Glycated albumin produced in diabetic hyperglycemia promotes monocyte secretion of inflammatory cytokines and bacterial adherence to epithelial cells


Background and Objectives: The prevalence and severity of periodontal disease increase in patients with insulin-deficient or insulin-resistant forms of diabetes. A common characteristic of diabetes is the presence of hyperglycemia. A critical consequence of hyperglycemia is the nonenzymatic glycation and oxidation of proteins and lipids, resulting in the irreversible formation of advanced glycation end-products (AGEs). A central means by which AGEs are believed to impart their pathogenic effects is via interacting with specific cellular receptors; the best-characterized of these is receptor for AGE (RAGE). The major consequences of the AGE–RAGE interaction are the generation of enhanced cellular oxidant stress, hypersecretion of inflammatory mediators and altered subgingival flora. The aim of this study was to elucidate the influence of glycated albumin (G-alb), with or without lipopolysaccharide (LPS) isolated from periodontal pathogens, on the secretion of inflammatory cytokines by cultured monocytic cells and also to investigate the role of G-alb in adherence of bacteria to epithelial cells.

Material and Methods: Activated THP-1 cells (1 x 10^6 cells) were incubated for 24 h with G-alb or normal albumin (N-alb), with or without LPS isolated from two periodontal pathogens. Supernatant fluids were collected and assayed for the cytokines interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) by ELISA. For bacterial adhesion assays, S-G epithelial cells were grown on cover slips and incubated with G-alb (10 μg/mL) or N-alb (control) for 30 min. The cover slips were rinsed and then incubated with bacteria for 2 h. The number of adherent bacteria was determined by counting 20 epithelial cells chosen randomly under a light microscope.

Results: The secretion of IL-1β, TNF-α and IL-6 by THP-1 cells was greater in the presence of G-alb than in the presence of N-alb. The amounts of cytokines secreted were even greater when THP-1 cells were incubated with G-alb and LPS of periodontal pathogens. The effect of G-alb and LPS was reduced when RAGE was blocked by its antibody. Coating the cultured epithelial cells with G-alb resulted in increased bacterial adherence.
Conclusion: This study demonstrated the role of G-alb in stimulating cultured monocytic cells to secrete inflammatory cytokines. The stimulation was found to be greater when cells were incubated with LPS in addition to G-alb. The over-expression of inflammatory cytokines as a result of the combined effects of G-alb and bacterial LPS may contribute to the severity of periodontal disease in diabetic subjects.

Diabetes affects more than 18 million individuals in the USA and more than 171 million individuals worldwide, and has reached epidemic status (1). The disease is characterized by an increased susceptibility to infection, poor wound healing and increased morbidity and mortality associated with disease progression. Diabetes is also recognized as an important risk factor for more severe and progressive periodontitis, infection or lesions resulting in the destruction of tissues and supporting bone that form the periodontal attachment. Both diseases are thought to share a common pathogenesis which involves an enhanced inflammatory response that can be observed at both local and systemic levels (2–6). The inflammatory response in diabetic subjects is mainly caused by the chronic effects of hyperglycemia and specifically by the formation of biologically active glycated proteins and lipids that promote inflammatory responses (7,8).

Evidence has accumulated supporting a role for advanced glycation end-products (AGEs) in exacerbating diabetic systemic complications and periodontal disease severity associated with a chronic and intense inflammatory response. Moreover, AGEs have been associated with enhanced oxidant stress (9,10) and the subsequent endothelial expression of vascular cell adhesion molecule 1 (11) and altered structure and function of the basement membrane in vitro (12), which are detected in tissues from diabetic animals and humans (8,11,12). AGEs also appear to up-regulate the expression of proinflammatory cytokines, such as interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), as well as growth factors such as platelet-derived growth factor (13–16).

In addition, in-vitro studies of monocytes isolated from people with diabetes have shown a hyper-responsive phenotype with over-expression of IL-1β, TNF-α and prostaglandin E2 (3,17). Patients with periodontitis and diabetes were found to have significantly higher levels of local inflammatory mediators compared with systemically healthy individuals with periodontal disease (18–20). In 1991, Sekizuka et al. (21) reported that the serum levels of IL-6 were significantly higher in patients with type 2 diabetic nephropathy than in diabetic patients without nephropathy. Collectively reported data suggest that the cytokines appear to play a role in the pathogenesis of diabetes.

