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Original Article

Porphyromonas gingivalis-derived lipopolysaccharide promotes mesangial cell fibrosis via transforming growth factor-beta1/Smad signaling pathway in high glucose

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Abstract *Background/purpose:* Periodontitis has been documented to increase the risk of diabetic nephropathy. However, the specific mechanisms through which periodontitis affects renal function remain unclear. This study aimed to investigate the mechanism by which an inflammatory reaction stimulated by periodontal pathogens affects mesangial cell fibrosis under hyperglycemic conditions *in vitro*.

Materials and methods: Murine mesangial cells were stimulated with 1,000 ng/mL of *Porphyromonas gingivalis*-derived lipopolysaccharide (PgLPS) in a control or high glucose (HG) medium. Activation of the extracellular signal-regulated kinase (ERK1/2) and expression of alpha-smooth muscle actin (α -SMA) and collagen type 1a2 (*Col1a2*) were analyzed for fibrosis and transformation via the transforming growth factor (TGF)- β 1/Smad signaling pathway.

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Results: PgLPS stimulation significantly upregulated TGF- β 1 expression and Smad3 phosphorylation in the HG group compared to the control group. Additionally, activation of ERK1/2 and expression of *Col1a2* and α -SMA were significantly elevated in the HG group compared to the control following PgLPS stimulation. The TGF- β 1 inhibitor significantly suppressed Smad3 phosphorylation and mRNA expression of *Col1a2* in the HG group.

Conclusion: Under HG conditions, PgLPS may aggravate fibrosis in mesangial cells via the TGF- β 1/Smad signaling pathway, leading to nephrosclerotic modifications. The presented study may support the association between periodontitis and chronic kidney disease, mediated by hyperglycemia.

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Introduction

Periodontal disease is characterized by local inflammation due to infection with pathogenic bacteria, such as *Porphyromonas gingivalis* (*P. gingivalis*), which leads to alveolar bone resorption and tooth loss. Diabetes has been known to demonstrate a two-way relationship with periodontal disease,^{1–4} which was evidenced to be a risk factor for the development and progression of microvascular complications in patients with Type 2 diabetes mellitus (T2D).^{5–7} A systematic review reported that periodontitis enhances the risk of diabetic nephropathy.⁸ Accumulating evidence exists regarding the effects of periodontitis on kidney disease.^{9–13} Cohort studies in the general population have shown that patients with periodontitis have an increased risk of decline in renal function.^{14,15} A meta-analysis indicated that patients with periodontitis are at significantly higher risk of developing chronic kidney disease.¹⁶ However, few studies have investigated the effects of periodontitis on renal function in patients with diabetes.

Renal fibrosis is associated with chronic kidney disease and diabetic nephropathy, leading to a decline in renal function. The Smad signaling pathway is implicated in the fibrosis of mesangial cells in the kidney.¹⁷ Specifically, the Smad2 and 3 are primarily activated by transforming growth factor beta (TGF- β). In this context, the TGF- β /Smad pathway is a central driver of fibrosis and a potential therapeutic target.¹⁸

The mechanisms through which periodontal disease affects renal function remain unclear. Previous *in vivo* studies using diabetic rodents have discovered that experimental periodontitis induced by lipopolysaccharide (LPS) derived from *P. gingivalis* causes histological modifications in the glomeruli, leading to renal dysfunction.^{19,20} Mesangial cells play an important role in maintaining glomerular structure and function, and their fibrosis compresses the glomerular capillaries and hinder glomerular filtration.²¹ This prompted the hypothesis that periodontal inflammation triggered by *P. gingivalis* affects the fibrosis of mesangial cells under hyperglycemic conditions.

This study aimed to investigate the mechanism by which an inflammatory reaction stimulated by periodontal pathogens affects mesangial cell fibrosis through TGF- β /Smad signaling under hyperglycemic conditions *in vitro*.

