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Effects of cranberry components on human aggressive periodontitis gingival fibroblasts

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Background and Objective: Aggressive periodontitis (AgP) causes rapid periodontal breakdown involving AgP gingival fibroblast production of cytokines [i.e. interleukin (IL)-6, a bone metabolism regulator], and matrix metalloproteinase (MMP)-3. Lipopolysaccharide upregulates fibroblast IL-6 and MMP-3, via transcription factors (i.e. NF-κB). Cranberry (V*accinium macrocarpon*) inhibits lipopolysaccharide-stimulated macrophage and normal gingival fibroblast activities, but little is known of its effects on AgP fibroblasts. Objectives of this study are to use AgP fibroblasts, to determine cytotoxicity of cranberry components or periodontopathogen (*Fusobacterium nucleatum, Porphyromonas gingivalis*) lipopolysaccharide ± cranberry components, and effects of cranberry components on lipopolysaccharide-stimulated NF-κB activation and IL-6 and MMP-3 production.

Material and Methods: AgP fibroblasts were incubated $\leq$ 6 d with high molecular weight non-dialyzable material (NDM) (derived from cranberry juice (1–500 μg/mL) or lipopolysaccharide (1 μg/mL) ± NDM. Membrane damage and viability were assessed by enzyme activity released into cell supernatants and activity of a mitochondrial enzyme, respectively. Secreted IL-6 and MMP-3 were measured by ELISA. NF-κB p65 was measured via binding to an oligonucleotide containing the NF-κB consensus site. Data were analyzed using analysis of variance and Scheffe's F procedure for *post hoc* comparisons.

Results: Short-term exposure to NDM, or lipopolysaccharide ± NDM caused no membrane damage. NDM ($\leq$ 100 μg/mL) or lipopolysaccharide ± NDM had no effect on viability $\leq$ 7 d exposure. NDM (50 μg/mL) inhibited lipopolysaccharide-stimulated p65 ($P \leq 0.003$) and constitutive or lipopolysaccharide-stimulated MMP-3 ($P \leq 0.02$). NDM increased AgP fibroblast constitutive or lipopolysaccharide-stimulated IL-6 ($P \leq 0.0001$), but inhibited normal human gingival fibroblast IL-6 ($P \leq 0.01$).

Conclusion: Lack of toxicity of low NDM concentrations, and its inhibition of NF-κB and MMP-3, suggest that cranberry components may regulate AgP fibroblast inflammatory responses. Distinct effects of NDM on AgP and gingival fibroblast production of IL-6 (which can have both positive and negative effects on bone metabolism) may reflect phenotypic differences in IL-6 regulation in the two cell types.

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Aggressive periodontitis (AgP) is characterized by generalized or localized extreme periodontal destruction, which involves pro-inflammatory cytokines, including interleukin (IL)-1β and IL-6 (1–3). IL-6 stimulates bone resorption, possibly through stimulating osteoclast formation (4,5). Gingival fibroblasts stimulated by periodontopathogen lipopolysaccharide produce cytokines such as IL-6, and play an important role in local inflammation. The expression of IL-6 is increased at periodontally diseased sites, including gingival soft tissue (4,6,7). Earlier work in this laboratory showed that AgP gingival fibroblasts produce greater amounts of IL-6 than normal human gingival fibroblasts, and that IL-6 production by these cells is upregulated by lipopolysaccharide (and IL-1β) (8,9).

AgP fibroblasts also produce matrix metalloproteinases (MMPs), including MMP-3, which degrades many extracellular matrix components. A study in this laboratory suggested that human AgP gingival fibroblasts might contribute to periodontal tissue destruction via increased production of MMP-3 (10). MMP-3 in gingiva is associated with periodontitis, and it may be a key enzyme causing tissue destruction in other inflammatory diseases, including arthritis (11,12).

Transcription of the IL-6 and MMP-3 genes (and a large number of other genes involved in inflammation and tissue damage) are regulated by the transcription factor nuclear factor-kappa B (NF-κB) (13). Work in this laboratory and others showed that cyclo-oxygenase-2 inhibitors inhibit NF-κB activation (14), which suggested that they might be useful in treating periodontal inflammation. However, cyclo-oxygenase-2 inhibitors have been linked to heart and renal failure, limiting their use as anti-inflammatory agents (15,16). These findings have led to renewed attention to natural products potentially useful in treating the inflammation and tissue destruction associated with periodontal diseases (17,18).

The focus of this research was the cranberry (*Vaccinium macrocarpon*), which contains polyphenolic compounds, particularly proanthocyanidins with A-type linkages. Cranberry components have long been used to promote human health, and have the ability to prevent urinary tract infections, reduce salivary bacterial levels, and inhibit biofilm formation, among many other effects (19–21).

Research by Bodet *et al*. showed that a high-molecular-weight fraction prepared from cranberry juice concentrate (termed non-dialyzable material or NDM) inhibited production of pro-inflammatory cytokines and chemokines by macrophages exposed to lipopolysaccharide from a number of periodontopathogens (22). This group also showed that NDM inhibited IL-6, IL-8, prostaglandin E_2 (PGE_2), and MMP-3 production by *Aggregatibacter actinomycetemcomitans* lipopolysaccharide-stimulated normal human gingival fibroblasts, as well as certain intracellular signaling proteins related to the transcription factor AP-1 (23,24). Their research, and that of others (25–27), have suggested that AP-1 may work together with NF-κB to regulate gingival fibroblast production of these molecules. Work in our laboratory has shown that IL-1β stimulation of MMP-3 production in AgP fibroblasts may be partially regulated by NF-κB, in addition to AP-1 (28). While there are studies on the effects of cranberry components on normal human gingival fibroblasts, there is no similar information on the effects of cranberry components on lipopolysaccharide-mediated NF-κB activation and production of IL-6 and MMP-3 in AgP human gingival fibroblasts, which may be phenotypically distinct from normal human gingival fibroblasts (8).

