Original

Macrophage Migration Inhibitory Factor Promotes Inflammation in Human Dental Pulp

Takahiro Watanabe1, Naoto Kamio1, Tatsu Okabe1,2, Tomomi Hayama3, Joji Fukai3, Arata Watanabe3, Hiroyuki Okada4,5 and Kiyoshi Matsushima3

1 Department of Endodontics, Nihon University School of Dentistry at Matsudo, Matsudo, Japan
2 Research Institute of Oral Science, Nihon University School of Dentistry at Matsudo, Matsudo, Japan
3 Department of Histology, Nihon University School of Dentistry at Matsudo, Matsudo, Japan

(Accepted for publication, November 13, 2019)

Abstract: Macrophage migration inhibitory factor (MIF) has emerged as an essential proinflammatory cytokine in inflammatory and immune responses. We investigated the expression of MIF in human dental pulp tissue and the function of MIF in human dental pulp fibroblast-like cells. MIF was expressed in areas of dental pulp characterized by a robust inflammatory response, for instance, in human dental pulp tissues that exhibited pathological signs of purulent inflammation. MIF stimulated the expression of prostaglandin-endoperoxide synthase 2 (PTGS2) mRNA and prostaglandin E2 production in human dental pulp fibroblast-like cells. These effects of MIF on the expression of PTGS2 mRNA and prostaglandin E2 production were attenuated in the presence of the CXCR2 and CXCR4 antagonists SCH527123 and WZ811. These results suggest that MIF is involved in inflammation by activating CXCR2 and CXCR4 in human dental pulp.

Key words: Dental pulp, Fibroblast, Macrophage migration inhibitory factor, PTGS2, Pulpitis

Introduction

Pulpitis is a typical inflammatory disease of dental pulp. It is characterized by local accumulation of inflammatory mediators, including cytokines and chemokines1. Bacterial infection leading to dental caries, mechanical and thermal stimulation due to cavity formation, and chemical stimulation by drugs cause dental pulp inflammation, triggering the release of inflammatory mediators, such as tumor necrosis factor, prostaglandin, prostaglandin-endoperoxide synthase (PTGS), and interleukin-1β (IL-1β)2,3. Some of these proinflammatory cytokines, such as IL-1β and Tumor Necrosis Factor-α (TNF-α), may stimulate PTGS2 mRNA and protein expression in human dental pulp cells4. PTGS2 catalyzes the conversion of arachidonic acid to PGH2. This is key in the stepwise biosynthesis of prostanoids.

Fibroblasts and macrophages in inflamed pulp tissues may participate in the production of prostaglandin through PTGS2 expression and likely play a role in the pathogenesis of irreversible pulpitis5. In contrast, because human dental pulp cells show increased ALP activity when exposed to low concentrations of PGE2, it has also been reported that stimulation with low concentrations of PGE2 promotes third dentin formation6. These observations indicate that inflammatory cytokines and mediators take part in destructive and reparative processes in the pulp. Therefore, understanding the mechanism of inflammation development and control in the dental pulp can help to develop new drugs for preventive and restorative treatment of dental pulps.

Macrophage migration inhibitory factor (MIF) has been described initially as an important chemokine-like function chemokine with an essential role in monocyte recruitment and arrest, but it is now known as a potent inflammatory cytokine with chemokine-like functions, such as chemotaxis and leukocyte arrest7. Studies of MIF expression in vivo have established a critical role for MIF in the host response to endotoxic shock8, delayed type-hypersensitivity reactions9, and inflammatory pathologies responsible for arthritis10,11, glomerulonephritis12,13, and adult respiratory distress syndrome14. MIF expression induced by glucocorticoids suppresses strong anti-inflammatory effects of glucocorticoids and potentiates the effects of inflammatory cytokines. This indicates that MIF acts as a counter-regulatory mediator that balances the immune system15.

In experiments using rheumatoid arthritis synovial cells, it has been reported that stimulation with MIF activated PLA2 and increased PTGS2 expression16. Besides, studies using mouse fibroblasts have also shown that MIF activates PLA2, releases arachidonic acid from membrane phospholipids, and produces inflammatory lipid mediators17. Thus, understanding the effects of MIF may be useful for controlling inflammation.

Furthermore, it has been revealed that MIF is ubiquitously expressed in not only the cells of the immune system but in various other organs and cells18. However, the expression and role of MIF in the human dental pulp have not yet been elucidated in detail.