Material and methods

Cell culture

Primary mouse mesangial cells²² were cultivated in Ham's tissue culture medium.²³ The cell isolation from animal isolated and were approved by the Institutional Animal Care and Use Committee of Osaka Medical and Pharmaceutical University (OMPU) (No. AM23-003). This animal care and use protocol adhered to the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions by the Ministry of Education, Culture, Sports, Science and Technology (Notice No. 71 issued on June 1, 2006), the Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain by the Ministry of the Environment (Notice No.84 issued on August 30, 2013), and the Act on Welfare and Management of Animals (the last revision on September 5, 2012) in Japan. This study conformed with the ARRIVE guidelines. Subsequently, the cells were incubated in Dulbecco's Modified Eagle Medium supplemented with glucose concentrations of either 5.5 mM (control medium; Cont) or 25 mM (high glucose medium; HG) for 72 h before the experiment.

LPS stimulation

Interleukin-6 (IL-6) activation was studied due to its reported involvement in the glomeruli induced by hyperglycemia.²⁴ The cells were stimulated with 1000 ng/mL of *P. gingivalis*-derived lipopolysaccharide (PgLPS) for 3 h. mRNA of *Il-6* expression was measured via real-time quantitative polymerase chain reaction (qPCR). Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). The extracted RNA was reverse transcribed to cDNA using Reverse transcription of total RNA was performed using a Prime Script RT Reagent Kit (Takara, Shiga, Japan). qPCR was performed with a Thermal Cycler Dice 1 Real Time System II (Takara) in reactions with a standard real-time PCR reagent SYBR1 Premix Ex Taq II (Takara). Gene expression levels were normalized to 18s ribosomal RNA (18s rRNA). The primer sequences are listed in Table 1.

Table 1 Primers used for qPCR analysis.

	Gene name	Forward	Reverse
MGI:MGI:97943	m18s rRNA	GCTTAATTTGACTCAACACGGA	AGCTATCAATCTGTCAATCCTGTC
MGI:MGI:96559	<i>Il-6</i>	ATAGCTCCCAGAAAAGCAAGC	CACCCCGAAGTTCAGTAGACA
MGI:MGI:98725	<i>Tgfβ1</i>	CAACGCCATCTATGAGAAAACC	AAGCCCTGTATTCCGTCTCC
MGI:MGI:88468	<i>Col1a2</i>	CAGAACATCACCTACCACTGCAA	TTCAACATCGTTGGAACCTG

m18s rRNA, 18s ribosomal RNA; *Il-6*, interleukin-6; *Tgfβ1*, transforming growth factor beta1; *Col1a2*, collagen type1a2.

NF-κB activation was measured by p65 expression after 1 h following *Pg*LPS stimulation by Western Blotting. The cells were fractionated and extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, USA). The protein of nuclear fractionation was electrophoresed in a sodium dodecyl sulfate-polyacrylamide gel and electrotransferred to nitrocellulose membranes. The membranes were incubated in a 1:1,000 dilution of NF-κB p65 (C22B4, Cell Signaling Technology, Danvers, MA, USA) antibodies at 4 °C overnight. Protein expression was standardized by proliferating cell nuclear antigen (PCNA) at 1:2000 dilution. The membranes were incubated in a 1:5,000 dilution of donkey anti-rabbit (sc-2313, Santa Cruz Biotechnology, Dallas, TX, USA) and goat anti-mouse immune globulin G (IgG)-HRP secondary antibody (SA00001-1 Proteintech, Tokyo, Japan) at room temperature for 1 h. The enhanced chemiluminescence reaction generated using the enhanced chemiluminescence (ECL) Western Blotting (Thermo Fisher Scientific).

Transforming growth factor (TGF)-β1 expression

Tgfβ1 mRNA expression was measured after 3 h following *Pg*LPS stimulation using qPCR. TGF-β1 expression in the supernatant was measured after 12 h following *Pg*LPS stimulation by an enzyme-linked immunosorbent assay (ELISA) (Enzyme-linked immunosorbent assay, Proteintech) per manufacturer instructions.