Regulation of gingival fibroblast pro-inflammatory reactions has been suggested as a means to prevent or control the progression of periodontitis. Cranberry components may have the potential to treat periodontitis, in the form of a rinse, toothpaste, or as a subgingival medicament. To assess this potential fully, *in vitro* studies of its effects on oral cells such as gingival fibroblasts, as well as *in vivo* studies, are needed to determine its safety and efficacy. The present study has determined the toxic effects of cranberry NDM on human AgP gingival fibroblasts *in vitro*, as well as its effect on NF-κB and production of IL-6 and MMP-3 when stimulated by periodontopathogen lipopolysaccharide.

Material and methods

Human gingival fibroblasts

A human gingival fibroblast cell line derived from a patient with AgP was used in this study (29). This patient presented with > 80% generalized alveolar bone loss and generalized erythematous, edematous gingival tissue. The cell line was established from gingival explants using standard techniques (30). In some experiments, three normal human gingival fibroblast cell lines (designated GN23, GN56, and GN60) were also used and were similarly established from explants of non-inflamed gingival tissue from healthy individuals. The cells were routinely cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Grand Island, NY, USA) supplemented with 10% (v/v) newborn calf serum (Life Technologies) and 100 μg/mL gentamicin (Sigma-Aldrich Co., St Louis, MO, USA) (complete medium), at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells between passages 5 and 14 were used in the experiments described below.

Cranberry components (non-dialyzable material) and lipopolysaccharide

Lyophilized high molecular weight NDM derived from cranberry juice, was provided courtesy of Dr. I. Ofek (Tel Aviv University). This material was prepared from concentrated juice of *Vaccinium macrocarpon* (Ocean Spray Cranberries, Inc., Lakeville-Middleboro, MA, USA) (20) by exhaustive dialysis against distilled water at 4°C in 15,000 MW cut-off dialysis bags. This material was composed of 5 6.6% carbon and 4.14% hydrogen, 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-arabinoside) and 65.1% proanthocyanidins (32). NDM was reconstituted at 2 mg/mL in DMEM and sterilized by filtration (0.2 μ; MILLEX-GS; Millipore Corp., Billerica MA, USA). Lipopolysaccharide was prepared from bacteria obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA): *Fusobacterium nucleatum* subsp. *polymorphum* ATCC 10953, and *Porphyromonas gingivalis* ATCC 25260. The bacteria were grown anaerobically for 48 h, centrifuged, and bacterial cell pellets were lyophilized. The lipopolysaccharide fractions were prepared by the hot phenol–water extraction method as described by Westphal and Jann (33). The purity and protein contamination of the isolated lipopolysaccharide was confirmed by 0.1% sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. The endotoxin activities of the isolated lipopolysaccharide fractions were measured using the Limulus assay (34).

Determination of effects of cranberry non-dialyzable material or lipopolysaccharide on human gingival fibroblast cell viability and membrane integrity

Lipopolysaccharide or cranberry NDM 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, using a kit from Boehringer Mannheim Corp. (Indianapolis, IN). Individual wells of 96-well microtiter tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) were seeded with 2.5×10^4 cells in complete medium. The cells were incubated overnight at 37°C. The medium was then removed, the cells were washed with phosphate-buffered saline (PBS), and serum-free medium supplemented with 100 μg/mL gentamicin (DMEM-gentamicin), containing lipopolysaccharide (0.1–5 μg/mL), or NDM (1–500 μg/mL) was added to the wells. The cells were exposed to lipopolysaccharide or NDM for as long as 7 d before the addition of MTT (0.5 mg/mL). The cells were then incubated for 4 h at 37°C, and purple formazan crystals produced from the MTT by metabolically active cells were solubilized by overnight exposure at 37°C to a solubilization solution provided in the kit. Absorbance was read at 595 nm using a microtiter plate spectrophotometer. Results were expressed as a percentage of control ($A_{595\ nm}$ in cells exposed to DMEM-gentamicin only).

Lipopolysaccharide- or NDM-induced cytotoxicity leading to plasma membrane damage was measured using the Cytotoxicity Detection Kit (LDH) (Boehringer Mannheim). Lactate dehydrogenase (LDH), a stable cytoplasmic enzyme found in all cells, is quickly released upon damage to the plasma membrane. Individual wells of 96-well microtiter tissue culture plates were seeded with 2.5×10^4 cells in DMEM-gentamicin + 1% newborn calf serum and cultured for 24 h at 37°C. (A low serum concentration was used to reduce background absorbance because animal sera contain various levels of LDH.) The medium was removed, the cells were washed with PBS, and DMEM-gentamicin was 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 (Boehringer Mannheim). Spontaneous LDH release (low control) was determined by incubating the cells with serum-free DMEM-gentamicin. The cells were exposed to lipopolysaccharide or NDM for 3 h. [When performed after a brief 3-h exposure, the LDH assay specifically measures the ability of the test agent to damage the integrity of the plasma membrane (35); for longer exposure times, LDH release serves as a general cytotoxicity assay]. 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, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride, was reduced to a red formazan salt. Absorbance was read at 490 nm using a microtiter plate spectrophotometer. Results were expressed as percentage cytotoxicity [(experimental value-low control/high control-low control) × 100].

