

## **Effects of Rac1 on the Production of MMP-3 by TNF- $\alpha$**

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## Abstract

**Purpose:** Inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were previously shown to be secreted in pulpitis tissue during the caries process. Matrix metalloproteinases (MMPs) such as MMP-1, 2, 3, and 14 were also shown to be expressed in inflamed dental pulp. MMP-3 can degrade the extracellular matrix (ECM) and activate other MMPs. MMP-3 is considered to be involved in wound healing, inflammation, and tumor initiation. Dental pulp destruction may be regulated, in part, by MMP-3, and other MMPs activated by MMP-3 have been shown to regulate the degradation and regeneration of dental pulp. Ras-related C3 botulinum toxin substrate 1 (Rac1) is a pleiotropic regulator of many cellular processes, including cell growth, cytoskeletal reorganization, and the activation of protein kinases. We hypothesized that Rac1 may negatively regulate the production of MMP-3 from human pulp fibroblasts (HPFs). To test this hypothesis, we isolated and purified HPFs from healthy donors and stimulated them with TNF- $\alpha$ .

**Methods:** HPFs were incubated in serum-free  $\alpha$ -MEM containing TNF- $\alpha$  (0, 10, 20, 50, or 100 ng/mL) for 24 h with or without the Rac1 inhibitor, NSC23766. The production of MMP-3 and activation of Rac1 by TNF- $\alpha$  were evaluated by the phosphorylation of Rac1 and MMP-3 antibodies using western blot analysis.

**Results:** We demonstrated that MMP-3 was produced from HPFs in response to TNF- $\alpha$  in a Rac1-dependent manner. TNF- $\alpha$ -induced the production of MMP-3 without affecting the total production of MMP-2. Blocking Rac1 activation with NSC23766 significantly enhanced the TNF- $\alpha$ -induced production of MMP-3 without affecting the total production of MMP-2.

**Conclusion:** These results suggest that Rac1 prevents pulpitis by negatively regulating the production of MMP-3 in HPFs.

**Key words:** Matrix metalloproteinase-3, Rac1, Human pulp fibroblasts

## Introduction

Dental pulp destruction associated with pulpitis is believed to be regulated, in part, by matrix metalloproteinases (MMPs) and the tissue inhibitors of MMPs (TIMPs). The connective tissue of pulp is composed of an extracellular matrix (ECM) and is degraded by MMPs. MMPs have been classified into MMP-1 and -8 (tissue collagenase), MMP-2 and -9 (gelatinase A and B), MMP-3, -10, and -11 (stromelysin-1, -2 and -3), MMP-7 (matrilysin), membrane-type MMPs (MT-MMPs), and other MMPs <sup>1, 2)</sup>. MMP-1, MMP-2, MMP-3, and MT1-MMP levels were previously shown to be significantly higher in pulpitis tissues and periapical lesions than in healthy tissues <sup>3)</sup>. MMP-3 was also shown to activate other MMPs such as MMP-1, MMP-7, and MMP-9 and has been implicated in a wide range of physiological and pathological processes, including degradation, morphogenesis, wound healing, and angiogenesis in inflamed tissue <sup>4-8)</sup>. A previous study showed that MMP-3 was expressed in pulpitis tissue, in which it plays an important role <sup>9)</sup>. MMP-3 has also been shown to induce angiogenesis, fibroblast wound healing, and reparative dentin formation in dental pulp tissue <sup>10)</sup>.

Dental caries can result in an inflammatory response in the dental pulp, which is characterized by the accumulation of inflammatory cells, resulting in the release of host inflammatory cytokines, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) <sup>11-13)</sup>.

Ras-related C3 botulinum toxin substrate 1 (Rac1) is a small signaling G protein and was reported to be a pleiotropic regulator of many cellular processes, including the cell cycle, cell-cell adhesion, motility, and epithelial differentiation <sup>14,15)</sup>.

However, the potential relationship between MMP-3 expression and the signaling pathway including Rac1 has not yet been determined in TNF- $\alpha$ -stimulated HPFs.