It has been noted that diabetic patients experience many oral infections and tooth loss (22). Epidemiologic studies have shown that the risk for developing periodontal disease is higher in patients with diabetes than in nondiabetic individuals (23,24). The role of glycated proteins in bacterial colonization of the oral cavity surfaces has not been investigated extensively.

Therefore, the purpose of this in-vitro investigation was to study the direct effect of glycated albumin (G-alb), along with lipopolysaccharide (LPS) from two periodontal pathogens, on the secretion of the inflammatory cytokines IL-1β, TNF-α and IL-6 by cultured monocytic cells. In addition, we also studied the influence of G-alb on bacterial adhesion to epithelial cells.

Material and methods

Albumin

Human G-alb and normal albumin (N-alb) were purchased from Sigma Aldrich (St Louis, MO, USA). The albumin products were confirmed to be endotoxin-free by the Limulus assay (25).

Bacteria

The periodontal pathogens Porphyromonas gingivalis 25260 and Prevotella denticola 33,185 were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA) and grown in thioglycollate broth (Difco, Detroit, MI, USA) for 72 h under anaerobic conditions. The ATCC strain of Actinomyces naeslundii 12104 was grown in Trypticase Soy Broth (Difco) for 24 h.

LPS preparation

Porphyromonas gingivalis and P. denticola were grown in liter batches of thioglycollate broth for 72 h in anaerobic jars. The bacteria were harvested by centrifugation, the cell pellets were lyophilized and the LPS was extracted using the hot phenol–water method as described by Westphal and Jann (25). The purity and protein contamination of the isolated LPS preparations were confirmed by gel electrophoresis and staining with silver nitrate. The endotoxin activity of LPS was measured using the Limulus assay (26).

Cultured monocytes

THP-1 are mature cells in the monocyte/macrophage lineage with a normal diploid karyotype, which produce several cytokines in response to purified endotoxin. These nonadherent cells were maintained in continuous culture in RPMI-1640 (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco) and 0.05 mM 2-mercaptoethanol (Gibco) in an atmosphere of 5% CO₂ at 37°C. One milliliter of THP-1 cells (1 × 10⁶
cells) was dispensed into 24-well microtiter plates and then treated with phorbol 12-myristate 13-acetate (PMA; 10^{-7} m; Calbiochem Co., La Jolla, CA, USA) to induce maturation of the monocytes and become macrophage-like. The differentiated macrophages were identified by morphological features and their ability to adhere to plastic surfaces. The viability of cultured cells was routinely monitored using the Trypan Blue dye-exclusion assay before and after treatment with test reagents.

**Treatment of the mononuclear cells with albumin and measurement of the secreted cytokines**

The mononuclear cells (1 \times 10^6 cells) were incubated with varying amounts of G-alb (50–500 \mu g/mL) or N-alb (100 \mu g/mL), with or without LPS (10 ng/mL), for 24 h. Supernatant fluids were collected and centrifuged. The concentrations of the three secreted cytokines – IL-1\( \beta \), TNF-\( \alpha \) and IL-6 – were measured using ELISA kits obtained from R & D Systems, Inc. (Minneapolis, MN, USA), according to the recommended protocol of the manufacturer.

**Effect of anti-RAGE serum on secretion of cytokines by THP-1 cells in response to G-alb**

Activated THP-1 cells (1 \times 10^6 cells) were incubated with 100 \mu g/mL of rabbit anti-RAGE serum (Abcam, Cambridge, MA, USA) or with normal serum in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) for 2 h at 37°C. After incubation, the cells were rinsed with fresh DMEM and then incubated for 24 h with 100 \mu g/mL of G-alb in DMEM, as described previously. The concentration of each cytokine in the cell-culture supernatants was assayed using ELISAs, as described above.

**Epithelial cells**

Human gingival epithelial cells (S-G cells) were obtained from F.H. Kasten, East Tennessee State University (Quillen College of Medicine, Johnson City, TN, USA). The cells were grown in DMEM supplemented with 10% (vol/vol) newborn calf serum (Gibco) and 100 \mu g/mL of gentamicin (Sigma-Aldrich) (term complete growth medium) at 37°C in a humidified atmosphere of 5% CO\(_2\) in air. The cells used in these studies were between the 30th and the 36th passages.