Fibrosis pathway

Activation of TGF-β1/Smad pathway can mediate extracellular matrix formation in Diabetic kidney disease (DKD).²⁵ Activation of extracellular signal-regulated kinase (ERK1/2) is associated with increasing extracellular matrix in mesangial cells.²⁶ The cells were harvested by scraping with a lysis buffer (RIPA buffer, FUJIFILM Wako Pure Chemical, Osaka, Japan), protease inhibitor cocktail (Roche, Basel, Switzerland), and a phosphatase inhibitor (Sigma–Aldrich, St. Louis, MO, USA). Phosphorylation of ERK1/2 and Smad3 and expression of alpha-smooth muscle actin (α-SMA) were evaluated by Western blotting at 3, 12, or 24 h following *Pg*LPS stimulation. Antibodies of ERK1/2 (#4695 S, Cell Signaling Technology), pERK (#9101 S, Cell Signaling Technology), Smad2/3 (#8685, Cell Signaling Technology), pSmad3 (#9520, Cell Signaling Technology), and α-SMA (#117614, SIGMA, Burlington, MA, USA) were applied in a 1:1,000 dilution at 4 °C overnight. Protein expression was standardized by comparing the signal from a monoclonal horseradish peroxidase (HRP)-conjugated anti-β-actin antibody at 1:1000 dilution. The secondary antibody was applied

in a 1:5,000 dilution for 1 h at room temperature. The ECL reaction was analyzed as described above. The mRNA of Collagen type 1a2 (*Col1a2*) expression was measured 12 h after *Pg*LPS stimulation with or without SB-525334 (Selleck Biotech, Tokyo, Japan), a selective inhibitor of TGF-β1 to examine TGF-β1/Smad pathway on mesangial cells.

Statistical analysis

Data are presented as means ± standard deviation (SD).

Tukey–Kramer test was used for comparisons between multiple groups. *P* values of less than 0.05 were considered to be statistically significant.

Results

LPS stimulation

Incubation in HG conditions with *Pg*LPS increased expression of mRNA levels of *Il-6* compared to HG without *Pg*LPS

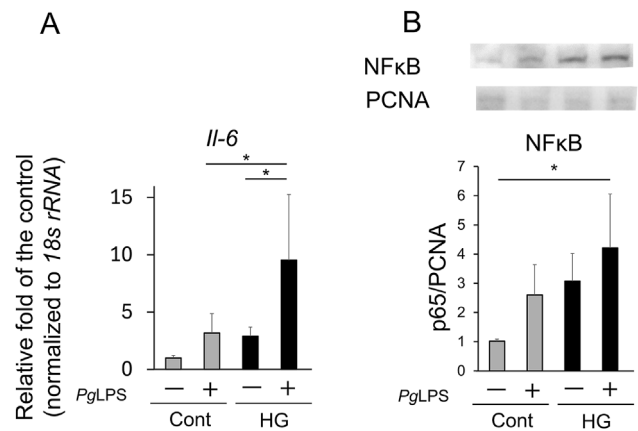


Figure 1 The mRNA expressions after 3 h of *Porphyromonas gingivalis*-derived lipopolysaccharide (*Pg*LPS) stimulation in mouse mesangial cells cultured in 5.5 mM (Cont) and 25 mM glucose medium (HG). (A) Expression levels of interleukin-6 (*Il-6*) were measured with real-time qPCR. The data were standardized by 18s ribosomal RNA (18s rRNA) and presented as the mean ± standard deviation (*n* = 4). (B) The protein expression of nuclear factor-kappa B (NF-κB) was evaluated by western blotting after 1 h of *Pg*LPS stimulation and compared to that of the control. Representative immunoblots of lysates standardized by proliferating cell nuclear antigen (PCNA). Protein expression was quantified by densitometry and expressed as the ratio in the Cont group without *Pg*LPS stimulation. The data are presented as the mean ± standard deviation (*n* = 4). **P* < 0.05 (Tukey–Kramer test).

(4.05 ± 1.69 -fold, $P = 0.012$) (Fig. 1A). The activation of NF- κ B was significantly elevated in HG with PgLPS compared to Cont (4.15 ± 1.52 -fold, $P = 0.03$) (Fig. 1B).

