The Effect of *Porphyromonas gingivalis* Augmented Invasion by TNF-α on Gingival Fibroblasts Derived from Down Syndrome

TNF-α によって侵入が増大した *Porphyromonas gingivalis* の Down 症候群由来歯肉線維芽細胞への影響

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The Effect of *Porphyromonas gingivalis* Augmented Invasion by TNF-α on Gingival Fibroblasts Derived from Down Syndrome

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Abstract

It is well known the high prevalence of periodontal disease in Down syndrome (DS). Tumor necrosis factor alpha (TNF-α) augments invasion of *Porphyromonas gingivalis (P. gingivalis)* in human gingival epithelial cells. We examined the effect of TNF-α on invasion of *P. gingivalis* to gingival fibroblasts derived from individuals with DS (DGF) and the influence of the cellular response in DGF pretreated with TNF-α prior to inoculation of *P. gingivalis*. The invasion assay was performed using gingival fibroblasts derived from individuals with non-DS (NGF) and DGF treated with TNF-α prior to inoculation of *P. gingivalis*. The mRNA expression of interleukin 6 (IL-6) and intercellular adhesion molecule 1 (ICAM-1) were quantified using real time PCR. Furthermore, the protein expressions of TNF-α receptor (TNFR) I and II, and phosphorylation of p65 nuclear factor-κB (NF-κB) and phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 were also performed in NGF and DGF pretreated with TNF-α prior to inoculation of *P. gingivalis* by Western blotting. The number of invasive *P. gingivalis* in TNF-α pretreated DGF was more than that in NGF. IL-6 and ICAM-1 mRNA expressions in DGF pretreated with TNF-α prior to inoculation of *P. gingivalis* were significantly higher than those in NGF. The relative signal intensity of TNFR I, phospho p-65 NF-κB, and phospho ERK1/2 were significantly higher than those in NGF. It was considered that TNFR I, phospho p65 NF-κB, and phospho ERK1/2 in DGF pretreated with TNF-α may contribute to enhancement of ICMA-1 mRNA expression and the infection of *P. gingivalis*. The collaboration of

these protein and P. gingivalis may be a key factor for development of severe periodontal disease in DS.

Introduction

Down syndrome (DS) is a genetic disorder caused by all or a part of a third copy of chromosome 21 (1). DS associated with abnormal immune function and immune defects is highly susceptible to bacterial and viral infections (2). It is well known the high prevalence of periodontal disease initiated by subgingival infection with Gram-negative bacteria in DS (3). Previous studies indicate that severe periodontitis in individuals with DS can not be explained by poor oral hygiene (4). Many investigators suggest that immunodeficiency of individuals with DS due to abnormal genetic background causes severe periodontitis (5, 6). We have indicated the enhancement of inflammatory mediators in gingival fibroblasts derived from individuals with DS (DGF) stimulated by lipopolysaccharide (LPS) from *Porphyromonas gingivalis* (*P. gingivalis*), which is one of the major periodontal pathogens, compared to those in gingival fibroblasts derived from individuals with non-DS (NGF) (7, 8). However, early-onset and rapid progression of periodontitis in individuals with DS remain unclear. *P. gin*givalis is considered a major etiological agent in periodontitis that destroys tooth-supporting tissues (9). *P. gingivalis*, an asaccharolytic and highly proteolytic Gram-negative anaerobe, can invade human gingival fibroblasts (hGF) and survives inside (10). Murakami *et al*. (11) indicates that the adhesion

and invasion efficiencies of *P. gingivalis* are significantly greater with DGF compared to NGF. According to Kato *et al*. (12), tumor necrosis factor alpha (TNF-α) augments invasion of *P. gingivalis* in human gingival epithelial cells (hGE). TNF-α plays an important role in the initial and long-term host immune protection against bacteria (13). Murphy *et al*. (14) demonstrates the overexpression of TNF-α of thymuses in individuals with DS compared to age-matched individuals with non-DS. Furthermore, Zhang *et al.* (15) reported that the individuals with DS had significantly increased circulating TNF-α levels compared to individuals without DS by meta-analysis of 19 studies from Pubmed and Web of Science databases. In addition, interleukin-6 (IL-6) plays an important role in immune response, inflammation, and bone metabolism in periodontitis (16). Intercellular adhesion molecule 1 (ICAM-1), which belongs to the Ig superfamily, is widely expressed on leukocytes, endothelial cells, epithelium, and fibroblasts (17). ICAM-1 is crucial for regulating the early phase of inflammatory responses in periodontitis (17). It is well known that IL-6 and ICAM-1 expressions are induced by TNF-α and bacterial endotoxin (17-19). The signaling pathway by TNF-α via TNF-α receptor (TNFR) I induce a proinflammatory pathway through NF-κB and MAPK and apoptosis through caspase (20).

