J Periodont Res 2007; 42; 159–168

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2006.00929.x

Inhibition of host extracellular matrix destructive enzyme production and activity by a high-molecular-weight cranberry fraction

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Bodet C, Chandad F, Grenier D. Inhibition of host extracellular matrix destructive enzyme production and activity by a high-molecular-weight cranberry fraction. J Periodont Res 2007; 42: 159–168. © 2006 The Authors. Journal compilation © 2006 Blackwell Munksgaard

Background and Objective: Periodontal diseases are a group of inflammatory disorders that are initiated by specific gram-negative bacteria and lead to connective tissue destruction. Proteolytic enzymes, including matrix metalloproteinases (MMPs) and elastase, produced by resident and inflammatory cells in response to periodontopathogens and their products, play a major role in gingival tissue destruction. The aim of this study was to investigate the effect of a high-molecular-weight fraction prepared from cranberry juice concentrate on MMP-3, MMP-9 and elastase activities, as well as on MMP production by human cells stimulated with lipopolysaccharide of *Actinobacillus actinomycetemcomitans*.

Material and Methods: MMP-3 and MMP-9 production by gingival fibroblasts and macrophages treated with the cranberry fraction and then stimulated with lipopolysaccharide was measured by enzyme-linked immunosorbent assay. MMP-3, MMP-9 and elastase activities in the presence of the cranberry fraction were evaluated using colorimetric or fluorogenic substrates. The changes in expression and phosphorylation state of fibroblast intracellular signaling proteins induced by *A. actinomycetemcomitans* lipopolysaccharide and the cranberry fraction were characterized by antibody microarrays.

Results: The lipopolysaccharide-induced MMP-3 and MMP-9 responses of fibroblasts and macrophages were inhibited in a dose-dependent manner by the cranberry fraction. This fraction was found to inhibit fibroblast intracellular signaling proteins, a phenomenon that may lead to a down-regulation of activating protein-1 activity. MMP-3, MMP-9 and elastase activities were also efficiently inhibited by the cranberry fraction, even when it was used at low concentrations.

Conclusion: These results suggest that cranberry compounds offer promising perspectives for the development of novel host-modulating strategies for an adjunctive treatment of periodontitis.

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Key words: cranberry; elastase; matrix metalloproteinase; periodontitis

Accepted for publication July 4, 2006

Periodontal disease comprises a group of inflammatory disorders that lead to the destruction of tooth-supporting tissue; and is initiated by a group of gram-negative anaerobic bacteria. Among these bacteria, *Actinobacillus actinomycetemcomitans* has been implicated as an etiological agent of localized agressive periodontitis (1,2). The host response to these bacteria and to their products, such as lipopolysaccharide, is a critical determinant for the initiation and progression of periodontitis. The lipopolysaccharide of *A. actinomycetemcomitans* has been proposed to contribute to alveolar bone loss and connective tissue degradation in periodontal disease (3,4). It is also one of the most potent inducers of inflammatory mediator production among lipopolysaccharide of major periodontopathogens (5). The continuous and high secretion of matrix metalloproteinases (MMPs), such as MMP-2, MMP-3, MMP-8 and MMP-9, by host cells after stimulation by periodontopathogens is known to contribute to periodontal tissue destruction (6,7). Indeed, MMP levels and their activities are significantly increased in both gingival tissue and gingival crevicular fluid of periodontitis subjects (8,9). MMPs are produced by the major cell types found in human periodontal tissue, including neutrophils, macrophages and fibroblasts (6,7). Inhibition of MMP production and activity in periodontitis patients may contribute to reduce periodontal tissue destruction. Interestingly, the use of a subantimicrobial dose of doxycycline, which down-regulates MMP activity, is indicated as an adjunctive treatment for periodontitis and confers clinical benefits to patients with periodontitis (10). In addition, elastase activity is increased in gingival crevicular fluid from periodontitis sites (11) and has been suggested as an indicator of periodontal disease progression (12). Recently, active compounds endowed with a capacity to modulate the host inflammatory response have received considerable attention because they may represent potential new therapeutic agents for the treatment of periodontal disease (13).

Polyphenolic compounds are able to inhibit both MMP expression and activity and have been proposed as new therapeutic molecules in various diseases (14–17). The cranberry is a polyphenolic rich berry fruit exhibiting several beneficial properties for human health, such as inhibition of human cancer cell line proliferation (18,19), reduction of dental biofilm formation (20,21) and prevention of urinary tract colonization by infectious agents (22). Previously, we reported the anti-inflammatory activity of a high-molecular-weight fraction prepared from cranberry juice concentrate (5). The aim of this study was to investigate the effect of cranberry on MMP activity and expression, as well as on elastase activity. More specifically, the capacity of a high-molecular-weight cranberry fraction to inhibit the production of MMP-3 and MMP-9 by macrophages and gingival fibroblasts, following stimulation with lipopolysaccharide from *A. actinomycetemcomitans*, was examined. In addition, changes induced by *A. actinomycetemcomitans* lipopolysaccharide and the cranberry fraction in the expression and the phosphorylation state of fibroblast intracellular signaling proteins were characterized by antibody microarrays. The effect of this cranberry fraction on the activities of the host extracellular matrix degradative enzymes MMP-3, MMP-9 and elastase was also investigated.