Fibrosis pathway

In mesangial cells, mRNA levels of *Tgfb1* were significantly elevated in HG condition with PgLPS compared to HG without PgLPS at 3 h (1.70 ± 0.78 -fold, $P = 0.022$), and protein levels of TGF- β 1 were significantly elevated in HG condition with PgLPS compared to HG without PgLPS at 12 h (1.19 ± 0.11 $P < 0.0001$) (Fig. 2A and B). The phosphorylation of ERK1/2 was significantly elevated in HG condition with PgLPS compared to HG without PgLPS at 3 h (1.43 ± 0.36 -fold, $P = 0.027$) (Fig. 2C). The phosphorylation of Smad3 was significantly elevated in HG conditions with PgLPS group compared with that in HG conditions without PgLPS at 12 h (by 2.55 ± 1.90 -fold, $P = 0.012$). TGF- β 1 receptor selective inhibitor SB-525334 reduced HG-

induced increases in pSmad3 (0.55 ± 0.20 -fold, $P = 0.025$) indicating that it occurred downstream of TGF- β 1 (Fig. 3A). Extracellular matrix, *Col1a2*, and α -SMA were significantly elevated in HG condition with PgLPS compared to HG without PgLPS at 12 h (3.54 ± 1.04 -fold $P < 0.0001$) and at 24 h (1.16 ± 0.46 -fold $P = 0.005$). SB-525334 significantly reduced the *Col1a2* (0.34 ± 0.87 -fold $P < 0.0001$) but not significant difference in α -SMA expression (Fig. 3B and C).

