Oral Diseases

HIF-1α controls palatal wound healing by regulating macrophage motility via S1P/S1P$_1$ signaling axis

Running title: Palatal wound healing in HIF-1α deficient mice.

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KEYWORDS: HIF-1α, S1P receptor, hypoxia, wound healing, M1/M2 macrophage, DMOG

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Abstract

Objectives: To investigate the role of hypoxia-inducible factor 1α (HIF-1α) signaling, the expression profile of M1 and M2 macrophages, and the role of the sphingosine 1-phosphate (S1P)/S1P receptor system in palatal wound healing of heterozygous HIF-1α-deficient (HIF-1α HET) mice.

Materials and methods: HIF-1α HET and wild-type (WT) littermates underwent palatal tissue excision at the mid-hard palate. Histological analysis, immunostaining, real-time PCR, Western blotting (WB), and cellular migration assays were performed to analyze wound closure and macrophage infiltration.

Results: DMOG pretreatment showed an acceleration of palatal wound closure in WT mice. In contrast, the delayed palatal wound closure was observed in HIF-1α HET mice with diminished production of Col1a1, MCP-1, and MIP-1α, compared with WT mice. Decreased infiltration of M1 macrophage ($F4/80^+TNF\text{-}\alpha^+$, $F4/80^+iNOS^+$) and M2 macrophage ($F4/80^+Arginase\text{-}1^+$, $F4/80^+CD163^+$) was observed. The numbers of $F4/80^+S1P_1^+$ macrophages of HIF-1α HET wounded tissues were significantly lower compared with WT tissues. S1P treatment of bone marrow macrophages (BMMs) significantly upregulated expression of $S1P_1$ in WT mice compared with HIF-1α HET. Phosphorylation of MAPK rapidly decreased in BMMs of HIF-1α HET mice than in BMMs of WT mice by S1P stimulation. Moreover, S1P enhanced HIF-1α expression via $S1P_1$ receptors to affect macrophage migration.

Conclusions: HIF-1α deficiency aggravates M1 and M2 macrophage infiltration and controls macrophage motility via S1P/$S1P_1$ signaling. These results suggest that HIF-1α

signaling may contribute to the regulation of palatal wound healing.

1. INTRODUCTION

After primary surgery for cleft palate, patients often exhibit growth impairment and a narrow collapse in the mid-facial region. These effects are often considered to be a consequence of palatal wound healing which involves wound contraction and scar formation (Ross, 1987). Wound healing is a site of inflammation and possible infection, and is characterized by low levels of oxygen for extended periods of time. Despite this hypoxic environment, macrophages accumulate in large numbers at sites of wound healing. It has been hypothesized that hypoxic conditions modulate the biological activities of macrophage (Murdoch, Muthana, & Lewis, 2005).

In most pathological situations, a hypoxic and inflammatory microenvironment activates hypoxia-inducible factors (HIFs) (Lin & Simon, 2016). Following activation, HIF forms a heterodimeric complex that consists of an α-subunit and a β-subunit. The α subunit mainly consists of HIF-1α or HIF-2α, and it primarily regulates HIF signaling. Meanwhile, the β-subunit is an aryl hydrocarbon receptor nuclear translocator (ARNT) protein. The HIF-1α subunit promotes inflammatory gene expression and can reprogram the metabolism of inflammatory cells. Correspondingly, HIF-1α is expressed in several types of innate immune cells (e.g., macrophages, neutrophils, and dendritic cells) (Corcoran & O'Neill, 2016; Cramer et al., 2003; Jantsch et al., 2008; Walmsley et al., 2005).

Activation of M1 or classic macrophages via HIF-1α leads to metabolic reprogramming involving pro-inflammatory mediators such as interleukin (IL)-1β, IL-6,

IL-12, and tumour necrosis factor (TNF)-α. Reactive oxygen and nitrogen species are generated and T cells are activated. In contrast, M2 macrophages or macrophages with an alternatively activated phenotype exhibit anti-inflammatory activities by producing activated transcription factors (STAT6, IRF6, CD206, CD163), specific chemokines (CCL17, CCL18), and Arginase-1 or -2 to limit nitric oxide (NO) production (Galvan-Pena & O'Neill, 2014; Krzyszczyk, Schloss, Palmer, & Berthiaume, 2018; Rius et al., 2008).

