Journal of Dental Sciences 17 (2022) 1321–1328

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Original Article

Staphylococcus aureus enhances gelatinase activities in monocytic U937 cells and in human gingival fibroblasts

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Received 5 April 2022; Final revision received 19 April 2022
Available online 8 May 2022

KEYWORDS
Fibroblasts;
Matrix metalloproteinases;
Staphylococcus aureus;
U937 cells

Abstract *Background/purpose: Staphylococcus aureus* (*S. aureus*) has been suggested to be an initiative pathogen in peri-implantitis because of the solid affinity to titanium. However, the detail pathogenesis for the peri-implantitis initiation by *S. aureus* is still lacking. This study aimed to *in vitro* examine the gelatinases' activities of monocytic U937 cell and human gingival fibroblast after challenges with *S. aureus* lipoteichoic acid (LTA) and peptidoglycan (PGN).
Materials and methods: Releases of gelatinases, including matrix metalloproteinase (MMP)-2 and −9, from cells were measured by zymography. The releases were further examined after being given the *S. aureus* LTA/PGN. Roles of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways on the enzyme releases were examined by administrating inhibitors.
Results: S. aureus LTA and PGN increased the activities of pro-MMP-9 from U937 cells and pro-MMP-2 and MMP-2 from gingival fibroblasts. By giving the NF-κB inhibitor, the enhanced gelatinase activities in both cells were attenuated. In U937 cells, the enhanced pro-MMP-9 could further be attenuated by MAPK inhibitors, including extracellular signal-regulated kinase 1 and 2 (ERK1/2), P38 MAPK, and c-Jun N-terminal kinase (JNK) inhibitors; however, the attenuation by MAPK inhibitors could not be observed for MMP-2 in gingival fibroblasts. Nevertheless, in gingival fibroblasts, the pro-MMP-2 could be attenuated by JNK inhibitor.
Conclusion: S. aureus could enhance gelatinase activities of gingival fibroblasts and U937 cells, via NF-κB. The MAPK pathway was also involved in MMP-9 activity of

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https://doi.org/10.1016/j.jds.2022.04.014

U937 cells; however, the involvement of MAPK in MMP-2 activity of gingival fibroblasts was questioned.

Introduction

Staphylococcus aureus (*S. aureus*) is a Gram-positive opportunistic bacterium, which has a strong affinity to titanium surfaces, and is considered as a major pathogen which is associated with medical device-related infections,[1] especially when they occur on implanted materials.[2] For instance, *S. aureus* is a common infection source related to metal-biomaterial, joint, bone, and soft tissue.[3] Studies have also reported the association between the presence of *S. aureus* and the peri-implantitis although it is not considered to be the periodontal pathogen.[4–9]

The cell wall of *S. aureus*, unlike that of *Porphyromonas gingivalis* (a common periodontal pathogen), does not contain lipopolysaccharides (LPS) structure but has lipoteichoic acid (LTA) and multilayer peptidoglycan (PGN), which can also activate a variety of signals, including nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways.[10–13] The activated NF-κB and MAPK translocate to the nucleus and play an important role in the production of varied cell mediators, including matrix metallopeptidases (MMPs).[14–19]

MMPs, a family of zinc-containing endopeptidases, act as the turnover of the extracellular matrix. Besides, they are considered to play important roles in morphogenesis, tissue remodeling, and diseases, including periodontitis.[20,21] Gelatinases are members of MMPs, which have been mainly associated with basement membrane degradation under pathological conditions. There are two forms of gelatinase: the type A (MMP-2) typically express in normal epithelial cells, endothelial cells, and fibroblasts including gingival fibroblasts;[22] the type B (MMP-9) mainly express in inflammatory cells including monocytes and macrophages.[23] The special attention has been paid to the role of gelatinases, released from the local gingival, periodontal and recruited inflammatory cells, in periodontitis and peri-implantitis.[24,25] Up to date, however, the knowledge related to the gelatinase activities in gingiva after *S. aureus* infection is still lacking.