In this study, we investigated *P. gingivalis* invasion to NGF and DGF pretreated with rTNF-α in order to prove the hypothesis that TNF-α augments the bacterial invasion and enhances the inflammatory response in DGF as compared with NGF.

Materials and Methods

Microorganism

P. gingivalis strain ATCC 33277 was cultured in Tryptic soy agar containing L-cysteine (800 mg/L), hemin (5 mg/ml), menadione (10 mg/L), 0.5 % yeast extract and sheep blood (50 ml/L) at 37 °C under anaerobic conditions. The bacterial counts in cell suspensions were measured using a spectrophotometer (BioSpectrometer; Eppendorf, Germany) at a wavelength of 600 nm by measuring their opacity and adjusted (OD600 =1.0) for our experiments. *P. gingivalis* was washed twice with sterilized phosphate-buffered saline (PBS) and suspended in Dulbecco's modified Eagle's medium (D-MEM) (Sigma, USA) for experiments.

Cell isolation

We collected the gingival tissues from 4 donors with DS (age range: 8-12 years) and with non-DS (age range: 16-18 years). We cultured DGF and NGF according to Somerman *et al* (21) with some modification. This study was performed under the research ethics committee (ethical approval number: EC16-15-011-1). The cells were cultured in D-MEM (Sigma, USA) containing 10% fetal bovine serum (FBS) and antibiotics (50 units/ml penicillin, 50 µg/ml streptomycin: GIBCO, USA) at 37°C in 95% air and 5% CO_2. The cells cultured through 6-10 passages were used for further experiments.

Informed consents from donors were obtained before starting the experiments.

Pretreatment of rTNF-α

DGF and NGF were cultured in 6-well culture plate at a density of 2×10^5 cells/well in D-MEM containing 10 % FBS for invasion assay and Western blotting. The cells were also cultured in a 60 mm culture dish at a density of 4×10^5 cells/dish to analyze mRNA expressions. After culturing them for 18 hours, the culture medium was replaced with fresh D-MEM without both FBS and antibiotics. The cells were pretreated with 10 ng/ml of rTNF-α (Sigma, USA) in D-MEM for 3 hours (12).

Invasion assay

Invasion of *P. gingivalis* was performed as described by Murakami *et al*. (11) and Kato *et al*. (12). Briefly, after NGF and DGF were precultured with rTNF-α for 3 hours, *P. gingivalis* was added to the wells at an MOI of 1:100 and cultured for 1.5, 4, and 24 hours in D-MEM without both FBS and antibiotics. The cells were washed 3 times with sterilized PBS and incubated for 1 hour in D-MEM containing with 200 μg/ml of metronidazole and 300 μg/ml gentamicin for killing the extra cellular *P. gingivalis* (11, 12). The cells were washed twice with sterilized PBS, and then 1 ml of sterilized distilled water was added for cell lysis. The intracellular *P. gingivalis* were plated on a Tryptic soy agar plate containing L-cysteine (800 mg/L), hemin (5 mg/ml), menadione (10 mg/L), 0.5 % yeast

extract and sheep blood (50 ml/L) at 37 ℃ under anaerobic conditions for 7 days. The colonies were counted to determine the number of colony-forming units (CFUs) of invasive *P. gingivalis* in fibroblast cells. Invasion assays were performed 3 times for each sample.

Real-time PCR

NGF (n=4) and DGF (n=4) were cultured each in 3 wells. The experimental groups are four, the cells pretreated with only rTNF-α, the cells non-pretreated with rTNF-α prior to inoculation of *P. gingivalis* (inoculated with only *P. gingivalis*), the cells pretreated with rTNF-α prior to inoculation of *P. gingivalis*, and the cells without rTNF-α nor *P. gingivalis* (control). Total RNAs were extracted from cultured cells at 1.5 and 4 hours after inoculation of *P. gingivalis* using the RNeasy Mini Kit® (Qiagen, Germany). cDNAs were synthesized using a QuantiTect Reverse Transcription Kit ® (Qiagen, Germany). The mRNA expressions were analyzed by real-time PCR (EcoTMReal Time PCR System; Illumina, USA) using a KAPA SYBR FAST qPCR Kit® (Kapa Biosystems, USA) with primers for IL-6 and ICAM-1 (Table 1). These expression levels were normalized using the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The relative expression levels were using the $2^{-\Delta\Delta C(T)}$ method (22).

