Chronic hyperglycemia reduces the expression of intercellular adhesion molecules and increases intercellular hyperpermeability in the periodontal epithelium

Yuki Narukawa[1,5], Naoyuki Sugiyama[2,5], Jiro Miura[3], Rentaro Yamashita[3], Shotaro Tominaga[1], Yoshihiro Izumi[4], Takeshi Bamba[4], Yasushi Ishihama[2], Yoichiro Kashiwagi[1,5*], and Shinya Murakami[1*]

[1] Department of Periodontology, Division of Oral Biology and Disease Control, Osaka University Graduate School of Dentistry, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan

[2] Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku Kyoto, Japan 606-8501

[3] Division of Interdisciplinary Dentistry, Osaka University Dental Hospital, 1-8 Yamadaoka, Suita, Osaka, Japan.

[4] Division of Metabolomics, Medical Institute of Bioregulation, Kyushu University, Higashi-ku, Fukuoka, 812-8582, Japan

[5] These authors contributed equally to this work.

[*]Corresponding author:

Yoichiro Kashiwagi, Shinya Murakami

Address: 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan

Tel: +81 6 6879 2932

Email: kashiwagi.yoichiro.dent@osaka-u.ac.jp & murakami.shinya.dent@osaka-u.ac.jp

Keywords: hyperglycemia, gingival epithelium, intercellular adhesion molecules, permeability, barrier function

Abstract:

Background/Aims: Hyperglycemia in diabetes is closely associated with periodontal disease progression. This study aimed to investigate the effect of hyperglycemia on the barrier function of gingival epithelial cells as a cause of hyperglycemia-exacerbated periodontitis in diabetes mellitus.

Methods: The abnormal expression of adhesion molecules in gingival epithelium in diabetes was compared between db/db and control mice. To study the effects of hyperglycemia on interepithelial cell permeability, the mRNA and protein expressions of adhesion molecules were investigated using a human gingival epithelial cell line (epi 4 cells) in the presence of either 5.5 mM glucose (NG) or 30 mM glucose (HG). Immunocytochemical and histological analyses were performed. We also studied HG-related intracellular signaling to assess abnormal adhesion molecule expression in the cultured epi 4 cells.

Results: The results of the proteomic analysis implied the abnormal regulation of cell–cell adhesion, and mRNA and protein expression assessments revealed the significant downregulation of Claudin1 expression in the gingival tissues of db/db mice ($P < 0.05$ *vs.* control). Similarly, the mRNA and protein expressions of adhesion molecules were lower in epi 4 cells cultured under HG conditions than in those cultured under NG conditions ($P < 0.05$). Three-dimensional culture and transmission electron microscopy revealed reduced thickness of the epithelial cell layers with no flattened apical cells and heterogeneously arranged intercellular spaces among adjacent epi 4 cells under the HG. These results were consistent with the increased permeability of epi 4 cells under the HG relative to that of cells under the NG. This abnormal expression of intercellular adhesion molecules under the HG was related to the increased expression of receptors for advanced glycation end products (AGEs) and oxidative stress relative to that seen under the NG, along with stimulation of ERK1/2 phosphorylation in epi 4 cells.

Conclusions: High glucose–induced impairment of intercellular adhesion molecule expression in gingival epithelial cells was related to the intercellular permeability of gingival cells, representing a

possible link to hyperglycemia-related AGE signaling, oxidative stress, and ERK1/2 activation.

1. Introduction

Diabetes mellitus is a critical risk factor for the development and progression of periodontitis. A close association between chronic hyperglycemia in diabetes and the progression of periodontal disease has been reported in many preclinical and clinical studies[1, 2]. Hyperglycemia is associated with an increased incidence of alveolar bone loss, which is characteristic of advanced periodontal disease. It is generally accepted that impaired leukocyte function and abnormal collagen content and glucose metabolism in periodontal cells, together with gingival microvascular dysfunction, are related to the progression of periodontal disease[3].

The gingival epithelium directly confronts periodontal bacteria in the periodontal pocket, whereas the physical barrier function of the gingival epithelium is maintained by adhesion molecules between epithelial cells, as seen with other epithelial tissues. Tight junctions are composed of the main components of adhesion molecules i.e., Claudin1, occludin, and ZO-1, whereas E-cadherin acts as the adhesion molecule at adherens junctions. Recently, hyperglycemia was reported to negatively affect the physical barrier function in the intestinal epithelium[4]. Thus, the effects of hyperglycemia on the barrier function of the gingival epithelium must be studied to protect against bacterial invasion via the epithelial cells.

Glucose metabolism in some cells is not regulated by insulin. Therefore, the large amount of glucose taken up by cells under hyperglycemic conditions activates various intracellular glucose metabolic pathways, such as increased polyol metabolism[5], accumulation of advanced glycation end products (AGEs)[6], abnormal activation of protein kinase C (PKC) signaling[7], mitochondrial superoxide anion production, and formation of reactive oxygen species (ROS), resulting in increased oxidative stress in these cells[8]. However, no definite evidence suggests that hyperglycemia affects the barrier function of the gingival epithelium. Therefore, in the present study, we investigated the effect of hyperglycemia on the barrier function of gingival epithelial cells to identify the possible mechanisms underlying hyperglycemia-exacerbated periodontitis in diabetes mellitus.

2. Materials & methods

2.1. Cell culture

Human gingival epithelial cells (HGECs (epi 4)) were established and cultured as previously described[9-12]. The cultured epi 4 cells were seeded in a density similar to that of each experiment in the culture plates. After reaching sub-confluency, epi 4 cells were cultured in 30 mM (high glucose, HG), 5.5 mM (normal glucose, NG), or 5.5 mM glucose with 24.5 mM mannitol (hyperosmolar control for HG group) for 14 or 21 d[13-15]. Some wells were cultured in HG conditions for 7 or 14 d and then in NG conditions for another 14 or 7 d. Cells were maintained in the culture medium, which was replaced every 3 d. In some experiments, epi 4 cells were pretreated with 1 mM N-acetylcysteine (NAC), an antioxidant agent, in the presence of HG conditions. The optimal exposure time to HG and NAC concentrations was determined based on preliminary experiments.

