

Alterations in the function of circulating mononuclear cells derived from patients with Crohn's disease treated with mastic

Andriana C Kaliora, Maria G Stathopoulou, John K Triantafyllidis, George VZ Dedoussis, Nikolaos K Andrikopoulos

Andriana C Kaliora, Maria G Stathopoulou, George VZ Dedoussis, Nikolaos K Andrikopoulos, Department of Science of Dietetics-Nutrition, Harokopio University, Athens, Greece
John K Triantafyllidis, Department of Gastroenterology, Saint Panteleimon General State Hospital, Nicea, Athens, Greece
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Correspondence to: Dr. Andriana C Kaliora, Department of Science of Dietetics-Nutrition, Harokopio University of Athens, 70 El. Venizelou ave., Kallithea 17671, Athens, Greece. akaliora@hua.gr

Telephone: +30-210-9549303

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Abstract

AIM: To assess the effects of mastic administration on cytokine production of circulating mononuclear cells of patients with active Crohn's disease (CD).

METHODS: The study was conducted in patients with established mildly to moderately active CD, attending the outpatient clinics of the hospital, and in healthy controls. Recruited to a 4 wk treatment with mastic caps (6 caps/d, 0.37 g/cap) were 10 patients and 8 controls, all of who successfully completed the protocol. Interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- α), monocyte chemotactic protein-1 (MCP-1), macrophage migration inhibitory factor (MIF) and intracellular antioxidant glutathione (GSH) were evaluated in peripheral blood mononuclear cells (PBMC) before and after treatment.

RESULTS: Treating CD patients with mastic resulted in the reduction of TNF- α secretion (2.1 ± 0.9 ng/mL vs 0.5 ± 0.4 ng/mL, $P = 0.028$). MIF release was significantly increased (1.2 ± 0.4 ng/mL vs 2.5 ± 0.7 ng/mL, $P = 0.026$) meaning that random migration and chemotaxis of monocytes/macrophages was inhibited. No significant changes were observed in IL-6, MCP-1 and GSH concentrations.

CONCLUSION: This study shows that mastic acts as an immunomodulator on PBMC, acting as a TNF- α inhibitor and a MIF stimulator. Although further double-blind, placebo-controlled studies in a large number of patients is required to clarify the role of this natural product, this finding provides strong evidence that mastic might be an important regulator of immunity in CD.

INTRODUCTION

Crohn's disease (CD) is a chronic relapsing inflammatory condition of unknown cause^[1]. Although the exact pathogenesis of CD is poorly understood, infection, environmental factors, heredity and immunological defects have been proposed as causes^[2]. In one or another scenario, a variety of cytokines, such as tumor necrosis factor-alpha (TNF- α), are secreted at the site of inflammation by intestinal lamina propria and attract and activate effector cells^[3-5]. Apart from the intestinal mucosa, TNF- α concentration is also raised in serum in patients with active Crohn's disease, compared to normal controls^[6,7] and inactive disease^[8]. The mechanism through which localized inflammation in the gastrointestinal tract in active CD is associated with systemic manifestations remains unconfirmed, but can involve activated mononuclear cells that migrate *via* the peripheral blood (PBMC) to other tissues. PBMC are highly activated in active CD and secrete higher quantities of proinflammatory mediators^[9-11]. Altered production of cytokines by PBMC is noted in patients with CD when compared to healthy subjects^[12].

As the name implies, monocyte chemotactic protein 1 (MCP-1) acts as a potent chemoattractant and activator of monocytes/macrophages^[13], as well as of NK cells, T cells, eosinophils, and basophils^[14-17]. During acute inflammatory processes the expression of MCP-1 is increased. Once activated, cells produce an assortment of immunoregulatory cytokines that influence the course of the ensuing immune response. Interleukin 6 (IL-6) expressed among others by T cells and monocytes/

macrophages, stimulates T- and B-cell proliferation and differentiation^[18]. The production of IL-6 in these various cells may be regulated by TNF. TNF- α is an important mediator of inflammation, found in substantial amount in the mucosa and stools of subjects with CD. It is a proinflammatory cytokine produced by monocytes, macrophages, and T cells that can affect proliferation, differentiation, and function of every cell type. Therefore, TNF inhibitors, which may be useful in the treatment of CD, have recently been developed^[19]. Among these, infliximab has been developed as a therapeutic agent for TNF- α -mediated diseases^[20]. Macrophage migration inhibitory factor (MIF) is a cytokine with dissimilar functions; inhibition of macrophage migration^[21] or proinflammatory activity^[22-25].