Macrophage cells are rapidly recruited to a wound site to infiltrate hypoxic tissue, initiate healing, and remove cellular debris. Wound sites exhibit marked hypoxia due to a lack of perfusion caused by vascular damage and the metabolic activity of inflammatory cells. In response to hypoxic conditions, macrophages alter their gene expression to induce a metabolic program of glycolysis and resistance to apoptotic stimuli (Cramer et al., 2003; Murdoch, Giannoudis, & Lewis, 2004). Previous studies have demonstrated a beneficial role for the hydroxylase inhibitor dimethyloxalylglycine (DMOG) in inflammatory conditions, including experimental colitis, by regulating the activity of HIF-1 and NF-κB (Cummins et al., 2008). DMOG treatment of mice promotes M2 polarization in macrophages within the peritoneal cavity, resulting in the downregulation of pro-inflammatory cytokines such as TNF-α (Hams et al., 2011). However, the mechanistic details of M1/M2 macrophage activation and functions under hypoxic conditions in the palatal wound tissue remain unclear. Further studies are needed to facilitate the development of novel targeted therapies for treatment of inflammatory diseases, especially delayed wound closure.

Sphingolipid metabolite sphingosine 1-phosphate (S1P), an essential bioactive lysophospholipid, is induced when tissue homeostasis is disrupted. It has recently been demonstrated that the S1P/S1P receptor system is a crucial regulator of immunity and controls immune cell trafficking (Cyster & Schwab, 2012; Spiegel & Milstien, 2011). S1P also plays a role in macrophage activation. During inflammation, S1P levels in affected tissues increase. As a result, macrophages are exposed to multiple signals from their environment. S1P production, S1P receptor expression, and signaling mechanisms are adjusted to achieve the needed output (Weigert, Olesch, & Brune, 2019).

Kalhori et al. (2013) investigated whether S1P serves as a non-hypoxic inducer of HIF-1α expression in thyroid cancer cells (Kalhori et al., 2013). Subsequently, Yang et al. (2015) reported the powerful migratory action of S1P on bone marrow macrophages (BMMs) via S1P receptor 2 ($S1P_2$) and $S1P_3$ in a cholestatic liver injury model (Kalhori et al., 2013; Yang et al., 2015). Hughes et al. (2008) also reported that during acute inflammation, S1P is able to switch the phenotype of macrophages from a pro-inflammatory phenotype to an anti-inflammatory phenotype via regulation of $S1P_1$ (Hughes et al., 2008).

In the present study, we demonstrate that wound closure in the palatal tissue of HIF-1α HET mice is delayed, and is accompanied by reduced expression of pro-inflammatory mediators and M1/M2 macrophage infiltration. We also provide evidence that S1P/$S1P_1$ signaling regulates the infiltration of macrophages into a wound site in HIF-1α HET mice.

2. MATERIALS AND METHODS

2.1 Mouse model

Twelve-week-old HIF-1α-deficient heterozygous (HIF-1α HET) mice and their wild-type (WT) littermates (C57BL/6J background: backcrossed more than ten times) for a total of 30 mice respectively were provided by Shuhei Tomita (Osaka City University) (Tomita et al., 2003). During the *in vivo* experiments performed, mice remained anaesthetised and all efforts were made to minimise their suffering. Mice were euthanised by cervical dislocation after CO_2 exposure. The Ethics Committee of the Tokushima University for Animal Research approved the experiments conducted for this study (approval number: T28-85).

2.2 Preparation of wound tissue

Mice were anesthetised with an intraperitoneal (i.p.) injection of sodium pentobarbital (25 mg/kg) in physiological saline prior to excision of tissue samples with a surgical blade. Excised tissue samples measured approximately 1.0 × 3.0 mm^2 and were obtained from the mid-hard palate between the upper molars as previously described (Jinno, Takahashi, Tsuchida, Tanaka, & Moriyama, 2009; Yoneda, Yasue, Watanabe, & Tanaka, 2013).

2.3 Reagents

Antibodies used in the present study included: TNF-α (#3707), Arginase-1 (#9819), phosphorylation (p) of -ERK (#4370), -Akt S473 (#9271), -p38 (#9216) (Cell Signaling Technology, Danvers, MA, USA), iNOS (#ab3523), CD68 (#ab955), CD163 (#ab182422), $S1P_3$ (#ab38324) (Abcam), $S1P_1$ (#10005228, Cayman Chemical, Ann

Arbor, MI, USA), F4/80 (Alexa Fluor 488 anti-mouse #123120, BioLegend, San Diego, CA, USA), and HIF-1α (#NB100-479, Novus Biologicals, Centennial, CO, USA). Antibodies recognizing β-actin (#A5441, Sigma Aldrich, St. Louis, MO, USA) and reagents, prolyl hydroxylase inhibitor dimethyloxalylglycine (DMOG) and sphingosine 1-phosphatase (S1P) (Cayman Chemical), were also purchased.