Determination of effects of non-dialyzable material on constitutive and lipopolysaccharide-stimulated production of interleukin-6 and matrix metalloproteinase-3

Individual wells of 24-well culture plates (Corning Inc., Corning, NY, USA) were seeded with 5×10^4 cells in complete medium and cultured overnight at 37°C. The medium was removed, the cells were washed with PBS, and then DMEM-gentamicin was added, with or without *F. nucleatum* or *P. gingivalis* lipopolysaccharide (0.1–5 μg/mL). Aliquots of 0.5 mL were collected at days 3 and 6 and stored frozen at −80°C until assayed. The production of IL-6 or MMP-3 was measured in the cell supernatants using the Human DuoSet IL-6 ELISA Development System (R&D Systems, Minneapolis, MN, USA), and the Human DuoSet MMP-3 ELISA Development System (R&D Systems), following manufacturers' instructions. To determine effects of NDM, these experiments were essentially carried out as described as above, except that in some cases, NDM (5–50 μg/mL) was added concurrently with *F. nucleatum* or *P. gingivalis* lipopolysaccharide (1 μg/mL only).

Determination of effects of non-dialyzable material on constitutive and lipopolysaccharide-stimulated NF-κB activation

The effect of *F. nucleatum* or *P. gingivalis* lipopolysaccharide on NF-κB activation was determined using the TransAM™ NF-κB p65 assay kit (Active Motif, Carlsbad, CA, USA). Individual wells of six-well plates (Costar Corp., Cambridge, MA, USA) were seeded with 1.5×10^6 cells in complete medium and cultured

overnight at 37°C. The medium was removed, cells were washed with PBS, and then DMEM-gentamicin was added, with or without *F. nucleatum* or *P. gingivalis* lipopolysaccharide (1 μg/mL). Cells were exposed to lipopolysaccharide for 30–105 min. Nuclear and cytoplasmic fractions were isolated using the Nuclear Extract Kit (Active Motif). The cells were harvested by scraping and pelleted by centrifugation for 5 min at 16 × g at 4°C. The cells were resuspended in hypotonic buffer supplied in the kit and incubated on ice for 15 min. Then detergent supplied in the kit was added, and the tubes were vortexed for 10 s and centrifuged for 30 s at 14,000 *g* at 4°C. Supernatants (cytoplasmic fractions) were collected in pre-chilled microcentrifuge tubes and stored at −80°C until assayed. To prepare the nuclear extracts, the nuclear pellets obtained above were resuspended in complete lysis buffer supplied in the kit, vortexed for 10 s, and incubated on ice for 30 min on a rocking platform. The tubes were then vortexed for 30 s and centrifuged at 14,000 *g* at 4°C for 10 min. The supernatant fluids (nuclear extracts) were added to pre-chilled microcentrifuge tubes and stored at −80°C until assayed.

To assay the samples for p65, 30 μL of complete lysis buffer, supplied in the kit, was added to microtiter plate wells pre-coated with an oligonucleotide containing an NF-κB consensus binding site. The active form of NF-κB specifically binds to this oligonucleotide. Cytoplasmic or nuclear extract samples (20 μL) were added to the wells; blank wells received 20 μL complete lysis buffer and the positive control wells received 20 μL complete lysis buffer containing 2.5 μL of a nuclear extract supplied in the kit. The plate was then incubated at room temperature for 1 h with mild agitation. After washing, 100 μL primary antibody (rabbit anti-p65 that recognizes an epitope on p65 accessible only when NF-κB is activated and bound to its target DNA) was added to each well and incubated at room temperature for 1 h. The wells were washed, and 100 μL secondary antibody [anti-rabbit horseradish peroxidase-conjugated IgG] was added to each well and incubated at room temperature for 1 h. After washing, the substrate solution was added to all wells, incubated at room temperature for 5 min, and stop solution was then added. Absorbance at 450 m was measured using a microtiter plate spectrophotometer. Results were calculated as A_{450}/mg protein in the cell monolayer (36), and expressed as percentage control (the value obtained in extracts of non-stimulated cells, set at 100% at all times).

To determine effects of NDM, these experiments were carried out as described above, except that in some cases, NDM (50 μg/mL) was added concurrently with *F. nucleatum* or *P. gingivalis* lipopolysaccharide.

Statistical analysis

All experiments were performed with triplicate samples. The data were expressed as mean ± standard deviation and were analyzed using a one-way analysis of variance and Scheffe's *F* procedure for *post hoc* comparisons, using STATVIEW® software.

Results

Effects of lipopolysaccharide and non-dialyzable material ± lipopolysaccharide on cell viability and membrane integrity

Effects of *F. nucleatum* lipopolysaccharide, *P. gingivalis* lipopolysaccharide, and NDM ± lipopolysaccharide on cell viability and membrane integrity were measured before determining their effects on other cellular functions. NDM ≤ 100 μg/mL had no significant effect on AgP gingival fibroblast viability at exposure times ≤ 7 d (Fig 1). NDM at 250 μg/mL decreased viability ≥ 40% at ≥ 96 h ($P < 0.008$). Finally, NDM at 500 μg/mL decreased viability of these cells ≥ 40% at times ≥ 24 h ($P < 0.008$), and by 100% at 144 h ($P < 0.0001$). Because these experiments were performed in serum-free medium, the reduction in viability is likely not related to a decrease in