Western blotting

The cultured cells were washed 3 times with cold sterilized PBS at 15 mins after inoculation of *P. gingivalis* and treated with RIPA buffer (Fuji Film Wako chemical, Japan) containing with a protease inhibitor Cocktail®（Thermo Fisher Scientific, USA）and a phosphatase inhibitor Cocktail Solution® (Fuji Film Wako Chemical, Japan). The proteins were transferred to a nitrocellulose membrane after 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis. The membrane with transferred proteins was then probed with a specific primary antibody and reacted with a secondary antibody labeled with horseradish peroxidase. We used specific antibodies to TNFR I and II, p-65 NF-κB, ERK 1/2, and housekeeping protein β-actin as shown in Table 2. All primary antibodies were reacted at ratio 1:500, and secondary antibodies were reacted at a ratio of 1:5000. All assays were performed 3 times for each sample. The band of target proteins was quantified using an image analysis software, ImageJ (NIH, USA). The target proteins were normalized using β-actin and calculated as the relative signal intensity. The reference ratio of target protein and β-actin were calculated for compensation. The signal intensity of target protein in each sample was divided by the reference ratio (X). The signal intensity of β-actin in each sample was also divided by reference ratio (β). The relative intensity of Target protein in each sample was X/B.

Statistical analysis

Statistical analysis was performed using SPSS Version 26.00 (IBM, USA). Data were subjected to two

way ANOVA for comparing 4 experimental group, the cells pretreated with rTNF-α, the cells inoculated with only *P. gingivalis*, the cells pretreated with rTNF-α prior to inoculation of *P. gingivalis*, and control in NGF or in DGF. And then Tukey-Kramer's test was used to compare 4 experimental groups in DGF and NGF.

Results

The influence of rTNF-α on invading of P. gingivalis to DGF and NGF

Black-pigmented colonies were observed on the blood agar plates in samples from NGF and DGF pretreated with rTNF-α at 1.5, 4 hours after inoculation of *P. gingivalis*. The number of CFUs was decreased in a time-dependent manner in all plates (Fig. 1). There were no colonies at 24 hours on any plates from NGF and DGF. The numbers of invasive *P. gingivalis* in DGF were significantly more than those in NGF. In NGF and DGF pretreated with rTNF-α, the numbers of CFUs were increased compared to those inoculated with only *P. gingivalis*. In addition, the efficiencies of rTNF-α on the invasion of *P. gingivalis* into gingival fiborolasts were significantly greater in DGF than NGF (Fig.1).

mRNA expressions of NGF and DGF pretreated with rTNF-α prior to inoculation of P. gingivalis

IL-6 expressions in NGF pretreated and non-pretreated with rTNF-α were increased by inoculation of *P. gingivalis* in a time-dependent manner, and the expression level in the cells was higher than those

in the cells inoculated with only *P. gingivalis* and pretreated with only rTNF-α (Fig. 2A). On the contrary, IL-6 expressions were not increased by the inoculation with only *P. gingivalis* in DGF. However, the expressions were increased by pretreatment of rTNF-α and pretreatment of rTNF-α and the inoculation of *P. gingivalis* in a time dependent manner. In addition, the expression levels were significantly higher in DGF then those in NGF, pretreated with rTNF-α and inoculated with *P. gingivalis* (Fig 2A).

ICAM-1 expressions in DGF non-pretreated with TNF-α were significantly higher than those in NGF (Fig. 2B). The expressions of ICAM-1 were significantly increased by the inoculation of *P. gingivalis* compared with no inoculation in NGF and DGF pretreated with rTNF-α, and the expression levels were significantly higher in DGF than those in NGF at 1.5 and 4 hours after the inoculation of *P. gingivalis* (Fig. 2C). However, the expressions of ICAM-1 were slightly and significantly increased by the inoculation of *P. gingivalis* compared with no inoculation in NGF and DGF non-treated with rTNF-α (Fig. 2B), but the expression levels were significantly lower than those in all conditions of NGF and DGF pretreated with rTNF-α at 1.5 and 4 hours after inoculation of *P. gingivalis* (Fig. 2C).