2.4 Histological analysis

Palatal tissues were prepared for histological analysis with a fixation step in 4% paraformaldehyde, followed by decalcification in 14% EDTA/PBS for 20 days. After dehydration, the tissues were embedded in paraffin. Serial sagittal sections were cut (HM360 microtome, Carl Zeiss, Jena, Germany) and then stained with hematoxylin and eosin (HE). A DAKO Envision kit (Glostrup, Denmark) was used for immunohistochemical staining with primary antibodies diluted in PBS/0.1% bovine serum albumin. After sections were incubated with primary antibodies overnight (4 °C), they were rinsed with PBS and then incubated with appropriate secondary antibodies for 1 h (at room temperature).

For immunofluorescent analysis, sections were double stained with first antibodies of anti-F4/80 with anti-TNFα or anti-iNOS for detecting M1 macrophages and anti-F4/80 with anti-Arginase-1 or anti-CD163 for detecting M2 macrophages. Corresponding Alexa Fluor anti-rabbit 488 (#4412) or anti-mouse 555 (#4409) were used as secondary antibodies (Cell Signaling Technology). Nuclei were stained with DAPI (#D212, Cellstain, Dojindo Molecular Technologies, Inc., Rockville, MD, USA). After the

sections were mounted, they were imaged with a fluorescence microscope BioRevo BZ-9000 and colocalization is visualized using color overlays (Pike, Styles, Rappoport, & Heath, 2017) BZ-II analyzer software (KEYENCE, Osaka, Japan). The quantification of positive-cells were analyzed using ImageJ software version 1.8.0 (NIH, Bethesda, MD; http://imagej.nih.gov/ij) (Wilkinson et al., 2019).

2.5 Western blot (WB) analysis

To prepare extracts for WB analysis, BMMs or palatal tissue samples were incubated with RIPA lysis buffer supplemented with a freshly prepared protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL, USA). The extracts were separated by SDS-PAGE and transferred to PVDF membranes. Proteins of interest were bound by corresponding primary and secondary antibodies. The latter included horseradish peroxidase-conjugated anti-mouse (#AP124P, Millipore, Billerica, MA, USA) and anti-rabbit (#7074, Cell Signaling Technology) antibodies. The LumiGLO Western Blot Detection System (#7003, Cell Signaling Technology) was used to visualise bound antibodies.

2.6 RT-PCR

ISOGEN (Nippon Gene, Tokyo, Japan) was used to extract total RNA from BMMs and palatal mucosa, according to the manufacturer's instructions. A NanoDropND-2000 instrument (NanoDrop Technologies, Wilmington, DE, USA) was used to estimate RNA concentrations. Samples with an optical density ratio (260 nm/280 nm) > 1.8 were

subjected to a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). In a 20-μL reaction mix containing 50 μM primers (Table 1), 10 ng cDNA, and 10 μL PowerSYBR Green PCR Master Mix, levels of *HIF-1α*, *Col1a1*, *MIP-1α*, *MCP-1, Sphk1, and* $S1P_{1\text{-}5}$ were measured with a 7500 Real Time PCR system (Applied Biosystems). Levels were normalised to GAPDH and quantitated according to the comparative cycle threshold method (ΔΔCt).

2.7 Cell culture of BMMs

BMMs were isolated and characterized as described previously (Muller, von Bernstorff, Heidecke, & Schulze, 2017; Takeshita, Kaji, & Kudo, 2000). In brief, cells flushed from the whole bone marrow of tibiae and femurs were collected in petri dishes and cultured in α-MEM containing: 10% heat inactivated fetal bovine serum (FBS, Japan Bioserum Co. Ltd., Fukuyama, Japan), 100 U/ml penicillin, 100 μg/ml streptomycin, and macrophage colony-stimulating factor (M-CSF; 20 ng/ml). Cells were incubated in an atmosphere containing 5% CO_2 kept at 37℃ for 3 d and were then washed with sterilized PBS and trypsinized with 1×trypsin/EDTA (Invitrogen). BMMs were counted using a hemocytometer and adjusted the concentration to 2×10^6 cells/mL and cultured in the same medium. BMMs were starved overnight before the following treatment including cell signaling experiment.

2.8 Migration assay

Chemotaxis was stimulated by S1P and DMOG in BMM cell cultures derived from HIF-1α HET and WT mice. Innocyte^{TM} Monocyte Cell Migration Assay kits (Calbiochem, Millipore, Temecula, CA, USA) were used according the manufacturer's directions. Pretreatment with inhibitors, including FTY720 (BioVision, San Francisco, CA, USA) and suramin (Sigma Aldrich), was performed 1 h prior to migration assays.

2.9 Statistical analysis

Experiments were independently repeated at least two or three times; and for each set of conditions, experiments were performed at least in triplicate. Statistical analyses were performed by applying one-way analysis of variance (ANOVA) with post-hoc Tukey's honest significant differences test or Student's *t*-test, as appropriate. P-values less than 0.05 were considered statistically significant.

