Effect of lipopolysaccharide stimulation on stem cell-associated marker-expressing cells in rat incisors

Y. Sueyama [1], T. Kaneko [2], T. Ito [1] & T. Okiji [2]

[1] Division of Cariology, Operative Dentistry and Endodontics, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan [2] Pulp Biology and Endodontics, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan

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Correspondence: Tomoatsu Kaneko, Pulp Biology and Endodontics, Tokyo Medical and Dental University (TMDU), Yushima 1-5-45, Bunkyo-Ku, Tokyo 113-8549, Japan (Tel: +0081-3-5803-5494; e-mail: tomoendo@tmd.ac.jp)

Abstract

Aim To examine the effect of inflammatory stimuli on the proliferation/migration of dental pulp stem cells by assessing the responses of stem cell-associated marker-expressing cells in rat incisors to lipopolysaccharide (LPS) stimulation *in vivo*.

Methodology The crowns of rat incisors were removed, and the coronal pulp chamber was instrumented. After haemostasis, an absorbent point soaked in LPS was inserted into the cavity, which was then sealed. At 3 h, 12 h, and 48 h after LPS application, pulp tissues were subjected to double-immunoperoxidase labeling using two of the antibodies against microtubule-associated protein 1B (MAP1B), CD146, and STRO-1. For gene expression analysis, total RNA was extracted, and mRNA expression levels of stem cell factor (SCF), stromal-derived factor 1 (SDF-1), CD146, and MAP1B were analyzed with real-time polymerase-chain reaction. SCF and SDF-1 protein levels were also assessed by western blot. Statistical analysis was performed by Kruskal-Wallis non-parametric analysis of variance, followed by Mann-Whitney U test with Bonferroni correction.

Results The density of $MAP1B^{+}CD146^{+}$ cells and $STRO\text{-}1^{+}CD146^{+}$ cells in LPS-stimulated pulp tissue increased significantly at 3 h and exhibited a 4- to 6-fold increase at 48 h as compared with their density observed in normal pulp tissue ($P < .05$). The expression of CD146 mRNA in LPS-stimulated pulp showed significant upregulation at 3 h as compared with that observed in normal pulp tissue ($P < .05$). MAP1B, SCF, and SDF-1 mRNA levels also showed significant upregulation at 3 h and 72 h ($P < .05$), and western blot analysis revealed increases in SCF and SDF-1 following LPS stimulation.

Conclusions LPS-stimulated pulp tissue exhibited upregulation of stem cell-differentiation/migration markers and showed increases in the number of

MAP1B^{+}CD146^{+} and STRO-1^{+}CD146 stem-like cells.

Introduction

Dental pulp tissue harbours mesenchymal stem cell (MSC)-like cell populations. There are two major cell populations in the dental pulp that are capable of self-renewal and possess differentiation potential: dental pulp stem cells, which were first isolated from human third molars (Gronthos *et al*. 2000), and stem cells from human exfoliated deciduous teeth (Miura *et al*. 2003). Despite advances in dental stem cell research, *in vivo* characteristics of stem cells in the dental pulp remain unclear.

Dental pulp tissue plays a crucial role in tooth homeostasis by conferring pulpul vitality and controlling pulp-defense functions (Tatullo *et al*. 2014). Therefore, the conservation of pulp tissue is critical for tooth longevity (Caplan 1991). Clinically, root canal treatment is performed on the majority of teeth exhibiting irreversible pulpitis to remove whole pulp tissue, although a large portion of the pulp remains viable (Barthel *et al*. 2000). Such treatment causes significant dentine loss and might increase the risk of tooth fracturing (Trope *et al*. 1986, Oliveira *et al*. 1987), which inevitably results in tooth extraction (Touré *et al*. 2011). If lost pulp tissue could be regenerated, extraction could be prevented. Therefore, dental pulp regeneration using stem cells with or without biomaterials is considered an important strategy. Furthermore, if autologous dental pulp stem cells can be obtained from these inflamed pulp tissues for re-transplantation into the same tooth, this would be optimal for dental pulp regeneration. However, the effect of pulp inflammation on dental pulp stem cells remains unclear and should be examined, because inflammation could adversely influence the differentiation potential of human MSCs (Gilbert *et al.* 2000).

