The Actions of Vegetable-derived Celadrin® on Monocyte-mediated Cytokine Response

Hatice Hasturk, DDS. Ph. D.

Boston University Goldman School of Dental Medicine

Department of Periodontology and Oral Biology

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Background:

Periodontitis is a local inflammation that occurs as a result of host response against specific microorganisms and eventually leads to the tissue destruction and systemic complications (Page & Schroeder, 1981). Once periodontal inflammation is initiated, the cascade of inflammatory events includes an amplified loop until the infection is contained and injury is confined. The early actions of the host response are later replaced by more specific mechanisms and eventually become redundant. Thus, it is important, as in are all biologic processes, to limit the response and to allow the inflammation to resolve in periodontal disease. While it has been shown that many molecules participate in the initiation and development of the host defense mechanisms, recent paradigm in periodontal disease pathogenesis emphasizes the importance of counterregulatory molecules in the control of inflammatory response to control its magnitude and duration (Van Dyke & Serhan, 2003).

Several inflammatory mediators including cytokines (i.e. IL-1 beta and IL-6) and lipid mediators are associated with periodontal disease. Among the lipid mediators are the arachidonic acid-derived products, including leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2) (Offenbacher et al, 1992). Indeed, some of the pathophysiological events that occur in periodontal diseases can be explained by the activities of these lipid mediators. Cytokines play crucial roles in the maintenance of tissue homeostasis, a process which requires a delicate balance between anabolic and catabolic activities. Although it is still controversial that excessive and/or continuous production of cytokines in inflamed periodontal tissues is responsible for the progress of periodontitis and periodontal tissue destruction, particularly, inflammatory cytokines such as IL-1β, IL-6, and IL-8 are present in the diseased periodontal tissues, and their unlimited production seems to play a role in chronic leukocyte recruitment and tissue destruction. Hence, it is possible that monitoring cytokine production or its profile may allow us to diagnose an individual's periodontal disease status and/or susceptibility to the disease or determine the effectiveness of periodontal therapy. Pathophysiological roles of inflammatory cytokines in periodontal tissue destruction are attributable for each cytokines. For example, IL-1 is known to play a role in the enhancement of bone resorption, stimulation of metalloproteinase production, plasminogen activator and prostaglandin synthesis while IL-6 primarily is responsible from cell activation resulting in non-specific antibody and IL-1 production in conjunction with enhancement of bone resorption. Similarly,

TNF-α plays a role in the enhancement of bone resorption synergistically with IL-1. On the other hand, IL-8 stimulation results in attraction and activation of neutrophils.

Monocytes/macrophages

Macrophages constitute from 5 to 30% of the infiltrating cells in inflamed periodontal lesions. In vitro studies have shown that macrophages produce cytokines such as IL- 1, IL-6, IL-10, IL-12, IL-13, TNF-α, and IFN-α. In particular, it is believed that macrophages play a central role in the production of IL-1 in inflamed sites (Oppenheim et al., 1986; Le and Vilcek, 1987; Page, 1991). Furthermore, mRNA expression of TNF-α, IL-6, and IL-8 was also detected in the macrophages in inflamed periodontal tissue. Several studies have shown that periodontopathic bacteria-such as Porphyromonas gingivalis (Pg), Actinobacillus actinomycetemcomitans (Aa), and Fusobacterium nucleatum (Fn)-induce expression of IL-1 in mononuclear cells/monocytes (Lindemann and Economou, 1988; Lindemann et al., 1988; Garrison and Nichols, 1989; Walsh et al., 1989; Bom-van Noorloos et al., 1990; McFarlane et al., 1990; Hanazawa et al., 1991; Gemmell and Seymour, 1993). These investigators have also shown that whole Gram-negative bacteria, their LPS, lipoteichoic acid, and fimbriae had stimulatory effects on IL-1 production by monocytes. A study of peripheral mononuclear cells from periodontitis patients demonstrated that unstimulated monocytes and monocytes stimulated by LPS derived from Aa produced more IL-1 than did similarly stimulated monocytes from healthy controls (McFarlane et al., 1990).

Fatty acids have been shown to regulate a variety of enzymatic processes that control chronic inflammatory disease (Zagola and Marik, 2001). In addition, it has also been shown that fatty acids can decrease the amount of arachidonic acid in cell membranes reducing eicosanoid production via cyclooxygenase and lipoxygenase. It is the integration between arachidonic acid byproducts and their involvement with leukotriene and prostaglandins that lead to inflammation control (Bandeira-Melo et al, 2002, Calder et al, 2002, Fabre et al, 2002, and Kroetz and Zeldin, 2002). These mechanisms have been shown to play important roles in the development of periodontal inflammation (Van Dyke and Serhan, 2003). Moreover, high epithelial penetration ability of fatty acids through gingival epithelium (Tanajo et al, 1997) suggests that the local application may be favorable in the treatment of periodontal inflammation.