Material and methods

Cranberry fraction

Concentrated juice from the American cranberry, *Vaccinium macrocarpon*, was obtained from Ocean Spray Cranberries, Inc. (Lakeville-Middleboro, MA, USA). The juice was exhaustively dialysed, in 14,000-molecular weight cut-off dialysis bags, at 4°C against distilled water and then lyophilized. The resulting material was named nondialysable material and dissolved in distilled water. Chemical analyses of the nondialysable material were carried out by Robin Roderick (Ocean Spray Cranberries, Inc.) and revealed that this fraction is devoid of sugars and acids and contains 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.

Lipopolysaccharide preparation

A. actinomycetemcomitans ATCC 29522 (serotype b) was grown in Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 1% yeast extract. The bacterial culture was incubated at 37°C under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) for 2 d. Lipopolysaccharide was isolated by the method of Darveau & Hancock (23), which is based on protein digestion of a whole-cell extract by proteinase K and successive solubilization and precipitation steps. The lipopolysaccharide preparation was freeze dried and kept at −20°C. The presence of contaminant proteins in the lipopolysaccharide preparation was evaluated by using a protein assay kit (Bio-Rad Laboratories, Mississauga, ON, Canada) with bovine serum albumin as a control, and was less than 0.001%.

Monocyte and macrophage cultures

U937 cells (ATCC CRL-1593.2), a human monoblastic leukemia cell line, were cultured at 37°C in an atmosphere of 5% CO_2, in RPMI-1640 (HyClone Laboratories, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/ml of penicillin–streptomycin. Monocytes (2×10^5 cells/ml) were incubated for 48 h with RPMI containing 10% heat-inactivated fetal bovine serum and 10 ng/ml of phorbol myristic acid (Sigma, St Louis, MO, USA) to induce differentiation into adherent macrophage-like cells, as previously reported (24). Following treatment with phorbol myristic acid, the medium was replaced with fresh medium, and differentiated cells were incubated for an additional 24 h before use. Adherent macrophages were suspended in RPMI-1640, containing 10% heat-inactivated fetal bovine ser-

um, and then centrifuged at 200 *g* for 8 min. Cells were washed, suspended in RPMI-1640 (containing 1% heat-inactivated FBS) to a density of 1×10^6 cells/ml and then seeded in a six-well plate (2×10^6 cells/well in 2 ml) at 37°C in a 5% CO_2 atmosphere before treatment with the nondialysable material and stimulation with *A. actinomycetemcomitans* lipopolysaccharide.

Fibroblast cultures

Human gingival fibroblasts (HGF-1; ATCC CRL-2014) were cultured in an atmosphere of 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium containing 4 mM L-glutamine (HyClone Laboratories), 10% heat-inactivated fetal bovine serum and 100 μg/ml penicillin–streptomycin. Cells used in all experiments were between passages 5 and 10. For the experiments, fibroblasts were seeded at a concentration of 25×10^3 cells/cm^2 in a 24-well plate and cultured for 24 h at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum. The medium was replaced with Dulbecco's modified Eagle's medium containing 1% heat-inactivated fetal bovine serum before treatments were carried out.

Treatments of fibroblasts and macrophages

Fibroblasts and macrophages were treated with 10, 25 or 50 μg/ml of the cranberry nondialysable material, and incubated at 37°C in 5% CO_2 for 2 h before stimulation with a final concentration of 1 μg/ml of lipopolysaccharide. After 24 h of incubation, conditioned media were removed and stored at −20°C until use. Cells incubated in culture medium, with or without the cranberry fraction (10, 25 or 50 mg/ml), but not stimulated with lipopolysaccharide, were used as controls.

Cell viability

The viability of nondialysable material-treated fibroblasts and macrophages was evaluated by a test with 3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (i.e. MTT), according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany).

MMP-3 and MMP-9 production

Commercial enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems, Minneapolis, MN, USA) were used to quantify MMP-3 and MMP-9 in the conditioned media, according to the manufacturer's protocols. The absorbance at 450 nm was read using a microplate reader (Bio-Rad Laboratories) with a wavelength correction set at 550 nm. MMP-3 and MMP-9 concentrations were determined in triplicate using a standard curve prepared for each assay. These assays determine both the active and latent forms of the MMP. The sensitivities of the commercial ELISA kits were 0.156 ng/ml for MMP-3 and 0.313 ng/ml for MMP-9.