The aim of this study was to *in vitro* evaluate the effect of cell wall components from the *S. aureus* (LTA and PGN) on the gelatinase activities, as well as the involvement of NF-κB and MAPK pathways, in monocytic U937 cells, a human histiocytic lymphoma cell line, and in human gingival fibroblasts.

Materials and methods

U937 cells and gingival fibroblasts

In this study, the human monocytic U937 cells used were purchased from American Type Culture Collection, whereas the gingival fibroblasts were gained from three systemically healthy patients of 40—70 years of age. Biopsies were taken during crown lengthening surgeries and the fibroblasts were harvest as described previously.[26] Briefly, the epithelium of collected gingiva specimens were removed after being immersed in Leibovitz medium (Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Invitrogen) and 2 mg/mL Dispase II (Roche Diagnostics, Indianapolis, IN, USA) for 48 h. Gingival fibroblasts migrated from the explants which were incubated in Dulbecco's modified Eagle medium/Nutrient Mixture F-12 (Invitrogen) containing 10% FBS with the pre-treatment in medium containing type I bacterial collagenase (Sigma—Aldrich, St Louis, MO, USA) and 10% FBS at 37 °C for 24 h. Fibroblasts between passages 2 and 6 were selected. All procedures were approved by IRB of Taipei Tzu Chi Hospital, New Taipei City, Taiwan (No. 10-X-013). For all the following experimental procedures, the U937 cells and fibroblasts were maintained in Roswell Park Memorial Institute 1640 medium (Invitrogen), pH 7.4, supplemented with 10% FBS and incubated at 37 °C in a humidified atmosphere of 5% CO_2.

Cell viability by MTS assay

U937 cells and gingival fibroblasts were seeded in 96 well plates, with a density of 5000 cells/well, respectively. Cells were treated with the concentrations of *S. aureus* LTA (0—10 μg/ml) and PGN (0—100 μg/ml) (InvivoGen) for 24hr and 48hr. The MTS reagent (20 μl) (Promega, Madison, WI, USA) was added to each well, and cells were incubated at 37 °C for a further 2hr. The absorbance was detected at 490 nm with a Microplate Reader.

Gelatin zymography

Using the zymography, the gelatinase releases in culture supernatants were examined. Gelatin zymography was performed using sodium dodecyl sulfate-polyacrylamide gels (SDS—PAGE, 10%) copolymerized with 0.1% gelatin. After being measured with the BCA protein assay (Pierce, Rockford, IL, USA), the protein was loaded into the gel lane. Gels were subjected to electrophoresis at 90 V for 120 min and then washed twice for 20 min in renaturing buffer (2.5% Triton X-100) at room temperature, followed by 16—18 h at 37 °C in developing buffer. The resulting gels were stained with 0.5% Coomassie Brilliant Blue (Sigma—Aldrich). The enzymatic activities of gelatinases (pro-MMP-9, pro-MMP-2, and MMP-2) were detected as 92, 72, and 66 kDa bands over the dark background respectively. The gel images were then scanned directly and quantified by densitometric analysis with software ImageJ.

Experimental design

U937 cells, with a density of 10^6 cells/ml, were put in each well of the 48 well plates. In the experiment of gingival fibroblasts, a density of$2x10^4$ cells was selected. Before studies, the starvation was accomplished in a serum-free medium for 24hr. After being treated with different concentrations of *S. aureus* LTA and PGN for 48hr, the supernatants were collected, centrifuged, and stored at −80 °C for later analysis. The ranges of 0–10 μg/ml and 0–100 μg/ml were selected for LTA and PGN, respectively, according the previous report.[27] In the experiment treated with NF-κB inhibitor (100 μM PDTC) (Sigma–Aldrich) and MAPK inhibitors (10 μM SCH7 72984 for extracellular signal-regulated kinase 1 and 2, ERK 1/2; SB203580 for P38 MAPK; and SP600125 for c- Jun N-terminal kinase, JNK) (TargetMol, Boston, MA, USA), cells were pretreated with inhibitor 1–2 h prior to stimulation. Then, *S. aureus* LTA or PGN (10 μg/ml in each) was added for 48 h. The supernatants were collected, centrifuged and stored at −80 °C for later zymography.