The influence of P. gingivalis and rTNF-α on TNFR I, II, and the phosphorylated response of p-65 NF-κB and ERK 1/2 by Western blotting

In the preliminary experiment, we confirmed the phosphorylation of NF-κB and ERK1/2, family of mitogen activated protein kinase (MAPK), was detected within 15-30 mins after adding *P. gingivalis* by Western blotting (Data not shown). Therefore, we decided collecting the proteins at 15 mins after inoculation of *P. gingivalis*.

The relative signal intensity (rSI) of TNFR I were significantly increased by pretreatment with rTNF-α, non-pretreatment with rTNF-α and inoculation of *P. gingivalis,* and pretreatment with rTNF-α and inoculation of *P. gingivalis* compared with non-treatment and non-inoculation in NGF and DGF. The expression level was higher in pretreatment with rTNF-α and inoculation of *P. gingivalis* than others in DGF (Fig. 3A). There were no significant differences at all conditions in rSIs of TNFR II from both NGF and DGF (Fig. 3B).

The rSIs of phospho-p-65 NF-κB were significantly increased by pretreatment with rTNF-α and pretreatment with rTNF-α and inoculation of *P. gingivalis* compared with non-treatment and non-inoculation in NGF and DGF. The expression level was higher in pretreatment with rTNF-α and inoculation of *P. gingivalis* than those in DGF (Fig. 3C). The rSIs of phospho ERK1/2 were significantly increased by pretreatment with rTNF-α, non-treatment with TNF-α and inoculation of *P. gingivalis*, and pretreatment with rTNF-α and inoculation of *P. gingivalis* compared with non-treatment and non-inoculation in NGF and DGF. The expression level was higher in pretreatment with rTNF-α and inoculation of *P. gingivalis* than others in DGF (Fig. 3D). The visualized data of these

proteins are shown in Fig. 3E.

Discussion

We examined the invasion assay of *P. gingivalis* in the rTNF-α pretreated cells and non-pretreated cells at various incubation hours. The number of invasive *P. gingivalis* was increased in NGF and DGF pretreated with rTNF-α rather than that in the cells non-treated with rTNF-α at 1.5 and 4 hours. The colonies were not observed at 24 hours. The number of invasive *P. gingivalis* at 4 hours decreased in NGF and DGF pretreated and non-pretreated with rTNF-α. It is considered that *P. gingivalis* in GF were eliminated by an autophagic degradation process. Our results indicated that TNF-α could augment *P. gingivalis* invasion in hGF similarly as in hGE. Also, the number of invasive *P. gingivalis* in DGF pretreated with rTNF-α was significantly higher than that in NGF. It is considered that TNF-αplays an important role in the increased susceptibility to *P. gingivalis* infection by individuals with DS. *P. gingivalis* can enter and exit host cells using the cellular endocytosis pathway (23). Previous studies reported the dysfunction of autophagy and endosomal-lysosomal pathway in various cells derived from individuals with DS (24-26). Further, some studies suggested that the abnormally accelerated endocytosis associated with rab5, a small guanosine triphosphatase (GTPase) that regulates the early stages of endocytosis (27, 28). Kato *et al.* (12) indicated that rab5 mediated endocytosis of *P. gingivalis* and TNF-α induced level of the active form of rab5. Therefore, it is

considered that abnormal endocytosis in DS was involved in augmenting *P. gingivalis* invasion by pretreatment of rTNF-α in DGF.

We next examined the effect of *P. gingivalis* augmented invasion by TNF-α to the cellular response. Interestingly, IL-6 mRNA expressions stimulated with only *P. gingivalis* did not differ from control in DGF at 1.5 and 4 hours. It was considered that live *P. gingivalis* could not induce secondary cytokine IL-6 mRNA expression by itself in DGF. The LPS, which is a major virulence factor of Gram-negative bacteria, contribute to the enhancement of cytokines, like IL-6. Recently, it is said that the LPS derived from *P. gingivalis* plays different role from LPS derived from other Gram-negative bacteria (29, 30). The LPS derived from *P. gingivalis* play as antagonist to Toll-like receptor 4 (TLR4) in the condition of high concentration of hemin and play as agonist to TLR4 in the condition of low concentration of hemin and high temperature, over 39℃ (29, 30). In our study, *P. gingivalis* was added in D-MEM without hemin and at 37℃. Therefore, it is considered that *P. gingivalis* played as antagonist to TLR4. However, IL-6 mRNA expression in NGF was increasing in a time dependent manner. It is possible that DGF is high susceptibility to *P. gingivalis*. In addition, it is known that repeated exposure of the host cells to bacteria or their virulence factors leads to the tolerance. Although GF do not develop tolerance against the Lipopolysaccharide, repeated stimulation of live *P. gingivalis* reduced cytokines expressions in GF (31, 32). Therefore, it is considered that one-time stimulation of live *P. gingivalis* induced non responsiveness of IL-6 expressions like tolerance in DGF. Otherwise, there were some

other signaling pathways in DGF. It is needed further study to be clear of this phenomenon.

