Effect of glycated albumin and cranberry components on interleukin-6 and matrix metalloproteinase-3 production by human gingival fibroblasts

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Background and Objective: Gingival fibroblasts have the potential to participate in periodontal inflammation and breakdown, producing interleukin (IL)-6 and matrix metalloproteinase (MMP)-3. Advanced glycation end products (AGEs), formed during diabetic hyperglycemia, might aggravate periodontal inflammation. The cranberry contains anti-inflammatory polyphenols, which inhibit proinflammatory activities of lipopolysaccharide (LPS)- and IL-1β-stimulated human cells. Little is known of its effects on gingival fibroblast IL-6 or MMP-3 production stimulated by AGEs. The objectives were to determine cranberry effects on IL-6 and MMP-3 production by gingival fibroblasts exposed to the representative AGE, glycated human serum albumin (G-HSA), or LPS/C6-G-HSA.

Material and Methods: Cranberry high molecular weight non-dialyzable material (NDM), was derived from cranberry juice. Normal human gingival fibroblasts were incubated with G-HSA or normal HSA or Porphyromonas gingivalis LPS (1 μg/mL) ± G-HSA, in the presence or absence of preincubation with NDM. IL-6 and MMP-3 were measured by enzyme-linked immunosorbent assay. Data were analyzed using one-way analysis of variance and Scheffe’s F procedure.

Results: IL-6 production was stimulated by G-HSA or LPS (p < 0.01), which was inhibited in both cases by NDM (p < 0.002). [G-HSA+LPS] synergistically stimulated IL-6 production (p < 0.0001), which was inhibited by NDM. MMP-3 levels were not stimulated by G-HSA but were decreased by LPS (p < 0.02). [G-HSA+LPS] increased MMP-3 production significantly, vs. LPS (p = 0.0005). NDM inhibited MMP-3 levels in the presence of G-HSA or LPS, and in the presence of [G-HSA+LPS] (p < 0.0001).

Conclusions: G-HSA ± LPS may have differential effects on IL-6 and MMP-3 production by human gingival fibroblasts, but both are inhibited by NDM. The study suggests that cranberry phenols may be useful in regulating the host response and perhaps treating periodontitis in patients with poorly controlled diabetes.
In 2012, an estimated 21 million people in the USA had been diagnosed with diabetes mellitus (1). The US population is growing older and more obese (2,3), and both are associated with diabetes. Diabetes is a risk factor for periodontitis, and periodontitis is usually considered a sixth complication of diabetes (in addition to retinopathy, nephropathy, neuropathy, heart disease and stroke) (4–6). Moderate and severe periodontitis occur in approximately 10% of the US population (aged 20–64), but the prevalence may be underestimated due to factors such as the absence of consensus of a complete case definition of periodontitis and measuring parameters at a limited number of sites (7). While aging is associated with increased incidence of periodontitis, it is not considered a risk factor (8). These statistics suggest that periodontitis in the US diabetic population will continue to be a growing public health problem.

Exaggeration of periodontitis and development of long-term degenerative complications of diabetes may be due in large part to non-enzymatic glycation of proteins and other molecules due to hyperglycemia (9). This leads to the formation and accumulation of advanced glycation end products (AGEs). Several proteins can bind AGEs, including the “receptor for AGEs” (RAGE) expressed on many cells. Serum and gingival AGE levels are associated with periodontal destruction, and AGE-RAGE interaction may upregulate oxidative damage, inflammatory cytokines and tissue-destructive matrix metalloproteinases (MMPs), which can exacerbate periodontitis and other complications of diabetes (10,11). Furthermore, AGE-RAGE binding enhances bacterial colonization of the oral cavity and subsequent inflammatory response to lipopolysaccharide (LPS) of the periodontal microflora (12), including production of mediators (such as interleukin [IL]-6 and MMP-3) by host cells in the periodontium, which cause most of the tissue destruction in periodontitis. The cytokine IL-6, shown to be important in regulating the immune response in periodontitis, induces osteoclast formation, promotes bone resorption, and is closely related to the clinical severity of periodontitis (13). MMP-3 is a key protease causing tissue destruction in periodontitis and other inflammatory diseases (14).