In this study, we demonstrated that TNF- $\alpha$  induced the production of MMP-3 in HPFs through the signaling cascade involving the Rac1-mediated phosphorylation of MAP kinase p38.

## Materials and methods

### Cell culture

Human pulp fibroblasts (HPFs) were grown from explants of the healthy pulp tissue of healthy donors. Primary cultures were grown in minimum essential medium alpha modification ( $\alpha$ -MEM; Wako Pure Chemical Industries, Japan) containing with 10% fetal bovine serum (FBS; Equitech-Bio, Inc., CA, USA), penicillin G sodium 100 units/mL, streptomycin 100  $\mu$ g/mL, L-glutamine 292  $\mu$ g/mL (Invitrogen Co., CA, USA) in an atmosphere of 5% CO<sub>2</sub>-95% air at 37°C. The first subcultures were obtained 20 to 30 days later, maintained in an atmosphere of 5% CO<sub>2</sub>-95% air at 37°C, and routinely subcultured after using trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA • 4NA, Invitrogen) for cell release. Experiments with HGFs were performed between passage 3 and 10. This study was approved by the Ethical Review Board of Osaka Dental University. (Approval No. 110751)

### Reagents and inhibitors

Human TNF- $\alpha$  (Miltenyi Biotec Inc. USA) was dissolved in deionized sterile-filtered water and the Rac1 inhibitor NSC2766 (CALBIOCHEM) was dissolved in H<sub>2</sub>O.

### Gelatin zymography

Gelatin zymography was performed according to previously described methods<sup>16)</sup>. HPFs were placed in a 24-well plate ( $5.0 \times 10^5$  cells/well) and incubated using  $\alpha$ -MEM (10% FBS) for 24 h. Each well was washed using PBS, and cells were incubated using  $\alpha$ -MEM (serum-free) with 0, 5, 10, 20, 50, or 100 ng/mL of TNF- $\alpha$  for 24 h. The culture solution in each well was centrifuged at 10,000 rpm for 10 min, and the sample buffer (0.0645 M Tris buffer (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, and bromophenol blue (BPB)) was then added to 10  $\mu$ L of the supernatant to make an electrophoresis sample. In 10% SDS-polyacrylamide gel electrophoresis (PAGE) including 1 mg/mL gelatin, 15  $\mu$ L of sample was added to each lane, and electrophoresis was performed with a constant voltage of 200 mV for 60 min in concentration and isolation gels, respectively. After electrophoresis, the gel was washed at room temperature using 2.5% Triton X-100 (Katayama Kagaku Kogyo, Osaka, Japan) for 20 min. After 20 min, 2.5% Triton X-100 was exchanged, and the gel was washed at room temperature for 20 min, soaked in 10 mL of activation buffer (200 ml H<sub>2</sub>O, 10 mL zymosin (200 mM Tris-HCl (pH 7.5) + 100 mM CaCl<sub>2</sub> + 20 mM ZnCl<sub>2</sub> and 2.5 mL Triton X-100)) at 37°C for 16 h. The gel was washed using 2.5% Triton X-100 for 5 min, soaked in

Coomassie blue solution for 3 h, destained using destain buffer (methanol : acetic acid : water, 50 : 10 : 40) and scanned (EPSON GT-9600; Seiko-Epson, Tokyo, Japan) to confirm MMP-2.

### **Western Blot analysis for MMP-3**

HPFs were incubated in serum-free  $\alpha$ -MEM containing TNF- $\alpha$  (0, 5, 10, 20, 50, or 100 ng/mL) for 24 h. Conditioned media were collected, centrifuged to remove debris, and concentrated in Amicon Centriprep concentrators (Millipore Corporation, Bedford, MA) up to 10-fold to visualize proteins by Western blotting. Total cell lysates were prepared by dissolving cells in sodium dodecyl sulfate (SDS)-sample buffer. In some experiments, HPFs were incubated in 2% FBS  $\alpha$ -MEM with NSC23766 (0, 1, 5, and 10  $\mu$ M) and TNF- $\alpha$  (100 ng/mL) for 24 h. Samples were separated on 8% SDS polyacrylamide gels (by SDS-polyacrylamide gel electrophoresis [SDS-PAGE]) under reducing conditions. Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated for 1 h with MMP-3 antibodies (Cosmo Bio Co., Tokyo) in PBS containing 0.05% Tween 20 and 5% BSA. A peroxidase-conjugated secondary antibody was used at a 1 : 1,000 dilution, and immunoreactive bands were visualized using a substrate. Signals on each membrane were analyzed with Versa Doc 5000 (Bio-Rad, Hercules, CA).