According to Murphy *et al*. (14), thymuses of individuals with DS overexpress mRNAs of IFN-γ and TNF. These cytokines induce ICAM-1 expression on cultured human thymic epithelial cells. In this study, the ICAM-1 mRNA expressions of DGF in control were significantly higher compared to NGF at 1.5 and 4 hours. Increased levels of IFN-γ and TNF-α might contribute to the enhanced expression of ICAM-1 in DGF. It was also considered that ICAM-1 was expressed constitutively in DGF. In addition, it is reported that ICAM-1 mediated endocytosis by stimulation of one of endocytic proteins, Dynamin 2 (Dyn2) via protein kinase C (33). Dyn2, which is a GTPase, regulates membrane constriction and fission coupled with GTP hydrolysis. Thus, it was considered that the constitutive expression of ICAM-1 in DGF helped augment *P. gingivalis* invasion to the cell. According to previous studies, high glucose condition also increases the invasion ability of *P. gingivalis* in hGF and then increases ICAM-1 and IL-6 via mitogen-activated protein kinase (MAPK) and NF-κB (34-36). Kato *et al.* (12) shows that TNF-α augments *P. gingivalis* invasion via NF-κB and MAPK. They suggest that TNF-α regulated activity of rab5 through the JNK pathway not through ERK. In our study, the rSI of phospho p-65 NF-κB in NGF and DGF treated with rTNF-αprior to inoculation of *P. gingivalis* were higher than that in NGF and DGF inoculated with only *P. gingivalis*. In addition, the rSI of phospho p-65 NF-κB in DGF treated with rTNF-α prior to inoculation of *P. gingivalis* was higher than that in NGF. These results seemed to indicate that the phosphorylation of p-65 NF-κB was enormously

influenced by pretreatment of TNF-α prior to inoculation of *P. gingivalis* in DGF. It was also considered that the escalated phosphorylation of p-65 NF-κB contributed to the enhancement of ICAM-1 mRNA expression in DGF. The rSI of phospho p-ERK in DGF treated with rTNF-αprior to inoculation of *P. gingivalis* was higher than that in DGF inoculated with only *P. gingivalis*, although there was no significant difference in the rSI of phospho p-ERK between NGF pretreated with rTNF-α prior to inoculation of *P. gingivalis* and NGF inoculated with only *P. gingivalis*. A previous study showed that DYRK1A, which is located on the 21st chromosome and is one of the responsible genes for DS, enhanced the MAPK, including ERK cascade (37). This abnormal genetic background is possibly associated with the increased rSI of p-ERK in DGF in this study.

The binding of TNF-α to TNFR I or TNFR II activates signaling pathways that regulate inflammatory and immune responses, and apoptosis (38). In general, TNFR I is expressed on almost all cells and TNFR II is expressed in immune cells (39). TNF-α induces immune modulation and tissue regeneration (39). Kato et al. (12) indicated that hGE expressed TNFR I but not TNFR II. However, it has been reported that TNFR I and II were expressed in GF (40, 41). In this study, TNFR I and II were detected in NGF and DGF treated with rTNF-α prior to inoculation of *P. gingivalis*. The expression levels of TNFR I in NGF were low, and there was no significant difference between the rSI in the cell treated with rTNF-α prior to inoculation of *P. gingivalis* and in the cells non-treated with rTNF-α. However, the expression levels of TNFR I in DGF were highly detected in the cells pretreated and

non-pretreated with rTNF-α prior to inoculation of *P. gingivalis*. In DGF inoculated with only *P. gingivalis,* TNFR I expression was higher than that in DGF pretreated with only TNF-α. It was considered that TNF-αwas administrated 3 hours prior to add *P. gingivalis.* However, rSIs of phospho p-65 NF-κB and phospho ERK 1/2 in the cells inoculated with only *P. gingivalis* were lower than those in the cells with only TNF-α. It was possible the difference of location of target proteins. TNFR I and II were located in cell membrane. NF-κB and ERK 1/2 were located in cytoplasm. The rSI in the cells pretreated with rTNF-α was significantly higher than that in the cells non-pretreated with rTNF-α. A previous study showed constitutive levels of TNF-α was high in the thymus from individuals with DS. It is considered that TNF-α expressed constitutively in DGF, the same as in the thymus and it induces TNFR I expression. There were no significant differences at all conditions in rSIs of TNFR II from both NGF and DGF. These results were different from that of Kato et al. (12). There is a possible difference in cell types between gingival fibroblasts and gingival epithelial cells. It is considered that *P. gingivalis* augmented invasion by rTNF-α has no effect on TNFR II in NGF and DGF.