Nowadays, various immunosuppressive therapies such as azathioprine, mercaptopurine, and methotrexate, are available. Nevertheless, the treatment of patients with CD still remains a clinical challenge. Furthermore, in view of the increased number of CD patients, there is a considerable scientific and commercial interest in the discovery of novel classes of therapeutic compounds. In particular, plants represent a good source of novel molecules. *Pistacia lentiscus* var. *Chia* (*Anacardiaceae*) is an evergreen shrub widely distributed in the Mediterranean region. Mastic, the resinous exudate, has been reported to possess antioxidant^[26] and antibacterial^[27] activity, to be effective against peptic ulcers^[28], to be hepatoprotective in tetrachloride-intoxicated rats^[29] and to suppress the extent of iron-induced lipid peroxidation in rat liver homogenates^[30]. We have previously shown that mastic administration resulted in the improvement of the clinical course and in the regulation of inflammatory biomarkers in plasma obtained from mildly to moderate active CD patients^[7]. The aim of the present work was to investigate the immunomodulatory effect of mastic treatment on cytokine secretion. Additionally, because inflammation results in oxidative stress and endogenous antioxidants, such as glutathione (GSH), do not counteract it with subsequent mucosal damage, intracellular GSH production from PBMC obtained from patients with mildly to moderately active CD was also measured.

MATERIALS AND METHODS

Setting and participants

Ten consecutive patients with active Crohn's disease and eight healthy controls were included^[7]. In brief, clinical evidence of mild to moderate Crohn's disease exacerbation was defined by a score of CD Activity Index (CAI) $150 < \text{CAI} < 400$. Mean CAI at baseline was 222.9 ± 18.7 (SE), while mean C-reactive protein (CRP) concentration was 40.3 ± 13.1 (SE) mg/mL. Exclusion criteria were elemental diet, parenteral nutrition or antioxidant/mineral supplementation and treatment with immunomodulators (biologic agents-infliximab) and/or corticosteroids. Controls were healthy volunteers, with normal concentrations of CRP [2.4 ± 0.7 (SE) mg/L] and albumin [42.1 ± 1.2 (SE) g/L], without chronic inflammatory disorder, BMI value < 30 (25.8 ± 3.3),

Table 1 Demographic characteristics and medications of patients with Crohn's Disease and controls

Characteristic	Patients	Controls
Age (yr)		
Mean	36.9	31.5
Range	18-73	25-45
Sex		
Female	5	4
Male	5	4
Duration of disease (yr)	6.4 (± 3.9)	-
Concomitant medication		-
None	3	
Mesalazine	3	-
Metronidazole	2	
Azathioprine	2	-
Location of Crohn's disease		
Small bowel	4	-
Small and large bowel	6	-
Fistulizing disease	3	-

none anti-inflammatory drug treatment or antioxidant vitamin/mineral supplementation. Informed consent was obtained from each subject included in the study. The Ethical Committees of both Harokopio University and Saint Panteleimon General State Hospital approved the protocol. Table 1 shows some demographic characteristics of patients and controls.

Intervention

The trial protocol was carried out as previously described^[7]. In short, participants were subjected to a 4-wk supplementation with mastic caps (0.37 g/cap, 2×3 caps/d, 2.2 g in total). Dietary instructions were given as to maintain consumption of foods rich in anti-inflammatory and antioxidant ingredients low as initially assessed by a food frequency questionnaire and 24 h recall interview and to refrain from mastic and mastic products. Blood samples were obtained prior and after the trial.

Cell cultures

PBMC were obtained from CD patients and controls as previously described^[31]. Viability of peripheral blood mononuclear cells (PBMC) was determined by Trypan blue exclusion test. PBMC were resuspended in complete medium consisting of RPMI 1640 medium supplemented with 10% fetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin. PBMC were added to each well of a 24-well plate at a density of 2×10^6 cells/mL and cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 18 h. At the end of incubation, conditioned media were collected and stored at -20°C until assayed, while cells were harvested for GSH measurement. All cultures were run in duplicate.