### **Proliferation experiment**

HPFs were seeded onto a 96-well plate at a density of  $1.0 \times 10^5$  cells/well and incubated for up to 24 h in  $\alpha$ -MEM (10% FBS) containing NSC23766 (0, 1, 5, and 10  $\mu$ M) in the presence of TNF- $\alpha$  (100 ng/mL). The cell proliferation reagent WST-1 (Roche Diagnostics, Basel, Switzerland) was used to assess cell proliferation by measuring absorbency (450/650 nm) with SpectraMax M 5 (Molecular Devices, Sunnyvale, CA, USA). The data were then analyzed using Tukey's test.

### **Western Blot analysis for MAP Kinase**

HPFs were preincubated with NSC23766 (10  $\mu$ M) at 37°C, and HPFs were placed in 2% FBS  $\alpha$ -MEM with 100 ng/mL TNF- $\alpha$  for 24 h and were then lysed by adding 100  $\mu$ L lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% tert-octylphenoxy polyethanol, 1 mM ethylenediaminetetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 20 mg/mL leupeptin, and 20 mg/mL aprotinin). Lysates were clarified by centrifugation at 12,000 revolutions per min for 10 min at 4°C. Samples were separated on 8% SDS polyacrylamide gels (by SDS-polyacrylamide gel electrophoresis [SDS-PAGE]) under reducing conditions. Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were

incubated for 1 h with p-p38 (Cell Signaling Technology) in PBS containing 0.05% Tween 20 and 1% skim milk proteins. A peroxidase-conjugated secondary antibody was used at a 1 : 1,000 dilution, and immunoreactive bands were visualized using a substrate. Signals on each membrane were analyzed with Versa Doc 5000.

## Results

### **TNF- $\alpha$ enhanced the production of MMP-3 in HPFs**

We evaluated the production of MMP-3 in HPFs in the presence of TNF- $\alpha$ . HPFs were incubated in the presence or absence of 5, 10, 20, 50, and 100 ng/mL TNF- $\alpha$ , as described in the Materials and Methods. When cells were cultured in the presence of TNF- $\alpha$ , the generation of MMP-3 was observed in a dose-dependent manner (Figure 1A). The same samples prepared were separated on gelatin zymography to visualize MMP-2 (Figure 1C). While unstimulated HPFs spontaneously produced the latent form of MMP-2 (Figure 1C), neither the total amount of production nor activation status of this enzyme was altered by stimulation with TNF- $\alpha$ . These results indicated that TNF- $\alpha$  specifically stimulated the production of MMP-3 from HPFs, and the production of MMP-2 served as a loading control for Figure 1.

### **Rac1 inhibitor suppressed the production of MMP-3 in TNF- $\alpha$ -activated HPFs.**

We added the Rac1 inhibitor to HPFs to investigate the effects of Rac1 on cell proliferation. HPFs were incubated with TNF- $\alpha$  (100 ng/mL) in a 96-well plate ( $1.0 \times 10^5$  cells/well), and 1, 5, or 10  $\mu$ M of NSC23766 was added 24 h later to investigate the effects of the Rac-1 inhibitor on HPFs proliferation. The results obtained showed that 10  $\mu$ M NSC23766 only affected the proliferation of HPFs (Figure 2).

NSC23766 markedly enhanced the production of MMP-3 in TNF- $\alpha$ -stimulated HPFs in a dose-dependent manner. Unstimulated HPFs spontaneously produced latent MMP-2, and its level was not altered by stimulation with TNF- $\alpha$ , with or without NSC23766. Thus, MMP-2 served as a loading control for Figure 3A.