2.2. Multilayer cell culture (airlift culture)

The epi 4 cells were exposed to an air–liquid interface for 3–4 weeks using clear polyester membrane inserts (Transwell®: 12-mm diameter, 0.4-mm pore size; Corning 3460; Corning, Corning, NY, USA) placed in 12-well culture dishes (Corning)[16]. The epi 4 cells were seeded onto the membrane insert at a density of 1×10^5 cells/well. HuMedia KG2 (KURABO, Osaka, Japan) was added to the lower wells, and the culture medium was replaced every other day.

2.3. Animals

Eleven-week-old male C57BLKS/Jlar-＋Leprdb/＋Leprdb (db⁄db) mice and C57BL/6 wild-type mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Housing conditions and anesthetization procedures were determined based on previous studies[17]. All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Dentistry (Permit Number: 27-022-0).

2.4. Quantification of alveolar bone resorption

Morphometric analysis of buccal alveolar bone resorption was performed using an R_mCT2 3D micro–

X-ray computed tomography (μCT) system designed for use on laboratory animals (Rigaku, Tokyo, Japan). An examiner, who was calibrated and blinded to the experimental groups, measured the linear distances from the cement-enamel junction (CEJ) to the alveolar bone crest (ABC) using the 3D image analysis software TRI/3D-BON (RATOC System Engineering Co., Ltd., Tokyo, Japan). Five point liners were measured on each molar root surface (three on the distal root and two on the mesial root). An increase in CEJ distance was considered as an onset and progress of periodontitis[18].

2.5. Proteomic analysis

The disrupted murine gingival tissues were suspended in 450 μL of methanol and then in 250 μL of water and 500 μL of chloroform. After vortex mixing, the suspension was centrifuged at 4,600 x *g* for 5 min, and then both methanol/water and chloroform phases were removed to yield the intermediate precipitates. The precipitates were dried under vacuum, and crude proteins extracted from the precipitates via the phase-transfer surfactant method[19] were subjected to reductive alkylation, followed by successive digestion with Lys-C endopeptidase and trypsin, as previously described[17]. After acidification with trifluoroacetic acid, the detergents were removed with ethyl acetate extraction. The peptide solution was desalted using a stage tip[20] equipped with an SDB-XC Empore disk membrane (3M, St. Paul, MN, USA). The desalted peptides were isotopically labeled with the TMTsixplex™ kit (Thermo Fisher Scientific, Waltham, MA, USA) via a nanoscale solid-phase labeling method[21] and then fractionated with a strong cation exchange chromatography through a combined acid salt gradient method[22].

The fractionated peptides were analyzed using nanoLC/MS/MS with an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) in data-dependent acquisition mode, coupled to an Ultimate3000 RSLCnano system (CTC Analytics, Zwingen, Switzerland) and an HTC-PAL autosampler (CTC). Peptides were identified via database searching using Mascot v. 2.7.0 (Matrix Science, London, UK) against the UniProtKB/ReferenceProteome (2017/11) of *Mus musculus*. Details of the sample preparation, nanoLC/MS/MS, and data analysis are described in the Supplementary Materials.

2.6. Quantitative analysis of mRNA levels

Total RNA isolation and cDNA synthesis were performed as previously described[11, 17]. Polymerase chain reaction (PCR) was carried out using the ABI 7300 real-time PCR system (Applied Biosystems, Waltham, MA, USA) with Power SYBR® Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. All reactions were performed in triplicate. The specific primers used are described in Supplementary Table S1.

2.7. Protein expression analysis

Cells were rinsed with ice-cold PBS and lysed with RIPA buffer (Thermo Fisher Scientific). Total proteins were extracted from frozen mouse gingival tissue using T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific) and then used for western blotting. Immunoblotting was performed using the following primary antibodies: Claudin1 (1:2500; 51-9000, Thermo Fisher Scientific), occludin (1:5000; 91131, Cell Signaling Technology Inc, Danvers, MA, USA), E-cadherin (1:500; 3195, Cell Signaling Technology Inc), ERK1/2 (1:1000; 9194, Cell Signaling Technology Inc.), phospho ERK1/2 (1:1000; 8544, Cell Signaling Technology Inc.), and β-actin (1:10000; A5416, Sigma-Aldrich, St. Louis, MO, USA). The membranes were briefly washed and incubated with HRP-conjugated anti-rabbit IgG antibodies (1:10000; NA934, GE Healthcare, Chicago, IL, USA). Immunoreactive bands were visualized using ECL chemiluminescent substrate (Thermo Fisher Scientific). Immunoreactive proteins were detected using the western blotting detection system ImageQuant LAS 4000 (GE Healthcare) and densitometrically analyzed using the image analysis program WinROOF (Mitani Corporation, Fukui, Japan).

2.8. Permeability assay

To assess the barrier function of epi 4 cells via the permeability assay method, cells were cultured in the upper compartment (Transwell® Permeable Supports; 12 mm insert, 0.4 mm pore size; Corning) ($1x10^5$ cells/well). After 14 d of culture, the passage of 5 mg/mL of fluorescein isothiocyanate (FICT)

dextran (4 kDa) (Sigma-Aldrich) from the upper compartment to the lower compartment was measured. After 2 h of incubation, the fluorescence intensity of the lower part of the medium was measured at 485 nm excitation and 538 nm emission using a microplate reader (Fluoroskan Ascent; Thermo Fisher Scientific Inc.) to determine permeability.

2.9. Immunocytochemical analysis

Epi 4 cells (9×10^5 cells/well) were cultured on a glass-bottom dish (Matsunami Co., Bellingham, WA, USA) mounted on a 35-mm plate. Confluent monolayers were cultured for 48 h in the presence of 1.5 mM Ca^{2+}. The cells were then fixed for 10 min with 4% paraformaldehyde (PFA). After blocking with 1% BSA, the cell monolayer was incubated with rabbit anti-human Claudin1 (1:250; 51-9000, Thermo Fisher Scientific Inc.) and then with rabbit anti-human E-cadherin (1:200; 3195, Cell Signaling Technology) in PBS for 60 min at room temperature(25 ° C). The cells were washed three times with 1% BSA in PBS between each step. The cells were incubated with Alexa Fluor 488-conjugated sheep anti-rabbit antibody (Thermo Fisher Scientific) for 30 min and counter-stained with 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich) for nuclear staining. Photographs were obtained using a confocal laser scanning microscope (Leica TCS SP8, Leica Microsystems, Wetzlar, Germany).

