We previously reported that bone marrow-derived CD11b positive (BM-derived CD11b+) cells are recruited into tumors and contribute to tumor blood vessel remodeling after irradiation when tumors recur in hypoxic areas (5). Moreover, it is thought that surgical interventions cause LN metastasis due to the activation of hypoxia-inducible factor-1 (HIF-1), however, the detailed mechanism remains unclear yet. Thus, in this study, we investigated whether surgery induces cervical LN metastasis of oral cancer using an orthotopic tongue cancer mouse model. The transition of the microenvironment in local

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Key Words: Oral cancer, LN metastasis, CD11b+ cell, lymphangiogenesis, hypoxia.
tumors and LNs was analyzed to determine the molecular and cellular mechanisms of LN metastasis for the development of novel treatment strategies.

Materials and Methods

Cell lines. OSC-19 (JCRB0198) and SAT (JCRB1027) cell lines (human squamous cell carcinoma of the tongue) were purchased from the Japanese Collection of Research Bioreresources (Osaka, Japan). OSC-19 is a cell line with a high rate of metastasis, but SAT has non-metastatic characteristics (6, 7). Retroviral transduction of both cells was carried out with the luciferase gene, as described previously (8). Cells were maintained in Dulbecco’s modified eagle medium containing 10% fetal bovine serum.

Orthotopic-xenografted tongue tumor model. For the orthotopic oral tumor model, OSC-19 (3x10^4 cells/30 μl) or SAT (4.5x10^4 cells/30 μl) cells were inoculated into the left edge of the tongue of 8-week-old female nude mice. After confirmation of tumor growth, mice were randomly divided into a surgical treatment (glossectomy) and control groups (6-7 mice per group). In the surgical treatment group, mice were anesthetized, and partial glossectomy was performed on day 14 using a scalpel, since the right side of the tongue carried almost half of the tumor; this resulted in a partially persistent tumor (8, 9).

In vivo real-time optical imaging. Real-time tumor growth was monitored by optical imaging using IVIS cooled CCD optical system (PerkinElmer, Downers Grove, IL, USA). Mice were anesthetized using 3% isofluorane after intraperitoneal injection of 150 mg/kg body weight of D-Luciferin (PerkinElmer). Five min after the injection of the D-Luciferin, images were acquired for 10 to 30 sec using Living Image analysis and acquisition software, Living Image (PerkinElmer). A photographic image was taken, onto which a pseudocolor image representing the spatial distribution of photon counts was projected. For bioluminescence imaging (BLI) plots, the photon flux was calculated for each mouse by using a square region of interest encompassing the head of the mouse in a supine position (5).

Tumor sample preparation. The bilateral neck LNs and tongue were excised when the mice started to lose weight or on days indicated in the results. Tumor samples were fixed with 4% paraformaldehyde for cardiac perfusion, and then, embedded in OCT compound (Sakura Finetek, Torrance, CA, USA). The tongue and cervical LNs were also stained with hematoxylin-eosin and evaluated for the presence of local tumors and metastases.

Immunohistochemistry. Frozen sections (6 μm) were incubated with the following primary anti-mouse antibodies overnight at 4°C; CD11b (BioLegend, San Diego, CA, USA) and LYVE-1 (RELIA Tech, Wolfenbüttel, Germany). They were then incubated with secondary antibodies for 30 min at room temperature, including Alexa Flour 488 and 546 (Thermo Fisher Scientific, Waltham, MA, USA). Hypoxic areas were detected using pimonidazole hydrochloride staining (Hypospyprobe™-1 Omni kit, Natural Pharmacia International, Burlington, MA, USA) according to the manufacturer’s instructions. All sections were mounted in ProLong® Gold Antifade Mountant with DAPI (Thermo Fisher Scientific) and viewed with a Leica DMI 4000B fluorescent microscope (Leica Microsystems, Wetzlar Hesse, Germany). Isotype-matched IgG was used as a negative control.