Antibody microarray analyses

Changes induced by the *A. actinomycetemcomitans* lipopolysaccharide and the nondialysable cranberry fraction in the expression and phosphorylation state of fibroblast signaling proteins were characterized by the Kinex™ Antibody Microarray service (Kinexus, Vancouver, Canada). This antibody microarray tracks over 600 different cell-signaling proteins in duplicate for more than 250 different phospho sites, 240 protein kinases and 110 other cell-signaling proteins, in two samples. In the initial screen, fibroblasts were treated with 1 μg/ml of *A. actinomycetemcomitans* lipopolysaccharide for 3 h and compared with unstimulated (control) cells. In the second screen, fibroblasts were treated with 50 μg/ml of the nondialysable material and incubated at 37°C in 5% CO_2 for 2 h before stimulation with 1 μg/ml of lipopolysaccharide for 3 h. Fibroblasts stimulated for 3 h with 1 μg/ml of lipopolysaccharide were used as a control. After the incubation period, cell lysates were prepared according to the manufacturer's protocol (Kinexus). In brief, cells were washed twice with ice-cold phosphate-buffered saline and homogenized at 4°C in a buffer containing 20 mM MOPS (pH 7.0), 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 60 mM β-glycerophosphate (pH 7.2), 1 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 3 mM benzamidine, 5 μM pepstatin A, 10 μM leupeptin, 0.5% Triton X-100 and 1 mM dithiothreitol. Cells were broken by sonication on ice, and cell lysates were centrifuged (LE-80K ultracentrifuge; Beckman, Mississauga, ON, Canada) at 36,000 *g* for 30 min at 4°C. Protein concentrations of the cell lysate supernatant fractions were estimated by using the Bradford assay (Bio-Rad Laboratories) and adjusted to a concentration of 2 mg/ml. Kinex™ antibody microarray analyses of the supernatants were performed by Kinexus.

Inhibition of MMP-3 and MMP-9 activities

Human recombinant MMP-3 and MMP-9 were used (Calbiochem, San Diego, CA, USA). MMPs were prepared at a concentration of 20 μg/ml in 5 mM Tris HCl, pH 7.5, containing 0.1 mM $CaCl_2$, 0.005% Brij 35 and 10% glycerol. MMPs were activated in 50 mM Tris-HCl, pH 7.5, containing 0.5 mM *p*-aminophenylmercuric acetate, at 37°C for 1 h for MMP-9 and overnight for MMP-3. Activated MMPs were diluted to a final concentration of 1 μg/ml for MMP-3 and 10 μg/ml for MMP-9 in the reaction buffer (50 mM Tris-HCl, 200 mM NaCl, 5 mM $CaCl_2$ and 0.2% Brij 35), in the presence or absence of increasing concentrations of the nondialysable material (10, 25, 50, 75, 100 or 150 μg/ml) or with the metalloproteinase inhibitor, ilomastat (25 μM; Calbiochem). MMPs were incubated at 25°C for 15 min before addition of the appropriate fluorogenic substrates. MMP-9 activity was quantified with the fluorogenic substrate, fluorescein isothiocyanate-labeled DQ™ gelatin (Molecular Probes, Eugene, OR, USA) and MMP-3 activity was quantified with the fluorogenic substrate 6-(7-nitro-benzo[1,2,5]oxadiazol-4-ylamino)-hexanoyl-Arg-Pro-Lys-Pro-Leu-Ala-Nva-Trp-Lys(7-dimethylaminocoumarin-4-yl)-NH_2 (Bachem

Bioscience Inc., King of Prussia, PA, USA). The assay mixtures were incubated for 2 h at 37°C in the dark with the substrate at a final concentration of 50 μg/ml. The fluorescence was measured by means of a fluorometer (VersaFluor model; Bio-Rad Laboratories) at excitation and emission wavelengths of 490 nm and 520 nm for MMP-9 and 360 nm and 460 nm for MMP-3. Fluorescent substrates alone, or with the nondialysable material, were used as controls.

Inhibition of elastase activity

The capacity of the nondialysable cranberry fraction to inhibit human neutrophil elastase (Calbiochem) was assessed. The assay mixtures contained 2 μl of elastase (50 μg/ml), 25 μl of elastase colorimetric substrate I (4 mM; Calbiochem) and 73 μl of reaction buffer (100 mM Tris-HCl, 500 mM NaCl, pH 7.5), with or without the nondialysable cranberry fraction (at final concentrations of 10, 25, 50, 75, 100 or 150 μg/ml). Assay mixtures were incubated in 96-well plates at 37°C for 1 h. Hydrolysis of the chromogenic substrate was determined by measuring the absorbance at 415 nm using a microplate reader (Bio-Rad Laboratories). Chromogenic substrate alone, or with nondialysable material, was used as a control.