2.10. Histological examinations and ultrastructural evaluation with transmission electron microscopy (TEM)

Multilayered epi 4 cells were cultured on Transwell^R, fixed in 4% PFA for 12 h at 4 °C, and embedded in paraffin. Tissue sections were cut at 4 μm thickness and stained with hematoxylin and eosin (H&E). Cultured cells were fixed with 3% glutaraldehyde (Fujifilm Wako Pure Chemical Corp., Tokyo, Japan) and 2% osmium tetroxide and then dehydrated in an ascending series of ethanol. The specimens were embedded in epoxy resin (Quetol 812; Nisshin EM, Tokyo, Japan). Curing samples were sectioned to a thickness of 70 nm using a diamond knife and mounted on copper grids using an ultramicrotome (Ultrome V; LKB, Bromma, Sweden). The sections were stained with an EM stainer (Nisshin EM, Tokyo, Japan) and 0.4% lead citrate for TEM (H800; Hitachi, Tokyo, Japan).

2.11. Measurement of intracellular ROS levels

To analyze oxidative stress under HG conditions, ROS accumulation was measured by fluorescence intensity using Fluoroskan Ascent (Thermo Fisher Scientific; excitation wavelength: 480 nm, fluorescence: 530 nm). Intracellular ROS levels were measured using the OxiSelect^{TM} Intracellular ROS Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA).

2.12. Statistical analysis

Data are expressed as mean ± SEM. Differences between multiple experimental conditions were analyzed using one-way analysis of variance with Tukey's post hoc test. All other comparisons between the two groups were analyzed using Welch's t-test. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Severity of alveolar bone resorption in diabetic db/db and control mice

Blood glucose and body weight under *ad libitum* feeding in db/db mice were significantly ($P < 0.05$) higher than those in wild-type (WT) mice (Fig. S1). To assess the severity of periodontitis, bone loss on the buccal side of the maxillary alveolar bone was measured at five points via μCT image analysis. db/db mice exhibited a more significant increase ($P < 0.01$) in alveolar bone resorption than WT mice at 11 weeks of age (Fig. 1a, b).

3.2. Differential proteomic expression in the gingival epithelium between WT and db/db mice

Proteomic profiles of the gingival tissues from normal and db/db mice were quantitatively analyzed using nanoLS/MS/MS-based proteomics. In total, 20,911 peptide-spectrum match (PSM), 6,904 unique peptides, and 1,742 unique proteins were identified (Supplementary table S2). Of these, 38 proteins were upregulated by more than 1.2-fold in db/db mice, while 17 proteins were downregulated by less than 0.83-fold (Figure 1C and Supplementary Table S2). Gene ontology enrichment analysis of the highly differentially expressed proteins in db/db mice using the DAVID Gene Functional Classification Tool[23, 24] revealed that molecules that play a role in the adherence junction or in biological processes related to the response to oxidative stress (such as oxidative phosphorylation and glutathione metabolism) were substantially enriched (Supplementary Table S2).

The expression of the IQ motif-containing GTPase-activating protein 1 (IQGAP1; IQGA1_MOUSE), which is a molecule that functions in adherence junctions, was slightly upregulated 1.35-fold in db/db mice. In addition, phosphoglycerate kinase 1 (PGK1_MOUSE) and aldo-keto reductase family 1 member A1 (AK1A1_MOUSE) were also upregulated in the glycolysis/gluconeogenesis pathway in db/db mice (Table 1,2).

3.3. HG induced reductions in the expressions of intercellular adhesion molecules of the gingival epithelium in db/db mice than those in WT mice

Through shotgun proteomic analysis of gingival tissue, we found that the expression of intercellular adhesion-related proteins in the db/db mice gingival epithelium was different from that in WT mice. To determine whether the expression of cell–cell adhesion–related molecules was downregulated in the gingival tissues obtained from db/db mice compared to those from WT mice, mRNA and protein expression levels were measured. Claudin1, Zo-1, and tricellulin mRNA expression levels were significantly lower in the db/db mice ($P < 0.05$) than in the WT mice. However, both E-cadherin and occludin mRNA expressions tended to be lower in the gingival tissues excised from db/db mice than in those from WT mice; however, these differences were not significant between the two groups of mice (Fig. 2a-e). Consistently, western blot analysis revealed lower levels of Claudin1 protein expression in the db/db mice ($P < 0.01$) than in the WT mice (Fig. 2f).

3.4. Effects of HG in the expression of intercellular adhesion molecules on epi 4 cells

We speculated that HG may modify the expression of intercellular adhesion molecules in epi 4 cells, as previously described in mouse gingiva. Thus, we investigated the effects of hyperglycemia on the viability of gingival epithelial cells. The epi 4 cells were cultured for 14 d under NG and HG conditions, and the amount of ATP in viable cells was measured by luminescence intensity. No significant differences in luminescence intensity were observed between the NG and HG conditions, indicating that hyperglycemia did not affect the viability of gingival epithelial cells (Fig. S2). To evaluate whether the expression of intercellular adhesion molecules in epi 4 cells was affected by HG levels, we collected cell lysates 14 d after the incubation of cells in the NG or HG conditions. *Claudin1* ($p < 0.001$), *ZO-1* ($p < 0.01$), *tricellulin* ($p < 0.001$), and *E-cadherin* ($p < 0.001$) mRNA expressions were significantly lower in epi 4 cells cultured under HG conditions for 14 d than in those cultured under NG conditions. However, the reductions in *occludin* mRNA expression were not significant between the HG and NG conditions (Fig. 3a-e). Western blot analysis also revealed lower levels of Claudin1, E-cadherin, and occludin protein expressions in epi 4 cells cultured in the HG conditions ($P < 0.05$) than in those cultured in the NG condition (Fig. 3f). Immunohistochemical analyses indicated downregulated Claudin1 and E-cadherin expressions in epi 4 cells cultured under the HG conditions than in those under NG

conditions (Fig. 3g-h). To investigate whether exposure time to HG conditions affects Claudin1 protein expression, we incubated epi 4 cells for three weeks under HG conditions, for two weeks under HG conditions, and then one week under NG conditions, or for one week under HG conditions and then two weeks under NG conditions. The results showed that the one-week HG exposure with two-week NG exposure yielded higher expression of Claudin1 than three-week exposure to HG conditions. Thus, we speculated that the reduction of Claudin1 protein expression observed under HG culture was dependent on the duration of HG exposure. Conversely, the Claudin1 expression might be time-dependently recovered, to some extent, after changes in medium glucose condition to NG. (Fig. 3i). To examine the contribution of hyperosmolarity to the effects of hyperglycemia, epi 4 cells were cultured for 21 d in medium containing NG, HG, or 5.5 mM glucose with 24.5 mM mannitol (Fig. 3j). There were no changes in the Claudin1 protein expression in epi 4 cells cultured in the high mannitol condition.