In this study, we found *P. gingivalis* invasion was augmented by rTNF-α in NGF and DGF, the same as in hGE. DGF is subject to TNF-α on the invasion of *P. gingivalis* compared to NGF. In addition, *P. gingivalis* augmented the invasion by TNF-α enhanced the inflammatory responses in DGF. It was considered that these phenomena may be caused by abnormal genetic background and led to severe periodontitis in individuals with DS.

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Conflict of Interest

The authors have no conflicts of interest directly relevant to the content of this article.

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Tables

Table 1 Primer lists of tagert genes

Gene name	Forward (5'-3')	Reverse (5'-3')
IL-6	AAGCCAGAGCTGTGCAGATGAGTA	TGTCCTGCAGCCACTGGTTC
ICAM-1	TCGGCAAAAGCACTATAT	ACAGGACAAGAGGACAAGGC
GAPDH	GTAGAGGCAGGGATGATGT	TCCAAAATCAAGTGGGGCGA

Table 2 Antibodies to target protieins

	Target protein	Company	Source
TNFR I	TNF Receptor I	Abcam	Rabbit monoclonal
TNFR II	TNF Receptor II	Abcam	Rabbit polyclonal
phospho-p-65 NF-kB	phospho-NF-kB p65 (Ser536) (93H1)	Cell Signaling	Rabbit monoclonal
phospho-ERK 1/2	phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204)	Cell Signaling	Rabbit polyclonal
β-actin	β-actin	Sigma	mouse monoclonal

Figure legend

Fig. 1

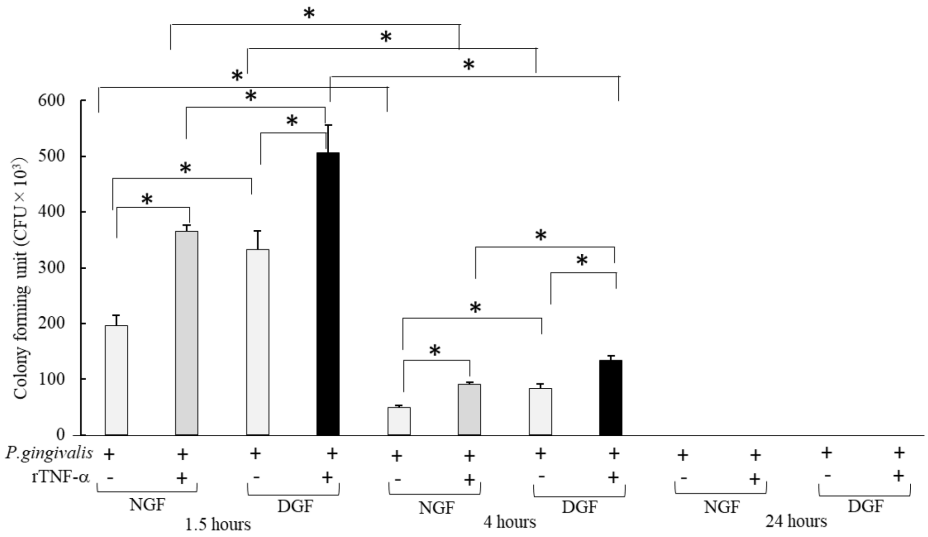

Fig. 1 The influence of rTNF-α on invading of *P. gingivalis* to DGF and NGF

P. gingivalis, +: the cells non-pretreated with rTNF-α prior to inoculation of *P. gingivalis*

rTNF-α, +: the cells treated with 10 ng/ml rTNF-α (3 hours) prior to inoculation of *P. gingivalis* MOI of 1:100.