3. RESULTS

3.1 HIF-1α regulates palatal wound healing

The hydroxylase activity of prolyl hydroxylase domain (PHD) proteins depends on the availability of O_2, Fe (II), and 2-oxoglutarate. The PHD inhibitor DMOG, a 2-oxoglutarate analogue, inhibits the interaction between 2-oxoglutarate analogue and PHDs, resulting in decreased PHD hydroxylase activity and stabilization of HIFs. To test our hypothesis that stabilization of HIF-1α proteins before palatal surgery augments wound healing, we first analyzed the effects of DMOG pretreatment in a murine palatal wound healing model. In a previous study, when DMOG (400 mg/kg body weight (b.w.))

was systemically administered 48 h prior to surgery, protection against ischemia was observed (Takaku et al., 2012). Therefore, we assessed whether DMOG can also accelerate palatal wound closure (Figure 1a). When mice (C56BL/6J) were pretreated with DMOG, palatal wound closure was effectively promoted (Figure 1b and c). Moreover, a significant decrease in gap distance was observed at the wound site in DMOG-treated mice compared to the control group (Figure 1d). The latter results are consistent with previous *in vivo* studies of skin flap survival (Takaku et al., 2012). To evaluate the role of HIF-1α in palatal wound closure, we excised palatal tissue from HIF-1α HET mice on days 4, 5, and 6 after wounding. The highest difference in percentage wound closure was observed on day 5. The gaps were observed with real-time images and histological analysis of HE stained sections (Figure 1e and f). Wound closure was found to be significantly delayed in a time-dependent manner and was gradually catching up on day 7 in the HIF-1α HET mice compared with WT littermates (Figure 1g). This reduction of wound healing in HET mice was significantly restored by DMOG pretreatment (Figure 1e, f, and g). Relative mRNA expression in palatal wound tissue samples was also analyzed. Expression levels of *HIF-1α*, *MCP-1*, *Col1a1*, and *MIP-1α* were significantly decreased in HIF-1α HET mice compared with WT mice (Figure 1h). Thus, HIF-1α appears to be essential for the predicted healing outcome marker, Col1a1, and also for sustained expression of MCP-1 and MIP-1α, two main components of monocyte recruitment.

3.2 HIF-1α affects the infiltration of pro-inflammatory M1 macrophage

To investigate how HIF-1α deficiency promotes delayed palatal wound closure, we examined critical features of macrophage biology which are important for initiating activation of foci inflammation in wound closure. As shown in Figure 2a, expression of TNF-α and iNOS, markers of pro-inflammatory mediators of M1 macrophage, were detected in an immunohistochemistry analysis. At the wound healing site, the numbers of cells expressing TNF-α and iNOS were significantly decreased in HIF-1α HET mice compared with WT mice (Figure 2b). Double immunofluorescent staining was also performed to detect protein expression of infiltrating M1 macrophages at day 5 (Figure 2c). There were significantly fewer numbers of M1 macrophage expressing $F4/80^{+}TNF\text{-}\alpha^{+}$ and $F4/80^{+}iNOS^{+}$ in HIF-1α HET mice than in WT mice (Figure 2c and d).

3.3 HIF-1α deficiency affects infiltration of anti-inflammatory M2 macrophages

To identify the effect of HIF-1α deficiency on anti-inflammatory M2 macrophages, M2 macrophage marker expressions were analyzed in palatal wound tissue. When expressions of $F4/80^{+}Arginase\text{-}1^{+}$ and $F4/80^{+}CD163^{+}$ macrophage were detected by immunofluorescence analyses of palatal wound sites of HIF-1α HET and WT mice (Figure 3a), the numbers of infiltrating M2 macrophage were significantly decreased in HIF-1α HET mice compared with WT mice (Figure 3b). Protein levels of Arginase-1, CD163, and CD68 confirmed by WB analysis were significantly lower in palatal wound tissue extracts prepared from HIF-1α HET mice than from palatal wound tissue extracts prepared from WT mice (Figure 3c).

3.4 S1P receptors expression in palatal wound closure tissues of HIF-1α HET mice

Macrophages need to migrate to an inflammation site to maintain wound healing homeostasis. S1P is produced by macrophage and it affects both their activation and migration. Moreover, S1P-dependent macrophage migration is tightly dependent on S1P receptors (Weigert et al., 2019). Therefore, we investigated the effect of S1P on palatal wound closure in HIF-1α-deficient macrophages. First, we examined mRNA levels of *Sphk1* and all five receptors of *S1P* in palatal tissues at the wound site. Interestingly, HIF-1α deficiency might be associated with decreased mRNA expression of the *S1P1* and *S1P3* receptors (Figure 4a). When double staining with F4/80 was performed to analyze both $S1P_1$ and $S1P_3$ expression respectively in palatal wounds of HIF-1α HET mice (Figure 4b), it was observed that $F4/80^+$ macrophages expressed $S1P_1$ were significantly low number in HIF-1α HET mice. On the other hand, $F4/80^+$ macrophages expressed $S1P_3$ were unchanged between WT and HIF-1α HET mice. Moreover, the quantification of cells positive for $F4/80^+S1P_1^+$ in HIF-1α HET tissues was significantly lower compared with WT tissues (Figure 4c).