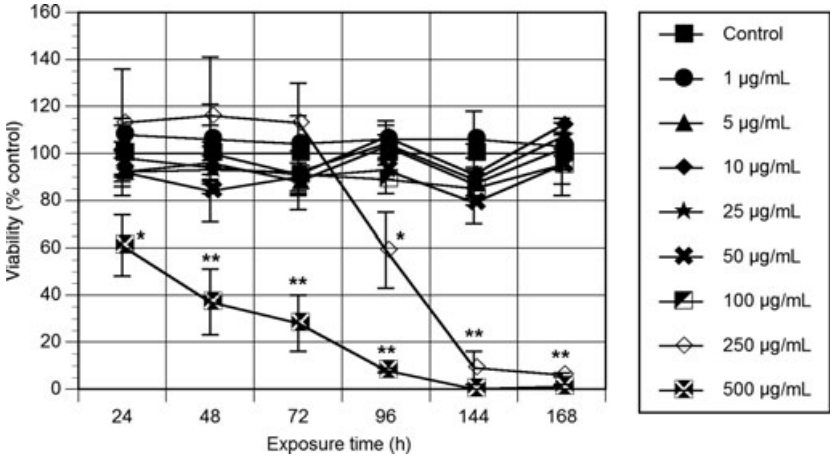

Fig. 1. Effects of non-dialyzable material (NDM) on aggressive periodontitis gingival fibroblast viability. Aggressive periodontitis fibroblasts were seeded at 2.5 × 10^4 cells/well in 96-well plates in complete Dulbecco's modified Eagle medium (DMEM). After overnight incubation at 37°C, the medium was removed, the wells were washed with phosphate-buffered saline, and NDM (1–500 μg/mL) in serum-free DMEM-gentamicin was added and incubated for 24, 48, 72, 96, 144, or 168 h. Cytotoxicity of NDM was determined by measuring its effect on the ability of the cells to cleave the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide to a colored formazan dye. Absorbance was read at 540 nm using a microtiter plate spectrophotometer. Results of multiple experiments with triplicate samples were expressed as percentage control ($A_{540\ nm}$ in cells exposed to DMEM-gentamicin only) ± standard deviation. *$P < 0.008$; **$P < 0.0001$.

proliferation as these cells, like other adult human fibroblasts, require serum for growth *in vitro* (37). *Fusobacterium nucleatum* or *P. gingivalis* lipopolysaccharide (0.1–5 μg/mL) did not reduce AgP fibroblast viability after 7 d, and *F. nucleatum* or *P. gingivalis* lipopolysaccharide [1 μg/ml ± NDM ($\leq$ 100 μg/mL)] (conditions under which effects on other cellular functions were determined) also did not reduce viability of these cells (data not shown). Short-term (3–6 h) exposure to NDM ($\leq$ 500 μg/mL) or *F. nucleatum* or *P. gingivalis* lipopolysaccharide (1 μg/mL) ± NDM caused no significant membrane damage to AgP fibroblasts (data not shown).

Effects of lipopolysaccharide and non-dialyzable material ± lipopolysaccharide on interleukin-6 production

Both *F. nucleatum* and *P. gingivalis* caused concentration-dependent increases in IL-6 production by AgP fibroblasts, which were statistically significant at higher concentrations [$P < 0.03$ for *F. nucleatum* lipopolysaccharide (Fig. 2A); $P < 0.02$ for *P. gingivalis* lipopolysaccharide) (Fig. 2B)]. *P. gingivalis* lipopolysaccharide appeared to be a better stimulator of IL-6, increasing production approximately twice as much as *F. nucleatum* lipopolysaccharide, at concentrations $\geq$ 0.25 μg/mL. Data shown were obtained after a 6-d incubation with lipopolysaccharide; 3-d data were similar but of lesser magnitude (not shown). NDM alone, at higher concentrations only, slightly increased AgP fibroblast IL-6 production, which did not reach statistical significance after 3- or 6-d incubation (data not shown). Concurrent exposure of AgP cells to NDM and *F. nucleatum* and *P. gingivalis* lipopolysaccharide (1 μg/mL) for 6 d further increased lipopolysaccharide-stimulated IL-6 production by AgP fibroblasts ($P \leq 0.0001$) (Fig. 3). With both *F. nucleatum* and *P. gingivalis* lipopolysaccharide, the effect of NDM was dose-dependent. In the case of *F. nucleatum* lipopolysaccharide, the stimulation appeared to reach a maximum when the cells were exposed concurrently to 10 μg/mL NDM (≈20-fold vs. *F. nucleatum* lipopolysaccharide only) (Fig. 3A). On the other hand, when combined with *P. gingivalis* lipopolysaccharide, lower NDM concentrations ($\leq$ 10 μg/mL) increased lipopolysaccharide-stimulated IL-6 levels by ≈5–6-fold, rising to ≈14-fold at 25 μg/mL NDM, and finally to ≈27-fold at 50 μg/mL NDM (Fig. 3B). These effects were also evident after 3 d of concurrent exposure to NDM and both *F. nucleatum* and *P. gingivalis* lipopolysaccharide, but of lesser magnitude (data not shown).

Three normal gingival fibroblast cell lines (designated GN23, GN56, and GN60) were also tested for effects of *F. nucleatum* or *P. gingivalis* lipopolysaccharide (1 μg/mL) ± NDM (10 or 50 μg/mL) on IL-6 production (Table 1). Lipopolysaccharide stimulated IL-6 production by 30–60% in two of the cell lines, reaching statistical significance in GN56. In the third cell line, lipopolysaccharide decreased IL-6 by ≈5–30% but these effects were not statistically significant. In contrast to the stimulatory effects of NDM on AgP fibroblast IL-6 production, non-cytotoxic concentrations of NDM generally inhibited constitutive and *F. nucleatum* and *P. gingivalis* lipopolysaccharide-stimulated IL-6 production by the three normal cell lines used in this study.