Detection of the localization of stem cells in a tissue may pose challenges, because they comprise a rare population and lack markers exclusively specific to these cells. In this regard, we recently immunolocalized stem-like cells co-expressing microtubule-associated protein 1B (MAP1B) and CD146 in rat dental pulp tissue (Kaneko *et al*. 2013). MAP1B belongs to a family of proteins that govern the dynamic state and organization of microtubules within cells (Halpain & Dehmelt 2006). Upregulation of MAP1B expression is related to the motility and proliferation of neural cells. MAP1B is expressed in undifferentiated human MSCs (Montzka *et al*. 2009), and bone-marrow MSCs from rats and humans are able to differentiate into neurons *in vitro* (Woodbury *et al*. 2000). CD146 expression constitutes a differentiation-potency marker in human MSCs, and it was reported that differentiation potency increases along with elevations in CD146 expression in human MSCs. Another well-known marker for human MSCs is STRO-1 (Shi & Gronthos 2003, Ning *et al.* 2011) STRO-1-expressing human adult dental pulp stem cells are able to differentiate into neuronal cells (Arthur *et al*. 2008). Therefore, it is reasonable to assume that the $MAP1B^{+}CD146^{+}$ and $STRO\text{-}1^{+}CD146^{+}$ cells from dental pulp tissue may represent populations of dental pulp stem cells, making double labeling useful for examining the effects of inflammation on dental pulp stem cells.

The purpose of this study was to examine the effects of inflammatory stimuli on the proliferation/migration of dental pulp stem cells by assessing the responses of stem cell-associated marker-expressing cells in rat incisors to lipopolysaccharide (LPS) stimulation *in vivo*. In addition to double-labeling immunohistochemistry to detect $MAP1B^{+}CD146^{+}$ and $STRO\text{-}1^{+}CD146^{+}$ cells, a quantitative gene-expression assay was performed to assess *CD146* and *MAP1B* mRNA expression in the pulp. Moreover, to investigate the mechanisms behind stem cell recruitment, mRNA and protein levels of

stem cell factor (SCF) and stromal-derived factor 1 (SDF-1) were assesed, which are major homing factors that have recently emerged as being important in the field of regenerative medicine (Urbich C *et al*. 2005, Kuang *et al*. 2008, Zaruba & Franz 2010).

Materials and methods

Sample preparation

All experiments were conducted in accordance with and following the approval of the Animal Care Committee, Niigata University. Five-week-old male Wistar rats (Charles River, Yokohama, Japan; $n = 52$) were used for all experiments.

Following intraperitoneal administration of 8% chloral hydrate anaesthesia (350 mg/kg), the crowns of the right maxillary incisors were removed at the gingival margin, and the coronal pulp chamber was instrumented to size 40 and to a 7-mm length using stainless steel files. After haemostasis, an absorbent point cut to 5-mm length and soaked in LPS (*Escherichia coli* 0111:B4; 10 mg/mL) was inserted into the cavity, which was then filled with a temporary filling material (Caviton; GC, Tokyo, Japan).

Prior to histological analysis, animals were euthanized by anaesthetic overdose at 3 h, 12 h, and 48 h ($n = 4$ teeth for each time point) after LPS application. Normal pulp tissues ($n = 4$ teeth) served as controls. The maxillary incisor pulp tissues were removed, fixed in periodate lysine paraformaldehyde for 3 h, and embedded as frozen blocks. Eight-micrometer-thick sections were cut in a cryostat (CM1900; Leica, Wetzlar,

Germany).