Invasion assay were performed in NGF (n=4) and DGF (n=4) in triplicate. *: $p<0.05$

Fig. 2

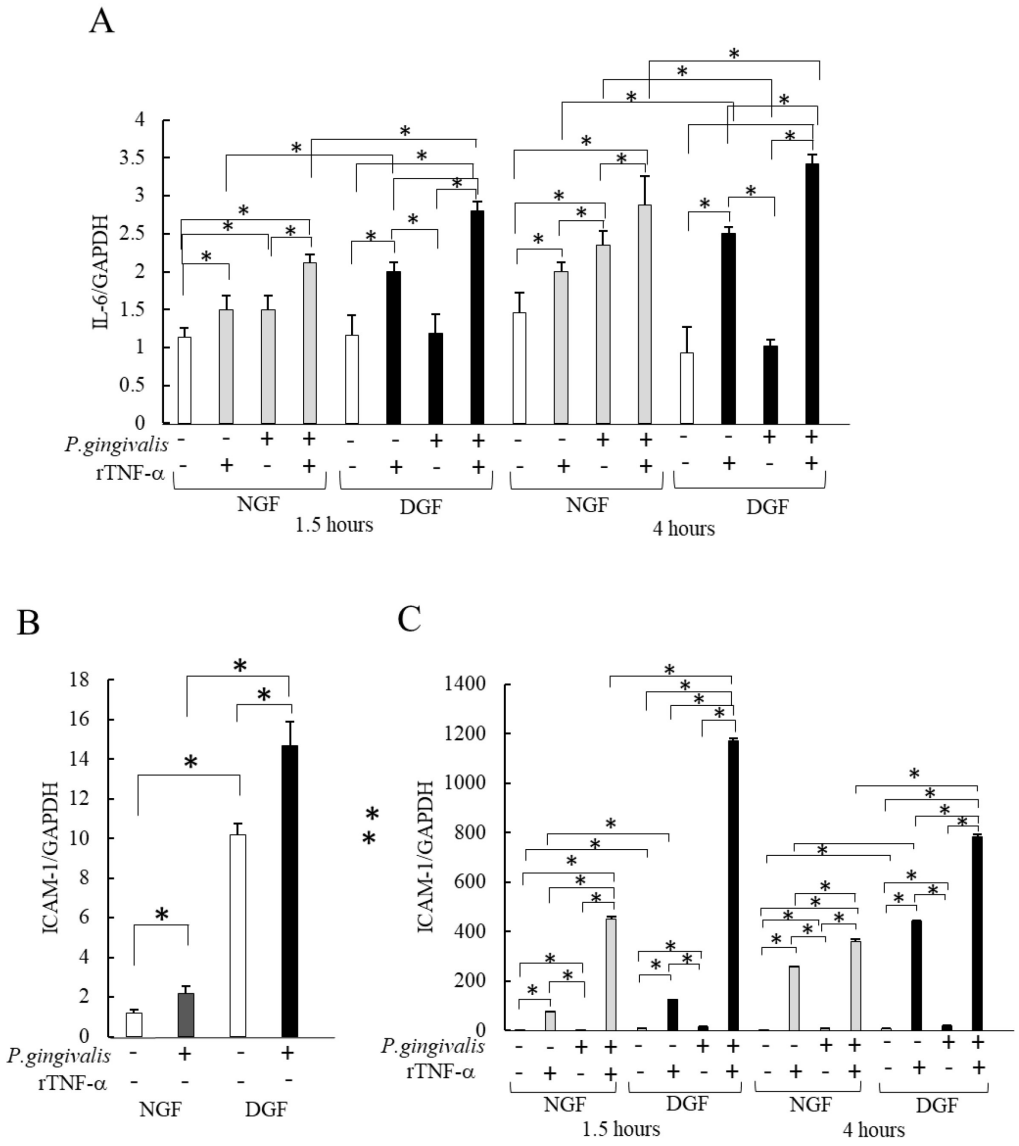

Fig. 2 mRNA expressions of IL-6 and ICAM-1 in NGF and DGF

A: IL-6 mRNA expressions, B: ICAM-1 mRNA expressions of control group in NGF and DGF at 1.5 hours incubation (excerpt of Fig2C), C: ICAM-mRNA expressions of all experimental group in NGF and DGF

P. gingivalis, +: the cells non-pretreated with rTNF-α prior to inoculation of *P. gingivalis*

rTNF-α ,+: the cells treated with 10 ng/ml rTNF-α (3 hours) prior to inoculation of *P. gingivalis* MOI of 1:100.