Statistical analyses

Data are expressed as the means ± standard deviations of three independent experiments. Analyses of variance were performed to compare the means of the different conditions. Differences were considered significant at a p-value of < 0.05. Protected Fisher's least significant difference and the Student's t-test were used for pairwise comparisons.

Results

Effect of the nondialysable material on MMP-3 and MMP-9 production by macrophages and fibroblasts

To investigate the effect of cranberry compounds on the production of MMP-3 and MMP-9 by gingival fibroblasts and macrophages, cells were treated with the nondialysable fraction, prepared from cranberry juice concentrate, before stimulation with the lipopolysaccharide of *A. actinomycetemcomitans*. This lipopolysaccharide showed a strong capacity to induce MMP production in fibroblasts (Fig. 1) and macrophages (Fig. 2).

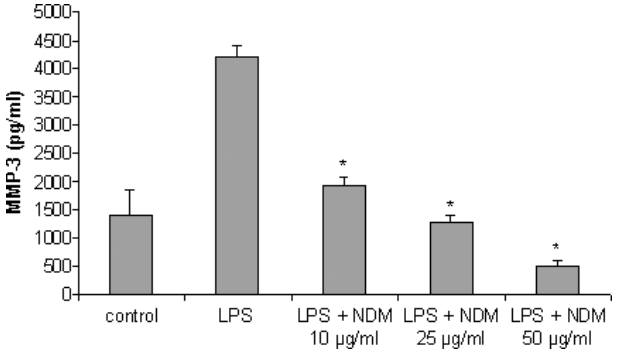

Fig. 1. Effect of the cranberry nondialysable material on matrix metalloproteinase-3 (MMP-3) production by human gingival fibroblasts stimulated with lipopolysaccharide (1 μg/ml) of *Actinobacillus actinomycetemcomitans* ATCC 29522. Fibroblasts were treated with the nondialysable material at 10, 25 or 50 μg/ml for 2 h before stimulation with lipopolysaccharide for 24 h. MMP-3 secretion was assessed by enzyme-linked immunosorbent assay (ELISA). The data represent the means ± standard deviations of triplicate assays for three independent experiments. *$p < 0.05$ compared with the untreated control. NDM, nondialysable material; LPS, lipopolysaccharide.

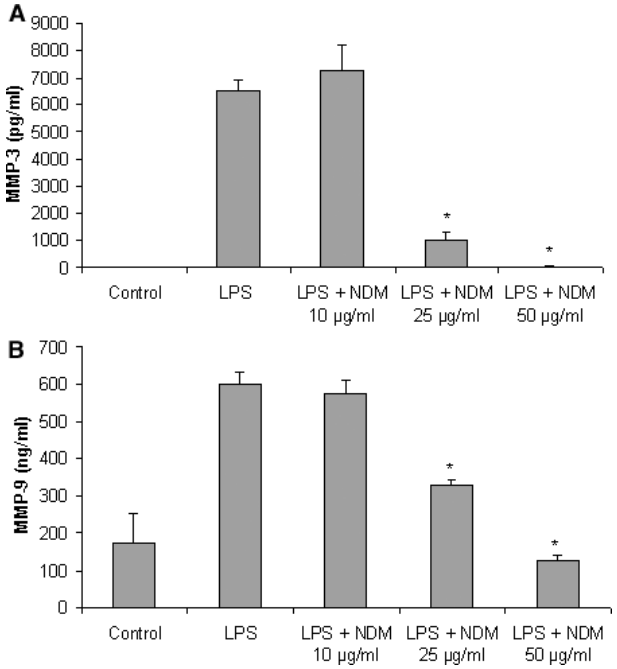

Fig. 2. Effect of the cranberry nondialysable material fraction on matrix metalloproteinase (MMP)-3 (A) and MMP-9 (B) production by human macrophages stimulated with lipopolysaccharide (1 μg/ml) of *Actinobacillus actinomycetemcomitans* ATCC 29522. Macrophages were treated with the nondialysable material at 10, 25 or 50 μg/ml for 2 h before stimulation with lipopolysaccharide for 24 h. MMP secretion was assessed by enzyme-linked immunosorbent assay (ELISA). The data represent the means ± standard deviations of triplicate assays for three independent experiments. *$p < 0.05$ compared with the untreated control. NDM, nondialysable material; LPS, lipopolysaccharide.

Although supernatants of lipopolysaccharide-stimulated fibroblasts were analyzed for both MMP-3 and MMP-9 production, only an MMP-3 response was observed. The MMP-3 response of fibroblasts stimulated with lipopolysaccharide of *A. actinomycetemcomitans* was significantly reduced ($p < 0.05$), in a dose-dependent manner, by the treatments with nondialysable material at all concentrations tested (Fig. 1). A 100% inhibition of the MMP-3 production induced by lipopolysaccharide was observed at a nondialysable material concentration of 25 μg/ml. At 50 μg/ml of nondialysable material, the amount of MMP-3 secreted by fibroblasts was below the basal level produced by unstimulated fibroblasts.