In a previous in vitro study, we have shown that Celadrin®, a novel cetylated fatty acid mixture, inhibits proinflammatory mediators including TNF-α, and IL-8 at 6 and 24 hours after stimulation by LPS *in vitro*. The results of this *in vitro* study clearly indicate that different cetylated fatty acids play role in different stages of inflammation by acting differently on inflammatory mediators. Based on our in vivo data (Hasturk et al. 2007), we have hypothesized that the control of inflammation can be mediated by the topical application of cetylated fatty acids. This *in vitro* study has shown that this hypothesis is valid and further indicated that various components of the previously tested compounds have different inhibitory effects on inflammatory mediators. The tested compounds show

cytokine-specific actions at different stages of inflammatory response and a blend of these fatty acids can complete and augment the inhibitory effect of a single cetylated fatty acid as shown in this study.

Objective:

We therefore aimed to further investigate the different sources of Celadrin®, a blend of cetylated fatty acids, in monocyte-mediated inflammatory response *in vitro*. Recently, it has become evident that using vegetable sources of these fatty acids would be more acceptable and convenient to process. The study objective was to test the *in vitro* anti-inflammatory actions of a newly formulated vegetable-derived Celadrin® as compared to original composition of Celadrin® (tallow source) *in vitro*.

Materials and Methods

Preparation of Celadrin® (both vegetable and tallow-derived)

In order to dissolve Celadrin® in an aqueous medium that can be utilized in carrying out the experiments the following material were used: ethanol (EtOH) and Methyl-β-cyclodextrin (Sigma-Aldrich, St. Louis, MO). Methyl-β-cyclodextrin in the amount of 750.0 mg was dissolved in 5.0 ml of nano-pure water to get a concentration 150.0 mg/ml (MBC). The MBC solution was stored in the refrigerator at a temperature of 4 ºC and was returned to room temperature before use.

Cytokine Release from Monocytes

In order to determine the anti-inflammatory actions of vegetable-derived Celadrin® using human primary monocytes, healthy subjects (n=5) with no medical condition, no periodontal or gingival inflammation and no known medication use were enrolled. All patient samples were obtained after the approval of the Institutional Review Board at Boston University Medical Center. None of the subjects were smokers and all subjects were of Caucasian origin with an age range of 24-51 years old. Fresh peripheral venous blood (~72 ml) was obtained by venipuncture into heparinized (10 U/ml) glass tubes. Monocytes were isolated using Ficoll-Hypaque density gradient centrifugation and separated from the other mononuclear cells (e.g., lymphocytes) by adherence over 2 hours. Pure cell cultures were treated with various doses (10^{-5}-10^{-9} M) of Celadrin® (tallow or vegetable) for 30 minutes. Vehicle (5% ethyl alcohol), which was used to dissolve the compound into an aqueous preparation, was used as the negative control while dexamethasone (1nM) was used as the positive control. After incubation with the test compound, half of the samples were treated with LPS from *E. coli* (100ng/ml) as the activator of cell cytokine release over various time points (24 hours) at 37°C under 5%CO_2. Supernatants were collected and stored at -80°C until analyzed. Each sample was prepared in triplicate. The cytokine release (IL-1β, TNF-α, IL-6, IL-8, MCP-1 and GM-CSF) was analyzed by xMAP multiplexing technology using Luminex 100 Platform. Data was presented as % inhibition over the vehicle's effect of LPS-mediated cell activation. Dexamethasone inhibition was taken as 100% inhibition.

Results:

This report presents data on 24-hour time points for monocyte/macrophages treated with *P. gingivalis* and both Celadrin® compounds (vegetable and tallow). The experiment as stated above has been repeated at least 3 times and data was presented as %-inhibition over the vehicle's effect of LPS-mediated cell activation. At least 10% inhibition is considered as inhibitory effect to demonstrate the potential impact of these compounds.

Monocyte/macrophage mediated cytokine release

Inhibition potential of vegetable-derived Celadrin® on monocyte-mediated cytokine release tested at different concentrations compared to tallow-derived Celadrin® is shown in Table 1. As shown, Celadrin® from both sources significantly inhibited IL-8, which is a strong chemoattractant and mainly released by neutrophils and MCP-1, which is also a strong chemokine for monocytes. TNF-α was also significantly inhibited by both types of Celadrin®, but the inhibition with tallow-derived Celadrin® was stronger. The other tested cytokines including IL-1β, IL-6 and GM-CSF were not inhibited by any of the tested compounds.