3.5. Hyperglycemia alters the intercellular permeability, 3D structure, and ultrastructure of gingival epithelial cells

Because the HG condition modified the expression of intercellular adhesion molecules in epi 4 cells, we speculated that HG conditions increased the intercellular permeability in those cells. As evaluated by the transepithelial flux of FITC-dextran compared to that in a monolayer culture under NG condition, a significant reduction in intercellular permeability was observed after 14 d of HG culture in epi 4 cells (Fig. 4a). We performed histological examination of epi 4 cells in more detail via the 3D culture method. Cells were grown on polycarbonate filters for airlift culture under NG or HG conditions. The experimental epithelium comprised approximately 5 to 8 cell layers after 30 d of culture (Fig. 4 b center or right panel) compared to the monolayer cells at the starting date (Fig. 4 b left panel). Most of the apical cells were smoothly layered (Fig. 4b center panel) with tight junctions under the NG condition. However, under HG conditions, the thickness of the cell layers decreased relative to that observed under NG conditions. Furthermore, apical cells exhibited a flattened structure under HG condition compared to their morphology under the NG condition (Fig. 4b right panel). Next, we investigated the effects of HG conditions on the 3D structure at the ultrastructural histology of epi 4 cells using TEM. It was

observed that the intercellular spaces in the adjacent parts of the cells were heterogeneously arranged under HG conditions but not under NG conditions (Fig. 4c).

3.6. Involvement of AGEs-RAGE and ROS in the Claudin1 expression of epi 4 cells cultured under HG conditions

We further investigated whether the observed effects on adhesion molecules in epi 4 cells cultured under HG conditions were mediated through AGEs and oxidative stress. To examine the response of epi 4 to an exposure of AGEs, we analyzed the expression of receptors for advanced glycation end products (RAGEs) that specifically recognize AGEs. The expression of RAGE mRNA was elevated in epi 4 cells under HG conditions (Fig. 5a). We also examined whether these effects were mimicked by carboxymethyl lysine (CML), a type of AGE, on the expression of Claudin1 mRNA. Claudin1 mRNA expression was suppressed in epi 4 cells exposed to CML for 14 d (Fig. 5b). Furthermore, we measured ROS levels in epi 4 cells after 14 d of culture under HG conditions. A significant increase in ROS production was observed in epi 4 cells in HG condition than in those in NG condition (Fig.5 c). Consistently, we found that the downregulation of Claudin1 mRNA and protein expressions in epi 4 cells cultured in the HG conditions was inhibited by 1 mM NAC, an antioxidant (Fig.5 d). These results indicated that HG-inhibited mRNA and protein expressions of intercellular adhesion molecule in epi 4 cells were mediated through oxidative stress.

3.7. Activation of cell signaling molecules through ROS production in epi 4 cells cultured under HG conditions

To evaluate whether MAPK activation in epi 4 cells cultured under HG condition was involved in HG-induced suppression of Claudin1 mRNA, MAPK phosphorylation was examined in epi 4 cells after 14 d of culture under HG conditions. As shown in Figure 5e, protein extracts prepared from epi 4 cells exposed to HG or NG conditions for 14 d were immunoblotted with antibodies against p-ERK1/2 and total ERK1/2. HG increased ERK1/2 phosphorylation after 14 d of incubation compared to that observed under NG conditions. In addition, protein extracts prepared from epi 4 cells exposed to HG or NG conditions for 14 d with NAC were immunoblotted with antibodies against p-ERK1/2 and total

295 ERK1/2. NAC significantly suppressed p-ERK1/2 activation in epi 4 cells under HG conditions (Fig.

296 5f).

297

4. Discussion

In the present study, to the best of our knowledge, we, for the first time, revealed impaired barrier function of gingival epithelial cells cultured under HG conditions with concomitant reduction in protein and mRNA expression of intercellular adhesion molecules such as Claudin1, Zo-1, E-cadherin, and tricellulin. The expression of occludin was also lower under HG conditions than under NG conditions. We also identified reduced expression of these adhesion molecules in the gingival tissues excised from markedly hyperglycemic diabetic db/db mice compared to that in the tissues excised from WT mice. Thus, abnormal barrier function in diabetes may be induced by hyperglycemic conditions in gingival tissues. Proteomic analysis of the gingival tissues excised from db/db mice showed that hyperglycemia was involved in several changes in proteins related to cell adhesion, glucose metabolism, and oxidative stress compared to that in the tissues excised from control mice. Specifically, we found an increased expression of IQGAP1, which was extracted as a protein related to cell adhesion; IQGAP1 has been reported as a negative regulator of E-cadherin-mediated cell adhesion and is involved in the regulation of ERK activity[25, 26]. Therefore, further studies to investigate the role of IQGAP1 in the reduced expression of intercellular adhesion molecules in the gingival tissues under diabetic condition are necessary.