The MMP-3 and MMP-9 responses of macrophages stimulated with lipopolysaccharide of *A. actinomycetemcomitans* were significantly reduced by treatment with nondialysable material at 25 and 50 μg/ml (Fig. 2A,B). Nondialysable material at a final concentration of 50 μg/ml completely inhibited the lipopolysaccharide-induced MMP-3 and MMP-9 responses of macrophages. No obvious cytotoxic effects following the treatment of fibroblasts and macrophages with nondialysable material were detected by an MTT test, indicating that the decrease in MMP levels was not related to cell toxicity (data not shown).

Effect of cranberry nondialysable material on the expression and phosphorylation state of fibroblast intracellular signaling proteins

Changes induced by the *A. actinomycetemcomitans* lipopolysaccharide and the cranberry nondialysable material in the expression and phosphorylation of fibroblast signaling proteins were characterized by antibody microarrays. Among the various changes induced by these two compounds, we selected only those for which the expression or phosphorylation of proteins was enhanced by the *A. actinomycetemcomitans* lipopolysaccharide and decreased by the presence of the cranberry nondialysable material during treatment with lipopolysaccharide (Table 1). On the one hand, the expression of mitogen-activated protein kinase protein-serine kinase 6 and Fos-c FBJ murine osteosarcoma oncoprotein-related transcription factor was enhanced by, respectively, 59% and 48% following treatment with the *A. actinomycetemcomitans* lipopolysaccharide. This lipopolysaccharide also enhanced strongly the phosphorylation state of Jun proto-oncogene-encoded AP1 transcription factor (+96%) and mitogen-activated protein kinase protein-serine kinase 3/6 (+84%). On the other hand, the cranberry nondialysable material strongly inhibited the phosphorylation of Jun N-terminus protein-serine kinases, Jun and Ras-related C3 botulinum toxin substrate 1 by, respectively, 115, 85 and 54%. Nondialysable material also reduced the phosphorylation of mitogen-activated protein kinase protein-serine kinase 3/6 and Fos by ≈30%. In addition, the expression of Rho-associated protein kinase 2, mitogen-activated protein kinase protein-serine kinase 6 and Fos were inhibited following treatment with nondialysable material by, respectively, 73, 41 and 23%.

Effect of cranberry nondialysable material on human MMP-3, MMP-9 and elastase activities

The effect of the cranberry nondialysable material on MMP-3, MMP-9 and elastase activities was also investigated. MMP-3 and MMP-9 activities were significantly inhibited by nondialysable material at all concentrations tested (Fig. 3A,B). A nondialysable material

Table 1. Changes induced by the *Actinobacillus actinomycetemcomitans* lipopolysaccharide and the cranberry nondialysable material in the expression and phosphorylation of gingival fibroblast intracellular signaling proteins

Protein			Signal (percentage change from control)	
Full name	Abbreviation	Phospho sites(s)	Unstimulated vs. LPS	LPS vs. LPS + NDM
Fos-c FBJ murine osteosarcoma oncoprotein-related transcription factor	Fos	Pan-specific	48	−23
Fos-c FBJ murine osteosarcoma oncoprotein-related transcription factor	Fos	T232	5	−28
Jun N-terminus protein-serine kinases [stress-activated protein kinase (SAPK)] 1/2/3	JNK	T183 + Y185	21	−115
Jun proto-oncogene-encoded AP1 transcription factor	Jun	S63	96	−85
Mitogen-activated protein kinase protein-serine kinase 3/6	MKK3/6	S189/S207	84	−36
Mitogen-activated protein kinase protein-serine kinase 6	MKK6	Pan-specific	59	−41
Ras-related C3 botulinum toxin substrate 1	Rac1/cdc42	S71	14	−54
Rho-associated protein kinase 2	ROCK2	Pan-specific	40	−73

Two antibody microarrays were performed to screen these changes. First, unstimulated fibroblasts were used as a control and compared with fibroblasts stimulated for 3 h with *A. actinomycetemcomitans* lipopolysaccharide (1 μg/ml). Second, fibroblasts stimulated for 3 h with *A. actinomycetemcomitans* lipopolysaccharide (1 μg/ml) were used as a control and compared to fibroblasts treated for 2 h with the cranberry nondialysable material (50 μg/ml) before stimulation for 3 h with *A. actinomycetemcomitans* lipopolysaccharide (1 μg/ml).
NDM, nondialysable material; LPS, lipopolysaccharide.