Statistics

All data were presented as mean ± standard deviation. The normality of the distribution of variables was assessed with the Kolmogorov–Smirnov test. One-way ANOVA with Tukey's HSD/Fisher's protected LSD test, or Kruskal–Wallis test was applied for intergroup comparisons. A P value < 0.05 was considered statistically significant. All data were obtained from at least 3 independent experiments.

Results

Cell viability (MTS assay)

The cell viabilities of U937 cells and gingival fibroblasts in different concentrations of *S. aureus* LTA (0–10 μg/ml) and PGN (0–100 μg/ml) were evaluated. All stimulants showed no cellular toxicity to U937 cells or gingival fibroblasts in 24hr and 48hr (Fig. 1).

Gelatinase activities in U937 cells/fibroblasts after LTA or PGN treatment

In U937 cells, *S. aureus* LTA and PGN significantly increased the activities of pro-MMP-9 if the concentration was over 10 μg/ml (Fig. 2A). Similar findings were also observed in gingival fibroblasts for pro-MMP-2 and MMP-2 activities (Fig. 2B).

Role of NF-κB pathway on enhanced gelatinases in U937 cells or gingival fibroblasts

The pro-MMP-9 activities were significantly increased in U937 cells after LTA and PGN treatments; however, the increased activities were reduced after being given the NF-κB inhibitor of PDTC (Fig. 3A). In gingival fibroblast, the pro-MMP-2 and MMP-2 activities presented similar: they increased after LTA or PGN treatment but reduced after NF-κB inhibitor (Fig. 3B).

Role of MAPK pathway on stimulated MMPs in U937 cells or gingival fibroblasts

In U937 cells, the LTA or PGN treatment significantly enhanced the pro-MMP-9 activity (Fig. 4A). However,