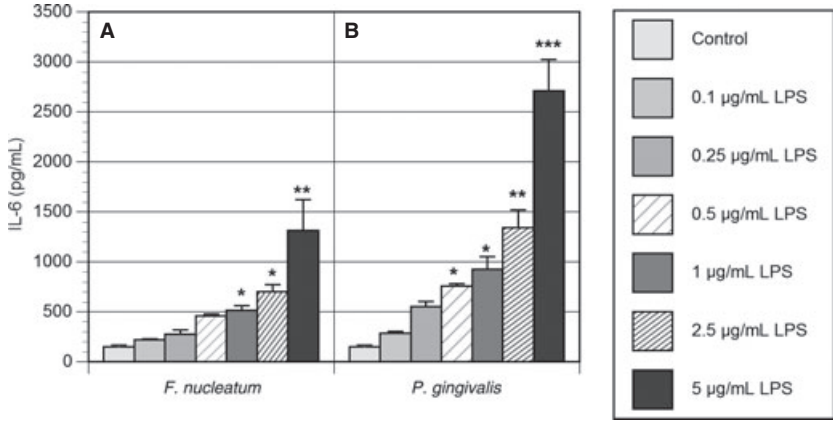

Fig. 2. Effect of *F. nucleatum* or *P. gingivalis* lipopolysaccharide on aggressive periodontitis gingival fibroblast production of interleukin (IL)-6. Aggressive periodontitis fibroblasts were seeded at 5×10^4 cells/well in 24-well plates in complete Dulbecco's modified Eagle medium and cultured overnight 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 *F. nucleatum* (A) or *P. gingivalis* (B) lipopolysaccharide (0.1–5 μg/mL) was added. After 6 d of incubation, levels of IL-6 were measured in the cell supernatants by ELISA. Results of multiple experiments with triplicate samples were expressed as pg/mL in the cell supernatants ± standard deviation. (A) $*P < 0.03$; $**P < 0.0001$. (B) $*P < 0.02$; $**P = 0.003$; $***P < 0.0001$. LPS, lipopolysaccharide.

Effects of lipopolysaccharide and non-dialyzable material ± lipopolysaccharide on matrix metalloproteinase-3 production

Exposure of AgP fibroblasts to non-toxic levels of NDM (5–50 μg/mL) inhibited constitutive MMP-3 production at some concentrations (Fig. 4A). Lower concentrations caused ≈25–35% inhibition compared to control which was not statistically significant. NDM at 25 and 50 μg/mL inhibited constitutive MMP-3 production by ≈85% and ≈95%, respectively ($P < 0.002$). Neither *F. nucleatum* nor *P. gingivalis* lipopolysaccharide alone had a consistent or statistically significant effect on MMP-3 production by this cell line (data not shown). When exposed concurrently for 6 d to lower concentrations of NDM and *F. nucleatum* lipopolysaccharide, MMP-3 production was not significantly inhibited, compared to levels produced by cells exposed to lipopolysaccharide

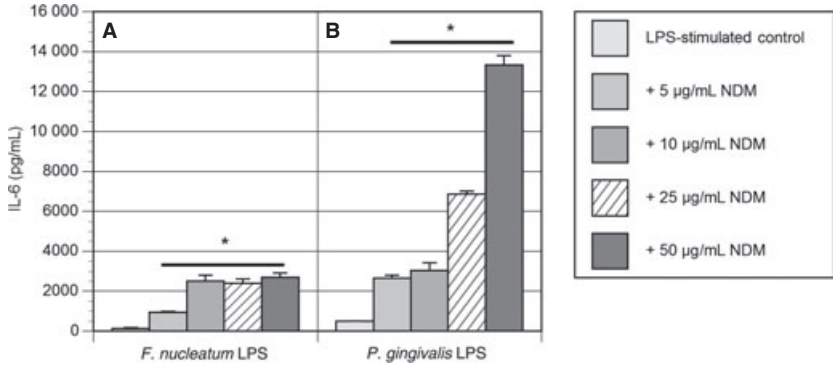

Fig. 3. Effect of NDM on *F. nucleatum* or *P. gingivalis* lipopolysaccharide-stimulated IL-6 production by aggressive periodontitis gingival fibroblasts. Aggressive periodontitis fibroblasts were seeded at 5×10^4 cells/well in 24-well plates in complete Dulbecco's modified Eagle medium and cultured 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 *F. nucleatum* (A) or *P. gingivalis* (B) LPS (1 µg/mL) ± NDM (5–50 µg/mL) was added. After 6 d of incubation, levels of IL-6 were measured in the cell supernatants by ELISA. Results of multiple experiments with triplicate samples were expressed as pg/mL in the cell supernatants ± standard deviation. $*P < 0.0001$. IL, interleukin; LPS, lipopolysaccharide; NDM, non-dialyzable material.

Table 1. Effect of non-dialyzable material (NDM) on normal gingival fibroblast interleukin (IL)-6 production

Treatment	GN23	GN56	GN60
Control (no lipopolysaccharide) [1]	100	100	100
+NDM 10 µg/mL [1]	61 (2)[b]	38 (6)[a]	87 (49)
+NDM 50 µg/mL [1]	12 (3)[a]	29 (2)[a]	17 (12)[d]
F. nucleatum LPS [1]	67 (13)	148 (21)[e]	130 (11)
+NDM 10 µg/mL [2]	112 (18)	33 (3)[a]	89 (10)
+NDM 50 µg/mL [2]	10 (3)[a]	25 (9)[a]	19 (4)[d]
P. gingivalis lipopolysaccharide [1]	94 (18)	160 (3)[c]	130 (13)
+NDM 10 µg/mL [3]	95 (0)	42 (6)[a]	73 (39)
+NDM 50 µg/mL [3]	15 (8)[a]	23 (8)[a]	40 (15)[d]