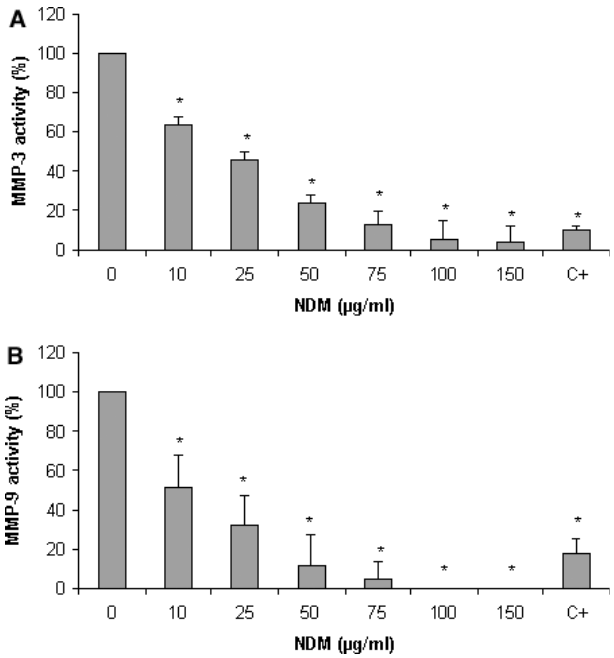

Fig. 3. Effect of the cranberry nondialysable material on matrix metalloproteinase (MMP)-3 (A) and MMP-9 (B) activities. MMPs were incubated for 15 min, with or without the nondialysable material (10, 25, 50, 75, 100 or 150 µg/ml), or with 25 µM ilomastat (C +), before addition of the fluorogenic substrates. MMP activity was measured with a fluorometer after 2 h of incubation at 37°C. The data represent the means ± standard deviations of three independent experiments. $*p < 0.05$ between various concentrations of the nondialysable material and the control (without the nondialysable material). NDM, nondialysable material.

concentration as low as 10 µg/ml significantly reduced the MMP-3 activity by ≈40% and the MMP-9 activity by ≈50%. A concentration of 100 µg/ml of nondialysable material totally inhibited the MMP-9 activity. The cranberry nondialysable material has been revealed to be an MMP inhibitor as potent as 25 µM ilomastat (a broad-spectrum MMP inhibitor), which was used as the positive control. Elastase activity was also significantly inhibited by the nondialysable material at all concentrations tested (Fig. 4). A 50% inhibition was observed with nondialysable material at a concentration of 10 µg/ml. The greatest inhibition (75%) was obtained with a nondialysable material concentration of 75 µg/ml.

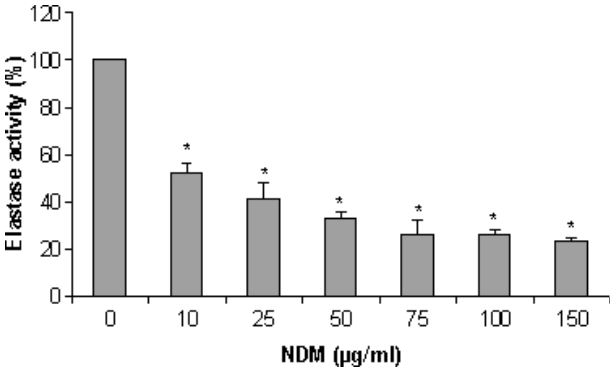

Fig. 4. Effect of the cranberry nondialysable material on elastase activity. Elastase was incubated for 15 min, with or without the nondialysable material (10, 25, 50, 75, 100 or 150 µg/ml), before addition of the chromogenic substrate. Elastase activity was measured using a spectrophotometer after 1 h of incubation at 37°C. The data represent the means ± standard deviations of triplicate assays for three independent experiments. $*p < 0.05$ between various concentrations of the nondialysable material and the control (without the nondialysable material). NDM, nondialysable material.

Discussion

The connective tissue of the periodontium is composed of fibrous proteins (collagen, elastin) and nonfibrous glycoproteins (laminin, fibronectin, proteoglycan), both of which are degraded during pathological conditions such as periodontitis. Accumulated evidence points to host-derived MMPs, produced by both infiltrating and resident cells of the periodontium, as key destructive enzymes in periodontal disease (25). Excessive MMP activity is a hallmark of human periodontal disease, leading to loss of gingival collagen, degradation of periodontal ligament and resorption of alveolar bone. Inhibition of MMP activities may thus be considered as an effective therapeutic approach for periodontitis patients. Gingival fibroblasts are the most abundant resident cells in periodontal tissue and can be considered as a major source of MMPs in periodontal diseased tissue (26). Macrophages, which constitute an important part of the inflammatory infiltrate in active periodontal lesions (27), are also potent MMP producers in gingival connective tissue from periodontitis patients (26). The significant role of MMP-3 and MMP-9 in the initial destruction of periodontal extracellular matrix macromolecules has been previously reported (7). Elastase, a protease found at high levels in periodontitis patients (11), also has the capacity to degrade matrix constituents. To demonstrate the potential of cranberry compounds to inhibit both MMP-3 and MMP-9 production, fibroblasts and macrophages were stimulated with lipopolysaccharide of *A. actinomycetemcomitans* following treatment with the cranberry nondialysable material. The effect of the nondialysable material on the activity of these MMPs, as well as on the elastase activity, was also tested.