Diabetic patients with poorer glycemic control may have a more rapid recurrence of periodontitis and a less favorable long-term response (15). This may be due to several factors, including the action of AGEs, impaired healing, and prolonged inflammatory conditions and osteo-astogenesis (16). A variety of adjunctive therapies are used to treat refractory forms of periodontitis (i.e. aggressive periodontitis) or in individuals with systemic disease such as diabetes. These include antibacterial topical agents, local drug delivery, systemic antibiotic therapy, host response modifiers, which inhibit inflammation and/or destructive host enzymes, and systemic doxycycline (17). Local subgingival doxycycline therapy combined with traditional mechanical treatment of periodontitis in diabetic patients improved clinical response for as long as 12 mo after treatment (5).

However, antibiotic resistance of subgingival bacteria in patients with chronic periodontitis has been reported (18), and anti-inflammatory drugs are associated with several adverse side effects. Therefore, natural products and essential oils are being viewed as alternatives for treatments for periodontitis (19). The focus of this study was the cranberry (Vaccinium macrocarpon), which, like other berry fruits, contain polyphenols, particularly proanthocyanidins, with antioxidant and anti-inflammatory activities (20–23). While several inflammatory cytokines and MMPs such as MMP-1 have important roles in periodontitis, we chose IL-6 and MMP-3 for this study for the reasons cited above and because our studies and those of others have shown that proanthocyanidin-enriched cranberry preparations can inhibit IL-6 and MMP-3 production by gingival fibroblasts and epithelial cells stimulated with LPS, IL-1β or IL-17 (20,24,25).

The purpose of this study was to investigate the effects of glycate human serum albumin (HSA) and periodontopathogen LPS on gingival fibroblast production of IL-6 and MMP-3, and modulation of this response by cranberry components.

Material and methods

Human gingival fibroblasts

Normal human gingival fibroblasts from a healthy patient with non-inflamed gingiva were derived from gingival explants in accordance with the UTHSC IRB protocol as described earlier (26). The cell line was identified as fibroblastic via cellular morphology and production of type I collagen (27–29). The cells were maintained in Dulbecco’s modified Eagle medium (DMEM, high glucose ~25 mm; Life Technologies, Grand Island, NY, USA) supplemented with 10% (v/v) newborn calf serum (Life Technologies), and 100 μg/mL gentamicin (Sigma-Aldrich, St. Louis, MO, USA) (complete medium). The cells were grown at 37°C in a humidified atmosphere of 5% CO2 in air and passaged by brief treatment with trypsin (0.25%) (Life Technologies). Fibroblasts between passages 2 and 10 were used.

Cranberry components, glycate albumin and lipopolysaccharide

Lyophilized high molecular weight non-dialyzable material derived from cranberry juice (NDM), was provided by Dr. I. Ofek (Tel Aviv University, Tel Aviv, Israel). This material was prepared from concentrated juice of Vaccinium macrocarpon (Ocean Spray Cranberries, Inc., Lakeville-Middleboro, MA, USA) (30) by exhaustive dialysis against distilled water at 4°C in 15,000 MW cut-off dialysis bags. The composition of this material was 56.6% carbon and 4.14% hydrogen, and it was highly soluble in water and devoid of proteins, carbohydrates and fatty acids (31). A similarly prepared cranberry fraction was analyzed and found to contain 0.35% anthocyanins, 0.055% cyanidin-3-galactoside, 0.003%
cyanidin-3 glucoside, 0.069% cyanidin-3-arabinoside, 0.116% peonidin-3-galactoside, 0.016% peonidin-3-glucoside and 0.086% peonidin-3-arabino-side) and 65.1% proanthocyanidins (32). NDM was reconstituted at 2 mg/mL in DMEM and sterilized by filtration (0.2 μm) (Millex-GS; Milli-pore Corp., Billerica, MA, USA). This study used glycated HSA (G-HSA; Sigma-Aldrich) as a representative AGE, and normal HSA (G-HSA) as control. LPS from *Porphyromonas gingivalis* (P.g.) (ATCC® 33277™) (American Type Culture Collection, Manassas, VA, USA) was isolated by the hot phenol method (33).