**Effect of G-alb on bacterial adhesion to epithelial cells**

S-G epithelial cells (1 \times 10^4 cells) were grown to confluence on 13-mm-diameter glass cover slips in 24 h by placing the cells in wells of a 24-multiwell tissue-culture plate containing 0.5 mL of medium (DMEM supplemented with fetal calf serum and gentamicin). The cells were then incubated for 60 min with G-alb or N-alb (each at 100–200 \mu g/mL), or phosphate-buffered saline (control), then rinsed and incubated for 2 h at 37°C with a suspension (100 \mu L) of freshly cultured A. naeslundii (1 \times 10^5 bacteria). Monolayer epithelial cells were washed to remove nonadhering bacteria and then fixed with methanol, gram stained and examined microscopically for adherent bacteria on 20 randomly chosen epithelial cells by two investigators.

**Statistical analysis**

All experiments were repeated a minimum of three times, with triplicate determinations at each data point. The error bars indicate the mean value \pm standard error. The data were analyzed with a one-way ANOVA and Scheffé’s F procedure for post-hoc comparisons, using STATVIEW\textsuperscript{\textregistered} software (SAS Institute, Cary, NC, USA). The significance level adopted was 5% (\( p < 0.05 \)). Control media obtained from THP-1 cells cultured without N-alb or G-alb served as the negative control.

**Results**

**Effect of LPS on secretion of cytokines by THP-1 cells in the presence of G-alb**

In order to test the combined effect of N-alb or G-alb and LPS, mononuclear cells were incubated with 50, 100 or 200 \mu g/mL of N-alb or G-alb, with or without 10 ng/mL of LPS, and then the amount of IL-1\( \beta \) secreted by the cells was measured by ELISA (Fig. 2). Monocytic cells incubated only with P. gingivalis LPS or P. denitrificans LPS (10 ng/mL) secreted 21 \pm 3 pg/mL and 24 \pm 5 pg/mL of IL-1\( \beta \), respectively (data not shown). The amount of IL-1\( \beta \) secreted by the
cells when stimulated with N-alb + LPS did not differ from the amount of IL-1β secreted by cells incubated with LPS alone. Incubation of cells with G-alb (100 μg/mL) + LPS (10 ng/mL) resulted in the secretion of significantly (p < 0.05) higher amounts of IL-1β (Fig. 2). THP-1 cells secreted 166 ± 24 pg/mL and 175 ± 21 pg/mL of IL-1β when incubated with 100 μg/mL of G-alb plus either *P. gingivalis* LPS or *P. denticola* LPS, respectively. Cells incubated with G-alb only secreted 71 ± 14 pg/mL of IL-1β. The amount of IL-1β secreted by cells when incubated with 200 μg/mL of G-alb, alone or with LPS, was found to be significantly higher (p < 0.05) than that secreted by cells incubated with 100 μg/mL of G-alb. There was no difference in the amount of IL-1β secreted when incubated with a higher concentration (500 μg/mL) of G-alb.

These results indicate that the presence of LPS and G-alb appears to amplify the secretion of IL-1β (Fig. 2).

The amounts of TNF-α and IL-6 secreted by monocytic cells when incubated with N-alb (100 μg/mL) were also found to be minimal, and the addition of LPS had no significant effect (Fig. 3). However, when the cells were incubated with G-alb (100 μg/mL) and LPS (10 ng/mL), the secretion of two cytokines significantly (p < 0.05) increased (Fig. 3). The addition of *P. gingivalis* LPS and *P. denticola* LPS to G-alb resulted in an increased secretion, from 31% to 66% for TNF-α and from 38% to 68% for IL-6, respectively, compared with cells incubated with the same amount of G-alb only (Fig. 3). LPS of *P. gingivalis* and *P. denticola* had a similar effect. The data collectively indicate that exposure of monocytic cells to G-alb, which is produced under hyperglycemic conditions, along with bacterial LPS, triggers the cells to secrete increased amounts of inflammatory cytokines.