Double-labeling immunohistochemistry

Prior to incubation with antibodies, sections were reacted with 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 15 min at 4°C to block endogenous peroxidase activity. Sections were then incubated with a primary monoclonal antibody against MAP1B (Novus Biologicals, Littleton, CO, USA; diluted 1:100) or STRO-1 (eBioscience, San Diego, CA, USA) for 2 h at room temperature. After washing with PBS, the sections were sequentially reacted with biotinylated horse anti-mouse IgG (rat adsorbed; Vector Laboratories, Burlingame, CA, USA; 1 h at room temperature) and the avidin-biotin-peroxidase complex (Elite ABC kit; Vector Laboratories; 30 min at room temperature). The sections were then developed with the EnVision+ system horseradish peroxidase (gray-black stain; Dako, Glostrup, Denmark) for MAP1B staining and the NovaRED substrate (red stain; Vector Laboratories) for STRO-1 staining. The stained sections were further incubated sequentially with a rabbit CD146 monoclonal antibody (GeneTex, Irvine, CA, USA; diluted 1:300), a biotinylated goat anti-rabbit IgG (Vector Laboratories), and the avidin-biotin-peroxidase complex. The CD146 immunoreaction was visualized with the Vina Green chromogen kit (green stain; Biocare Medical, Inc., Concord, CA, USA) for MAPIB-stained sections and a diaminobenzidine-H_2O_2 solution

substrate kit with nickel chloride enhancement (gray-black stain; Vector Laboratories) for STRO-1-stained sections.

Control staining was consistently performed in parallel by the omission of either or both of the primary antibodies.

For quantitative analysis, three typical sections selected at intervals of 10 serial sections were chosen from each specimen (n = 4 teeth for each time point). MAP1B/CD146 and STRO-1/CD146 double-stained cells were enumerated beneath the abscesses of the exposure site under a light microscope with the aid of a 10 mm × 10 mm ocular grid. To calculate the density of double-stained cells, digital pictures of the sections in the area surrounding the LPS-stimulated region (0–100 μm from the LPS-application site) were collected and stored as JPEG files. The area corresponding to each region in each specimen was determined using ImageJ software version 1.37 (National Institutes of Health, Bethesda, MD, USA).

Real-time polymerase chain reaction (PCR)

Gene-expression analysis was performed on LPS-stimulated and saline-treated pulp tissues from each of the three time points (n = 4 teeth for each time point). Normal pulp tissues were also analyzed (n = 4 teeth for each time point). After LPS application as described above or saline application instead of LPS, each pulp tissue was removed, and

the coronal portion ~1.0 mm from the LPS-application site was retrieved under a microscope. Each tissue was collected in an individual tube and then immersed in a storage reagent (RNAlater; Qiagen, Valencia, CA, USA) at 4°C.

Total RNA was extracted and purified from retrieved pulp samples as previously described (Kaneko *et al*. 2007). First-strand cDNA synthesis was performed using TaqMan reverse-transcription reagents (Applied Biosystems, Carlsbad, CA, USA). Probe and primer sets [Rn00576900_m1: CD146 (MCAM); Rn01494211_m1: MAP1B; Rn01502851_m1: KIT ligand (SCF); Rn00573260_m1: chemokine (C-X-C motif) ligand 12 (SDF-1); and Rn01775763_g1: glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] were obtained from Applied Biosystems. Total RNA at 1 μg/100 μL of reaction mixture was prepared using TaqMan universal master mix II with UNG (Applied Biosystems). The reactions were performed in a 48-well clear optical-reaction plate using the StepOne sequence-detection system (Applied Biosystems), and data were normalized to *GAPDH* levels. All reactions were performed in triplicate, and three independent experiments were performed to verify the reproducibility of results.