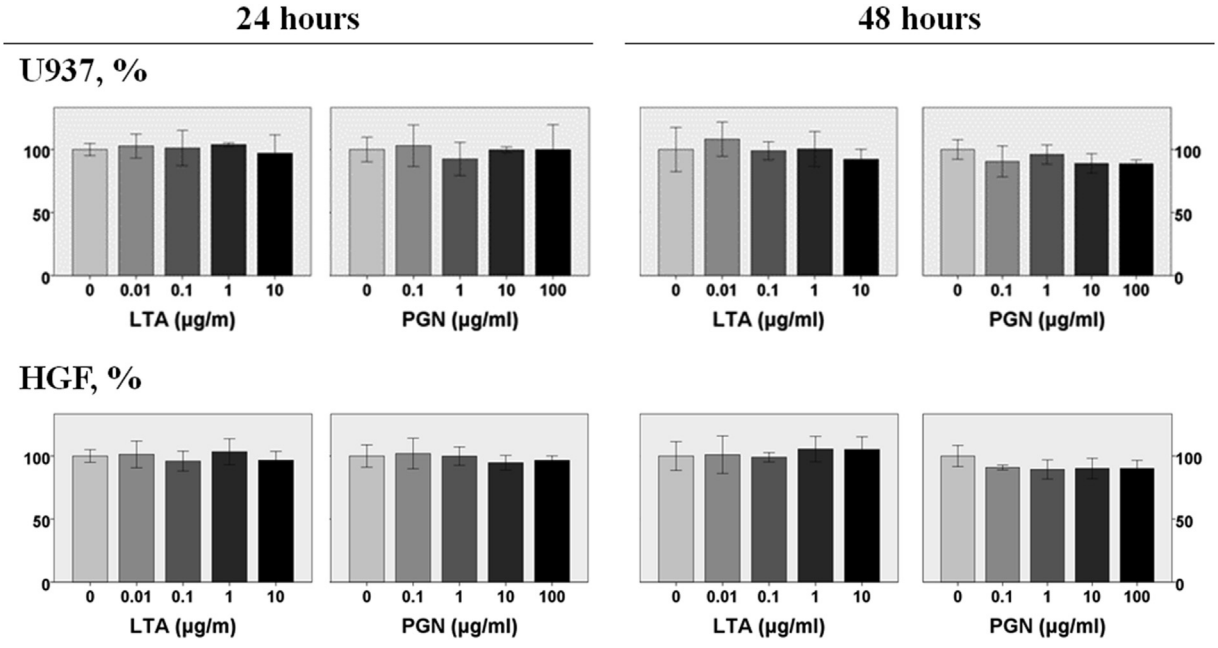

Figure 1 Viability of U937 cells and gingival fibroblasts after treatment with *Staphylococcus aureus* cell wall components of LTA or PGN. Data represent the percentage of cell viability relative to that of the control, and are given as mean ± SD (n = 5).