### **TNF- $\alpha$ activated transcription factors through the p38-dependent signaling pathway.**

Our previous study demonstrated that the activation of p38 played a key role in the production of MMP-1<sup>17</sup>). Therefore, we investigated whether Rac-1 affected the TNF- $\alpha$ -induced phosphorylation of p38 in HPFs. NSC23766 enhanced the TNF- $\alpha$ -induced phosphorylation of p38 MAP kinase in HPFs (Figure 4). The total amount of p38 was not affected under any of the experimental conditions (Figure 4).

## Discussion

In this study, we demonstrated that TNF- $\alpha$  enhanced the production of MMP-3 in conditioned media of HPFs. However, TNF- $\alpha$  did not affect the production or activation of MMP-2 under our experimental conditions. MMP-2 has been shown to play a critical role in the development of inflammatory periapical lesions <sup>18)</sup>, while MMP-3 has a broad substrate specificity to ECM <sup>19)</sup> and induced angiogenesis, fibroblast wound healing, and reparative dentin formation in dental pulp tissue <sup>10)</sup>. Pulpitis develops through the stimulation of TNF- $\alpha$  <sup>20)</sup> and vascular endothelial growth factor expression <sup>21)</sup>. The production of MMP-3 stimulated by TNF- $\alpha$  could play an important role in pulpitis; therefore, we examined the signaling cascade that produced MMP-3 in TNF- $\alpha$ -activated HPFs.

A previous study showed that the activation of Rac1 played a key role in the expression of MT1-MMP <sup>22)</sup>. Rac1 was reported to stimulate hypertrophic phenotype markers such as the gene expression of MMP-9 and -13 in primary chondrocytes <sup>23)</sup>. However, the attenuation of Rac1 activation by a specific inhibitor (NSC23766) inhibited the proliferation of HPFs,

NSC23766 markedly enhanced the production of MMP-3 by TNF- $\alpha$ , which implicated Rac1 as a downstream target of the stimulation with TNF- $\alpha$ .

Although Rac1 plays an essential role in the production of MMP-3 by TNF- $\alpha$  stimulation, it is also involved in many other mechanisms, such as cell growth, vesicle trafficking, and epithelial differentiation, which can potentially be modulated by opioids in other model systems and lead to the disruption of homeostasis <sup>24)</sup>. We previously showed that p38 MAP kinase regulated the production of MMP-1 in human gingival fibroblasts <sup>17)</sup>. Therefore, we investigated the relationship between Rac1 and p38 in TNF- $\alpha$ -stimulated HPFs. The Rac1 inhibitor increased the phosphorylation of p38 MAP kinase induced by the TNF- $\alpha$  stimulation in HPFs. These results suggest that Rac1 specifically regulated the production of MMP-3 in TNF- $\alpha$ -stimulated HPFs.

Taken together, our results suggest that the abrogation of Rac1 may have enhanced the production of MMP-3, as detected by elevations in the phosphorylation of p38 MAP kinase in TNF- $\alpha$ -stimulated HPFs. Rac GTPases act as molecular switches by cycling between active GTP-bound and inactive GDP-bound forms. This cycling is regulated by guanine nucleotide exchange factors, which serve as activators, and GTPase activating proteins and GDP dissociation inhibitors, which negatively regulate Rac GTPase activity <sup>25)</sup>. These findings also support our conclusion that p38 MAP is downstream of Rac, by inhibiting Rac1-GTP, leading to activation of p38 MAP via inhibition of Rac1-GTPase



Rac1 negatively regulated TNF- $\alpha$ -induced MMP-3 production via p38 MAP kinase-activated signaling pathways in HPFs, and thereby stimulated the degradation of surrounding collagen, leading to alterations in the inflamed ECM structure, and potentially the promotion of angiogenesis.