Quantification of expression. Quantitative analyses for CD11b were performed by determining the number of positive cells. The signal density of LYVE-1 was measured using Image J. All analyses were performed in at least three randomly photographed fields using x20 or x40 objectives and x10 eyepieces of the fluorescence microscope. Three to six mice were used per group, and the error bars shown in the figures represent standard deviations.

Statistical analysis. Comparisons between two groups were done using the Student’s t-test (GraphPad Prism software version 6.03). p-Values (exact significance) less than 0.05 were considered statistically significant.

Results

Surgical tumor reduction (glossectomy) increases LN metastasis of tongue cancer in an orthotopic mouse model. There were no significant differences between parent and luciferase-transduced cells in morphology, growth, and metastasis rates in vitro and in vivo (data not shown). The injection of OSC-19-Luc into the tongues of nude mice formed tumors in all mice and yielded some instances of LN metastases by day 14 (35%, Figure 1A and C). As shown in Figure 1A, surgical tumor reduction by glossectomy stimulated cervical LN metastases. The incidence of LN metastases of OSC-19 in the glossectomy group was approximately 80% (Figure 1C). These cervical LN metastases were confirmed microscopically (Figure 1B).

The influx of CD11b+ cells into the primary and cervical LNs is increased after glossectomy in highly metastatic tongue cancer. We next investigated whether CD11b+ cells are recruited into tumors after glossectomy in this model. As shown in Figure 1D, glossectomy tumors exhibited significantly increased levels of CD11b+ cell influx at the primary site. The average number of CD11b+ cells in the glossectomy group was 18, while that of the control group was 3 (Figure 1E). Notably, influx of CD11b+ cells was also significantly higher in the glossectomy group in cervical LNs (Figure 1D and E). We then investigated the microenvironmental changes in this oral cancer model. To do this, we first examined the recruitment of CD11b+ cells in a glossectomy model with no tumor inoculation. As shown in Figure 2A and B, a small number of CD11b+ cells were recruited into the tumor in the glossectomy group without tumors. However, this increase was not significant, when compared with the control group. We next examined if signals from the cancer cells were important for both CD11b+ cell influx and LN metastasis. To this direction, we utilized the SAT cell line, a non-metastatic tongue cancer cell line, in the glossectomy model. As shown in Figure 2A and B, there was no significant difference in the recruitment of CD11b+ cells.
Figure 1. Surgical tumor reduction (glossectomy) induces CD11b+ cell influx into the primary tumors and cervical lymph nodes (LN), and increases LN metastases. A) Real-time tumor growth and LN metastasis were monitored by optical imaging as described in Materials and Methods. Representative tumor images on day 14 in the control and on day 28 in the glossectomy group are shown. Arrow indicates cervical LN metastasis. B) Tumors in the tongue and cervical LN. C) The rate of LN metastasis in OSC-19 orthotopic mouse model. D) Representative images of immunohistochemistry for CD11b staining in primary tumors and LN of the control and glossectomy groups in OSC-19 orthotopic tumor model. E) Quantification of CD11b cell influx into the tongue and LN. Data shown are means±SD.
in the control and glossectomy groups. LN metastasis was observed neither in the control group nor in the glossectomy group in the SAT model. These results indicate that surgical intervention and signals from the persistent tumor are involved in local microenvironmental changes, leading to the metastasis of oral cancer into cervical LNs.

CD11b+ cells are recruited into the hypoxic region by surgical tumor reduction. As glossectomy in the no tumor model slightly increased CD11b+ cell influx, and as other investigators have shown that tissue repair after surgical invasion is more hypoxic, hypoxia might have caused the microenvironmental changes in our model (10). To test this, we determined the level of hypoxia and CD11b+ cell recruitment into the primary site. We found that CD11b+ cells were rapidly recruited into tumors in the glossectomy group within a day. The CD11b+ cells on day 1 after glossectomy were localized at the surgical edge, close to the hypoxic area (Figure 3). On day 10, the area of CD11b+ cells and hypoxia were colocalized. These results suggest that CD11b+ cells were recruited into the hypoxic area after glossectomy.