In the present study, db/db mice exhibited higher alveolar bone resorption than that exhibited by WT mice. Diabetes mellitus is a disease associated with high blood glucose levels, resulting in poor peripheral circulation and high chances of infection. Therefore, this model is believed to mimic human periodontitis with diabetes to some extent. Hyperglycemia was previously reported to inhibit intercellular adhesion and promote intestinal epithelium permeability[4]. We investigated the effect of hyperglycemia on the intercellular permeability of cultured epithelial cells in vitro using epi 4 cells. When epi 4 cells were cultured in the presence of 30 mM D-glucose for 21 d, the dextran permeability increased compared to that in the control group. This indicates that hyperglycemia functionally reduces cell–cell adhesion and increases leakiness in the gingival epithelium. This impairment in the barrier

function of epi 4 cells corresponded to the abnormal expression of adhesion molecules under HG conditions. An increase in osmotic pressure has been reported to increase tight junctions in renal tubular epithelial cells[27]. However, we did not observe increased barrier function in epi 4 cells cultured under high osmolar condition induced by a high concentration of mannitol, which was different from the HG condition in renal tubular epithelial cells. These suggested that epi4 cells exhibit a distinct mechanism of action compared to renal tubular epithelial cells, specifically regarding the osmotic effect of glucose.

The barrier function of the epithelium is based on the intercellular adhesion mechanism, which is mainly composed of tight and adherens junctions. The epi 4 cells cultured under HG conditions, as well as gingival tissues excised from hyperglycemic db/db mice, showed a significant reduction in mRNA and protein expression of various adhesion proteins. In addition, this study was focused on the protein expression of Claudin1, which is a component of tight junctions that contributes to the outermost barrier mechanism in the epithelium[28]. Our results showed that the protein expression of Claudin1 significantly decreased *in vivo* and *in vitro*. Immunostaining of epi 4 cells showed that Claudin1 was expressed in the intercellular space of the NG group, but the staining was less evident in the HG group. These results suggest that barrier function is impaired under hyperglycemic conditions because of reduced intercellular adhesion molecule expression. In the present study, we found that the reduction of Claudin1 protein expression under HG condition was dependent on the duration of HG exposure. Conversely, the Claudin1 expression may be recovered by exposure to medium glucose or NG conditions in a time-dependent manner (Fig. 3i). However, abnormalities in adhesion molecules because of long-term hyperglycemia and reversibility with glycemic control were not assessed this study.

Previous reports have described a gingival tissue model that mimics the oral junctional epithelial interface using gingival epithelial cells and gingival fibroblasts in a 3D co-culture system *in vitro*[29, 30]. Therefore, in the present study, epi 4 cells were cultured under HG conditions in a 3D culture system. We found that the uniform cell arrangement of the epithelial cell layer was impaired under HG condition and the cell layer was thinner than that in the NG group. Moreover, TEM revealed that intercellular

spaces were irregularly and heterogeneously arranged under HG culture conditions. Decreased expression of functional intercellular adhesion molecules in hyperglycemia may alter the morphology of the intercellular spaces and weaken the barrier function. All these abnormalities could be related to abnormal barrier function in cultured epi 4 cells under HG conditions.

In terms of the biological significance of impaired barrier function in epithelial cells, hyperglycemia reportedly affects the physical barrier function in the intestinal epithelium[4]. Similarly, hyperglycemia-mediated barrier disruption has also been reported to cause a systemic influx of microbial products and enhanced dissemination of enteric infection. Treatment with hyperglycemia or intestinal epithelial-specific GLUT2 deletion restores barrier function and bacterial containment[4, 31]. The gingival epithelium directly confronts periodontal bacteria in the periodontal pocket, and the physical barrier function of the gingival epithelium is preserved by the integrity of gingival epithelial cells, which is maintained by the adhesion molecules between epithelial cells similar to other epithelial tissues. Therefore, impaired integrity of the gingival epithelium may permit the chronic penetration of periodontal bacteria in the subepithelial space and the gingival vascular bed. Furthermore, systemic translocation of infectious oral bacteria may cause systemic microinflammation, which is a risk factor for the progression of insulin resistance, diabetes, and cardiovascular disease[32-35].

Insulin is less involved in the glucose metabolism in non-insulin-sensitive cells than in insulin-sensitive muscle cells, adipocytes, or hepatocytes. Glucose influxes into epithelial cells are dependent on the concentration of glucose in the extracellular matrix. Glucose taken up by cells is used for energy production by glycolysis and tricarboxylic acid cycle; however, under hyperglycemic conditions, collateral glucose metabolism may be activated in epithelial cells including polyol, hexosamine, PKC, AGEs, and the pentose phosphate pathway[36]. Previously, serum AGEs were reported to be significantly associated with periodontitis progression in patients with type 2 diabetes[37]. Histological studies using human gingival epithelial tissue samples have shown that RAGE expression is increased in the gingival epithelium of patients with periodontal disease compared to that in healthy gingiva, and further enhanced RAGE expression has also been reported in diabetic patients with periodontal disease[38]. In

this study, we used CML, an AGE, as a ligand for RAGE. CML is an indicator of long-term oxidative stress. We hypothesized that CML is involved in the reduction of intercellular adhesion molecules. HG treatment increased the expression of RAGE in epi 4 cells, suggesting that cellular responsiveness to AGEs is enhanced. After 14 d of stimulation with CML instead of HG, a significant reduction in Claudin1 mRNA expression was observed, suggesting that AGEs were involved in the downregulation of intercellular adhesion molecule expression in gingival epithelial cells. A previous study reported that AGEs induce ERK1/2 phosphorylation and decrease E-cadherin expression in the renal tubular epithelium[39], and another study reported that AGEs decrease the expression of ZO-1 in retinal microvascular endothelial cells[40]. Moreover, activation of collateral pathways of the glycolytic system in epithelial tissues under HG conditions has resulted in the production of AGEs, and ERK1/2 phosphorylation is involved in intracellular signaling[41]. Another report revealed that the expression of E-cadherin is downregulated in renal tubular epithelial cells because of increased ROS levels and the activation of ERK1/2[42] and that ERK1/2 is involved in the downregulation of ZO-1 expression in corneal epithelial cells[43, 44]. In this study, proteomic analysis of the gingival tissues excised from db/db mice implied a change in expression of proteins involved in oxidative stress. We demonstrated that ROS production and ERK1/2 phosphorylation significantly increased in epi 4 cells under HG conditions. These results imply that hyperglycemia increases oxidative stress and activates ERK1/2 signaling, downregulating the expression of intercellular adhesion molecules in gingival epithelial cells. Furthermore, the addition of NAC, an antioxidant, significantly protected against HG-dependent Claudin1 mRNA repression and upregulation of ERK phosphorylation. To confirm the direct significance of ERK signaling in the downregulation of Claudin1 under HG conditions, experiments with an ERK1/2 inhibitor should be performed. However, because of the cytotoxicity of ERK1/2 inhibitors in this experiment, we could not demonstrate that ERK1/2 signaling was directly related to Claudin1 downregulation under HG conditions.