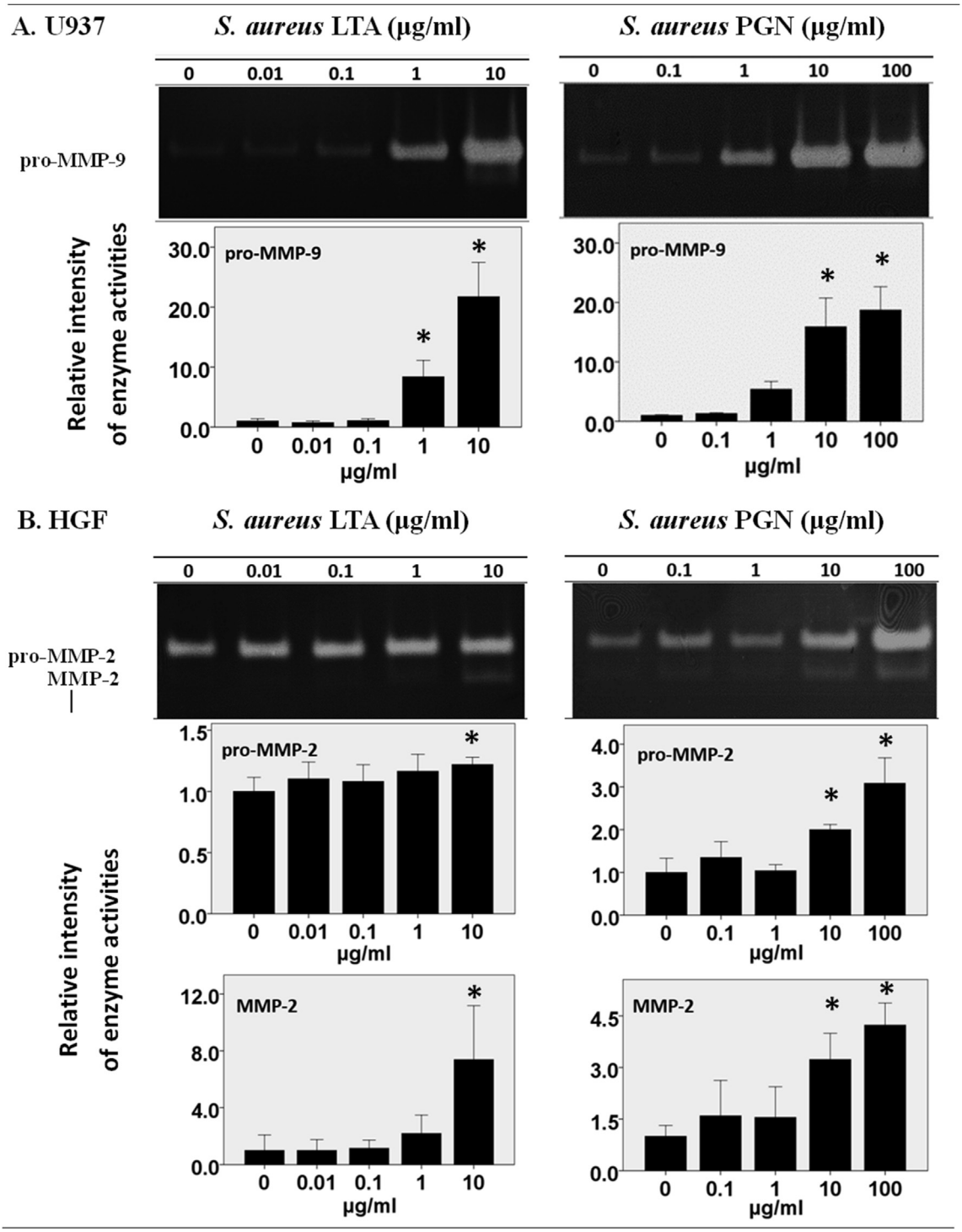

Figure 2 Effect of *Staphylococcus aureus* LTA or PGN on activities of gelatinases released from U937 cells and gingival fibroblasts. Effect of LTA (left) and PGN (right) on pro-MMP-9 activities released from U937 cells (A), and on pro-MMP-2/MMP-2 activities released from gingival fibroblasts (B). The gel images showed the enzyme activities released from the cells after 48 h treatment by zymography, whereas the underneath plots represented the statistical results. Data represent the relative intensity of enzyme activities, and the statistical results are given as mean ± SD (n = 3) (*: significance at $P < 0.05$).

the enhanced activities could be reduced by any of the MAPK inhibitors, despite that for ERK1/2, P38 MAPK, or JNK.

In gingival fibroblasts, LTA-enhanced pro-MMP-2 activity could be attenuated after being given any of the MAPK inhibitors (statistically indifferent noticed for ERK1/2)