**Determination of effects of glycated or normal human serum albumin on human gingival fibroblast cell viability and membrane integrity**

G-HSA or HSA influence on cell viability was assessed by determining their effects on the ability of the cells to cleave the tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) to a formazan dye by mitochondrial succinate dehydrogenase (Roche Diagnostics Corp., Indianapolis, IN, USA). Individual wells of 96-well microtiter tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) were seeded with 2.5 × 10⁴ cells in complete medium. The cells were incubated overnight at 37°C. The medium was then removed, the cells were washed with PBS and DMEM-gent containing PBS and DMEM-gent containing 100 μg/mL gentamicin (DMEM-gent), containing G-HSA or HSA (7.8–250 μg/mL) added to the wells. To determine maximum LDH release (high control), some cells were solubilized with a final concentration of 1% (w/v) Triton X-100 (Roche Diagnostics). Spontaneous LDH release (low control) was determined by incubating the cells with serum-free DMEM-gent. The cells were exposed to the test materials for 3 h, a period in which plasma membrane damage can be specifically detected (34). Cell-free supernatants were removed and transferred to clean 96-well plates. LDH activity was assayed in the supernatants by a reaction in which the tetrazolium salt, INT, was reduced to a red formazan salt. Absorbance was read at 490 nm using a microtiter plate spectrophotometer. Results were expressed as % cytotoxicity [experimental value – low control(high control – low control) × 100].

**Effects of glycated or normal human serum albumin + non-dialyzable material on production of interleukin-6 and matrix metalloproteinase-3**

Individual wells of 24-well culture plates (Corning Inc., Corning, NY, USA) were seeded with 5 × 10⁴ cells in complete medium and cultured overnight at 37°C. The medium was removed, cells were washed with PBS and then fibroblasts were exposed to G-HSA or HSA (7.8–250 μg/mL) in serum-free DMEM-gent. Cell culture supernatants were collected at days 1, 3 and 6. In some experiments, the cells were exposed to 100 μg/mL AGE-HSA, ± 2 h preincubation with NDM (10–50 μg/mL); cell supernatants were collected at day 6 for assays. IL-6 and MMP-3 levels were measured in the cell supernatants using the Human DuoSet IL-6 and MMP-3 enzyme-linked immunosor-bent assay (ELISA) Development Systems (R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s instructions.

**Statistical analysis**

All experiments were performed with triplicate samples. Data were expressed as a mean ± standard deviation and analyzed using a one-way analysis of variance and Scheffe’s F procedure for post hoc comparisons (StatView®; SAS Institute, Inc., Cary, NC, USA).

**Results**

**Cytotoxicity and viability**

Short-term exposure to G-HSA or HSA caused no significant cytotoxic membrane damage resulting in LDH release (data not shown). In addition, our previous studies showed that
NDM (≤ 500 µg/mL) and LPS (1 µg/mL), to which the cells were also exposed, caused no significant membrane damage to gingival fibroblasts (25,35). While neither G-HSA nor HSA decreased cell viability, after 3 d exposure all G-HSA concentrations increased MTT assay values by ~20–30% compared to control, but these values had returned to control level by day 6 (data not shown). Furthermore, exposure of the fibroblasts to NDM concentrations ≤ 100 µg/mL for as long as 6 d had no significant effect on cell viability, but after 6 d exposure 250 µg/mL significantly decreased viability by ~40%, and 500 µg/mL caused ~80% decrease (IC50 = 275 µg/mL at 144 h) (25). P.g. LPS also had no effect on gingival fibroblast viability (35).

Effects of glycated human serum albumin ± non-dialyzable material on interleukin-6 and matrix metalloproteinase-3 production

G-HSA but not HSA caused concentration- and time-dependent stimulation of IL-6 production (Fig. 1). In the absence of G-HSA or HSA, levels of IL-6 increased four-fold over a 6 d period (7 pg/mL [day 1]–29 pg/mL [day 6; p < 0.0001 vs. control and day 3 level]). Concentrations of G-HSA < 62.5 µg/mL had no significant effect on IL-6 production at any time period. At higher concentrations (generally, ≥ 75 µg/mL) G-HSA significantly stimulated IL-6 production (p < 0.01). To determine the effect of NDM on G-HSA-stimulated IL-6 production, the cells were preincubated with NDM for 2 h before stimulation with 100 µg/mL G-HSA for 6 d. NDM caused concentration-dependent inhibition of G-HSA-stimulated IL-6: ~20%, ~50% (p < 0.0001) and ~65% (p < 0.0001) inhibition caused by 10, 25 or 50 µg/mL, respectively (Fig. 2). As HSA caused relatively little or no stimulation of IL-6, the effects of NDM on IL-6 production in the presence of HSA were not determined.