In this study, we examined the expression of MIF in the human dental pulp and the effect of MIF in cultured dental pulp cells.

Materials and methods

Tissue preparation

This research plan was approved by the Nihon University Matsudo Dental Ethics Committee (approval number: EC19-19-003-1). The samples were obtained from four patients who visited Nihon University Matsudo Dental School for treatment. The third molar affected by caries that caused spontaneous pain was removed, and the tissue was used for
immunohistochemical staining. After extraction, the samples were immediately fixed in 4% paraformaldehyde for 2 days and further desalted with 10% EDTA for 4 weeks. The samples were processed through a series of graded ethanol and xylene solutions and embedded in paraffin. The 4-μm thick sections were sliced using a microtome and mounted on a slide glass. Hematoxylin and eosin staining and immunohistochemistry staining were performed using the obtained paraffin sections.

**Immunohistochemistry**

Paraffin sections were deparaffinized, rehydrated, and then steamed in 10 mM citrate buffer (pH 6.0) for 10 minutes for antigen activation. In addition, endogenous peroxidase activity was inhibited by incubating for 15 minutes with 3% H2O2 in methanol. After washing with 0.1% PBST (PBS + Tween 20), the sections were probed for 30 min in a moist chamber with a rabbit anti-MIF antibody (1:200, ab7207, Abcam, Cambridge, UK) at room temperature. Negative controls were not treated with the primary antibody. The primary antibody was revealed using Dako REAL® EnVision™/HRP, Rabbit/Mouse (Agilent Technologies Ltd., Tokyo, Japan), and the sections were visualized using Dako Liquid DAB + Substrate Chromogen System (Agilent Technologies Ltd., Tokyo, Japan). Counterstaining was performed with Mayer’s hematoxylin.

**Cell culture**

This research was conducted based on the approval of Nihon University Matsudo Dental Ethics Committee (approval number EC19-19-003-1). Human dental pulp was obtained from healthy third molars aseptically removed from four male and female patients aged 10 to 20 who visited the Nihon University Matsudo Dental Hospital for orthodontic treatment. The removed dental pulp was thoroughly washed with PBS and incubated in 35 mm culture dishes in α-essential medium (α-MEM, GIBCO BRL Life Technologies Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (GIBCO BRL Life Technologies Ltd., Tokyo, Japan) and 100 μg/ml penicillin G (Meiji Seika Pharma Co., Ltd., Tokyo, Japan). Human dental pulp (fibroblast-like) cells grown at 37 °C in the atmosphere of 95% air and 5% CO2 were used for experiments after 5 to 9 passages.

**Total RNA extraction**

Human dental pulp cells were cultured in a 10 cm culture dish until confluence as above, and then the medium replaced with α-MEM containing 1% FBS 24 hours before stimulation. Total RNA was extracted from Recombinant human MIF (GF180, Merck Millipore, DE, USA) stimulated or unstimulated dental pulp cells using an RNeasy® Mini Kit (QIAGEN, Hilden, Germany). The purity of the obtained total RNA was determined spectrophotometrically from the ratio of absorbance values at 260 nm and 280 nm.

**RT-PCR**

RT-PCR was performed using total RNA (100 ng), DNA primer (500 nM) and QIAGEN One Step RT-PCR kit (QIAGEN, Hilden, Germany) with TaKaRa PCR Thermal Cycler Dice Standard (TaKaRa Bio Inc., Shiga, Japan). Primer sequences are indicated in Table 1. The reaction conditions for RT-PCR were 30 minutes at 50 °C, one cycle at 95 °C for 15 minutes for DNA synthesis, and then DNA denaturation was carried out for 30 seconds at 94 °C. The elongation reaction was carried out at 55 °C for 30 seconds, and the elongation reaction was carried out for 30 seconds at 72 °C. This was carried out as one cycle for 28 cycles, and the final elongation reaction was carried out for 10 minutes at 72 °C. PCR amplification product was electrophoresed on a 2.0% agarose gel. After the experiment, ethidium bromide staining was performed, and gene amplification was confirmed under ultraviolet irradiation.