3.5 Signal crosstalk between HIF-1α and S1P/$S1P_1$ system controls migration of BMMs

To elucidate the mechanism by which S1P regulates the responses and movements of macrophages in HIF-1α HET mice, we first isolated and cultured BMMs from femur samples with M-CSF treatment. After 3 days, BMMs were treated with S1P before mRNA

was extracted and analyzed. Increased transcription of *HIF-1α*, *$S1P_1$*, and *$S1P_3$* was detected after S1P treatment in WT macrophage. S1P-stimulated BMMs trigger an enhancement of *$S1P_1$* and *iNOS* expression only in WT mice. The levels of S1P induced expression were not significant for *$S1P_1$* and *iNOS* mRNA in HIF-1α HET macrophage. Meanwhile, the levels of S1P induced expression were not significant for *$S1P_2$, and $S1P_3$* mRNA in HIF-1α HET macrophage (Figure 5a). *Arginase-1* mRNA expression in HIF-1α HET macrophage after S1P stimulation was significantly lower compared with WT macrophage. WB to detect levels of HIF-1α and phosphorylated forms of ERK, Akt, and p38 showed a time-dependent increase in S1P-induced HIF-1α activity of WT BMMs. In contrast, S1P treatment of HIF-1α HET BMMs did not upregulate expression of these proteins (Figure 5b). Finally, macrophage migration experiments were conducted with WT and HIF-1α HET BMMs which were treated with S1P, DMOG, FTY20, or suramin (Figure 5c). DMOG and S1P stimulation increased the migration of WT BMMs. FTY720 pretreatment inhibited the migration of S1P stimulated WT BMMs. In contrast, suramin pretreatment unchanged the migration rates of S1P induced macrophage migration. These findings indicate that S1P enhances HIF-1α expression via $S1P_1$ receptors to affect macrophage migration.

4. DISCUSSION

The results of the present study demonstrate that HIF-1α plays an essential role in wound closure and macrophage infiltration following palatal injury in HIF-1α HET mice. Previously, DMOG-treated mice promoted expression of HIF-1α in the skin flap with a

reduction in apoptotic cells and induction of endothelial and bone marrow progenitor cells. It was confirmed that HIF-1α HET mice, unlike WT mice, do not preserve an ischemic skin flap (Takaku et al., 2012). Thus, accelerated palatal wound closure observed in DMOG-treated mice is consistent with delayed palatal wound closure due to HIF-1α deficiency in HIF-1α HET mice. This delayed palatal wound healing in HET mice was also significantly restored by DMOG pretreatment (Figure 1). A detailed analysis of this evidence in oral mucosa remains to be performed since it has been observed that wound healing in oral mucosa and skin proceeds through similar stages. Oral mucosa wounds heal more rapidly with minimal complications or scar formation compared with skin wounds (Iglesias-Bartolome et al., 2018). Moreover, mucosa wounds heal under significantly decreased hypoxic conditions compared to skin wounds (Chen, Gajendrareddy, & DiPietro, 2012; Szpaderska, Walsh, Steinberg, & DiPietro, 2005).

In murine wound models, macrophages rapidly accumulate at the wound site to remove cellular debris, activate revascularization, and repair the wound. Initially, neutrophils infiltrate a wound within 24 to 48 h after the wound is established. Monocytes are subsequently recruited to the wound site (2–5 days after wounding). During this later stage, macrophage accumulation is significant (Stewart, Duley, Rosman, Fraser, & Allardyce, 1981). In the present study, the most significant gap distance in the palatal wounds was observed on day 5 after wounding (Figure 1). This evidence suggests that HIF-1α is likely to affect the cellular metabolism of infiltrating macrophages in the later stages of a wound site.