[1]Values shown are (IL-6) in pg/mL converted to percentage control (no lipopolysaccharide) (± SD).
[2]% *F. nucleatum* lipopolysaccharide-stimulated (± SD).
[3]Percentage *P. gingivalis* lipopolysaccharide-stimulated (± SD).
[a]$P < 0.001$.
[b]$P = 0.003$.
[c]$P = 0.006$.
[d]$P < 0.01$.
[e]$P = 0.03$.

only (set at 100%) (Fig. 4B). NDM at 25 and 50 µg/mL inhibited MMP-3, in the presence of *F. nucleatum* lipopolysaccharide, by ≈30% and ≈80%, respectively ($P < 0.02$), after 6 d of exposure. Only the highest concentration of NDM (50 µg/mL) significantly inhibited MMP-3 in the presence of *P. gingivalis* lipopolysaccharide (≈50% inhibition; $P = 0.0003$) after 6 d of exposure (Fig. 4C). These effects were also evident after 3 d of exposure to NDM and *F. nucleatum* or *P. gingivalis* lipopolysaccharide (data not shown).

Effects of lipopolysaccharide and non-dialyzable material ± lipopolysaccharide on NF-κB p65 levels

Based on earlier experiments, lipopolysaccharide concentrations of 1 µg/mL and NDM at 50 µg/mL were used in these experiments. AgP cells were exposed to NDM or *F. nucleatum* or *P. gingivalis* lipopolysaccharide ± NDM and NF-κB p65 levels were measured in nuclear extracts. In this assay, microtiter plate wells were coated with an oligonucleotide containing an NF-κB consensus binding site to which active p65 heterodimers specifically bind. Figure 5 shows that NDM alone had no significant effect on basal nuclear p65 levels. *Fusobacterium nucleatum* lipopolysaccharide appeared to be a stronger stimulator of NF-κB activation and translocation of p65 into the nucleus than *P. gingivalis* lipopolysaccharide. *Fusobacterium nucleatum* lipopolysaccharide stimulated nuclear p65 levels ≈12-fold ($P < 0.0001$) (Fig. 5A), while *P. gingivalis* lipopolysaccharide stimulated them ≈2.5-fold ($P < 0.01$) (Fig. 5B) (maximum stimulation at 75 and 60 min, respectively). Concurrent exposure to NDM inhibited *F. nucleatum* lipopolysaccharide stimulated nuclear p65 levels by ≈25% ($P < 0.003$) (Fig. 5A), while NDM inhibited *P. gingivalis* lipopolysaccharide-stimulated nuclear p65 levels by ≈80% ($P < 0.003$) (Fig. 5B). NDM also inhibited total cellular levels of *F. nucleatum* or *P. gingivalis* lipopolysaccharide-stimulated p65 ($P < 0.0001$) (data not shown).

Discussion

AgP is a form of periodontal disease characterized by rapid destruction of the periodontium, usually in individuals who are otherwise systemically healthy (38). Like other forms of periodontitis, AgP has a multifactorial etiology, with a host immunoinflammatory response to periodontopathogens modified by genetic, environmental, and behavioral factors. Several periodontal pathogens, including *A. actinomycetemcomitans*, *P. gingivalis*, and *F. nucleatum* may be elevated in the dental plaque of some patients with AgP (39,40). Traditional periodontal scaling and root planing can be ineffective in treating AgP and is often combined with local delivery of antimicrobial agents, including chlorhexidine, tetracycline, and minocycline (41,42). In recent

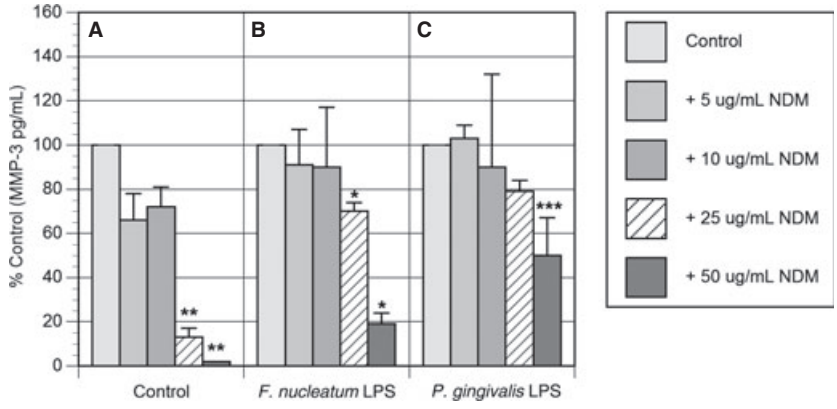

Fig. 4. Effect of NDM on aggressive periodontitis gingival fibroblast MMP-3 production in presence or absence of *F. nucleatum* or *P. gingivalis* lipopolysaccharide. Aggressive periodontitis fibroblasts were seeded at 5×10^4 cells/well in 24-well plates in complete Dulbecco's modified Eagle medium (DMEM) and cultured overnight at 37°C. The medium was removed, cells were washed with phosphate-buffered saline, and then DMEM-gentamicin (A) or DMEM-gentamicin containing *F. nucleatum* (B) or *P. gingivalis* (C) lipopolysaccharide (1 μg/mL) ± NDM (5–50 μg/mL) was added. After 6 d of incubation, levels of MMP-3 were measured in the cell supernatants by ELISA. Results of multiple experiments with triplicate samples were expressed as pg/mL in the cell supernatants ± standard deviation. $*P < 0.02$; $**P < 0.002$; $***P = 0.0003$. LPS, lipopolysaccharide; MMP, matrix metalloproteinase; NDM, non-dialyzable material.