Six-fold by day 6 (p < 0.0001 vs. day 1 and day 3 levels) (Fig. 3). In contrast to its effects on IL-6, G-HSA did not affect MMP-3 production or caused decreases/increases that were not statistically significant from control. At each time point tested, G-HSA had no significant effect on MMP-3 production compared to control, and cells exposed to a given concentration of G-HSA or HSA produced amounts of MMP-3 that were not significantly different from one another (Fig. 3). However, preincubation with NDM (10–50 µg/mL) caused concentration-dependent inhibition of MMP-3 levels after 6 d of exposure to G-HSA (~30%, ~60% [p < 0.0001] and ~75% [p < 0.0001] inhibition caused by 10, 25 or 50 µg/mL NDM, respectively) (Fig. 4) or HSA (not shown).

Effect of lipopolysaccharide, glycated human serum albumin or [glycated human serum albumin + lipopolysaccharide] on interleukin-6 and matrix metalloproteinase-3 production

As shown in Fig. 5, consistent with our results as seen above, the production of IL-6 was stimulated by G-HSA after 6 d of exposure (p < 0.0001), and IL-6 was stimulated to an even greater extent by P.g. LPS (11-fold; p = 0.0002 vs. control). Similar to its effect on G-HSA-stimulated IL-6 levels as noted above, NDM also inhibited LPS-stimulated IL-6 production (in this case by ~50%; p < 0.002). [G-HSA+LPS] synergistically increased IL-6 production compared to each alone (p < 0.0001). In initial experiments testing the effect of NDM (50 µg/mL) on G-HSA [100 µg/mL] +LPS [1 µg/mL]-stimulated IL-6 production, NDM did not alter it significantly. However, when the cells were exposed to G-HSA at the slightly lower concentration of 90 µg/mL and LPS at 1 µg/mL, there was still synergistic stimulation of IL-6 production, but this was inhibited by an increased NDM concentration of 100 µg/mL (p < 0.0001).

Consistent with the previous experiment above (Fig. 3), the production of MMP-3 was not significantly affected by G-HSA after 6 d of exposure (Fig. 6). In addition, unlike its effects on IL-6, P.g. LPS decreased MMP-3 production (p = 0.02 vs. control), which was further inhibited by
NDM. However, [G-HSA + LPS] increased MMP-3 production compared to G-HSA or LPS alone. This increase was significant vs. LPS alone \( (p = 0.0005) \), but not compared to G-HSA or control. NDM inhibited [G-HSA + LPS]-stimulated MMP-3 \( (p < 0.0001) \), in contrast to its lack of effect on [G-HSA + LPS]-stimulated IL-6.

**Discussion**

Exacerbated periodontal inflammation and long-term degenerative complications of diabetes may be due to proinflammatory activities of AGEs (9). We determined the *in vitro* effects of a representative AGE (G-HSA) on human gingival fibroblast production of IL-6 and MMP-3, key factors in periodontal breakdown.

Short (3 h) exposure of the cells to G-HSA caused no significant membrane damage, similar to other studies that showed AGEs caused no or minimal membrane damage to human fibroblasts and murine macrophages (36,37). Our finding that G-HSA caused no significant toxicity, measured using the MTT assay, is consistent with minor toxicity of glycated serum to murine macrophages (37), but other studies showed that G-HSA at some concentrations had toxic effects on gingival fibroblasts (38,39). Toxicity of AGEs appears to depend upon time of exposure, AGE concentration and type of cell, as well as the particular cell line, reflecting interindividual differences in response.