Cytokine assays

Plasma cytokines from patients with CD and controls were assessed by quantitative, sandwich, enzyme-linked, immunosorbent assays (ELISA) (R&D Systems Abingdon, UK) according to the manufacturer's instructions. Sensitivity of TNF- α ELISA was less than 1.6 pg/mL, of

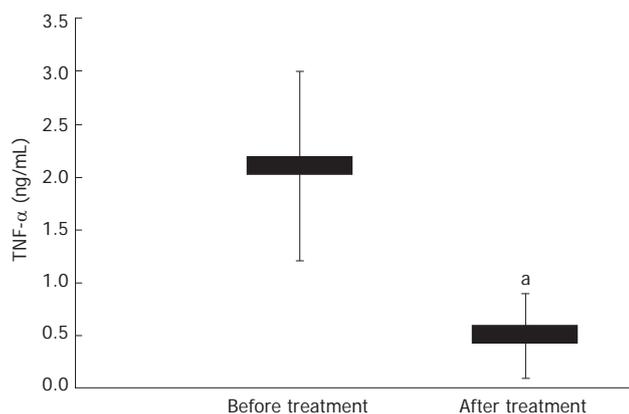


Figure 1 Secretion of tumor necrosis factor-alpha (TNF- α) was decreased in PBMC derived from patients with active Crohn's disease ($n = 10$) after 4-wk treatment with mastic caps ($^{\#}P < 0.05$). Horizontal bars represent the mean \pm SE.

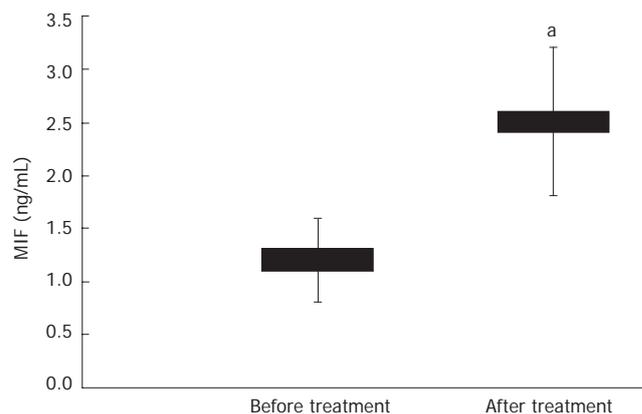


Figure 2 Secretion of macrophage migration inhibitory factor (MIF) was increased in PBMC derived from patients with active Crohn's disease ($n = 10$) after 4-wk treatment with mastic caps ($^{\#}P < 0.05$). Horizontal bars represent the mean \pm SE.

IL-6 was less than 0.70 pg/mL, of MIF less than 0.017 ng/mL and of MCP-1 less than 5.0 pg/mL.

Assay for GSH

At the end of incubation, cells were MPA-treated (5%) and then centrifuged at $2000 \times g$ for 10 min. The resulting supernatant was separated and the GSH assay was performed using the Colorimetric Assay for Glutathione as indicated by the manufacturer (OxisResearch Inc., Portland, USA). GSH concentration was evaluated using a standard curve of Absorbance Units *vs* GSH concentrations and expressed as $\mu\text{mol/L}$.

Statistical analysis

Results are expressed as mean \pm SE. The Mann-Whitney Test was used for comparing differences between patients and controls prior treatment. Differences reported primarily and at the end of the study within individual groups, were tested for significance by the Wilcoxon signed ranks test. A P value below 0.05 was regarded as limit of significance.

RESULTS

Secretion of TNF- α

Our data show that PBMC isolated from patients with active CD exhibited no significant difference in the production of TNF- α compared to controls prior to treatment (2.1 ± 0.9 ng/mL *vs* 1.2 ± 0.3 ng/mL, $P = 0.200$). TNF- α in controls administered mastic caps was not significantly changed (1.2 ± 0.3 ng/mL *vs* 0.5 ± 0.2 ng/mL, $P = 0.173$), while in patients it was significantly reduced (2.1 ± 0.9 ng/mL *vs* 0.5 ± 0.4 ng/mL, $P = 0.028$, Figure 1).