In HIF-1α HET palatal wound tissue, expression levels of *Col1a1*, *MIP-1α*, and *MCP-*

1 were decreased (Figure 1). Previously, mutant collagen mice (Col1a1$^{r/r}$) exhibited severely delayed wound closure due to a prolonged early response of inflammatory cells which was attributed to delayed re-epithelialization (Beare, O'Kane, Krane, & Ferguson, 2003). Meanwhile, wound healing in MCP-1$^{-/-}$ mice, yet not in MIP-1α$^{-/-}$ mice, has revealed a significant delay in collagen synthesis and wound re-epithelialization (Low et al., 2001). In a study of alveolar hypoxia, enhanced expression of HIF-1α, TNF-α, MCP-1, MIP-1β, and albumin was triggered by macrophage recruitment (Madjdpour et al., 2003). Taken together, these results suggest that HIF-1α regulates the primary interstitial collagen, Col1a1, as well as two potent mediators of macrophage movement and recruitment in palatal wound healing, MIP-1α and MCP-1.

Our immunohistochemistry analyses demonstrate that expression of TNF-α and iNOS in the HIF-1α HET mice palatal wound margin is attenuated (Figure 2). These data are consistent with the reduced inflammatory phenotype of HIF-1α-deficient macrophage (Aarup et al., 2016). Expression of surface markers, CD68, Arginase-1, and CD163, detected in WB analyses were found to be diminished in HIF-1α HET mice (Figure 3). These results are consistent with the strong relationship observed between CD68 and HIF-1α in tumour tissue, with low levels of HIF-1α associated with significantly fewer CD68^{+} macrophage (Li et al., 2016). Thus, it appears that macrophage infiltration into palatal wound closure is HIF-1α–dependent.

To be more precise, we further investigated whether HIF-1α affects the phenotype of M1/M2 macrophage. Macrophages were detected in wound tissue sections on day 5 by staining for the macrophage marker, F4/80 (Biswas, Tapryal, Mukherjee, Kumar, &

Mukhopadhyay, 2013). Immunohistofluorescent analyses demonstrated that palatal wounds in HIF-1α HET mice led to dysregulation of macrophage function, with fewer macrophages recruited to the wound site and down-regulation of $F4/80^{+}TNF\text{-}\alpha^{+}$, $F4/80^{+}iNOS^{+}$, $F4/80^{+}Arginase\text{-}1^{+}$, and $F4/80^{+}CD163^{+}$. HIF-1α participates in M1 polarization of macrophage through the induction of glycolysis metabolism, while M2 macrophage markers remain unaffected (Ke et al., 2019). In contrast, *in vitro* studies have revealed that in HIF-1α-null macrophages, M1-characteristic genes are diminished; yet M2-characteristic genes are unchanged (Aarup et al., 2016). During the wound healing process, macrophages are highly dependent on HIF-1α expression, which is accompanied by a decrease in pro-inflammatory cytokines. HIF-1α also affects macrophage metabolism by disrupting phenotypic polarization. In the late wound stage, the M2 macrophage phenotype (CD163 and Arginase-1) is also less frequent (Figure 3). These results suggest that in addition to common macrophage chemokines, other receptors may influence macrophage movement, such as the transmembrane lipid receptor, S1P.

Several studies have shown that hypoxia increases expression of Sphk1 and S1P. In human cancer cells, Sphk1 regulates hypoxia-induced stabilization of HIF-1α via Akt and GSK3β (Ader, Brizuela, Bouquerel, Malavaud, & Cuvillier, 2008). Meanwhile, S1P increases HIF-1α activity and expression in ML-1 thyroid cancer cells, while HIF-1 mediates S1P-induced cell migration (Kalhori et al., 2013). S1P has also been shown to regulate HIF-1α transcription in mouse T cells (Srinivasan et al., 2008) and macrophages (Herr et al., 2009). Since the ability of macrophages to infiltrate a wound site is strongly influenced by their migration capacity, we analyzed S1P expression in macrophages that

infiltrated to the wound site.

Sphk1 and all five S1P receptors were detected in the palatal wound tissues examined. However, palatal wound tissue in the HIF-1α HET mice exhibited the reduction of *S1P1* and *S1P3* mRNA levels. It is known that S1P provokes HIF-1α responses. For example, in macrophages, activation of $S1P_1$ and/or $S1P_3$ triggers HIF-1α expression (Herr et al., 2009). Our protein analysis of $F4/80^+S1P_1^+$ but not $F4/80^+S1P_3^+$ shows that in HIF-1α HET tissue, decreased activation of $S1P_1$ receptor occurred by post transcriptional regulation. We also observed that $S1P_1$ expression was detected in cells other than F4/80 macrophage in HIF-1α HET palatal wounds (Figure 4). Further experiments are needed to analyze the impact of S1P on T-cells during the late stages of palatal wound closure. It has been demonstrated that S1P acts through $S1P_1$ and HIF-1α to negatively regulate T-cell activation (Srinivasan et al., 2008). Furthermore, T-cells that lack HIF-1α exhibit enhanced pro-inflammatory cytokine expression (e.g., IFN-γ) (Lukashev et al., 2006).