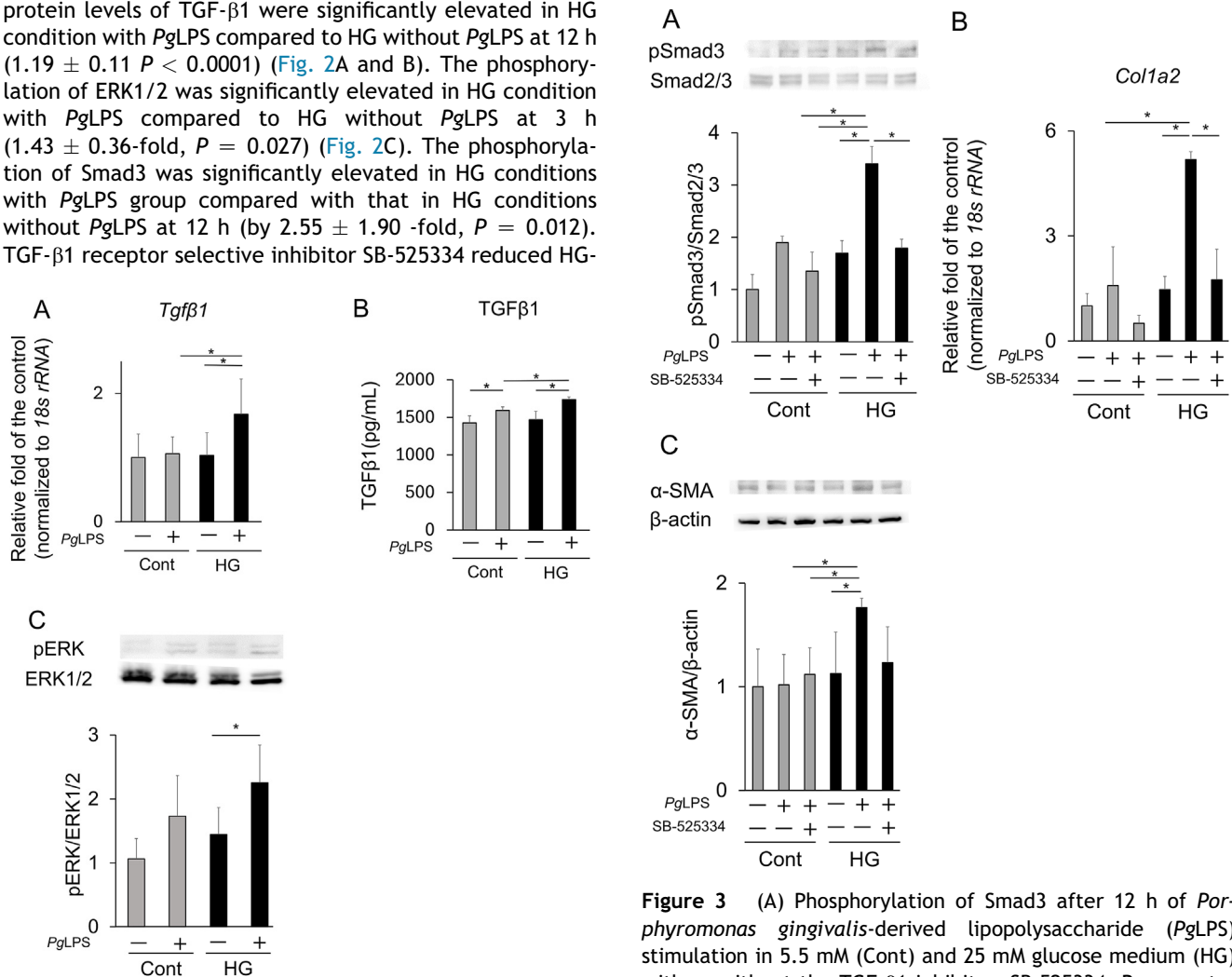


Figure 2 Enhancement of mesangial cell differentiation after 3 h of PgLPS stimulation in mouse mesangial cells cultured in 5.5 mM (Cont) and 25 mM glucose medium (HG). (A) Expression levels of transforming growth factor beta1 (*Tgfb1*) were measured with real-time qPCR. The data were standardized by 18s ribosomal RNA (18s rRNA) and presented as the mean $\pm$ standard deviation ($n = 4$). (B) The protein expression of TGF- β 1 in the supernatant was evaluated by ELISA after 12 h of PgLPS stimulation and compared to that of the control. (C) Phosphorylation of extracellular signal-regulated kinase1/2 (ERK1/2) after 3 h of PgLPS stimulation. Representative immunoblots of cell lysates standardized by ERK1/2 staining. Phosphorylation was quantified by densitometry and expressed as the ratio of phosphorylation in the Cont group without PgLPS stimulation. The data are presented as the mean $\pm$ standard deviation ($n = 4$) * $P < 0.05$ (Tukey–Kramer test).

Figure 3 (A) Phosphorylation of Smad3 after 12 h of *Porphyromonas gingivalis*-derived lipopolysaccharide (PgLPS) stimulation in 5.5 mM (Cont) and 25 mM glucose medium (HG) with or without the TGF- β 1 inhibitor, SB-525334. Representative immunoblots of cell lysates standardized by Smad2/3 staining. Phosphorylation was quantified by densitometry and expressed as the ratio of phosphorylation in the Cont group without PgLPS stimulation. The data are presented as the mean $\pm$ standard deviation ($n = 4$). (B) The mRNA expression of Collagen type 1 a2 (*Col1a2*) after 12 h of PgLPS stimulation in Cont and HG, with or without SB-525334. The data were standardized by 18s ribosomal RNA (18s rRNA) and presented as the mean $\pm$ standard deviation ($n = 4$). (C) Protein expression of alpha-smooth muscle actin (α -SMA) after 24 h of PgLPS stimulation in Cont and HG, with or without SB-525334. Representative immunoblots of lysates standardized by β -actin. Protein expression was quantified by densitometry and expressed as the ratio in the Cont group without PgLPS stimulation. The data are presented as the mean $\pm$ standard deviation ($n = 4$). * $P < 0.05$ (Tukey–Kramer test).

Discussion

In this study, we examined the effects of PgLPS on mesangial cells under hyperglycemic conditions and found that PgLPS promotes cell fibrosis via TGF- β 1/Smad signaling. To the best of our knowledge, this is the first study to report the pathway which glomerular fibrosis is promoted by the mesangial cells affected by diabetes and periodontitis.