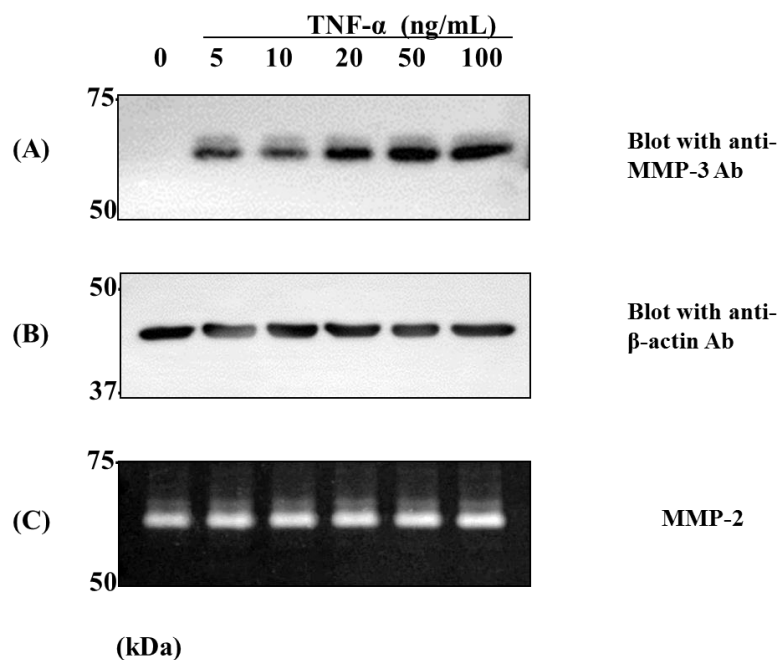
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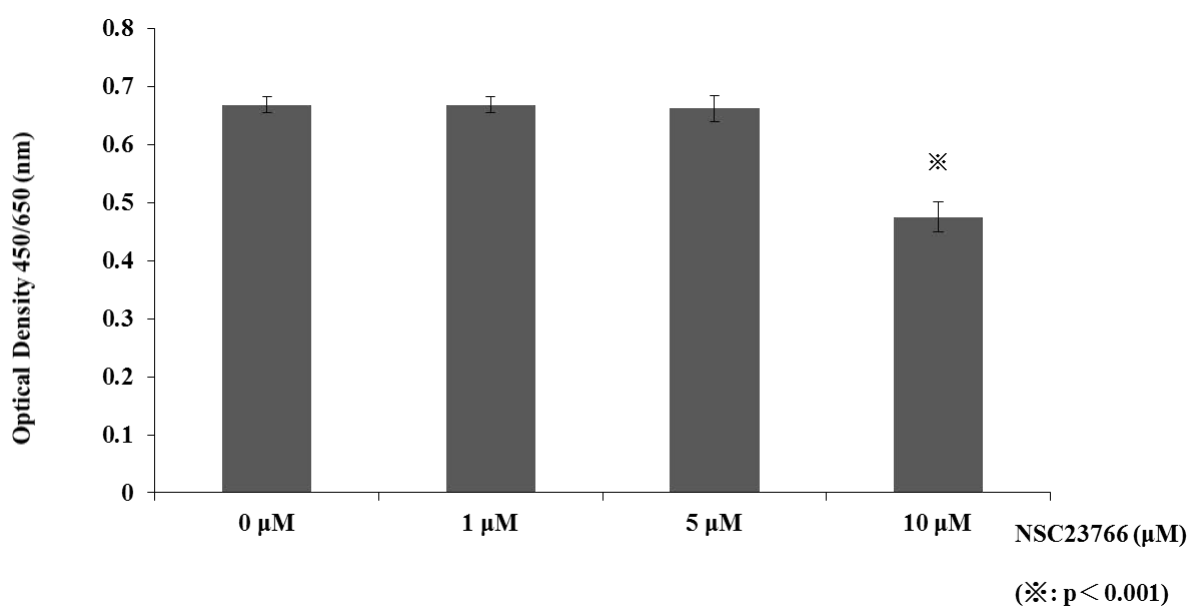


**Figure 1 Effect of TNF- $\alpha$  on the expression of MMP-3 by HPFs.**

HPFs were seeded in a 24-well plate ( $5.0 \times 10^5$  cells/well), incubated in  $\alpha$ -MEM (10% FBS) for 24 h, washed with PBS, and then incubated for a further 24 h with TNF- $\alpha$  (0, 10, 20, 50, or 100 ng/mL). A. The concentrated conditioned medium was separated by SDS-PAGE and analyzed by western blotting using an anti-MMP-3 antibody. Antibody binding was visualized using the Super Signal West Pico chemiluminescent substrate. Molecular markers (kDa) are shown in the left column. The 54 kDa band was MMP-3.