It has been reported that S1P influence on macrophage polarization (Muller et al., 2017). In this study, S1P stimulated BMMs trigger a significant enhancement of *S1P1* and *iNOS* expression only in WT mice. The levels of S1P induced expression were not significant for *S1P1* and *iNOS* mRNA in HIF-1α HET macrophage. On the other hand, mRNA expression of *Arginase-1* after S1P stimulation was significantly lower in HIF-1α HET macrophage (Figure 5). It has also been shown that HIF-1α induce M1 macrophages polarization (Takeda et al., 2010). This activation in macrophages subsequently limits the expression of pro-inflammatory cytokines (Hughes et al., 2008). These results suggest that signaling crosstalk between S1P/$S1P_1$ and HIF-1α might control macrophage

polarization. We further demonstrate that S1P regulates HIF-1α via activation of the MAPK phosphatases; ERK, Akt, and p38. It has previously been shown that inhibition of p38 reduces pro-inflammatory cytokines under hypoxic conditions (Sakiyama et al., 2003), and hypoxia-induced p38 phosphorylation is partially dependent on HIF-1α expression (Ke et al., 2019). Cell migration in relation to HIF-1α and S1P/S1P receptor signaling was also characterized with use of the S1P receptor agonist, FTY720 and the $S1P_3$ antagonist, suramin. We observed that DMOG and S1P affect BMM migration via $S1P_1$. Taken together, these results suggest that crosstalk between HIF1α and S1P/ $S1P_1$ signaling has a regulatory function in the migration of macrophages, and further study of this function is warranted.

In conclusion, the results of this study provide an analysis of macrophage infiltration during a delayed palatal wound healing under hypoxia. To the best of our knowledge, this is the first study to provide such evidence. In addition, we demonstrate that HIF-1α controls the bifurcation of anti-inflammatory and pro-inflammatory M1/M2 macrophages and the motility of macrophage via the S1P/$S1P_1$ system.

ACKNOWLEDGEMENTS

This work was supported by the grants provided by JSPS KAKENHI (Grant Numbers. 17K19758, 18H03011, 18KK0464 to T.I.), The Nakatomi Foundation, Smoking Research Foundation, Takeda Science Foundation, to T.I., and LPPM Sultan Agung Islamic University to I.H.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS CONTRIBUTIONS

Study design: IH, TI, ST, and ET
Data acquisition: IH, TK-O, TS, AI, and TI
Data analysis: All authors
Writing of first draft: IH, TI, and ET
Manuscript revision and approval of final manuscript: All authors

FIGURE LEGENDS

FIGURE 1. Effects of DMOG and HIF-1α deficiency on palatal wound healing. (a) C57BL/6J mice were randomly assigned to a control group or an experimental group. The latter received an intraperitoneal (i.p.) injection of DMOG (400 mg/kg body weight (b.w.)). Palatal wound healing was evaluated on day 5 after a palatal excisional wound approximately 1.0 mm in width was made in the hard palate. (b) Images of palatal wound closure obtained at day 0 and day 5. Representative images of three to five mice in each group are shown. (c) HE staining was performed to analyze wound closure distance at day 0 and day 5. Representative images show three to five mice of each group. (d) The wound closure distances of palatal tissues at day 5 in WT and HIF-1α HET mice (μm). The results represent the mean ± SD of three to five mice from each group. (e) Representative images of palatal wounds at day 5 after surgery. Representative images of three to five mice in each group are shown. (f) Representative histological sections stained

with HE at day 0 and day 5. Representative images show three to five mice of each group. (g) The ratio of wound closure to original wound gap for palatal tissues at indicated time-points in WT and HIF-1α HET mice with or without DMOG injection. The results represent the mean ± SD of three to five mice from each group. (h) Total mRNA was collected 5 days after palatal surgery. Expression levels of *HIF-1α*, *Col1a1*, *MCP-1*, and *MIP-1α* were measured. Data are presented as mean ± SD of three to five mice from each group. Scale bar: 100 μm. **p < 0.01, *p < 0.05.

FIGURE 2. Expression profiles of M1 macrophages marker in palatal wound healing of HIF-1α HET mice. (a, b) Representative immunohistochemical images and quantification of M1-related markers, TNF-α- and iNOS-, in the outer perimeter of a wound site. Representative images and quantifications of three to five mice in each group are shown. (c) Representative double stained immunohistofluorescent images of M1 macrophage-related markers in a palatal wound area. Wounded sections were stained with F4/80 (green) for TNF-α (red) or iNOS (red) and counterstained with DAPI (blue) respectively. Representative images of three to five mice in each group are shown. (d) The numbers of $F4/80^{+}$ macrophages and positive for TNF-α or iNOS in palatal wound areas. Data are presented as mean ± SD (4–5 sections from 3–5 individual animals for each group). Scale bar: 100 μm. **p < 0.01.