**Real-time RT-PCR**

Real-time PCR was performed using a One Step SYBR® RT-PCR kit and II Perfect Real Time (TaKaRa Bio Inc., Shiga, Japan) and Thermal Cycler Dice Real Time System (TaKaRa Bio Inc., Shiga, Japan). Reactions contained 8.5 μl of RNase-free dH2O, forward and reverse primers at 400 nM each, 12.5 μl of 2× One-Step SYBR® RT-PCR buffer 4, 1 µl of PrimeScript One-step Enzyme Mix 2, and 1 µl of RNA (100 ng total). The primers used in the experiment are shown in Table 2. After performing reverse transcription for 10 minutes at 95 °C, heat denaturation for 10 seconds at 95 °C, annealing and elongation reaction for 60 minutes. Fifty cycles were performed in a two-step method of 30 seconds at 95 °C. ΔΔCt was used as the determination method.

**Western blotting**

Human dental pulp cells were cultured in 10 cm culture dish until confluence as described in Cell culture section above and then incubated in α-MEM containing 1% FBS 24 hours before stimulation. MIF-stimulated and unstimulated human dental pulp cells were dissolved with Celloytic M Cell lysis reagent (Sigma-Aldrich, Co., St. Louis, MO, USA) containing 2 mM EDTA, 0.2 mM EGTA, 100 μM phenylmethylsulfonyl fluoride. After adjustment of the amount of protein by the Bradford method, sodium dodecyl sulfate (SDS) sample buffer (New England Biolabs Japan Inc., Tokyo, Japan) was added, the mixture was boiled for 5 minutes, and then the supernatant was centrifuged at 15,000 rpm for 1 minute. The sample was electrophoresed on a 7.5% SDS polyacrylamide Mini-PROTEAN TGX gel (Bio-Rad Laboratories Inc., CA, USA). Then, the samples were transferred by the transfer device (NIHON EIDO Corp., Tokyo, Japan) to nitrocellulose membrane (Bio-Rad Laboratories Inc., CA, USA), blocked with skimmed milk (Becton Dickinson Co., NJ, USA) for 30 minutes at room temperature, and reacted with the primary antibody for 2 hours at room temperature while shaking. The secondary antibody was similarly reacted at room temperature for 90 minutes. A mouse antibody against PTGS2 (1:1,000, sc-19999, Santa Cruz Biotechnology Inc., CA, USA) and a rabbit antibody against β-actin (1:1,000, 13E5, 4970S, Cell Signaling Technology Inc., MA, USA) were used as primary antibodies. HRP-conjugated anti-mouse IgG (1:3,000, 170-6516, Bio-Rad Laboratories Inc., CA, USA) or HRP-conjugated anti-rabbit IgG (1:2,000, 7074S, Cell Signaling Technology Inc., MA, USA) were used as secondary antibodies. Western blot results were visualized using the chemiluminescence detection system (Bio-Rad Laboratories Inc., CA, USA). Western blot images are shown in Figure 5.
Technology Inc., MA, USA) were used as secondary antibodies. Immunoreactivity was detected using an ECL prime Western Blotting detection system (GE Healthcare, NJ, USA) and an X-ray film (GE Healthcare, NJ, USA) was used to confirm the expression levels of the proteins of interest.

**PGE₂ secretion**

Human dental pulp cells were plated at a density of 5×10⁴ cells/well in a 24-well cell culture dish, and the medium was switched to α-MEM containing 1% FBS 24 hours before the action. After pre-incubation for 1 hour with CXCR2 and CXCR4 inhibitors SCH527123 (CS-0609, ChemScene, NJ, USA) and WZ811 (S2912, Selleck Chemicals, TX, USA), supernatants of the cultures stimulated with MIF for 3 hours were used for the experiments. The amount of secreted PGE₂ was determined by a Prostaglandin E₂ enzyme immunoassay kit (Oxford Biomedical Research Inc., MI, USA) measuring absorbance at an excitation wavelength of 450 nm.

**Statistical analysis**

Results are reported as the mean ± standard error of the mean of the
Results

Localization of MIF in human teeth affected by pulpitis

We examined the localization of MIF in human teeth affected by pulpitis using immunohistochemistry. Hematoxylin and eosin staining revealed evident dental pulp’s purulent inflammation (Fig. 1a). A strong MIF signal was detected at the site of intense neutrophil infiltration, consistent with pulpal fibroblasts (Fig. 1b, c-1). Expression of MIF was hardly detected in dental pulp located in areas with mild inflammation (Fig. 1b, d-1). Sections from the negative control did not indicate any specific immunoreactivity for MIF (Fig. 1c-2, d-2).