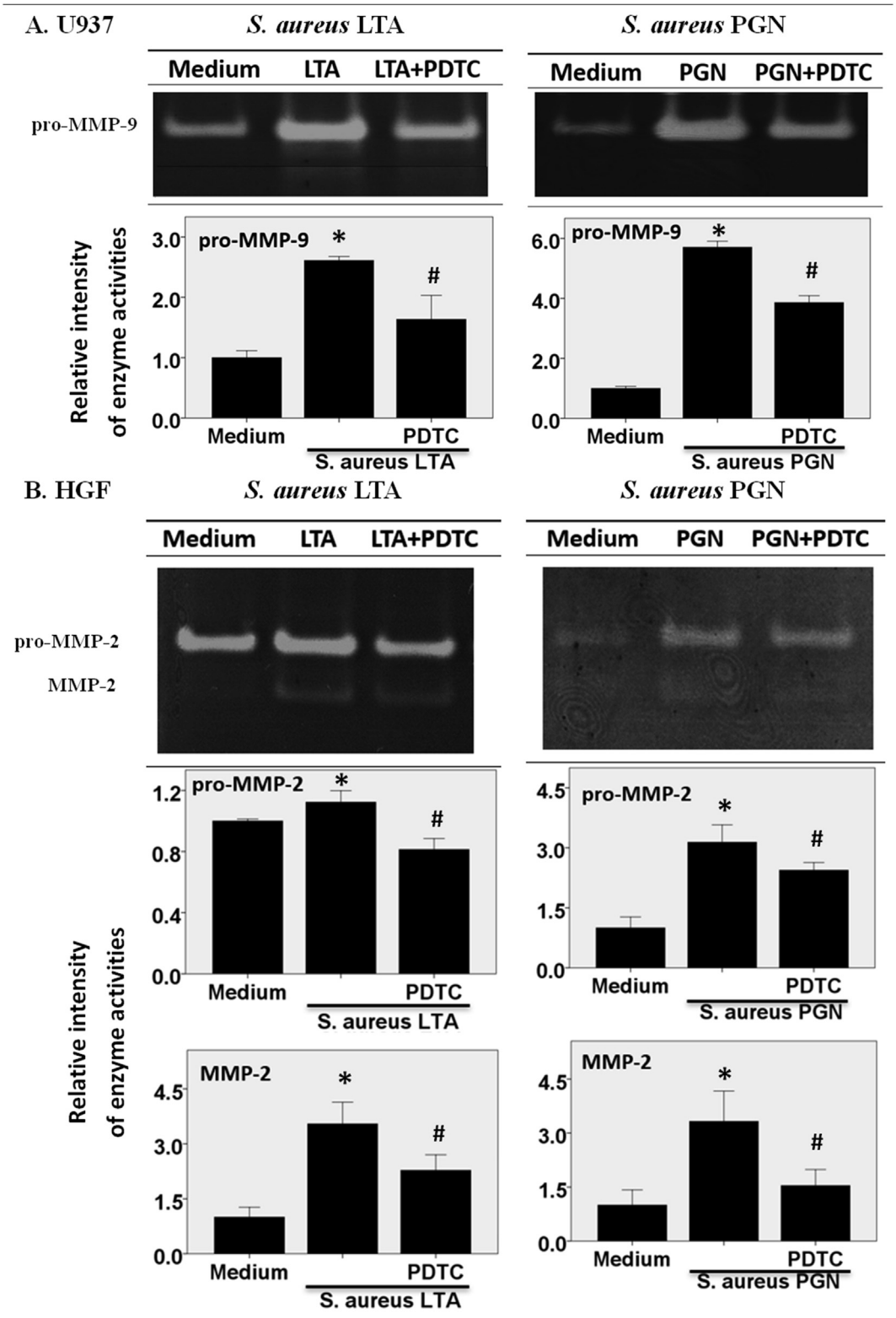

Figure 3 Role of NF-κB in the LTA- or PGN-enhanced gelatinases released from U937 cells or gingival fibroblasts. Role of NF-κB inhibitor (PDTC) in the enhanced pro-MMP-9 released from U937 cells (A), and in the enhanced pro-MMP-2/MMP-2 released from gingival fibroblasts (B). The gel images showed the enzyme activities released from the cells after 48 h treatment(s) by zymography, whereas the underneath plots represented the statistical results. Data represent the relative intensity of enzyme activities, and the statistical results are given as mean ± SD (n = 4) (* and #: significant different *vs* media and *vs* LTA/PGN treatment, respectively, at $P < 0.05$).