FIGURE 3. Expression of M2 macrophages related markers in palatal wound healing of HIF-1α HET mice. (a) Representative double stained immunohistofluorescent images of

M2 macrophage-related markers in palatal wound areas. Wounded sections were stained with F4/80 (green) for Arginase-1 (red) or CD163 (red) and counterstained with DAPI (blue) respectively. Representative images and quantifications of three to five mice in each group are shown. (b) The numbers of $F4/80^+$ macrophages and positive for Arginase-1 or CD163 in palatal wound areas. Data are presented as mean ± SD (4–5 sections from 3–5 individual animals for each group). (c) WB analysis of Arginase-1, CD163, and CD68 from palatal wound tissues at day 5. Scale bar: 100 μm. **p < 0.01 Samples are from three to five individual animals for each group.

FIGURE 4. Expression of Sphk1 and S1P receptors in palatal wound healing of HIF-1α HET mice. (a) Relative mRNA expression in palatal tissue was determined at day 5 after surgery. Data are presented as mean ± SD (4–5 sections from 3–5 individual animals for each group). (b, c) Representative double stained immunohistofluorescent images of F4/80 macrophages (green) expressing $S1P_1$ (red) or $S1P_3$ (red) and counterstained with DAPI (blue) in palatal wound areas. Positive cell numbers are indicated. Representative images of three to five mice in each group are shown. Quantification data are presented as mean ± SD. Scale bar: 100 μm. **p < 0.01, *p < 0.05.

FIGURE 5. Effect of HIF-1α deficiency on expression of S1P receptors and motility in BMMs. (a) Relative mRNA expression of *HIF-1α*, *$S1P_1$*, *$S1P_2$*, *$S1P_3$*, *iNOS* and *Arginase-1* in BMMs stimulated with or without S1P (10 μM). Data are presented as mean ± SD of

three to five mice from each group. (b) A time course WB analysis of phosphorylated forms and total of ERK, Akt, and p38 in WT and HIF-1α HET BMMs. The BMMs were cultured with M-CSF (20 ng/mL) for 3 d, were starved, and then were stimulated with S1P (10 μM) for an additional 0–30 min. HIF-1α, β-actin were exposed as loading controls. Samples are from three to five individual animals for each group. (c) The percentage of migrating BMMs from WT and HIF-1α HET mice which were incubated with DMOG alone or S1P alone or were pretreated for 1 h with FTY720 or suramin. Data are presented as the mean ± SD of three to five mice from each group. $^{**}p < 0.01$, $^{*}p < 0.05$.

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TABLE 1. Primer sequence used for RT-PCR

Gene	Sense	Antisense
HIF-1α	5’-GTCACCTGGTTGCTGCAATA-3’	5’-CATGATGGCTCCCTTTTTCA-3’
Col1a1	5’-CCACGTCTCACCATTGGGG-3’	5’-GCTCCTCTTAGGGGCCACT-3’
MCP-1	5’-GCATTAGCTTCAGATTTACGGGT-3’	5’-TAAAAACCTGGATCGGAACCAAA-3’
MIP-1α	5’-CAACGATGAATTGGCGTGGAA-3’	5’-TGTACCATGACACTCTGCAAC-3’
Sphk1	5’-GCTTCTGTGAACCACTATGCTGG-3’	5’-ACTGAGCACAGAATAGAGCCGC-3’
$S1P_1$	5’-CGCAGTTCTGAGAAGTCTCTGG-3’	5’-GGATGTCACAGGTCTTCGCCTT-3’
$S1P_2$	5’-TGTTGCTGGTCCTCAGACGCTA-3’	5’-AGTGGGCTTTGTAGAGGACAGG-3’
$S1P_3$	5’-GCTTCATCGTCTTGGAGAACCTG-3’	5’-CAGAGAGCCAAGTTGCCGATGA-3’
$S1P_4$	5’-GTGTATGGCTGCATCGGTCTGT-3’	5’-GAGCACATAGCCCTTGGAGTAG-3’
$S1P_5$	5’-AGACTCCTCCAACAGCTTGCAG-3’	5’-TAGAGCTGCGATCCAAGGTTGG-3’
iNOS	5’-GAGACAGGGAAGTCTGAAGCAC-3’	5’-CCAGCAGTAGTTGCTCCTCTTC-3’
Arginase-1	5’-CATTGGCTTGCGAGACGTAGAC-3’	5’-GCTGAAGGTCTCTTCCATCACC-3’
GAPDH	5’-GGTCCTCAGTGTAGCCCAAG-3’	5’-TGCCCAGAACATCATCCCT-3’