Table 1: Inhibition of cytokine release from peripheral blood monocytes (24 hours)

Compound	**TNF-α**	**IL-1β**	**IL-6**	**GM-CSF**	**IL-8**	**MCP-1**
Celadrin®-vegetable	√				√*	√*
Celadrin®-tallow	√*				√*	√*

*Strong inhibition

These results support our previous findings with tallow-derived Celadrin® in terms of cytokine inhibition including TNF-α, IL-8 and MCP-1. In addition, the results clearly demonstrated that vegetable-derived Celadrin® acts as synergistically as the tallow-derived compound on monocyte-mediated cytokine response. Both compounds significantly inhibited the release of TNF-α, IL-8 and MCP-1 at 24-hour period (Figure 1; panels a and b). While the inhibitory effect of Celadrin® (both vegetable and tallow derived) on MCP-1 and TNF-α was dose-dependent, the effect on IL-8 was more robust and independent of various doses.

In summary, both vegetable- and tallow-derived Celadrin® showed significant inhibitory actions on various and important pro-inflammatory cytokines indicating their potential role in different stages of inflammation.

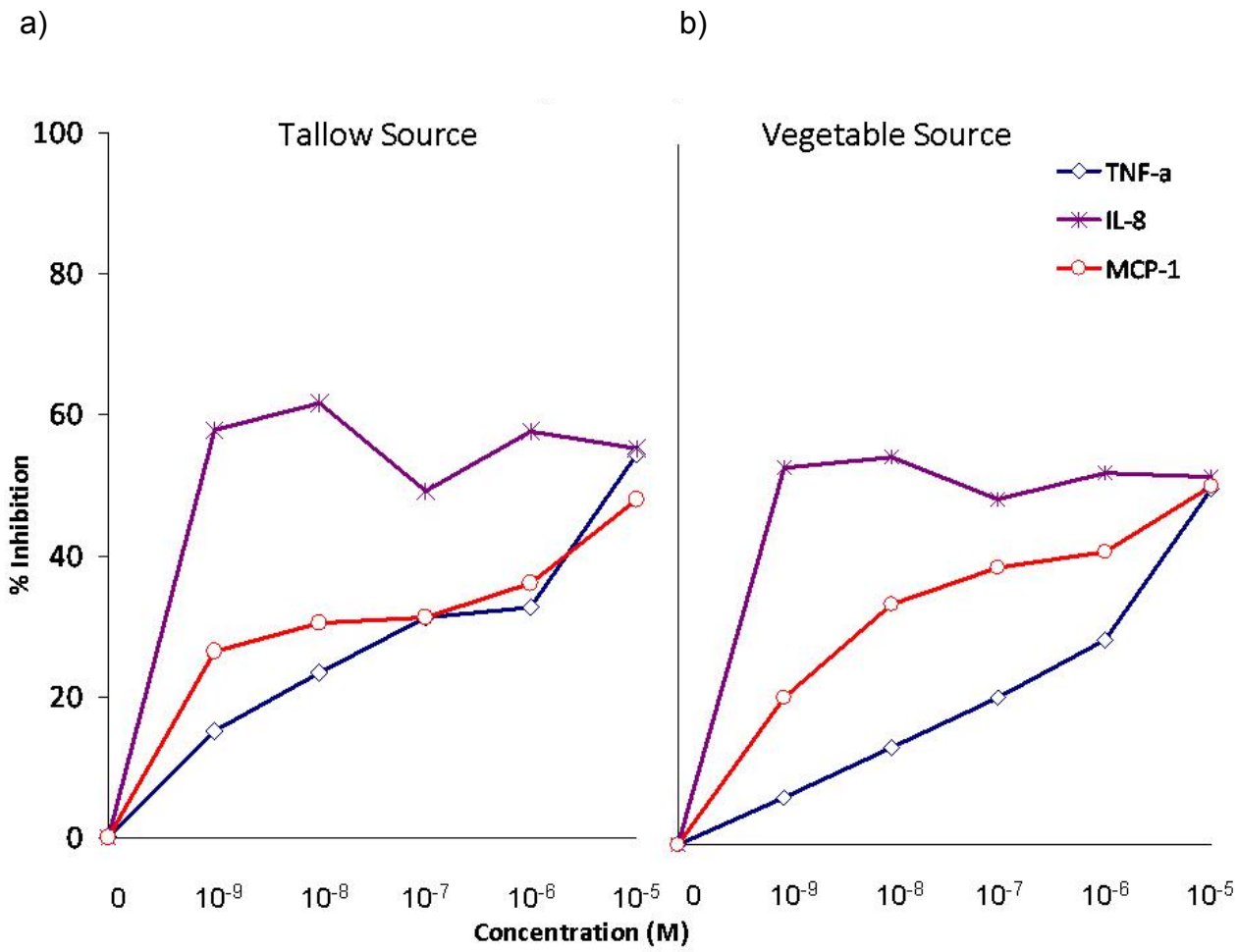

Figure 1. Inhibition of monocyte-mediated cytokine release by Celadrin® (24 hours)