**Effect of anti-RAGE serum on G-alb-mediated secretion of cytokines by THP-1 cells**

It is well accepted that the interaction of AGEs with their receptor, RAGE, leads to inflammatory reactions in diabetic individuals. In the present study we examined the cytokine-stimulatory effect of G-alb on THP-1 cells preincubated with anti-RAGE serum. The results (Fig. 4) show that when the cellular RAGE was blocked by antibody, the ability of G-alb to stimulate the THP-1 cells to secrete the three cytokines was significantly inhibited. In the absence of anti-RAGE serum, the cells secreted 66 ± 9 pg/mL of IL-1β but secreted only 21 ± 6 pg/mL of IL-1β when the cells were preincubated with anti-RAGE serum. Anti-RAGE serum also significantly inhibited the G-alb-mediated secretion of TNF-α (p < 0.006) and IL-6 (p < 0.003) by THP-1 cells (Fig. 4). The interaction between RAGE and G-alb appears to be crucial in initiating the inflammatory cascade, resulting in the secretion of excessive amounts of inflammatory cytokines, as observed in the present study.

**Influence of G-alb on bacterial adherence to epithelial cells**

S-G epithelial cells (1 x 10⁴ cells) grown on glass coverslips were treated with N-alb or G-alb for 30 min, rinsed with phosphate-buffered saline and then incubated with *A. naeslundii* (1 x 10⁹ bacteria) for 2 h. Nonadherent bacteria were washed off, the cells were stained and the number of adherent bacteria was counted on 20 randomly chosen cells. The results (Fig. 5) show that coating epithelial cells with G-alb significantly (p < 0.05) enhanced adhesion of bacteria compared with cells coated with N-alb. There was a slight, but significant (p < 0.03), difference in the number of bacteria...
secreted a higher level of IL-1 when the cells were incubated with LPS and G-alb (*p < 0.0025) compared with those stimulated with N-alb alone or N-alb + LPS. The cells stimulated with G-alb also secreted a higher level of IL-1β (>0.025) compared with those stimulated with N-alb alone or N-alb + LPS. The results are given as mean and standard error of triplicate measurements of multiple experiments, and are expressed as pg/mL of media obtained from the cell supernatants.

Fig. 2. Secretion of interleukin-1β (IL-1β) by THP-1 cells after stimulation with varying concentrations of normal albumin (N-alb) and glycated albumin (G-alb) plus 10 ng/mL of lipopolysaccharide (LPS) from the periodontal pathogens Porphyromonas gingivalis (Pg-LPS) or Prevotella denticola (Pd-LPS). The amount of IL-1β secreted by cells incubated with G-alb + LPS was significantly higher (*p < 0.006) than the amount secreted by cells incubated with G-alb alone or with N-alb + LPS. The results of our study clearly demonstrate that G-alb has a significant stimulatory effect on the secretion of IL-1β, TNF-α and IL-6 by cultured monocytes. Various cells have been demonstrated to possess RAGE, and it is believed that AGEs are the transi-tional pathological ligands that interact with RAGE, resulting in the onset of various physiological changes in the cell (34). Collison et al. (35) showed that by blocking the binding of AGE albumin to human neutrophil RAGE by excess soluble RAGE and by anti-RAGE serum, the degree of alteration of neutrophil functions by AGE albumin was reduced. We have also demonstrated that after blocking the RAGE with antibodies, the ability of G-alb to stimulate the THP-1 cells to secrete cytokines was greatly inhibited, suggesting the interaction of

Discussion

Numerous studies have indicated that patients with diabetes are at increased risk for the development of periodontal disease compared with their non-diabetic counterparts (27,28). Multiple factors, including altered bacterial flora, impaired host defenses and diminished function of polymorphonuclear leukocytes, are probably involved in the pathogenesis. The main cause of the pathological conditions has been linked to the formation of AGEs (29). An important component of the formation of certain classes of AGEs is their ability to produce reactive oxygen intermediates, a means ultimately by which to perturb cellular function by the activation of proinflammatory pathways inside the cell (30). The cellular receptor for AGEs, RAGE, is a multiligand member of the immunoglobulin superfamily of cell-surface molecules and is highly expressed on diabetic endothelial cell and implicated in accelerated inflammation (15,29). A number of inflammatory cytokines and mediators, including IL-1β, TNF-α, IL-6, C-reactive proteins and matrix metalloproteinases, have been implicated in inflammatory progression and complications of diabetes through altering systemic lipid metabolism and inducing insulin resistance (31–33). The results of our study clearly demonstrate that G-alb has a significant stimulatory effect on the secretion of IL-1β, TNF-α and IL-6 by cultured monocytes.

adhering to the cells incubated with 100 or 200 µg/mL of G-alb. The results (Fig. 5) suggest that G-alb promotes adherence of bacteria to epithelial cells; however, the exact mechanism of the role of G-alb needs to be explored.
G-alb with RAGE sites on the cultured monocytic cells.