Induction of PTGS2 mRNA expression by MIF

RT-PCR and real-time PCR were used to investigate the effect of MIF on PTGS2 expression in human dental pulp cells. RT-PCR with primers specific for PTGS2 generated a band of the expected size of 310 bp. This result indicates that the enzyme was expressed in human dental pulp cells (Fig. 2). The intensity of the band increased proportionally to MIF concentration (Fig. 2a). Furthermore, PTGS2 expression peaked after 1 hour of stimulation with MIF and then its level decreased (Fig. 2b). Similarly, the results of real-time PCR also showed that the expression of PTGS2 increased in a concentration-dependent manner in the...
MIF promoted inflammation via activation of CXCR2 and CXCR4 (Fig. 3). Furthermore, PTGS2 expression peaked in 180 minutes after treatment with 100 ng/ml MIF and then decreased (Fig. 3b).

Induction of PTGS2 protein expression by MIF

PTGS2 protein expression was increased in a concentration-dependent manner in cells stimulated with MIF for 180 min (Fig. 3a). Furthermore, PTGS2 expression peaked in 180 minutes after treatment with 100 ng/ml MIF and then decreased (Fig. 3b).

Coupling of PGE_{2} release induced by MIF to CXCR2 and CXCR4 activation

Stimulation of dental pulp cells with 100 ng/ml MIF for 180 min stimulated PGE_{2} release. However, when the cells were incubated in the presence of 1 μM SCH527123 and 1 μM WZ811, CXCR2, and CXCR4 antagonists, PGE_{2} release was significantly reduced, suggesting that MIF promoted inflammation via activation of CXCR2 and CXCR4 (Fig. 4).

Discussion

Acute pulpitis presents with intense clinical symptoms, and in order to prevent apical periodontitis as well as to alleviate the sequelae of the symptoms, dental pulp removal therapy (pulpectomy) is often chosen. Although this treatment remains one of the best for dental pulpitis, it is incompatible with long-term tooth preservation. Therefore, keeping dental pulp healthy is considered to prevent toothache and tooth loss, and prolong healthy life. Elucidation of the mechanisms of dental pulp inflammation should contribute to the prevention of tissue deterioration. Furthermore, there are reports about various roles of inflammation in pulpitis: in addition to the well-established fact that inflammation is harmful to dental pulp tissue, it is often considered that a certain degree of inflammation is necessary for third dentin formation.

In the present study, prompted by the reports showing that inflammatory cytokines participate in physiological and pathological processes in dental pulp, we investigated the relationship between pulpitis and MIF.

Immunohistochemical staining revealed that there was a stronger expression of MIF in the inflamed area than in the non-inflamed one and that the signal may be consistent with dental pulp fibroblasts. Fibroblasts are typically a significant component of connective tissue that has not been considered to substantially produce of inflammatory mediators, including chemokines. However, it has been reported that fibroblasts do produce chemokines when activated by substances released during tissue damage or those derived from infectious microorganisms. For example, plasmin stimulated the expression of the chemokine interleukin-8 (IL-8) mRNA and prostaglandin E_{2} release in human dental pulp cells.

Besides, in human dental pulp tissue, reports showed generation of PTGS2 by dental pulp fibroblasts also suggested that these cells are regulators of dental pulp tissue inflammation. Additionally, it has been reported that pretreatment of rats with an anti-MIF antibody attenuated neutrophil accumulation in the lung and 24 hours after administration of lipopolysaccharide. Furthermore, an anti-MIF antibody was shown to protect from liver damage by suppressing TNF-α production in acute hepatitis caused by the administration of Bacillus Calmette-Guérin and lipopolysaccharide to mice. These reports suggest that MIF generated at sites of a robust inflammatory response in dental pulp tissue stimulates the migration of neutrophils and macrophages and activates the pulpitis immune response.

Besides, we demonstrated that stimulation of human dental pulp culture cells with MIF promoted mRNA and protein expression of PTGS2 and PGE_{2} protein production. Peptidoglycan, which is a bacterial cell wall component, and IL-1β, TNF-α, which is an inflammatory cytokine, increase the amount of PGE_{2} in human dental pulp cultured cells in a concentration-dependent manner. Furthermore, it has been found that the amount of PGE_{2} in the pulp tissue increases with the progress of pulpitis. On the other hand, it is reported that high concentration of PGE_{2} inhibits hard tissue formation in cultured human dental pulp cells, but low concentration of PGE_{2} promotes hard tissue formation.