Glossectomy promotes local influx of CD11b+ cells at the primary site, followed by induction of lymphangiogenesis in the primary tumors and LNs. We then investigated the relationship between CD11b+ cell influx and lymphangiogenesis in the glossectomy model. As shown in Figure 4, CD11b+ cells were recruited into the surgical site

Figure 2. Glossectomy does not significantly stimulate recruitment of CD11b+ cells into the tumor either in the no tumor or the SAT tumor model. A) No tumor model and SAT models did not show significant lymphangiogenesis (LYVE-1; green) and recruitment of CD11b+ cells (CD11b; red) into the tumor after glossectomy. B) Quantification of CD11b+ cells influx in the primary tumor with or without glossectomy in the no tumor or SAT tumor model.
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Figure 3. Recruitment of CD11b cells into the hypoxic area following glossectomy. Representative images indicate the different time points of OSC-19 orthotopic tumor model after glossectomy; 1 h, 1 day, 10 days. Upper: control group, lower: glossectomy group. Glossectomy leads to hypoxia, stained by pimonidazole, and CD11b+ cells infiltrate into the hypoxic region.

Figure 4. Glossectomy promotes local influx of CD11b+ cells and induces lymphangiogenesis. The primary tumors and LNs were collected at different time points after glossectomy in OSC-19 orthotopic mice. Representative images show influx of CD11b+ cells in the primary tumor and cervical LNs and lymphangiogenesis. CD11b+ cells were recruited into the surgical site following glossectomy. In LNs, CD11b+ cells gradually moved from the lymph nodules to the medullary sinuses.
following glossectomy. The location of the lymph vessels was changed over time, and lymphangiogenesis seemed to take place in the site of CD11b+ cell infiltration in the primary tumor after glossectomy. In LNs, more CD11b+ cells were observed in mice that underwent glossectomy, and localization of CD11b+ cells was changed from the lymph nodules on day 1 and 7 to the medullary sinuses on day 14.

**Discussion**

It is widely known that cervical LN metastases occur in some OSCCs, and LN metastasis is one of the major prognostic factors in patients with OSCC. Although lymph vessels were initially thought to be the route of metastasis, recent studies have provided evidence that the lymphatic system is affected by tumor cells, the tumor microenvironment including stroma cells and immune cells, and inflammation. In fact, a high density of lymphatic vessels is associated with a poor prognosis of cancer patients (11). As the lymphovascular system is considered the first site of metastasis for most malignant tumors (12), targeting lymphangiogenesis or utilizing it for drug delivery is considered a promising strategy.

It was recently suggested that lymphangiogenesis in the process of LN metastasis is mainly caused by cancer cells, but also by the tumor microenvironment, which is composed of fibroblasts, vascular endothelial cells, lymphatic endothelial cells, pericytes, and immune cells. These cells communicate with each other intricately and regulate the formation of new vessels and vessel networks. VEGF-C expressed in CD11b+ cells plays a critical role in angiogenesis and lymphangiogenesis in mouse models of hind-limb ischemia (13). It has also been reported that BM-derived CD11b+ myeloid cells infiltrating the tumor site contribute to lymph vessel formation. In this study, we found that CD11b+ cells were increased in both the primary sites and LNs when the tongue tumors were metastasized into the cervical LNs. CD11b is widely expressed among myeloid cells. CD11b+CXCR2+Ly6G+Ly6C low cells, characterized as myeloid-derived suppressor cells (MDSCs), promote breast cancer growth and LN metastasis (14). Although the recruited CD11b+ cells in both the primary sites and metastatic LNs in this study were not characterized, these cells might be CD11b+ M2 macrophages, as we have previously reported that M2 macrophages are recruited into OSC-19 tumors and contribute to vasculogenesis following irradiation (15). Another study has shown that CD11b+CD68+ tumor-associated macrophages flocked near lymphatic vessels and expressed higher levels of VCEGF-C in an orthotopic urinary bladder cancer model (16). Moreover, Schledzewski et al. recently reported that LYVE-1 is expressed in a subset of CD11b+ F4/80+ tissue macrophages in a murine tumor model (17). Since this study showed different locations of CD11b+ cells and LYVE-1 expression in a short-term post-glossectomy period, the recruited CD11b+ cells might be different from locally existing tissue macrophages. Further analysis is required for the characterization of CD11b+ cells recruited into tumor sites and LNs in future studies.