In the present study, gingival fibroblast IL-6 production was stimulated by G-HSA but not by HSA, similar to a report of specific effects of glycated bovine serum albumin (G-BSA) on other types of cells, including human fibroblast-like synovial cells (40–42). Gingival fibroblasts express RAGE, and AGE-RAGE binding activates signaling pathways, including MAPK/ AP-1 and nuclear factor kappa B (NF-κB), which can regulate these cells’ IL-6 expression (24,43–45). Like G-HSA, *P. g.* LPS stimulated IL-6 production consistent with our previous study (35). Via toll-like receptors, LPS also activates the NF-κB and MAPK/AP-1 pathways in human gingival fibroblasts (44). [G-HSA + LPS] synergistically increased IL-6 production, consistent with another study using human umbilical vein endothelial cells (45). This may occur via crosstalk between toll-like receptors and RAGE signaling, involving various transcription factors, as the IL-6 gene promotor can contain binding sites for transcription factors other

![Fig. 2. Effect of NDM on G-HSA-stimulated production of IL-6 by gingival fibroblasts. Cells were seeded at $5 \times 10^6$ cells/well in 24-well plates in complete medium and cultured overnight at 37°C. The medium was removed, the cells were washed with PBS, and then DMEM-gent or DMEM-gent containing G-HSA (100 μg/mL) was added. In some cases, cells were preincubated with NDM (10, 25 or 50 μg/mL) for 2 h before the addition of G-HSA. After 6 d incubation, levels of IL-6 were measured in the cell supernatants by enzyme-linked immunosorbent assay. Results of multiple experiments with triplicate samples were expressed as percentage control (amount of IL-6 [pg/mL] produced by cells exposed to DMEM-gent, set at 100%) ± standard deviation (*p < 0.0001 vs. control; †p < 0.0001 vs. G-HSA-stimulated). DMEM-gent, Dulbecco’s modified Eagle medium-gentamicin; G-HSA, glycated human serum albumin; IL, interleukin; NDM, non-dialyzable material.](#)

![Fig. 3. Effect of G-HSA or HSA on gingival fibroblast production of MMP-3. Cells were seeded at $5 \times 10^6$ cells/well in 24-well plates in complete medium and cultured overnight at 37°C. The medium was removed, the cells were washed with phosphate-buffered saline, and then Dulbecco’s modified Eagle medium-gentamicin, with or without G-HSA or HSA (7.8–250 μg/mL) was added. Aliquots of the cell culture supernatants were collected at days 1, 3 and 6, and levels of MMP-3 were measured by enzyme-linked immunosorbent assay. Results of multiple experiments with triplicate samples were expressed as pg/mL in the cell supernatants ± standard deviation (*p < 0.0001 vs. day 1 and day 3 levels). G-HSA, glycated human serum albumin; HSA, human serum albumin; MMP-3, matrix metalloproteinase-3.](#)
than MAPK/AP-1 and NF-κB in some types of cells (44–49). Because IL-6 is increased at periodontally diseased sites and enhances bone resorption, [G-HSA+LPS] synergism in its expression could have deleterious effects on periodontal bone levels (13,50).

Several MMPs play a role in periodontal destruction, including MMP-3, which is associated with both aggressive and chronic periodontitis and other inflammatory diseases (14,51–53). In the present study, G-HSA generally had no significant effect on MMP-3 production, similar to findings that G-BSA had no effect on MMP-3 mRNA in human mesangial cells (54). However, in other studies G-BSA increased MMP-3 expression by human osteoarthritic chondrocytes and in mouse tissues (11,55), and glycated albumins increased expression of other MMPs in cells, including human gingival fibroblasts (11,39). MAPK and NF-κB can be involved in MMP-3 production (44,45,52), and AGEs may stimulate MMP production in some cells via RAGE activation of signaling pathways, including NF-κB (39).