As a limitation of the present study, mechanistic studies were exclusively represented by Claudin1 protein expression in epi 4 cells under HG condition. We also did not assess the protein expression of

other adhesion molecules in periodontal tissues of db/db mice in the present study. Thus, further studies are needed to determine whether the protein expression of other adhesion molecules in periodontal tissues is also downregulated in a time-dependent manner and reversed by modulation of those signaling molecules under HG condition in the future studies.

Taken together, the results of past studies show that retinal, renal tubular, intestinal, and gingival epithelial cells exhibit reduced expression of adhesion molecules under hyperglycemic conditions through AGEs-RAGE, oxidative stress, and ERK1/2 signaling derived from abnormal intracellular glucose metabolism.

5. Conclusions

We identified the impaired expression of intercellular adhesion molecules in gingival epithelial tissues in hyperglycemic db/db mice relative to that in WT mice, as well as in HGECs cultured under HG versus NG conditions. This abnormal gene expression was reflected in the reduction of protein expression of adhesion molecules and the leakiness of the intercellular barrier in cultured HGECs (epi 4 cells). The abnormal expression of intercellular adhesion molecules under HG conditions was related to AGE-RAGE signaling activation, oxidative stress, and ERK1/2 in gingival epithelial cells. Thus, the findings of the present study suggest that hyperglycemia-induced disruption of intercellular adhesion in gingival epithelial cells is related to the development and progression of periodontal disease in diabetes.

Authors' contributions

Y.N. and N.S. are the co-first authors and contributed equally to this work. Y.N. and Y.K. designed the study. Y.N., N.S., Y. Izumi., and Y.K. finalized the study protocols. Y.N., S.T., and Y.K. performed *in vivo* and HGEC experiments. J.M. acquired images using TEM. N.S., Y. Ishihama, and T.B. performed proteomic experiments and interpreted the analytical outcomes in close collaboration with Y. Izumi., T.B. Y.N., S.T., Y.K., and S.M. Y.N, N.S., and Y.K. wrote the manuscript. Y. Ishihama., T.B., and S.M. revised the manuscript.

All authors approved the final version of the thesis and manuscript. The authors declare that they have no conflicts of interest.

Acknowledgments

This study was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI (Grant Numbers JP18K09573 and JP21K09916 to Y.K. and JP21K21084 to Y.N.) and the Advanced Research and Development Programs for Medical Innovation (AMED-CREST) Grant Number JP18gm1010010 to S.M., YI, and BT.

Conflict of interest: The authors declare no conflict of interest related to this study.

Ethics statement: All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Dentistry (Permit Number: 27-022-0).

Data sharing and data accessibility: The MS raw data, peak list, and database search files for the proteome analysis have been deposited to the ProteomeXchange Consortium via the jPOST partner repository (https://jpostdb.org)[45] with the data set identifier PXD035613 (preview URL for reviewers: https://repository.jpostdb.org/preview/83357616262e368f73b605; Access key: 1552). The other data that support the findings of this study are available from the corresponding author upon reasonable request.

Figure legends

Fig. 1 Diabetes exacerbates alveolar bone loss and changes the proteomic expression phenotype in db/db mice compared to that in WT mice. The buccal-side maxillary alveolar bone loss (ABL, colored lines) was measured from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) at five points (a); distobuccal regions for the first maxillary molar (M1) (1), mesiobuccal regions (2), distobuccal regions for the second maxillary molar (M2) (3), mesiobuccal regions (4), and distobuccal (5) regions for the third maxillary molar (M3) in 11-week-old db/db mice or control mice. Comparison of the sum of the five CEJ-ABC linear distances (b). $n = 5$; ** $P < 0.01$. Data are shown as mean ± SEM. Proteome profiles of the gingival tissues of WT and db/db mice, using nanoLC/MS/MS, were measured in triplicate (c).

Fig. 2 mRNA and protein expression of intercellular adhesion molecules in the gingival epithelium of db/db or WT mice. mRNA expression of Claudin1 (a), ZO-1 (b), tricellulin (c), occludin (d), and E-cadherin (e). mRNA expression was determined using quantitative RT-PCR and normalized against the expression of *HPRT* mRNA. $n = 4$, * $P < 0.05$ vs control mice. Western blotting of Claudin1 expression in gingival epithelium excised from db/db and control mice (f). The bar graphs on the right show densitometric quantification of the levels of Claudin1 relative to those in the control (f). $n = 3$; ** $P < 0.01$ vs WT mice. β-actin was used as loading control.

Fig. 3 The mRNA and protein expression of intercellular adhesion molecules in the human gingival epithelial cell line (epi 4). mRNA expression of Claudin1 (a), ZO-1 (b), tricellulin (c), occludin (d), and E-cadherin (e) in epi 4 cells. mRNA expression was determined using quantitative RT-PCR and normalized against HPRT mRNA expression. $n = 4$, ** $P < 0.01$, *** $P < 0.001$, vs NG group (a-e). Western blot analysis of Claudin1, occludin, and E-cadherin on epi 4 cells (f). β-actin was used as a loading control. The bar graphs on the right show densitometric quantification of the levels of Claudin1,

occludin, and E-cadherin, relative to those of the NG group. $n = 3$; $^{*}P < 0.05$. Immunocytochemistry with Claudin1 (g) and E-cadherin (h) in epi 4 cells under HG or NG conditions. Scale bars: 50 μm. Western blot analysis of Claudin1 in epi 4 cells evaluating the effect of HG and its recovery by transfer of the cells to the NG condition. β-actin was used as the loading control. The bar graphs on the right show densitometric quantification of Claudin1 levels. $n = 3$; $^{**}P < 0.01$. The groups of 'NG (3W)' or 'HG (3W)' were cultured for three weeks of epi 4 cells under NG or HG conditions, respectively. The group of 'HG (1W) → NG (2W)' was cultured under HG conditions for one week and then changed to NG conditions for two weeks. The group of 'HG (2W) → NG (1W)' was cultured for two weeks under HG conditions and then changed to NG conditions for one week. $n = 3$; $^{**}P < 0.01$ (i). To examine the contribution of hyperosmolarity to the effects of high glucose, epi 4 cells were cultured for 21 d in a medium containing either 6 mM glucose (NG), 25 mM glucose (HG), or 5.5 mM glucose containing 24.5 mM mannitol as a hyperosmolar control group (j). $n = 5$; $^{**}P < 0.01$. Data are shown as mean ± SEM.