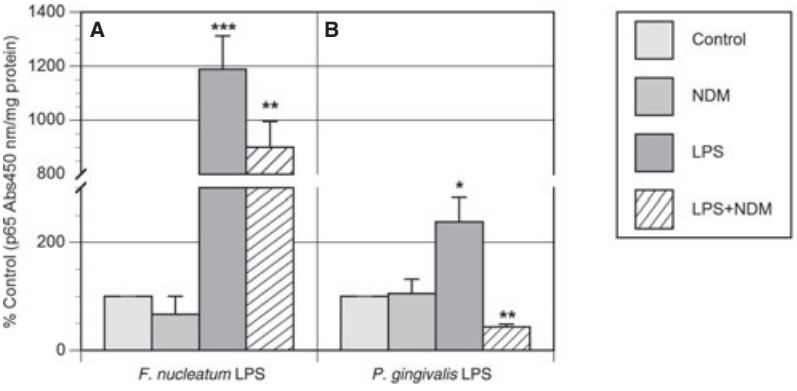

Fig. 5. Effect of NDM on constitutive or *F. nucleatum* or *P. gingivalis* lipopolysaccharide-stimulated nuclear NF-κB p65 levels. Aggressive periodontitis fibroblasts were seeded at 1.5×10^6 cells/well in six-well plates in complete DMEM and cultured overnight at 37°C. The medium was removed, the cells were washed with PBS, and then DMEM-gentamicin containing NDM (50 μg/mL) or *F. nucleatum* (A) or *P. gingivalis* (B) lipopolysaccharide (1 μg/mL) ± NDM was added. After incubation periods of 30–105 min, nuclear fractions were isolated and NF-κB p65 levels were measured using a colorimetric assay that detects free, active p65 binding to an oligonucleotide containing an NF-κB consensus binding site. Results of experiments each with triplicate samples are shown as percentage non-stimulated control (Abs450 nm/mg protein± standard deviation. $*P < 0.01$ (control vs. *P. gingivalis* lipopolysaccharide); $**P < 0.003$ (lipopolysaccharide vs. lipopolysaccharide + NDM); $***P < 0.0001$ (control vs. *F. nucleatum* lipopolysaccharide). LPS, lipopolysaccharide; NDM, non-dialyzable material.

years, because of several factors including antibiotic resistance, natural products that may be useful in treating the inflammation and tissue destruction of diseases such as AgP have received a great deal of study. The focus of the present research was the cranberry, which has beneficial antibacterial and anti-inflammatory effects. This study suggests that a high-molecular-weight cranberry preparation, enriched in proanthocyanidins, may regulate AgP gingival fibroblast inflammatory and proteolytic activities in response to lipopolysaccharide from periodontopathogens (*F. nucleatum* and *P. gingivalis*) associated with AgP. *Fusobacterium nucleatum* and *P. gingivalis* lipopolysaccharide stimulated AgP fibroblast IL-6 production in a dose-dependent manner. This is consistent with previous studies that used gingival fibroblasts from normal and chronic periodontitis gingiva (8,9,43). Lipopolysaccharide activates the NF-κB and MAPK/AP-1 signaling pathways and their associated inflammatory gene expression in fibroblasts, including human gingival fibroblasts (24,43). However, lipopolysaccharide activation of AP-1 in human gingival fibroblasts is controversial, and Jin *et al*. recently reported that in normal human gingival fibroblasts, *Escherichia coli* lipopolysaccharide stimulated NF-κB, but not AP-1 (45,46). The IL-6 gene promoter is under the regulation of AP-1 and NF-κB, and can contain binding sites for other transcription factors in some types of cells (47,48); both AP-1 and NF-κB are involved in IL-1-stimulation of IL-6 in human gingival fibroblasts (13,24,47,49). As discussed earlier, AP-1 may work together with NF-κB to regulate gingival fibroblast production of IL-6 and other mediators (22–27). In the present study, the focus was on the NF-κB signaling pathway, and it was found that both *P. gingivalis* and *F. nucleatum* lipopolysaccharide activated NF-κB in AgP fibroblasts, suggesting that this pathway may play a role in their increased production of IL-6 in response to the lipopolysaccharide of these bacteria. When combined with lipopolysaccharide, NDM at all concentrations significantly increased IL-6 production compared to levels produced in response to lipopolysaccharide alone. This effect occurred only in the presence of lipopolysaccharide; NDM alone did not significantly increase constitutive IL-6 production. This stimulatory effect was also observed under different experimental

conditions, including pre-incubating with NDM for 2 h or 24 h, then adding lipopolysaccharide with or without an intervening washing step. This is in contrast to another study, using normal human gingival fibroblasts, which found that NDM decreased *A. actinomycetemcomitans* lipopolysaccharide-stimulated IL-6 production (23). In the present study with AgP fibroblasts, NDM inhibited lipopolysaccharide-stimulated NF-κB activation, but despite this, NDM increased lipopolysaccharide-stimulated IL-6 production. This suggested involvement of other mechanisms of IL-6 regulation in these cells.

Three normal human gingival fibroblast cell lines were then also tested for effects of *F. nucleatum* and *P. gingivalis* lipopolysaccharide on IL-6 production in the presence or absence of NDM. Similar to the findings of Bodet *et al.* (23), and in contrast to our results with AgP fibroblasts, we found that non-cytotoxic concentrations of NDM inhibited constitutive and *F. nucleatum* and *P. gingivalis* lipopolysaccharide-stimulated IL-6 production by these particular normal cell lines. We have found that NDM also inhibits IL-6 production by human gingival epithelial cells, in the presence or absence of *F. nucleatum* or *P. gingivalis* lipopolysaccharide, IL-17, or IL-1β (50,51, unpublished observation).