We observed that lipopolysaccharide of *A. actinomycetemcomitans*

acts as potent inducer of MMP-3 and MMP-9 production by macrophages and fibroblasts, suggesting that it may participate in the gingival tissue destruction observed in periodontal disease. MMP-3 (stromelysin-1) and MMP-9 (gelatinase B) can degrade a wide range of extracellular matrix proteins, such as gelatin, elastin, proteoglycan, laminin, fibronectin, type II, III, IV, V, VII, IX and X collagen (7,28), and fibrin (29). In addition, these MMPs cleave α1-proteinase inhibitor, tumor necrosis factor-α precursor and interleukin-1β (28,30). Furthermore, it was reported that MMP-3 is an activator of latent pro-MMP-1, -8 and -9 (31–33), and an inactivator of plasminogen activator inhibitor I (34). Periodontal tissue destruction has also been associated with high levels of MMP-3 and MMP-9 in gingival tissue (35–38) and gingival crevicular fluid (39,40). The level of MMP-3 in gingival crevicular fluid has been proposed as a prognostic factor of attachment loss in established periodontitis sites (39) and is reduced after periodontal therapy (41). It was also suggested that the active form of MMP-9 could be a marker for the clinical severity of periodontal disease (37). All these data support the fact that MMP-3 and MMP-9 can contribute to destruction of connective tissues in periodontitis. In this study, the cranberry nondialysable fraction showed a strong capacity to inhibit lipopolysaccharide-induced MMP-3 and MMP-9 production by both macrophages and fibroblasts, suggesting that cranberry compounds may limit the overexpression of these MMPs in periodontitis patients. Nondialysable material was also shown to be a potent inhibitor of MMP-3 and MMP-9 activity and may thus contribute to limit MMP-3- and MMP-9-mediated destructive processes occurring in periodontal disease.

The expression of MMPs is regulated by a variety of factors, including cytokines, growth factors, chemical agents and physical stresses (42). It has been reported that inflammatory cytokines increase MMP secretion by human gingival fibroblasts (43,44). Prostaglandin E_2 also modulates MMP-3 expression by gingival fibroblasts. Ruwanpura *et al.* (45) reported that interleukin-1β-induced MMP-3 production was down-regulated by prostanglandin E_2 in human fibroblasts from healthy gingiva and up-regulated by prostanglandin E_2 in fibroblasts from periodontally diseased tissue. In addition, pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1β, selectively up-regulate the macrophage expression of MMP-9 (46). Previously, we reported that the cranberry nondialysable material inhibits inflammatory cytokine production by lipopolysaccharide-stimulated fibroblasts and macrophages (5) and prostaglandin E_2 production by lipopolysaccharide-stimulated fibroblasts (C. Bodet *et al.*, submitted). Therefore, the inhibition of MMP production by nondialysable material, observed in this study, can be related, in part, to anti-inflammatory properties of this cranberry fraction. It has been demonstrated that MMP inhibitors reduce alveolar bone loss in experimental periodontal disease (47). In addition, the efficiency of MMP inhibitors in chronic periodontitis patients is enhanced when combined with a non-steroidal anti-inflammatory drug (48). This suggests that therapeutic strategies based on the inhibition of diverse periodontal destruction pathways could be a successful approach for periodontitis treatment. The cranberry nondialysable material inhibits MMP production and activities, as well as pro-inflammatory mediator production, and thus by interfering with some of the destructive host mechanisms may have beneficial effects in slowing the disease progression.

