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Bromelain treatment decreases neutrophil migration to sites of inflammation

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Abstract Bromelain, a mixture of proteases derived from pineapple stem, has been reported to have therapeutic benefits in a variety of inflammatory diseases, including murine inflammatory bowel disease. The purpose of this work was to understand potential mechanisms for this anti-inflammatory activity. Exposure to bromelain *in vitro* has been shown to remove a number of cell surface molecules that are vital to leukocyte trafficking, including CD128a/CXCR1 and CD128b/CXCR2 that serve as receptors for the neutrophil chemoattractant IL-8 and its murine homologues. We hypothesized that specific proteolytic removal of CD128 molecules by bromelain would inhibit neutrophil migration to IL-8 and thus decrease acute responses to inflammatory stimuli. Using an *in vitro* chemotaxis assay, we demonstrated a 40% reduction in migration of bromelain- vs. sham-treated human neutrophils in response to rhIL-8. Migration to the bacterial peptide analog fMLP was unaffected, indicating that bromelain does not induce a global defect in leukocyte migration. *In vivo* bromelain treatment generated a 50–85% reduction in neutrophil migration in 3 different murine models of leukocyte migration into the inflamed peritoneal cavity. Intravital microscopy demonstrated that although *in vivo* bromelain treatment transiently decreased leukocyte rolling, its primary long-term effect was abrogation of firm adhesion of leukocytes to blood vessels at the site of inflammation. These changes in adhesion were correlated with rapid re-expression of the bromelain-sensitive CD62L/L-selectin molecules that mediate rolling following *in vivo* bromelain treatment and minimal re-expression of CD128 over the time period studied. Taken together, these studies demonstrate that bromelain can effectively decrease neutrophil migration to sites of acute inflammation and support the specific removal of the CD128 chemokine receptor as a potential mechanism of action.

Introduction

Bromelain is a natural mixture of proteolytic enzymes that is derived from pineapple stems and has been proposed be useful for therapy of immune-mediated diseases [1,2]. Bromelain has been used either alone or in a multi-enzyme preparation, most commonly combined with trypsin and rutin, in multiple clinical trials in both humans and animals. The level of proof, method of bromelain administration and dose, and quality of the studies vary, but beneficial effects were suggested or proven in a variety of inflammatory diseases and models of inflammation. These include the experimental allergic

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encephalomyelitis (EAE) model of the human autoimmune disease multiple sclerosis [3], carrageenan-induced pleurisy in the rat [4–6], immunologically mediated arteriosclerosis in rat aortic allografts [7], rheumatologic diseases in mice and humans [8–13], and allergic asthma [14] and rhinitis [15]. Some studies demonstrated that bromelain had efficacy similar to standard anti-inflammatory drugs such as dexamethasone [5,6] or non-steroidal anti-inflammatory agents (NSAIDs) [8,10,11,16]. Our previous studies showed that oral administration of 5 mg bromelain/day markedly decreased the development and severity of inflammatory bowel disease in IL-10$^{-/-}$ mice [17]. Bromelain was also anecdotally reported to induce remission in 2 patients with refractory ulcerative colitis [18].

Despite the promising results of bromelain treatment in animal models and human clinical trials, the mechanisms that are primarily responsible for its anti-inflammatory effects are still unclear. However, proteolytic activity is required for the anti-inflammatory effect of bromelain on T cell activation and cytokine secretion *in vitro* [19–21] and in murine models of inflammatory bowel disease *in vivo* [17]. We previously showed that *in vitro* bromelain treatment proteolytically removed at least 14 cell surface molecules that have been associated with leukocyte adhesion and/or activation [19,22]. Among the bromelain-sensitive molecules identified were CD62L (L-selectin), CD128a (CXCR1) and CD128b (CXCR2). The latter two molecules make up the receptor for IL-8, a chemokine that regulates neutrophil activation and chemotaxis to sites of acute inflammation [23]. Leukocyte migration to sites of inflammation is a complex process (reviewed in [24]) that first requires decreasing the velocity of leukocyte flow in the bloodstream through selectin-mediated rolling followed by chemokine-activated changes in integrin affinity that allow firm adhesion to the blood vessel wall. The binding of IL-8 to its receptor on neutrophils is thought to regulate the integrin affinity changes that result in firm adhesion, thus allowing the neutrophils to firmly adhere and then to transmigrate through the endothelium toward the source of chemoattractant. For example, IL-8 is increased in rectal dialysate from patients with ulcerative colitis, leading to the attraction and activation of neutrophils that drive the colonic inflammation that is characteristic of that disease [25]. Use of anti-IL-8 antibodies to decrease adhesion and migration of neutrophils has been shown to decrease the severity of the resulting inflammation in several different *in vivo* models (reviewed in [26]). The purpose of these studies was to determine the effects of bromelain on the migration of leukocytes, particularly neutrophils, and to further elucidate the mechanisms underlying these effects.