The mechanism(s) of NDM enhancement of lipopolysaccharide-stimulated IL-6 production by AgP fibroblasts are unknown. However, NDM also increased *F. nucleatum* and *P. gingivalis* lipopolysaccharide-stimulated PGE_2 in AgP fibroblasts, paralleling IL-6 increases (unpublished observation). This is in contrast to other studies using normal human gingival fibroblasts, which found that NDM inhibited *A. actinomycetemcomitans* lipopolysaccharide-stimulated PGE_2 (23). IL-6 can be upregulated by endogenous PGE_2 in human gingival fibroblasts and other types of cells (52,53). The increased PGE_2 production in response to lipopolysaccharide and NDM may thus act in an autocrine manner on AgP fibroblasts to stimulate their production of IL-6, influenced by factors, including types and prevalence of cell surface PGE_2 receptors. The specific roles of PGE_2 and NDM in enhancing lipopolysaccharide-stimulated IL-6 production in AgP but not the normal gingival fibroblasts used in this study remain to be determined. NDM may potentiate stimulatory pathways [e.g. Involving cAMP and/or proteinase kinase C (52,54)] in the AgP cells used in this study that override its inhibitory effects on NF-κB-mediated IL-6 production. Additional studies will also be needed to investigate fully the effects of cranberry polyphenols on IL-6 mRNA expression, regulation, and correlation with IL-6 protein expression levels. Because of the limited sample sizes of cell lines derived from AgP and normal gingiva, it was not possible to determine definitively if the cells derived from these two types of tissue are phenotypically different in this regard. While IL-6 is considered a pro-inflammatory cytokine, it also has anti-inflammatory/regenerative activities (55). The overall effects of IL-6 can depend upon the presence of the soluble IL-6 receptor and other IL-6-type cytokines. For example, IL-6 appears to activate osteoclast formation in the presence of soluble IL-6 receptor (5), but, with other IL-6 family members, it can also inhibit osteoclast formation and bone resorption (56) Therefore, NDM enhancement of lipopolysaccharide-stimulated IL-6 by AgP fibroblasts could inhibit osteoclastogenesis, depending on the local environment, *in vivo*. While *F. nucleatum* and *P. gingivalis* lipopolysaccharide both strongly stimulated AgP IL-6 production, neither lipopolysaccharide had a consistent or statistically significant effect on MMP-3 production. Work in this laboratory showed that *E. coli* lipopolysaccharide at comparable concentration (1 μg/mL) stimulated AgP MMP-3 production by < 50% (57,58), and in a study by others, *P. gingivalis, F. nucleatum,* and *A. actinomycetemcomitans* lipopolysaccharide (1 μg/mL) increased MMP-3 production by ≈25%, 60%, and ≈3-fold, respectively, in normal human gingival fibroblasts (59). These studies demonstrate that factors such as the specific type of target cell, and lipopolysaccharide source and concentration can influence cellular response. Unlike its stimulatory effects on IL-6 production by AgP fibroblasts, NDM at some concentrations inhibited constitutive and lipopolysaccharide-stimulated MMP-3 production, consistent with studies by others using human gingival fibroblasts and macrophages (24,60). While AP-1 is considered the dominant transcription factor for MMP-3 because there are AP-1 sites in its promoter region (27), there may also be AP-1-independent mechanisms of MMP-3 induction, including involvement of NF-κB, in several types of rabbit and human cells, including fibroblasts (61,62). An earlier study in our laboratory showed that in AgP fibroblasts, an NF-κB inhibitor (NBD peptide) specifically decreased IL-1β-stimulated nuclear p65 by ≈55%, and decreased IL-1β-stimulated MMP-3 production by a maximum of 26%, suggesting that in these cells, MMP-3 expression can be partially regulated by NF-κB (28). In the present study, NDM inhibited NF-**κ**B activation and MMP-3 levels. Others have shown that NDM inhibits *A. actinomycetemcomitans* lipopolysaccharide-stimulated signaling proteins in the AP-1 pathway in normal human gingival fibroblasts and macrophages (23,24,60), and we have found that NDM inhibits AP-1 activation in human gingival epithelial cells (unpublished data). However, this has not yet been shown for AgP fibroblasts.

This study has shown that cranberry NDM can inhibit *F. nucleatum* and *P. gingivalis* lipopolysaccharide-stimulated NF-κB activation in AgP fibroblasts, and the production of MMP-3, which has a wide range of substrates present in periodontal tissues. NDM appears to have different effects on lipopolysaccharide-stimulated IL-6 production, depending on the cell type. IL-6, in turn, can have anti-inflammatory or pro-inflammatory effects *in vivo*. While much *in vitro* and clinical investigation remain to be done, this study and others studies suggest that cranberry may have potential for the treatment and/

or management of periodontitis. This could be in the form of a cranberry-containing mouth rinse, as investigated by Weiss *et al*. (63). Cranberry polyphenols could also be used as a subgingival medicament; others have found that hydroxypropylcellulose is an efficient subgingival delivery system for controlled release of green tea catechins and tetracycline (64,65). Alternatively, cranberry components may be incorporated into microspheres similar to Arestin® (minocycline microspheres; OraPharma, Inc., Horsham, PA, USA) (66), or as cranberry procyanidin nanoparticles, as Zou *et al*. recently reported (67). Long-term safety and efficacy studies of cranberry polyphenols and evaluation of potential delivery systems, however, must be performed to determine their suitability for managing or treating periodontitis.

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