Fig. 4 Measurements of gingival epithelial barrier function and the structure of the experimental 3D-cultured epi 4 cells.

Permeability was analyzed as described in the Materials & methods section. Cells were cultured with NG (5 mM) or HG (30 mM) for 14 d. Results are expressed as fold change relative to the NG group. $^{*}P < 0.05$ compared to the NG group (a). A cross-section of the immortalized HGECs (epi 4 cells) cultured by the airlift multilayer model (approximately seven cell layers; apical side up) and stained with H&E. Scale bar, 100 μm (b). Transmission electron microscopy image of gingival epi 4 cells. The intercellular spaces in the adjacent parts of the cells are more heterogeneously arranged under HG conditions than under NG conditions, and adherence junction is reduced in the cell adhesion region (white arrowheads) (c, d). Original magnification (c); 6000×, (d); 12000× area in white rectangle in (c) respectively, Scale bar, 500 nm.

Fig. **5** Molecular mechanisms underlying HG-induced suppression of Claudin1 expression in epi 4 cells, AGE signaling, oxidative stress, and ERK1/2 activation. Cells were cultured with NG or HG for 14 days, mRNA expression of *RAGE* on epi 4 cells were determined using quantitative RT-PCR and normalized against HPRT mRNA expression. $n = 4$; $^{*}P < 0.05$ (a). Cells were cultured with NG condition with or without CML (1 ng/mL) for 14 d, mRNA expression of Claudin1 in epi 4 cells was determined. $n = 4$; $^{*}P < 0.05$ (b). Hyperglycemic gingival epithelial cells increase the generation of intracellular reactive oxygen species (ROS). ROS production was determined by H2DCFDA fluorescence in epi 4 cells., $n = 4$; $^{*}P < 0.05$ (c), and N-acetyl cysteine (NAC) reverses the damage to the gingival tight junction proteins compromised by hyperglycemia, $n = 4$; $^{*}P < 0.05$, $^{**}P < 0.01$ (d). Hyperglycemia upregulated the phosphorylated ERK (p-ERK) pathway in human gingival epithelial cells (e), and NAC attenuated this upregulation (f). Claudin1 mRNA expression in epi 4 cells under NG or HG conditions with or without NAC (1 mM). $n = 4$; $^{*}P < 0.05$, $^{**}P < 0.01$. Western blot analysis using the specific antibodies against p-ERK, total ERK, and β-actin. The bar graphs on the right show densitometric quantification of the amounts of p-ERK normalized to the amount of total ERK, relative to that in the NG group. Cells were cultured with NG (5 mM) or HG (30 mM) for 14 d. $n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$.

References

1. Emrich LJ, Shlossman M, Genco RJ. Periodontal disease in non-insulin-dependent diabetes mellitus. *J Periodontol* 1991;62:123-131.
2. Popławska-Kita A, Siewko K, Szpak P, et al. Association between type 1 diabetes and periodontal health. *Adv Med Sci* 2014;59:126-131.
3. Genco RJ, Graziani F, Hasturk H. Effects of periodontal disease on glycemic control, complications, and incidence of diabetes mellitus. *Periodontol 2000* 2020;83:59-65.
4. Thaiss CA, Levy M, Grosheva I, et al. Hyperglycemia drives intestinal barrier dysfunction and risk for enteric infection. *Science* 2018;359:1376-1383.
5. Chung SS, Ho EC, Lam KS, Chung SK. Contribution of polyol pathway to diabetes-induced oxidative stress. *J Am Soc Nephrol* 2003;14:S233-236.
6. Murata T, Nagai R, Ishibashi T, Inomuta H, Ikeda K, Horiuchi S. The relationship between accumulation of advanced glycation end products and expression of vascular endothelial growth factor in human diabetic retinas. *Diabetologia* 1997;40:764-769.
7. Inoguchi T, Li P, Umeda F, et al. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C--dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* 2000;49:1939-1945.
8. Yao D, Brownlee M. Hyperglycemia-induced reactive oxygen species increase expression of the receptor for advanced glycation end products (RAGE) and RAGE ligands. *Diabetes* 2010;59:249-255.
9. Oda D, Watson E. Human oral epithelial cell culture I. Improved conditions for reproducible culture in serum-free medium. *In Vitro Cell Dev Biol* 1990;26:589-595.
10. Kashiwagi Y, Yanagita M, Kojima Y, Shimabukuro Y, Murakami S. Nicotine up-regulates IL-8 expression in human gingival epithelial cells following stimulation with IL-1beta or P. gingivalis lipopolysaccharide via nicotinic acetylcholine receptor signalling. *Arch Oral Biol* 2012;57:483-490.
11. Kashiwagi Y, Takedachi M, Mori K, et al. High glucose-induced oxidative stress increases IL-8 production in human gingival epithelial cells. *Oral Dis* 2016;22:578-584.
12. Kusumoto Y, Hirano H, Saitoh K, et al. Human gingival epithelial cells produce chemotactic factors interleukin-8 and monocyte chemoattractant protein-1 after stimulation with Porphyromonas gingivalis via toll-like receptor 2. *J Periodontol* 2004;75:370-379.
13. Reining G, Baumgartner-Parzer S, Waldhäusl W. Impaired membrane translocation of thrombin stimulated PKC epsilon by high glucose. *J Endocrinol* 1998;157:R7-10.
14. Garcia Soriano F, Virág L, Jagtap P, et al. Diabetic endothelial dysfunction: the role of poly(ADP-ribose) polymerase activation. *Nat Med* 2001;7:108-113.
15. Peng J, Zheng H, Wang X, Cheng Z. Upregulation of TLR4 via PKC activation contributes to

impaired wound healing in high-glucose-treated kidney proximal tubular cells. *PLoS One* 2017;12:e0178147.