Western blot analysis

Western blot analysis was performed on LPS-stimulated and control pulp tissues from each of the three time points (n = 4 teeth for each time point). Each pulp sample was

retrieved and collected in an individual tube as mentioned above and resolved by polyacrylamide gel electrophoresis. The membranes were probed overnight at 4°C with anti-SCF (Signalway Antibody, College Park, MD, USA), anti-SDF-1 (Bioworld Technology, Inc., St. Louis Park MN, USA), or anti-GAPDH primary antibodies (GeneTex). Blots were exposed to appropriate peroxidase-coupled secondary antibodies, and proteins were visualized with enhanced chemiluminescence (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Levels of protein expression were compared following normalization against GAPDH expression.

Statistical analysis

Statistical analysis was performed by Kruskal-Wallis non-parametric analysis of variance, followed by Mann-Whitney U test with Bonferroni correction. The level of significance was set at $P < 0.05$.

Results

MAP1B$^+$CD146$^+$ and STRO-1$^+$CD146$^+$ cell localization in LPS-stimulated rat dental pulp tissue

After double labeling MAP1B (brown) and CD146 (green), MAP1B and CD146 expression were mainly observed on nerve fibres and blood vessels, respectively (Fig. 1e–h). MAP1B$^+$CD146$^+$ cells were predominantly detected as small and round cells distributed in the vicinity of blood vessels (Fig. 1e–h, m). In the double labeling of

STRO-1 (red) and CD146 (grey), STRO-1 expression was mainly observed in blood vessels (Fig. 1i–l, n) and some odontoblasts (data not shown). STRO-$1^{+}$$CD146^{+}$ cells predominantly showed a small and round shape and were detected in the vicinity of blood vessels (Fig. 1i–l, n). The density of $MAP1B^{+}$$CD146^{+}$ cells and STRO-$1^{+}$$CD146^{+}$ cells increased significantly ($P < 0.05$) from 3 h onwards and showed a ~4- and ~6-fold respective increase at 48 h following LPS stimulation as compared with the densities observed in normal pulp tissue (Fig. 1o, p).

Upregulated mRNA and protein expression in LPS-stimulated rat dental pulp tissue

CD146 mRNA levels in LPS-stimulated pulp tissues showed significant upregulation at 3 h after LPS stimulation as compared with levels observed in normal and control (saline-treated) pulp tissues ($P < 0.05$; Fig. 2a). At 48 h, there was no significant difference between *CD146* mRNA levels in LPS-stimulated pulp tissue and those observed in control (saline-treated) pulp tissue ($P > 0.05$). *MAP1B* mRNA levels in LPS-stimulated pulp tissues showed significant upregulation at 3 h and significantly higher levels at 12 h and 48 h after LPS stimulation as compared with levels observed in normal and control (saline-treated) pulp tissues ($P < 0.05$; Fig. 2b).

Expression levels of *SCF* and *SDF-1* mRNA showed significant and continuous increases up to 48 h after LPS stimulation as compared with levels observed

in normal and control (saline-treated) pulp tissue ($P < 0.05$; Figs. 2c and d).

Western blot analysis showed bands corresponding to approximate molecular weights of 40 kDa, 10 kDa, and 36 kDa in lysates exposed to SCF, SDF-1, and GAPDH antibodies, respectively. SCF and SDF-1 expression gradually increased continuously up to 48 h after LPS stimulation as compared with normal pulp tissue (Fig. 3).

Discussion

LPS has been widely applied to examine the inflammatory reaction of cells and/or tissues (Russo & Lutton 1977) and is a potent inducer of pulpitis (Warfvinge *et al*. 1985). LPS-stimulated dental pulp cells induce the expression of matrix metalloproteinases (MMP-2 and MMP-9) and various cytokines, such as interleukin (IL)-6 and IL-1β, which lead to host-tissue destruction (Hosoya & Matsushima 1997, Lee *et al*. 2011). However, the behaviour of stem cells in dental pulp tissue in response to inflammatory stimuli remains unclear, although MSCs are known to actively respond to stress or injury in a manner similar to how adaptive- and innate-immune cells, such as macrophages, respond to pathogen exposure or apoptosis (Dimarino *et al*. 2013). Therefore, in this study, the proliferation/recruitment of $MAP1B^{+}CD146^{+}$ and $STRO\text{-}1^{+}CD146^{+}$ cells in dental pulp tissue to LPS stimulation were examined *in vivo*. The results showed that *CD146* and *MAP1B* mRNA expression in pulp tissues was