MIF production

MIF production was significantly increased in control group before mastic treatment compared to patients (4.7 ± 1.0 ng/mL *vs* 1.2 ± 0.4 ng/mL, $P = 0.038$). MIF production in controls administered mastic caps was not altered (4.7 ± 1.0 ng/mL *vs* 4.7 ± 0.7 ng/mL, $P = 0.593$), while, as shown in Figure 2, it was significantly increased in patients after mastic treatment (1.2 ± 0.4 ng/mL *vs* 2.5 ± 0.7

ng/mL, $P = 0.026$).

IL-6 and MCP-1

PBMC isolated from patients with active CD exhibited significantly elevated secretion of IL-6 compared to controls prior to therapy (622.5 ± 130.1 pg/mL *vs* 56.7 ± 20.2 pg/mL, $P = 0.014$). No significant difference was observed in controls before and after treatment (56.7 ± 20.2 pg/mL *vs* 19.9 ± 7.5 pg/mL, $P = 0.285$). In patients, a trend towards statistical significance was observed in IL-6, before and after treatment although, differences did not reach statistical significance (622.5 ± 130.1 pg/mL to 519.9 ± 176.5 pg/mL, $P = 0.068$).

In the case of MCP-1, no significant difference was observed between patients and controls prior to treatment (3.0 ± 1.1 ng/mL *vs* 0.7 ± 0.3 ng/mL, $P = 0.302$). MCP-1 secretion from controls' PBMC (0.7 ± 0.3 ng/mL *vs* 0.6 ± 0.3 ng/mL, $P = 0.593$) or from patients' PBMC (3.0 ± 1.1 ng/mL *vs* 1.7 ± 1.0 ng/mL, $P = 0.463$) before and after the trial was not significantly changed.

Intracellular GSH

No significant difference in intracellular GSH was detected between controls and CD patients before mastic treatment (66.4 ± 20.1 $\mu\text{mol/L}$ *vs* 34.0 ± 15.8 $\mu\text{mol/L}$, $P = 0.144$). No significant difference was observed in GSH before and after treatment in controls (66.4 ± 20.1 $\mu\text{mol/L}$ *vs* 55.4 ± 9.7 $\mu\text{mol/L}$, $P = 0.285$), whereas, even though not statistically significant, a trend towards statistical significance was observed in patients (34.0 ± 15.8 *vs* 56.6 ± 10.3 $\mu\text{mol/L}$, $P = 0.075$).

DISCUSSION

Four-week mastic administration has been previously shown to effectively regulate the clinical course, inflammation, and oxidative stress in CD patients. It statistically decreased CD activity index and plasma concentrations of C-reactive protein and IL-6, while it increased plasma total antioxidant potential. Also, nutrition risk index (NRI), one of the most useful measures of nutritional status that incorporates albumin level and body

weight, was improved^[7]. In particular, the main element of NRI showing improvement was body weight gain, and since the daily energy intake was unchanged during the trial, increase in body weight and in NRI was attributed to decrease of liquid stools and consequent improvement in nutrient absorption. In two out of ten NRI was > 100 denoting adequate nutrient absorption and absence of nutritional risk. As a continuation of our research to evaluate the effect of mastic on CD and before conducting placebo-controlled studies in large cohorts, in the current study we demonstrated that mastic administration affects cytokine secretion from PBMC obtained from CD patients. Mastic acts as an immunomodulator (a) inhibiting the secretion of TNF- α and (b) inducing the secretion of MIF.

In the report of Grip and coworkers^[32] TNF- α was significantly elevated in plasma obtained from CD patients, but not in PMBC, compared to healthy controls. Accordingly, the difference in TNF- α concentrations between patients and controls at baseline was significant in plasma^[7], but insignificant in PBMC (present study). Interestingly though, secretion of TNF- α showed a significant decrease in CD patients subjected to mastic treatment (Figure 1). Even though the data reported about TNF- α is conflicting^[33,34], the anti-TNF- α treatment in TNF-mediated diseases is developing. The mechanism of mastic's anti-TNF activity in CD may be related to specific blockade of TNF- α secretion. Because cells were always viable in all the experimental conditions, the mechanism including complement mediated lysis of cells expressing membrane bound TNF- α ^[35], is fairly excluded. A possible approach would be *via* the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. By blocking HMG-CoA reductase on human monocytes, cells reduce the production of TNF- α ^[32]. TNF- α is suggested to regulate MCP-1 secretion *via* the activation of nuclear factor-kappa B^[36]. Yet, this is rather unlikely to be seen hereby, given that MCP-1 concentration was unaffected in CD patients administered with mastic. It is rather that the nuclear factor-kappa B pathway secondary to the decrease in TNF- α was not activated.