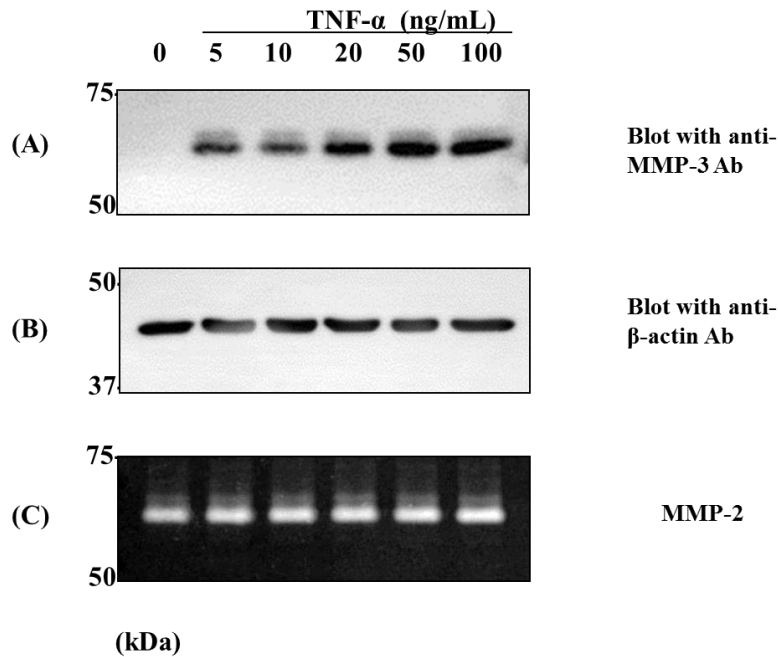
B. The same sample was lysed by adding 100  $\mu$ L lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% tert-octylphenoxy polyethanol, 1 mM ethylenediaminetetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 20 mg/mL leupeptin, and 20 mg/mL aprotinin). Lysates were analyzed by western blotting to assess the expression of  $\beta$ -actin (45 kDa).

C. The concentrated conditioned medium was analyzed by gelatin-zymography to assess the expression of MMP-2 as described in the Materials and Methods. The same samples prepared in Figure 1 A were separated on gelatin zymography to visualize MMP-2 (72 kDa).



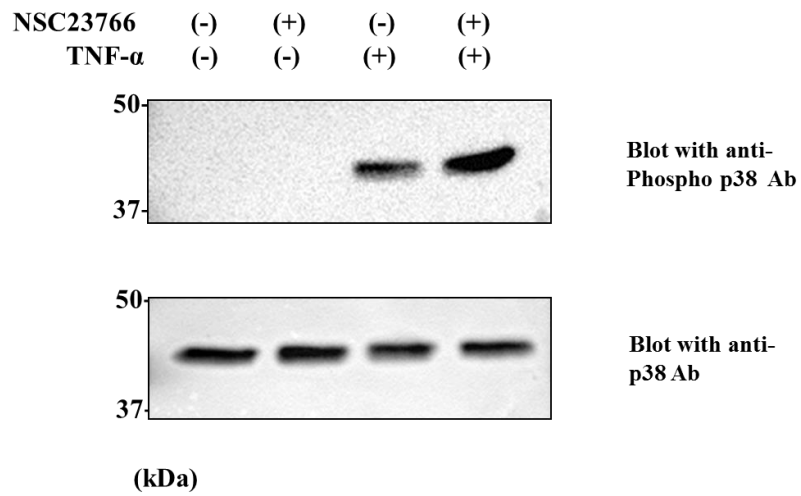
**Figure 2 Effect of NSC23766 on the proliferation of HPFs.**

HPFs were seeded onto a 96-well plate at a density of  $1.0 \times 10^5$  cells/well and incubated for up to 24 h in  $\alpha$ -MEM (10% FBS) containing NSC23766 (0, 1, 5, 10  $\mu$ M) in the presence of TNF- $\alpha$  (100 ng/mL). Cell proliferation was assessed by measuring absorbency at 450/650 nm. Cell counts reflect the average of data from 3 wells.