Furthermore, there are reports that PGE_{2} modifies the promotion of ALPase activity by bone morphogenetic protein -2 in human periodontal ligament cells. That means appropriate control of PGE_{2} production has the potential to alleviate the inflammatory symptoms of the pulp and promote the formation of hard tissues such as repaired dentin and dentine-bridge. Stimulation of rat pulmonary artery smooth muscle cells with MIF was previously shown to increase PTGS2 protein level in a concentration-dependent manner. MIF stimulation also increased PTGS2 mRNA expression and PGE_{2} secretion in microglial cells. Furthermore, MIF was found to promote PGE_{2} production from astrocytes after spinal cord injury. These reports collectively suggest that MIF acts as an inflammatory cytokine that initiates the arachidonic acid cascade, thereby being involved in the regulation of the inflammatory environment. However, it is difficult to uniformly consider the expression of MIF in dental pulp tissue and the production of PGE_{2} by MIF stimulation in human dental pulp cultured cells, and further investigation is necessary.

Furthermore, the effects of CXCR2 and CXCR4 inhibitors SCH527123 and WZ811 in this study demonstrated that CXCR2 and CXCR4 are involved in PGE_{2} production under MIF stimulation. CXCRs are receptors for the chemokines of the CXC chemokine family. CXCRs are expressed in a cell-type specific manner in subsets of leuko-
cytes, but also some non-hematopoietic cells, such as endothelial and epithelial cells\(^{31}\). It has been reported that CXCL1 enhances inflammatory pain and PTGS2 expression in spinal cord neurons via CXCR2 activation\(^{32}\). A positive correlation between CXCR4 and PTGS2 expression has been observed in basal cell carcinoma\(^{33}\). These reports suggest that CXC receptors are involved in PTGS2-mediated inflammatory responses and cancer progression.

MIF is a non-cognate ligand for CXC chemokine receptors. CXCL2/ MIF-CXCR2 signaling has been shown to promote mobilization of bone marrow-derived suppressor cells and to correlate with bladder cancer prognosis\(^{41}\). Furthermore, MIF-CXCR4 interaction was reported to be involved in tumor chemotaxis of human mesenchymal stem cells\(^{35}\). In addition, by activating both CXCR2 and CXCR4, MIF acts as a critical regulator of inflammatory cell migration and atherogenesis\(^{36}\). CXCR2 and CXCR4 signaling pathways have been shown to cross-activate and mutually enhance each other through positive feedback\(^{37}\), which was also observed in this study (data not shown). However, there are other reports on signaling pathways by MIF besides CXCR2 and CXCR4. In human placental villous epithelial cells, low concentrations of MIF activate Signal-regulated Kinase (ERK) 1/2 via CD74 to promote PGE\(_2\) production\(^{38}\). Moreover, in monocytes and T cells, it has been reported that CXCR2 and CXCR4 form a receptor complex with CD74, and MIF competes with each ligand to control migration and adhesion to the blood vessel wall\(^{39}\). Although CD74 has been reported not to have a cytoplasmic domain that induces signal transduction\(^{40}\), no clear conclusion has been reached regarding the MIF signaling pathway. Besides, whereas cultured human dental pulp cells are abundant in fibroblasts, they probably also contain other cell types, including odontoblast precursors, so it cannot be ruled out that they may influence the results of this study. Therefore, in vivo experiments need to be considered in the future.

In conclusion, MIF was detected in dental pulp tissue that was affected by caries and exhibited signs of purulent inflammation. In addition, MIF stimulated PGE\(_2\) production in human dental pulp cultured cells through the activation of CXCR2 and CXCR4. As mentioned above, control of PGE\(_2\) production in pulpitis is essential not only in suppressing inflammation progression but also in the formation of repaired dentin. MIF expression in the pulp tissue and the mechanism of PGE\(_2\) production by MIF. Notably, the elucidation of this mechanism could inspire drug discovery for prevention, treatment and regenerative medicine of pulpitis.

**Acknowledgments**

We would like to thank Editage (www.editage.jp) for English language editing. This study was supported by Grants-in Aid for Scientific Research (No. 15K11131, 17K17143) from the Japan Society for the Promotion of Science (JSPS).

**Conflict of Interest**

The authors have declared that no COI exists.

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