The results of the antibody microarray analyses showed that the cranberry nondialysable material inhibits the phosphorylation and expression of some fibroblast intracellular signaling proteins induced by the *A. actinomycetemcomitans* lipopolysaccharide. These results suggest that the nondialysable material can act by reducing the activity of activator protein-1, leading to the inhibition of MMP production. Activator protein-1 complexes are heterodimers of proteins of two proto-oncogene families (Jun and Fos) that regulate MMP gene expression (49). Indeed, the activator protein-1-binding site is found in the promoter region of inducible MMP-3 and MMP-9 genes (49). Transcriptional activity and protein stability of Jun is increased by phosphorylation of serine 63 by Jun N-terminus protein-serine kinases (50). In the present study, the phosphorylation of Jun on serine 63 was strongly reduced by the nondialysable material, suggesting that this may result in an inhibition of MMP gene transcription. The reduction of Jun phosphorylation that was induced by the nondialysable material was probably a consequence of the decreased level of the phosphorylated form of Jun N-terminus protein-serine kinases. Treatment of fibroblasts with *A. actinomycetemcomitans* lipopolysaccharide resulted in an increased production of Fos expression that is important for activator protein-1 activity, because Jun–Fos heterodimers are more stable than Jun–Jun homodimers (51), thus leading to a more stable activator protein-1 complex. The reduction of Fos expression induced by the nondialysable material decreases the formation of Jun–Fos heterodimers and probably participates in reduction of the activator protein-1 activity. We also observed that mitogen-activated protein kinase protein-serine kinase 3/6 phosphorylation and mitogen-activated protein kinase protein-serine kinase 6 expression – two dual-specificity kinases implicated in the p38 pathway activation – were inhibited by the nondialysable material. The p38 pathway also contributes to the activator protein-1 activity (50), as well as to MMP gene expression (49), suggesting that inhibition of these two major activators of the p38 pathway by the nondialysable material can participate in the inhibition of MMP production. In addition, Ras-related C3 botulinum toxin substrate 1/Cdc42 activates p38 mitogen-activated protein kinase cascades and can promote gene transcriptional changes (52). This upstream signaling molecule, which also stimulates the Jun N-terminus protein-serine kinases and Jun transcriptional activities, plays a critical role in controlling the Jun

N-terminus protein-serine kinase signaling pathway (53,54). This suggests that inhibition of this Rho GTPase by nondialysable material may be implicated in the reduction of MMP production by affecting activator protein-1 activity through both Jun N-terminus protein-serine kinases and the p38 pathway. Finally, it was reported that inhibition of Rho-associated protein kinase leads to a reduction in MMP-9 mRNA levels, probably by acting at the transcriptional level, possibly as a result of reduced transcription factor binding (55). The nondialysable material induced an important decrease of Rho-associated protein kinase expression, a phenomenon that may contribute to the reduction of the MMP expression observed following treatment with the nondialysable material. The cranberry nondialysable material seems to affect the phosphorylation and expression of various intracellular proteins that are implicated in MMP production. Our results strongly suggest that this cranberry fraction may act notably via a down-regulation of activator protein-1 activity, leading to the inhibition of MMP production. However, additional studies are required to identify the exact mechanism of action of the cranberry nondialysable material.

Excessive production and/or activity of MMPs is widely recognized as a potential therapeutic target in a number of host disorders, such as periodontal disease, arthritis and cancer. Consequently, there is intense interest in the development of MMP inhibitors for therapeutic application in many human diseases. For instance, modulation of MMP production and/or activity by several naturally occurring substances, such as flavonoids, green tea polyphenols and curcumin, represents novel potential therapeutic options for cancer (14). In addition, MMP inhibition can be considered as a promising approach in periodontal disease treatment. There are various potential strategies to reduce the level of MMPs, including blocking the production of MMPs, blocking activation of the pro-enzyme, blocking activity of the enzyme and increasing inhibitor production. This study suggests that cranberry compounds, by inhibiting both the production and the activity of MMPs, offer promising perspectives to develop novel and innovative therapies for the treatment of pathologic conditions characterized by excessive MMP activity.

Neutrophil elastase is a serine protease that degrades a wide variety of extracellular matrix proteins, including elastin, collagen, proteoglycan, fibronectin and laminin (56,57). Elastase has been observed at elevated levels in the gingival crevicular fluid of periodontitis patients (11,58) and is reduced after periodontal therapy (59). It was also reported that elastase remaining in periodontal tissue may impair the regenerative response initiated by periodontal ligament cells (60) and the gingival fibroblast-mediated host defense (61). In this study, we showed that the nondialysable material inhibited elastase activity, suggesting that cranberry components may reduce the adverse effect of high elastase activity on peridontal tissue.

One hypothesis, regarding periodontal disease pathogenesis, is that host cells stimulated directly or indirectly by components of the dental biofilm secrete proteinases, such as MMPs and elastase, which are associated with altered connective tissue remodeling and alveolar bone resorption. Consequently, it is logical to consider therapeutic approaches, based on host-proteinase inhibition, for managing adult periodontitis. Host modulators may be highly useful for individuals with a substantially increased risk for periodontitis (62). In this study, cranberry nondialysable material exhibited a high capacity to inhibit MMP-3, MMP-9 and elastase activities, as well as MMP-3 and MMP-9 production by macrophages and gingival fibroblasts. This suggests that cranberry compounds may contribute to limit extracellular matrix degradation and others pathologic processes implicating these enzymes. Therefore, the local application of cranberry nondialysable material or nondialysable material-derived molecules in diseased sites may offer perspectives for the development of novel adjunctive treatment for periodontal disease.

Acknowledgements

This work was supported by the Cranberry Institute (East Wareham, MA, USA). We thank Robin Roderick and Marge Leahy (Ocean Spray Cranberries, Inc.) for chemical analyses and for providing cranberry juice concentrate.

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