**Figure 3 Effect of the Rac1 inhibitor on TNF- $\alpha$ -induced activation of MMP-3.**

The effect of the Rac1 inhibitor NSC23766 (0, 1, 5, 10  $\mu$ M) on the TNF- $\alpha$  stimulation of MMP-3 expression by HPFs was analyzed by western blotting as described in Figure 1 A. The 54 kDa band was MMP-3 (Figure 3A). B. The same sample was lysed by adding 100  $\mu$ L lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% tert-octylphenoxy polyethanol, 1 mM ethylenediaminetetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 20 mg/mL leupeptin, and 20 mg/mL aprotinin). Lysates were analyzed by western blotting to assess the expression of  $\beta$ -actin (45 kDa). C. The concentrated conditioned medium was analyzed by gelatin-zymography to assess the expression of MMP-2 as described in the Materials and Methods. The same samples prepared in Figure 3 A were separated on gelatin zymography to visualize MMP-2 (72 kDa).



**Figure 4 Effect of the Rac1 inhibitor on TNF- $\alpha$ -induced phosphorylation of p38.**

HPFs were pretreated with or without 10  $\mu$ M NSC23766 for 24 hours and were then treated with 100 ng/mL TNF- $\alpha$  for 15 minutes. The activation of p38 activity was determined by western blot analysis using a phospho-specific antibody.

## 抄録

目的：う蝕の進行に伴い、歯髄組織では TNF- $\alpha$  などの炎症性サイトカインが産生され、歯髄炎が惹起される。また、刺激を受けた歯髄組織では細胞外マトリックス分解酵素であるマトリックスメタロプロテアーゼ (MMPs)などが産生され、歯髄組織を破壊し病態が進行する。可逆性歯髄炎は原因を除去することにより正常な歯髄に回復し得るため、歯髄に存在する細胞における炎症の発症機序や進行状況を解明することは歯髄の保存のために重要であると考えられる。また small G protein は、炎症に深く関わっているタンパク質で知られている。そこで今回、ヒト歯髄由来線維芽細胞における TNF- $\alpha$  刺激による MMP-3 産生に対する small G protein の関与について検討した。

材料・方法：大阪歯科大学医の倫理委員会に置いて承認を得た（大歯医倫 110751 号）。ヒト歯髄由来線維芽細胞を初代培養し以下の実験に用いた。ヒト歯髄由来線維芽細胞を  $\alpha$ -MEM (serum-free) にて培養後、TNF- $\alpha$  (0、5、10、20、50、100 ng/ml) および Rac1 inhibitor (NSC23766) を添加し、24 時間共培養を行った。刺激終了後、上清中の MMP-3 の産生および ERK1/2、p-38 のリン酸化をウェスタンブロット法にて検討した。また、培養上清を 1mg/ml の gelatin を加えた SDS-PAGE に供し電気泳動を行い、Comassie blue にて染色後 Destain buffer にて脱染色しザイモグラフィ法にて検討した。

結果：ヒト歯髄由来線維芽細胞において TNF- $\alpha$  濃度依存性に MMP-3 の産生は増強したが、MMP-2 の産生に影響は認められなかった。次に、TNF- $\alpha$  刺激により増強した MMP-3 の産生および p-38 のリン酸化は Rac1 阻害剤である NSC23766 により有意に増強した。

結論：以上より、ヒト歯髄由来線維芽細胞において TNF- $\alpha$  刺激による MMP-3 の産生は small G protein の Rac1 が関与していることが示唆された。

## キーワード

マトリックスメタロプロテアーゼ-3、Rac1、ヒト歯髄由来線維芽細胞