significantly upregulated following LPS stimulation, with significant increases in the density of both MAP1B^{+}CD146^{+} and STRO-1^{+}CD146^{+} cells. These findings suggested that recruitment and/or proliferation of stem cells takes place following LPS-stimulation in the rat incisor pulp.

It has recently been reported that stem-like cells co-expressing MAP1B and CD146 are distributed in the dental pulp of rat molars (Kaneko *et al*. 2013). In this study, rat incisors were investigated, because time-course tissue reaction following LPS stimulation is well investigated histologically as compared with molars (Kawanishi *et al*. 2004), and confirmed the presence of stem-like cells in the incisors. Notably, expression of *CD146* mRNA in the pulp tissue was upregulated at 3 h after LPS application when the pulp inflammation was at its initial stage (Kawashima *et al.* 2005). Subsequently, the density of both MAP1B^{+}CD146^{+} and STRO-1^{+}CD146^{+} stem-like cells increased gradually, whereas *CD146* mRNA expression in the pulp tissue decreased over the course of 48 h. At 48 h, pulp inflammation had almost ceased, and *CD146* mRNA expression reached a level comparable with that in normal pulp tissue. CD146 is mainly expressed in endothelial cells, and LPS-stimulated endothelial cells exhibit increased release of growth factors, such as vascular endothelial growth factor (VEGF) (Marx *et al*. 1999), which is important to stem cell activation and proliferation (Breier *et al*. 1992). VEGF-activated stem cells subsequently release additional growth factors to

further induce proliferation (Pedersen *et al*. 2014). In humans, these growth factors are known to stimulate endothelial progenitor cells, with the activated cells further producing growth factors, such as SCF, SDF-1, and nerve growth factor (Peplow 2014). Combinations of these growth factors, including VEGF, SCF, and SDF-1, subsequently reduce apoptosis, increase cell migration, and induce the formation of new blood vessels (Peplow 2014). The present results suggest that the increased expression of CD146 in the inflamed pulp tissue contributed to the continuous upregulation of *SDF-1* and *SCF* mRNA, which might be a mechanism involved in the recruitment and proliferation of stem-like cells.

MAP1B belongs to a family of microtubule-associated proteins that govern the dynamic state and organization of microtubules within cells (Halpain & Dehmelt 2006). Several reports suggested that MAP1B is involved in neuronal differentiation by regulating microtubule dynamics in the growing axon (Riederer *et al*. 1986, Pedrotti & Islam 1995). Therefore, MAP1B antibodies are normally used as a neuronal-fibre marker. MAP1B is expressed in odontoblasts (Bleicher *et al*. 2001, Maurin *et al*. 2005), and a recent study showed that MAP1B is also expressed in undifferentiated human MSCs (Montzka *et al*. 2009). Furthermore, bone marrow MSCs from rats and humans are capable of differentiating into neurons *in vitro* (Woodbury *et al*. 2000). Notably, the present results indicate upregulation of *MAP1B* mRNA following LPS stimulation,

which could potentially be involved in promoting stem cell proliferation.

STRO-1 was originally reported as a cell-surface marker to identify stromal cell precursors in human bone marrow (Simmons & Torok-Storb 1991). STRO-1 antibody has been used for the recognition and isolation of various types of MSCs, especially in dental tissues (Shi & Gronthos 2003, Arthur *et al*. 2008). Therefore, STRO-1 is considered among the best-known MSC markers. STRO-1 is also found on blood vessels in human dental pulps (Shi & Gronthos 2003), and a high level of STRO-1 expression is retained in inflamed dental pulp tissue (Alongi *et al.* 2010). Notably, it was observed the expression of STRO-1 in vessels and showed an increase in STRO-$1^{+}$$CD146^{+}$ cells following LPS stimulation. These results may support LPS-induced STRO-1-expressing stem-like cell recruitment, which reportedly occurs through complementary activation (Chmilewsky *et al.* 2015).