Several stimulatory and inhibitory pathways affecting MMP-3 production, including autocrine downregulation by transforming growth factor beta (TGFβ) 1 (56), may be activated by G-HSA, and while specific mechanisms remain to be determined, this resulted in no significant overall stimulatory or inhibitory effect on MMP-3 levels produced by fibroblasts in this study. On the other hand, P.g. LPS inhibited MMP-3, consistent with findings that P.g. LPS with tetra-acylated lipid A structures had no effect or decreased MMP-3 production in human gingival fibroblasts (57). Moreover, P.g. LPS can upregulate TGFβ (58) and as noted above, TGFβ can inhibit MMP-3 production in fibroblasts. Therefore, lack of significant effect of G-HSA on MMP-3 production and its inhibition by LPS may reflect involvement of competing, antagonistic pathways that stimulate MMP-3, also influenced by species-, tissue- and individual-specific cellular phenotype. In this study, the gingival fibroblasts were grown in DMEM containing high glucose 4500 mg/L (~25 mM), a commonly used formulation of this medium. The rate of formation of glycated albumin in the presence of high glucose in vitro appears to be low, approximately 0.005–0.008% per mM glucose/d (59). The cells produced IL-6 and MMP-3 constitutively, in the absence of added normal or glycated albumin, and we...
did not compare this to production in low glucose medium. In other studies, 25 mM glucose stimulated tumor necrosis factor alpha and IL-1β levels in gingival fibroblasts (60), and non-significantly stimulated IL-6 production by periodontal ligament cells (61), compared to low (5.5 mM) glucose. High glucose also upregulated expression of MMP-9 and MMP-13 mRNA, but not MMP-2 or MMP-8 in rat tendon cells (62). High glucose had no effect on MMP-2 activity of mouse cardiac fibroblasts but did increase expression and activity of MMP-2 in mouse adventitial fibroblasts (63,64); 25 mM glucose induced MMP-1 and MMP-2 expression and activity in human umbilical vein endothelial cells, but reduced MMP-3 expression (65). Specific effects of high glucose appear to depend on the type of cell and the particular type of molecule studied.

Several bioactive compounds in cranberry, including flavonoids and proanthocyanidins, have anti-inflammatory, antioxidant and/or antitumor activities and can inhibit production/activity of a number of MMPs (21,22,66). The cranberry preparation used in this study is enriched in proanthocyanidins, but it is not known which specific component(s) are responsible for the effects we observed on gingival fibroblasts, including toxicity. However, non-toxic levels of cranberry NDM significantly inhibited IL-6 and MMP-3 production in the presence of either G-HSA or LPS alone, consistent with another study that showed non-toxic levels of an A-type cranberry proanthocyanidin preparation had biological effects on cells (67). Our finding that cranberry NDM inhibited LPS-stimulated IL-6 production supports previous studies that showed NDM inhibited *Fusobacterium nucleatum* and *P.g.* LPS-stimulated IL-6 production in normal human gingival fibroblasts and human gingival epithelial cells (36) (D. Tipton, N. Zacharia, S. Rawal, M. Dabbous, unpublished studies), and it inhibited IL-6 production by *Aggregatibacter actinomycetemcomitans* LPS-stimulated human gingival fibroblasts (20,43). NDM inhibits activation of NF-κB and MAPK/AP-1 signaling pathways stimulated by G-HSA, LPS, IL-1 and other mediators, resulting in decreased expression of IL-6 (18,24,25,35). MMP-3 production is also under the regulation of NF-κB and AP-1 (44,52), and although not specifically determined in this study, the inhibition of IL-6 and MMP-3 production in the presence of LPS or AGE-HSA may occur via a similar mechanism. NDM inhibited the non-synergistic stimulation of MMP-3 by G-HSA+LPS, and it inhibited IL-6 levels synergistically stimulated by G-HSA and LPS, although a greater NDM concentration was required in the case of IL-6. This may reflect a stronger stimulation of NF-κB and MAPK/AP-1 signaling pathways that regulate IL-6 expression or potentiation by other IL-6 stimulatory pathways sensitive to inhibition by NDM (68,69).

G-HSA and [G-HSA+LPS] may therefore have differential effects on IL-6 and MMP-3 production by human gingival fibroblasts *in vitro*, but both are inhibited by NDM. More experimental work must be done to determine the mechanisms of inhibition and the signaling pathways involved. Additional *in vitro* and *in vivo* studies will be necessary to determine whether cranberry phenols may be useful in the regulation of the host response, including fibroblast inflammatory and catabolic reactions, and in helping to prevent or control the progression of periodontitis in patients with poorly controlled diabetes.

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