The mRNA expression analysis was performed by real time PCR and normalized by GAPDH. *: $p<0.05$

Fig. 3

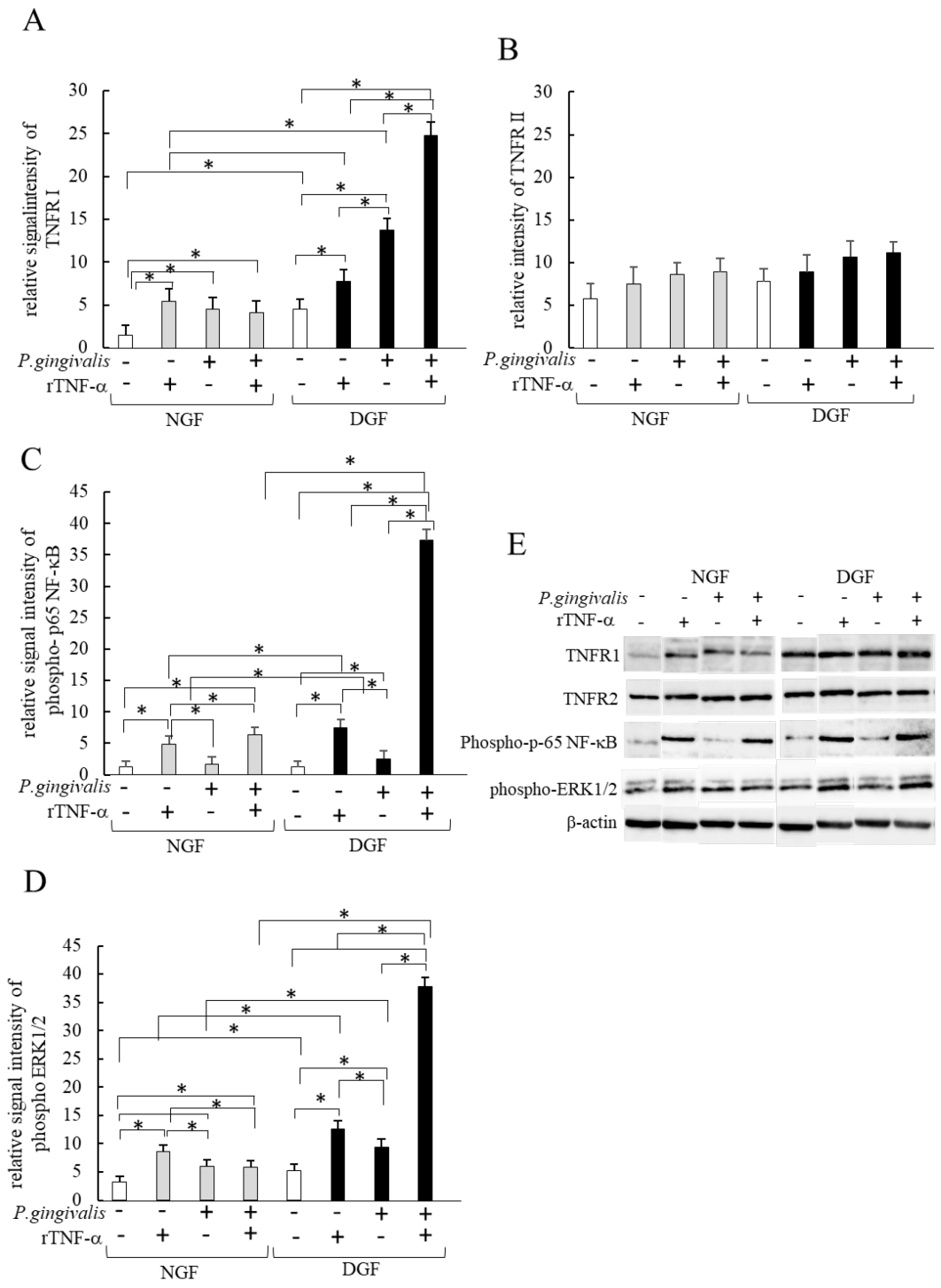

Fig. 3 Western blotting of TNFR I, II, and phosphorylated response of p-65 NF-κB and ERK 1/2
P. gingivalis, +: the cells non-pretreated with rTNF-α prior to inoculation of *P. gingivalis*

rTNF-α, +: the cells treated with 10 ng/ml rTNF-α (3 hours) prior to inoculation of *P. gingivalis* MOI of 1:100.

The signal of the target protein band was quantified using ImageJ and normalized by β-actin and calculated as relative signal intensity (rSI). A: rSI of TNFR I, B: rSI of TNFR II, C: rSI of phospho-p-65 NF-κB, D; rSI phospho-ERK 1/2, E: The band on the membrane of each target protein and β-actin from NGF and DGF. *: $p<0.05$