The chemokines SDF-1 and SCF are major stem cell homing factors expressed by endothelial cells, stromal cells, and MSCs (He *et al*. 2014). SDF-1 and SCF can mobilize stem cells and promote their movement toward homing sites. SDF-1 functions as the ligand for chemokine receptor 4, whereas SCF dimerizes with tyrosine kinase derived from the proto-oncogene *Kit*, resulting in phosphorylation and signal transduction related to proliferation and differentiation (Lennartsson *et al*. 1999). It is suggested that both the mitogen-activated protein kinase kinase/extracellular

signal-related kinase and the phosphoinositide 3-kinase pathways are involved in modulating the effects of SCF on cell behavior (Pan *et al*. 2013). It is also suggested that phosphatidylinositol 3-kinase and protein kinase C signaling pathways are involved in SDF-1α–mediated transmigration of stem cells from apical papilla. In this study, LPS stimulation induced upregulation of *SDF-1* and *SCF* mRNA expression and increased the number of $MAP1B^{+}CD146^{+}$ and $STRO\text{-}1^{+}CD146^{+}$ stem-like cells. Furthermore, SDF-1 and SCF protein levels were higher when $MAP1B^{+}CD146^{+}$ and $STRO\text{-}1^{+}CD146^{+}$ stem-like cells showed increased densities. These results suggest that LPS-stimulated upregulation of SDF-1 and SCF promoted the migration and proliferation of stem-like cells.

Conclusions

LPS-stimulated pulp tissue exhibited upregulation of stem cell differentiation/migration markers, and showed increases in the number of $MAP1B^{+}CD146^{+}$ and $STRO\text{-}1^{+}$CD146 stem-like cells.