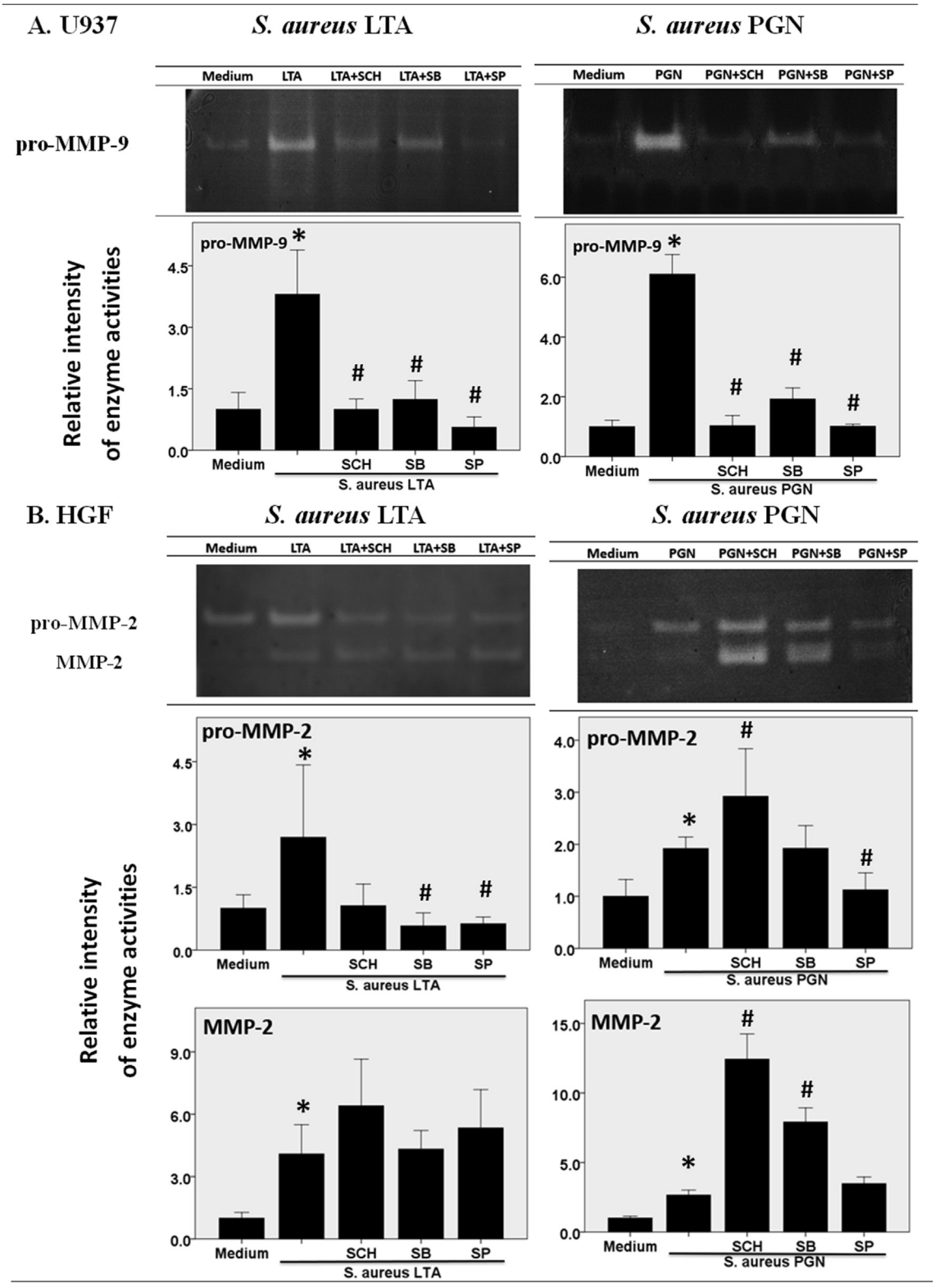

Figure 4 Role of MAPK in the LTA or PGN enhanced gelatinases released from U937 cells or gingival fibroblasts. Effect of MAPK inhibitors, including the inhibitors for ERK1/2 (SCH7 72984), P38 MAPK (SB203580), and JNK (SP600125), in the enhanced pro-MMP-9 released from U937 cells (A), and on the enhanced pro-MMP-2/MMP-2 released from gingival fibroblasts (B). The gel images showed the enzyme activities released from the cells after 48 h treatment(s) by zymography, whereas the underneath plots represented the statistical results. Data represent the relative intensity of enzyme activities, and the statistical results are given as mean ± SD (n = 4) (* and #: significant different *vs* media and *vs* LTA/PGN treatment, respectively, at $P < 0.05$).

(Fig. 4B). For PGN-increased activity, however, the reduction was observed only for JNK. Moreover, the LTA/PGN-enhanced MMP-2 activities were not attenuated by any of MAPK inhibitors (Fig. 4B).