To shed more light on the mechanisms by which mastic might work, we also investigated the potency of mastic caps in affecting MIF secretion. Originally, MIF was described as an inhibitor of migration and chemotaxis of monocytes/macrophages^[21]. Our findings of suppressed secretion of MIF in CD mononuclear cells compared to healthy prior treatment indicate that monocytes are sensitized to chemotaxis. Increased secretion after treatment (Figure 2) points to the inhibition of monocyte chemotaxis. The significance of this finding is that migration of chemokine or peptide or nonpeptide stimulated monocytes and differentiation to macrophages into the site of inflammation is limited and further trigger of inflammation is controlled. In some studies it is proposed to have proinflammatory properties and be the first cytokine appearing, followed by others^[37]. However, since in our present and previous study^[7] mastic administration was not followed by enhanced proinflammatory production, induction of MIF secretion should only be allied with inhibition of chemotaxis.

Even though insignificant, a marginal increment in intracellular GSH concentration ($P = 0.075$) was observed. GSH is the most abundant non-enzymatic antioxidant present in cells that plays an important role in the defence against oxidative-stress-induced cell injury^[38]. During inflammatory processes, cells of the immune system are exposed to large amounts of reactive oxygen intermediates, and, thus, an efficient GSH system to neutralize free radicals that otherwise disturb immune functions is essential^[39]. Mastic has been proven to induce GSH production in PBMC under oxidative conditions *in vitro*^[26], while in CD patients to increase plasma total antioxidant potential *in vivo*^[7].

IL-6 is thought to play a crucial role in the pathogenesis of CD^[40]. We hereby demonstrated that IL-6 secretion in PBMC from CD patients was significantly elevated compared to healthy controls ($P = 0.014$), evident of the cytokine role in CD inflammation. While in plasma IL-6 was statistically decreased with mastic administration^[7], in PBMC insignificant nevertheless decrease ($P = 0.068$) was reported, perhaps due to the small number of samples.

Cytokines play a central role in the modulation of the immune system and they have either proinflammatory, such as TNF- α , or antiinflammatory functions. In CD patients the imbalance between proinflammatory and antiinflammatory cytokines brings about the rationale for "anticytokine" treatment. It is however uncertain whether only one cytokine should be targeted or several pro- and antiinflammatory cytokines, or cytokine synthesis inhibitors, soluble receptors, receptor antagonists or receptor antibodies. In the case of mastic, the activity in CD shown previously^[7] and hereby may well be extremely interesting. However, further studies -now in progress- are needed as to determine the target and whether there is one or a class or more than one class of compounds acting synergistically to obtain this effect. As a final point, mastic might serve well in the regulation of immunity in CD patients.

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COMMENTS

Background

Mastic administration improves the clinical course and regulates plasma inflammatory and antioxidative mediators of patients with mildly to moderately active Crohn's disease (CD). We aimed to assess the effects of mastic administration on cytokine production of circulating mononuclear cells of patients with active CD.

Research frontiers

The exact pathogenesis of Crohn's disease (CD) is poorly understood; infection, environmental factors, heredity and immunological defects have been proposed as causes. Peripheral blood mononuclear cells (PBMC) are highly activated in active CD and secrete higher quantities of proinflammatory mediators. In view of the increased number of CD patients and of the severe side effects of the immunosuppressive therapies available there is a considerable scientific and commercial interest in the discovery of novel classes of therapeutic compounds.

Innovations and breakthroughs

This is the very first study regarding the effect of mastic administration on cytokine production of circulating mononuclear cells of patients with active Crohn's disease.

Applications

Although further double-blind, placebo-controlled studies in a large number of patients is required to clarify the role of this natural product, this finding provides strong evidence that mastic might be an important regulator of immunity in Crohn's disease.

Peer review

This study does provide some new information about a novel possible treatment for Crohn's disease.

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