Materials and methods

Reagents and approvals

Bromelain was purchased from Sigma-Aldrich (catalog #B-4882; St. Louis, MO) or obtained as a gift from Hong Mao Biochemicals Company, Ltd. (Nikompattana, Thailand). Antibodies were obtained from R & D Systems (Minneapolis, MN), BD PharMingen (San Diego, CA), or Caltag Laboratories (Burlingame, CA). Unless specified, all other reagents were obtained from Sigma-Aldrich (St. Louis, MO), VWR (Atlanta, GA), or Invitrogen (Carlsbad, CA). All aspects of this study that involved participation of humans in research, including the consent form, were approved by the Institutional Review Board of Duke University Medical Center. All animal studies were approved by the Institutional Animal Care and Use Committee of Duke University.

In vitro leukocyte migration assays

Healthy adults were recruited as blood donors after they gave informed consent for participation. Neutrophils (>90% purity) were obtained from their peripheral blood by density gradient centrifugation using Histopaque-1077 and -1119 (Sigma-Aldrich). The cells obtained were labeled with 1 μg/ml Calcein AM in PBS for 30 min at 37 °C, then treated with RPMI1640 media or 100 μg/ml bromelain at 37 °C for 30 min. Cells were washed, then 2×10^4 cells were added to the upper well of each chemotaxis chamber (ChemoTx microplates, Neuro Probe Inc, Gaithersburg, MD). The chemoattractants rhIL-8/CXCL8 (20 ng/ml) (Biosource, Camarillo, CA) or formyl-methionyl-leucyl-proline (fMLP, 10 nM) were placed in the lower chamber. Migration was allowed to occur for 3 h; cells present in the lower chamber were documented by flow cytometry. Results were expressed as a Migration Index, defined as the number of cells migrated in response to the stimulus divided by the number of cells migrated in response to media alone for each treatment condition. In some experiments, leukocyte migration was also confirmed by examining filters under fluorescence microscopy.

In vivo leukocyte migration assays

Saline or 200 μg bromelain (10 mg/kg) in saline was injected intravenously (i.v.) into the tail vein of 5–8 wk old female Balb/C mice (Jackson Laboratories, Bar Harbor, ME). Thirty minutes later, 1 ml of 3% (w/v) thioglycollate, 300 ng/ml rhIL-8, or 1 μM fMLP was injected intraperitoneally (i.p.) to provide an inflammatory stimulus. Mice were euthanized 6 (thioglycollate) or 4 (IL-8 or fMLP) h later, and peritoneal lavage was performed with 10 ml ice-cold media containing RPMI1640, 5% fetal bovine serum, 5 mM EDTA, 10 mM HEPES, and 10 U/ml heparin. A total cell count was obtained using a hemocytometer and cells were further characterized by light microscopy and flow cytometry. Cytocentrifuge preparations were stained with a modified Wright–Giemsa stain (Hema 3 stain set, Fisher Scientific, Kalamazoo, MI) and a differential was performed. Flow cytometric analysis of peritoneal lavage cells included antibodies against the following antigens: Gr-1/Ly-6C/G (neutrophils; clone RB6-8C5), CD3ε (T cells; clone 145-2C11), F4/80 (macrophages; clone BM8), and CD117/c-kit (mast cells; clone 2B8). Cells with the phenotype of Gr-1$^+$ F4/80$^-$ were identified as neutrophils. The cytokines and chemokines present in peritoneal lavage fluid were quantitated using a Luminex bead-based fluorescent multiplex immunoassay (BioRad, Hercules, CA).

Intravital microscopy

Peritoneal inflammation was elicited by injection of 1 ml of 3% thioglycollate i.p. 30 min after i.v. injection of 200 μg bromelain (10 mg/kg) in 0.1 ml saline or saline alone, as for the *in vivo* leukocyte migration assays. Six hours later, mice were anesthetized using Nembutal (80 mg/kg i.p.) and the tail vein was cannulated using a 30 1/2 gauge needle with PE10 tubing.