16. Dickinson BC, Moffatt CE, Hagerty D, et al. Interaction of oral bacteria with gingival epithelial cell multilayers. *Mol Oral Microbiol* 2011;26:210-220.

17. Kashiwagi Y, Aburaya S, Sugiyama N, et al. Porphyromonas gingivalis induces entero-hepatic metabolic derangements with alteration of gut microbiota in a type 2 diabetes mouse model. *Sci Rep* 2021;11:18398.

18. Maekawa T, Takahashi N, Tabeta K, et al. Chronic oral infection with Porphyromonas gingivalis accelerates atheroma formation by shifting the lipid profile. *PLoS One* 2011;6:e20240.

19. Masuda T, Tomita M, Ishihama Y. Phase transfer surfactant-aided trypsin digestion for membrane proteome analysis. *J Proteome Res* 2008;7:731-740.

20. Rappsilber J, Mann M, Ishihama Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat Protoc* 2007;2:1896-1906.

21. Ogata K, Tsai CF, Ishihama Y. Nanoscale Solid-Phase Isobaric Labeling for Multiplexed Quantitative Phosphoproteomics. *J Proteome Res* 2021;20:4193-4202.

22. Adachi J, Hashiguchi K, Nagano M, et al. Improved Proteome and Phosphoproteome Analysis on a Cation Exchanger by a Combined Acid and Salt Gradient. *Anal Chem* 2016;88:7899-7903.

23. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols* 2009;4:44-57.

24. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic acids research* 2009;37:1-13.

25. Kuroda S, Fukata M, Nakagawa M, et al. Role of IQGAP1, a target of the small GTPases Cdc42 and Rac1, in regulation of E-cadherin- mediated cell-cell adhesion. *Science* 1998;281:832-835.

26. Roy M, Li Z, Sacks DB. IQGAP1 binds ERK2 and modulates its activity. *J Biol Chem* 2004;279:17329-17337.

27. Canuto LP, Collares-Buzato CB. Increased osmolality enhances the tight junction-mediated barrier function in a cultured renal epithelial cell line. *Cell Biol Int* 2019;43:73-82.

28. Zihni C, Mills C, Matter K, Balda MS. Tight junctions: from simple barriers to multifunctional molecular gates. *Nat Rev Mol Cell Biol* 2016;17:564-580.

29. Bao K, Akguel B, Bostanci N. Establishment and characterization of immortalized gingival epithelial and fibroblastic cell lines for the development of organotypic cultures. *Cells Tissues Organs* 2014;199:228-237.

30. Takeuchi H, Sasaki N, Yamaga S, Kuboniwa M, Matsusaki M, Amano A. Porphyromonas gingivalis induces penetration of lipopolysaccharide and peptidoglycan through the gingival epithelium via degradation of junctional adhesion molecule 1. *PLoS Pathog* 2019;15:e1008124.

31. Martin A, Devkota S. Hold the Door: Role of the Gut Barrier in Diabetes. *Cell Metab* 2018;27:949-951.

32. Cheru L, Saylor CF, Lo J. Gastrointestinal Barrier Breakdown and Adipose Tissue Inflammation. *Curr Obes Rep* 2019;8:165-174.
33. Yuan JH, Xie QS, Chen GC, et al. Impaired intestinal barrier function in type 2 diabetic patients measured by serum LPS, Zonulin, and IFABP. *J Diabetes Complications* 2021;35:107766.
34. Rajakaruna GA, Negi M, Uchida K, et al. Localization and density of Porphyromonas gingivalis and Tannerella forsythia in gingival and subgingival granulation tissues affected by chronic or aggressive periodontitis. *Sci Rep* 2018;8:9507.
35. Fiorillo L, Cervino G, Laino L, et al., Periodontal and Systemic Implications: A Systematic Review. *Dent J (Basel)* 2019;7.
36. Madonna R, De Caterina R. Cellular and molecular mechanisms of vascular injury in diabetes--part I: pathways of vascular disease in diabetes. *Vascul Pharmacol* 2011;54:68-74.
37. Takeda M, Ojima M, Yoshioka H, et al. Relationship of serum advanced glycation end products with deterioration of periodontitis in type 2 diabetes patients. *J Periodontol* 2006;77:15-20.
38. Amir J, Waite M, Tobler J, et al. The role of hyperglycemia in mechanisms of exacerbated inflammatory responses within the oral cavity. *Cell Immunol* 2011;272:45-52.
39. Li JH, Wang W, Huang XR, et al. Advanced glycation end products induce tubular epithelial-myofibroblast transition through the RAGE-ERK1/2 MAP kinase signaling pathway. *Am J Pathol* 2004;164:1389-1397.
40. Song HB, Jun HO, Kim JH, Yu YS, Kim KW. Suppression of protein kinase C-ζ attenuates vascular leakage via prevention of tight junction protein decrease in diabetic retinopathy. *Biochem Biophys Res Commun* 2014;444:63-68.
41. Kido R, Hiroshima Y, Kido JI, et al. Advanced glycation end-products increase lipocalin 2 expression in human oral epithelial cells. *J Periodontal Res* 2020;55:539-550.
42. He T, Guan X, Wang S, et al. Resveratrol prevents high glucose-induced epithelial-mesenchymal transition in renal tubular epithelial cells by inhibiting NADPH oxidase/ROS/ERK pathway. *Mol Cell Endocrinol* 2015;402:13-20.
43. Jiang QW, Kaili D, Freeman J, et al. Diabetes inhibits corneal epithelial cell migration and tight junction formation in mice and human via increasing ROS and impairing Akt signaling. *Acta Pharmacol Sin* 2019;40:1205-1211.
44. Wang Y, Zhang J, Yi XJ, Yu FS. Activation of ERK1/2 MAP kinase pathway induces tight junction disruption in human corneal epithelial cells. *Exp Eye Res* 2004;78:125-136.
45. Okuda S, Watanabe Y, Moriya Y, et al. jPOSTrepo: an international standard data repository for proteomes. *Nucleic acids research* 2017;45:D1107-D1111.