We also found that CD11b+ cells were also recruited into the hypoxic area following glossectomy. Hypoxia is associated with metastasis and mortality due to its ability to induce angiogenesis and lymphangiogenesis. It has been reported that 4T1 breast cancer cells inoculated in the pre-irradiated site reduced angiogenesis, were more hypoxic, and metastasized to LNs (18). Influx of CD11b+c-Kit+Ly6G+Ly6C lowGr1+ myeloid cells correlated with higher levels of metastasis in an HIF-1 dependent manner. Our previous study also demonstrated that recruitment of CD11b+ myeloid cells is stimulated by activation of HIF-1α, and pharmacological inhibition of HIF-1α prevents the mobilization of CD11b+ cells and attenuates vasculogenesis (5). Although the molecular mechanism of CD11b+ cell influx in residual tumors following glossectomy is still unknown, HIF-1 is a solid candidate for stimulation of lymphangiogenesis through the alteration of the microenvironment after surgery in OSCC.

We also found in this study that a higher number of CD11b+ cells existed in LNs in mice that underwent glossectomy, and the localization of CD11b+ cells was changed from lymph nodules to medullary sinuses as the tumors grew following glossectomy, regardless of whether it was in the presence of metastatic cancer cells. This altered localization might be closely related to the pre-metastatic cancer cell niche phenomenon. Recent evidence has revealed that tumors induce pre-metastatic niches in distant organs, leading to metastatic conditions. Pre-metastatic niches represent a tumor-favorable microenvironment prior to the presence of cancer cells. Few studies have reported that the pre-metastatic niche in oral cancer is related to lymphangiogenesis within the cervical LN metastases (19, 20). Mayorca-Guiliani et al. reported that highly lymph metastatic OSCC cell line, SASL1m, that disseminates mainly via lymphatic vessels, induces an assemblage of blood vessels in tumor-draining LNs and SASL1m, that disseminates mainly via lymphatic vessels, induces an assemblage of blood vessels in tumor-draining LNs even without cancer cells, while non-metastatic cells fail to produce any change. We also found that highly lymph metastatic OSC-19 cells exhibit a higher number of CD11b+ cells and that CD11b+ cells localized in medullary sinuses in draining LNs. Moreover, our preliminary analysis using human OSCC specimens revealed that a higher number of CD11b+ cells in sentinel LNs with or without metastatic cancer cells as compared to LNs from metastatic-free neck dissections (N0 cases, data not shown). Although there was no significant difference between metastatic sentinel LNs and non-metastatic sentinel LNs in the accumulation of CD11b+ cells,
the stimulated signaling pathway might be remotely provided by the primary tumor for engraftment of metastatic cancer cells, even in metastasis-free LNs (21). The accumulated CD11b+ cells in the medullary sinuses need to be investigated to clarify whether this localization is for stimulation of lymphangiogenesis, and to explore its potential as a predictive marker and treatment target.

Conflicts of Interest

The Authors state that they have no conflicts of interest to declare in regard to this study.

Authors’ Contributions

KS analysed the immunohistochemical results, performed the statistical analyses, and drafted the manuscript. SN, IK, MO, and YN participated in the collection of specimens and carried out immunohistochemistry. KM coordinated the study and supervised the statistical analyses, and approved the final manuscript. MK designed the study, approved the results of experiments, and wrote the manuscript. All Authors read and approved the final manuscript.

Acknowledgements

The Authors are grateful to Dr. Tohnai for helpful suggestion; Ms. Taniguchi and Kusaka for help in generating tissue slices of OSCC patients. The Authors would like to thank Editage (www.editage.jp) for English language editing. This research was supported by Grant-in-Aid for Early-Career Scientists (no. JP18K17203) from the Japan Society for the Promotion of Science (JSPS), and by grants-in-aid from the Japanese Ministry of Education, Science, and Culture (to MK, no.17H04408).

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Received November 6, 2020
Revised November 14, 2020
Accepted November 17, 2020