Inflammation causes kidney fibrosis and reduces the glomerular filtration rate. The transformation of mesangial cells promotes fibrosis, which compresses the glomerular capillaries and reduces glomerular filtration. In this process, it is known that TGF- β 1 promotes the production of collagen type 1 via the Smad2/3 pathway.²⁷ When TGF- β binds to transforming growth factor-beta1 receptor (TGF β R) on the cell surface, the internal region of the TGF β R is phosphorylated and Smad2 and Smad3 are activated and translocated to the nucleus. The transnuclear Smad complex acts as a transcription factor and increases fibrosis-related genes (e.g., collagen and fibronectin) in renal mesangial cells.

The transnuclear Smad complex acts as a transcription factor and increases fibrosis-related genes (e.g., collagen and fibronectin) in renal mesangial cells. Mesangial cells undergo transformation into myofibroblasts in response to external stimuli, thereby inducing fibrosis of the underlying cellular substrate. It has been reported that α SMA is expressed in myofibroblasts as a critical marker of diabetic glomerular dysfunction.²⁸ Upon stimulation of mesangial cells with PgLPS under hyperglycemic conditions, an increase in TGF- β expression was observed. The phosphorylation of Smad3 was further enhanced in the hyperglycemic group, leading to a significant elevation in Col1a2 and α SMA expression. Subsequently, the phosphorylation of Smad3 and the expression of Col1a2 were inhibited by specific TGF- β inhibitors. However, α SMA expression was not significantly downregulated by TGF- β inhibition, which may suggest involvement of mechanisms independent of the Smad2/3 signaling pathway. For example, increased α SMA expression via the BMP4 and Smad1 pathways has been previously reported.²⁹ Another potential mechanism could involve the phagocytosis of mesangial cells. Mesangial cells are known to secrete cytokines that enhance phagocytosis in response to LPS, leading to fibrosis and apoptosis.³⁰ Further studies on myofibroblast transformation following *P. gingivalis*-derived LPS stimulation are needed.

In this study, LPS stimulation was used to model kidney involvement derived from periodontal disease. A previous report showed that patients with periodontitis exhibit elevated levels of LPS in the bloodstream.³¹ The translocation of LPS derived from pathogenic oral bacteria into the systemic circulation is considered one of the mechanisms by which periodontitis may influence various systemic diseases.³² A previous report³³ demonstrated the expression of Toll-like receptor 2 (TLR2) in the glomeruli of murine kidneys, suggesting that *P. gingivalis*-derived LPS may potentially reach the glomeruli via systemic circulation. Another potential consequence of periodontal disease affecting the kidney is an increase in renal macrophages due to elevated circulating tumor necrosis factor- α (TNF- α).³² To

investigate these potential mechanisms further, it would be beneficial to conduct co-culture experiments involving TNF- α -activated macrophages and mesangial cells.

Several limitations were acknowledged that may impact the findings of this study. First, we employed a model where mesangial cells under hyperglycemic conditions were stimulated with LPS from periodontal pathogens. However, this *in vitro* model is insufficient to account for the findings observed in the clinical study. To clarify the mechanisms underlying the clinical findings, *in vivo* studies are required to morphologically confirm kidney fibrosis or renal function decline in rodent models of experimental periodontitis. Second, as this was a preliminary investigation into the impact of Pg LPS on mesangial cell fibrosis, a relatively high LPS concentration of 1,000 ng/mL³⁴ was applied to emphasize the physiological response. However, this dose likely differs from levels encountered in a living organism. Future research will need to confirm the fibrosis pathways identified in this study using an *in vivo* model that more accurately reflects the pathophysiology. Well-designed studies are needed to investigate the detailed mechanisms and verify the renal protective effects of periodontal therapy in patients with diabetes.

In conclusion, a potential mechanism is that PgLPS may induce fibrosis in mesangial cells via the TGF- β 1/Smad signaling pathway, leading to nephrosclerotic modifications.

Declaration of competing interests

All the authors report no conflicts of interest related to this study.

Acknowledgments

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