Discussion

To elucidate the possible role of *S. aureus* on peri-implantitis, the effect of bacterial components of LTA and PGN on the gelatinase activities in gingival fibroblast and an inflammatory cell of monocytic U937 cell was evaluated *in vitro*. According to the cell wall structure and the reaction to the Gram stain, bacteria can be classified into two categories, including the Gram-positive or the Gram-negative.[28] The main structure of the bacteria cell wall is PGN, which maintains the shape and provides protection. Gram-positive bacteria contain thick layers of PGN, which enable to retain of the crystal violet dye during Gram staining. However, the Gram-negative bacteria have only a single thin layer of PGN with an outer membrane and LPS (endotoxin). *S. aureus*, as one of the Gram-positive bacteria, does not contain LPS but possesses LTA, which physiochemical properties similar to LPS.[29] These cell wall components including LTA, PGN, and LPS, can be recognized by toll-like receptor 2.[30,31] In the present study, we found that, after the treatment of *S. aureus* LTA or PGN, the gelatinase activities increased in U937 cells and gingival fibroblasts (Fig. 2). Similar to our results, Souza et al. showed that *S. aureus* LTA and PGN increase the MMP-9 activity of RAW 264.7 macrophages.[32] Kumar et al. also presented that intraocular injection of *S. aureus* LTA and PGN increased MMP-2 and -9 gelatinases expression in rat retina.[33]

Our results further showed, in U937 cells and gingival fibroblasts, the *S. aureus* LTA- or PGN-enhanced gelatinase activities could be regulated by the NF-κB pathway (Fig. 3). Besides, the MAPK pathway, including ERK1/2, p38 MAPK, and JNK subfamilies, were also involved with the enhanced pro-MMP-9 activity in U937 cells (Fig. 4). However, in gingival fibroblasts, the enhanced MMP-2 activities were not associated with the three MAPK sub-pathways, although the enhanced pro-MMP-2 activities were consistently regulated by JNK (Fig. 4). Our findings might indicate that the two different cell types of the U937 cells and the gingival fibroblasts examined in this study exhibited different mechanisms in reacts to the stimulants from *S. aureus* LTA and PGN. Tsai et al. showed that *S. aureus* enhanced MMP-9 level in human aortic endothelial cells via activation of NF-κB, ERK1/2, p38 MAPK, but not JNK pathway,[34] whereas Souza et al. revealed that *S. aureus* LTA increased MMP-9 expression on ERK rather than p38 MAPK or JNK pathway in RAW 264.7 macrophages.[32]

Besides, MMP activity could be regulated by several growth factors and cytokines. Studies have shown that the membrane-type I-matrix metalloproteinase (MMP-14) and the tissue inhibitors of metalloproteinase-2 (TIMP-2) participate in the pro-MMP-2 activation,[35] which reveal the complexity of mechanisms. These may partially explain the unexpected results in the experiment treated with MAPK inhibitors in LTA or PGN stimulated gingival fibroblasts. For instance, the LTA- and PGN-enhanced MMP-2 activities could not be attenuated by any of the MAPK inhibitors (Fig. 4B). Recent studies have further indicated a cross-talk between gingival fibroblasts and monocytes,[26,36] which may further affect the MMP activities observed in the present study. In the current experiment, two types of cells (U937 cells and the primarily cultured gingival fibroblasts) were selected and used. The U937 cells are a monocytic cell line derived from histiocytic lymphoma;[37] however, this cell line has been widely used to study MMP expression and activity.[22,38] In gingival fibroblasts, this is the first study that showed the MMP-2 activities could be enhanced after challenging *S. aureus* via the NF-κB pathway, while that via MAPK was still uncertain. Further detailed investigation is still indicated.

In conclusion, the present *in vitro* study indicated that the cell wall structures of *S. aureus* could enhance the gelatinase activities released from the monocytic U937 cells and the gingival fibroblasts, via the up-regulation of NF-κB. Our results further suggested that the MAPK pathway might involve in the *S. aureus* enhanced MMP-9 activities from U937 cells; however, the involvement of MAPK in the enhanced MMP-2 from gingival fibroblasts might still be questioned. A further detailed investigation is indicated.

Declaration of competing interest

The authors claim to have no conflicts of interest related to this paper.

Acknowledgments

This work was supported by a grant from Tri-Service General Hospital (TSGH-D-109046).

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