The results of our study demonstrated an additive effect of LPS and G-alb on the production of inflammatory cytokines. Following *P. gingivalis* LPS challenge in the presence of G-alb, an increase in production of up to 133% for IL-1β, 156% for TNF-α and 410% for IL-6 was observed compared with no LPS challenge. A similar increase was also observed following challenge with *P. denticola* LPS. It has been demonstrated that LPS, the main virulence factor for *P. gingivalis*, up-regulates the expression of toll-like receptors by periodontal ligament cells, which stimulates the production of inflammatory cytokines through the activation of nuclear factor-kappaB, leading to an overproduction of inflammatory cytokines such as IL-1β, IL-6 and TNF-α (34). This exaggerated inflammatory response to LPS is implicated in the tissue destruction observed in periodontitis (35). In fact, several studies have reported that individuals with periodontitis show increased levels of IL-1, TNF-α and IL-6 in gingiva and crevicular fluid (36,37).

A study by Chang *et al.* (31) demonstrated that toll-like receptors and RAGE were up-regulated by glycated matrix and also that *P. gingivalis* LPS could activate the expression of RAGE in periodontal ligament cells, implying that periodontopathogens potentially activate signaling pathways in parallel to AGEs via the same ligand–receptor pathway.

Pacios *et al.* (38) reported the relationship between the number of bacteria adhering to oral epithelium and the severity of inflammation. The bacterial strain used in our study for bacterial adhesion to the S-G epithelial cells was *A. naeslundii*. The type 1 fimbriae of *A. naeslundii* have been shown to mediate the adhesion of this organism to the tooth surface and to tissue collagen through adhesion–receptor binding (39,40). We suspect that the increase in the number of *A. naeslundii* adhering to the S-G epithelial cells was *A. naeslundii*. The type 1 fimbriae of *A. naeslundii* have been shown to mediate the adhesion of this organism to the tooth surface and to tissue collagen through adhesion–receptor binding (39,40). We suspect that the increase in the number of *A. naeslundii* adhering to the S-G epithelial cells was *A. naeslundii* adhering to the S-G epithelial cells, as observed in our study, may be a result of the possible alteration of the structure of the receptors on the epithelial cell surfaces for the fimbriae by G-alb or up-regulation of the expression of receptors, making them more available for bacterial adhesion. Subsequently, we expect that such an enhanced ability of bacteria to adhere to epithelial cells will result in more severe inflammation. A study by Chen *et al.* (41) described a role for RAGE...
in the adherence of *Helicobacter pylori* to gastric epithelial cells. They demonstrated that *H. pylori* may interact directly with RAGE in comparison with the pathogen’s usual binding to gastric epithelial cells via the pattern-recognition receptor toll-like receptor 4, and also reported that *H. pylori* infection increases RAGE expression, resulting in amplification of the inflammatory cascade (41). The exact mechanism mediated by G-alb promoting a higher degree of bacterial adherence needs to be studied further.

Within the limitations of the study, we demonstrated a clear stimulatory, additive effect of G-alb and LPS on monocytic cells to induce the proinflammatory cytokines, IL-1β, TNF-α and IL-6. In addition, we also demonstrated a role for G-alb in promoting bacterial adhesion to S-G epithelial cells, resulting in further secretion of inflammatory cytokines and perpetuation of the inflammation. Tang et al., (42) compared the levels of G-alb in diabetic patients with those in control populations. They reported that the serum G-alb level of the diabetic subjects was 169 ± 62 mg/dL. Our study demonstrated that a G-alb concentration of 50–100 μg/mL had a demonstrable effect on stimulating the cultured monocyte cells to secrete greater amounts of inflammatory cytokines, further validating our findings. Further research will be needed to elucidate the possible mechanisms involved in our finding, namely the interaction of G-alb with and without LPS from periodontal pathogens, to provide a better understanding of the relationship between periodontal disease and diabetes.

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