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Figure legends

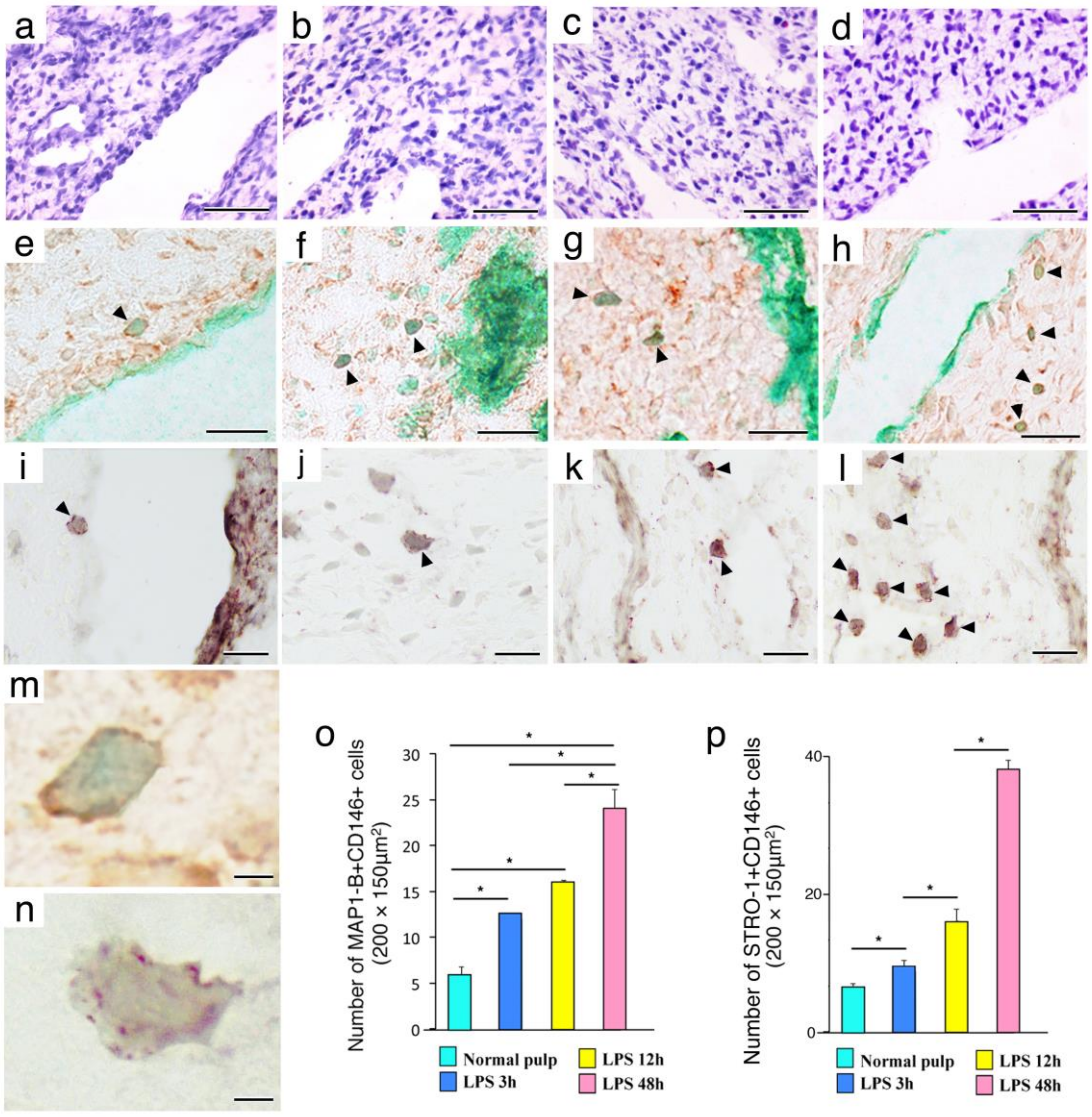
Figure 1 MAP1B$^+$CD146$^+$ and STRO1$^+$CD146$^+$cells in normal and inflamed pulp tissue. **(a–d)** Hematoxylin and eosin staining of dental pulp at (a) 0 (normal), (b) 3, (c) 12, and (d) 48 h after LPS stimulation (scale bar: 50 μm). **(e–h)** Distribution of MAP1B (brown) and CD146 (green) immunoreactivity at (e) 0 (normal), (f) 3, (g) 12, and (h) 48 h after LPS stimulation (scale bar: 50 μm). Arrow heads indicate cells co-expressing MAP1B (brown) and CD146 (green). Green stain, CD146$^+$ blood vessels. Brown stain, MAP1B$^+$ nerve fibers. **(i–l)** Distribution of STRO-1 (red) and CD146 (grey) immunoreactivity at (i) 0 (normal), (j) 3, (k) 12, and (l) 48 h after LPS stimulation (scale bar: 20 μm). Arrow heads indicate cells co-expressing STRO-1 (red) and CD146 (grey). Grey stain, CD146$^+$ blood vessels. Some blood vessels also expressed STRO-1. **(m)** Higher magnification of (e) (scale bar: 4 μm). **(n)** Higher magnification of (j) (scale bar: 4 μm). **(o-p)** Density of MAP1B$^+$CD146$^+$ cells (o) and STRO1$^+$CD146$^+$ cells (p) at 0 (normal), 3, 12, and 48 h after LPS stimulation (200 $\mu m^2 \times 150\ \mu m^2$). *$P < 0.05$ (Mann-Whitney U-test with Bonferroni correction).

Figure 2 Quantitative real-time PCR analysis of (a) *CD146*, (b) *MAP1B*, (c) *SDF-1*, and (d) *SCF* mRNA in normal and inflamed pulp tissue. *$P < 0.05$ (Kruskal-Wallis

non-parametric analysis of variance, followed by Mann-Whitney U test with Bonferroni correction).

Figure 3 Representative images of western blot analysis of SCF, SDF-1, and GAPDH. Values denote the ratio of band density as compared with that of GAPDH and are expressed in arbitrary units.

Figure 1


a
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Number of MAP1-B+CD146+ cells (200 × 150μm²)
30
25
20
15
10
5
0
*
Normal pulp
LPS 3h
LPS 12h
LPS 48h
p
Number of STRO-1+CD146+ cells (200 × 150μm²)
40
20
0
*
Normal pulp
LPS 3h
LPS 12h
LPS 48h

Figure 2

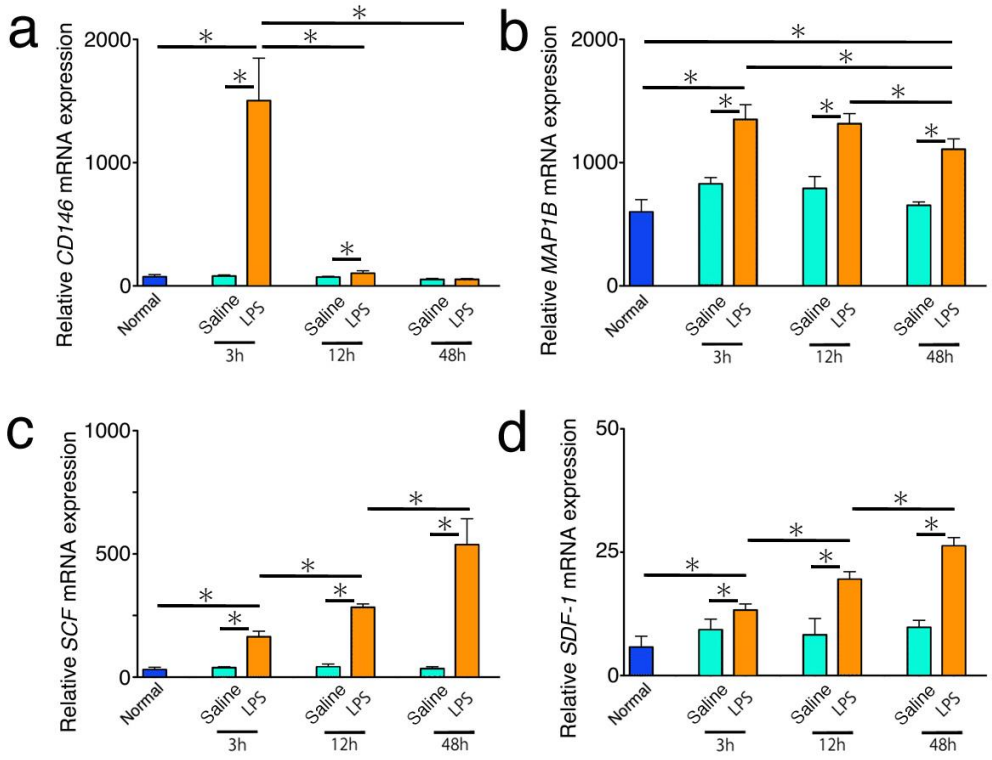
a
Relative CD146 mRNA expression
2000
1000
0
Normal
Saline
LPS
Saline
LPS
Saline
LPS
3h
12h
48h
b
Relative MAP1B mRNA expression
2000
1000
0
Normal
Saline
LPS
Saline
LPS
Saline
LPS
3h
12h
48h
c
Relative SCF mRNA expression
1000
500
0
Normal
Saline
LPS
Saline
LPS
Saline
LPS
3h
12h
48h
d
Relative SDF-1 mRNA expression
50
25
0
Normal
Saline
LPS
Saline
LPS
Saline
LPS
3h
12h
48h

Figure 3