Abstract: Taurocholic acid (TCA), a conjugation of cholic acid with taurine, is one of the main bile acids that is elevated in liver disease. Considering the epidemiologic linkage of periodontal disease to liver disease, the question arises about the possible effect of elevated TCA levels on periodontal cells. To answer this question, gingival fibroblasts and human oral squamous cell carcinoma cell line (HSC-2) were pretreated with interleukine1β (IL1β) and tumor necrosis factorα (TNFα) in the presence and absence of TCA. Also, mouse macrophages (RAW 264.7) were incubated with sterile-filtered human saliva with and without TCA. Inflammatory cytokines were measured by real time polynucleotide chain reaction (RT-PCR) and an immunoassay. The nuclear translocation of the p65 subunit was visualized by immunostaining. In pretreated gingival fibroblasts and HSC-2 cells, TCA considerably reduced the expression of IL1β, IL6, and IL8. In support of these observations, TCA lowered the salivá-induced expression of IL1α, IL1β and IL6 in RAW 264.7 cells. An immunoassay confirmed the capacity of TCA to diminish inflammation-saliva-induced expression of IL1α, IL1β, and IL6 in RAW 264.7 cells. Consistently, TCA blocked the nuclear translocation of p65 in fibroblasts. These findings suggest that TCA has anti-inflammatory activity in gingival fibroblasts, human oral squamous cell carcinoma cells and macrophages in vitro.

Keywords: cytokine, in vitro, liver cirrhosis, periodontitis, taurocholic acid

Introduction

Chronic inflammation is responsible for the catabolic events that lead to the loss of tooth-supporting structures [1]. The causes of periodontal disease are multifactorial and cannot be blamed on the local calculus and the microbial virulence factors [1]. Epidemiological studies revealed an association between periodontal disease and major systemic disorders including those affecting the cardiovascular system [2] and endocrine organs causing diabetes mellitus [3]. A less recognized association is that between periodontal disease and liver disease [4,5], even though there are other associations with risk factors such as alcohol abuse, obesity and viral hepatitis [6]. Preclinical models have focused on how periodontal disease affects liver disease [7-11]; however, whether or not liver disease causes the progression of periodontitis has not been investigated by preclinical models. There is reason to suggest that liver disease can affect periodontal disease.

Bile acids are synthesized in hepatocytes from cholesterol through classical and alternative pathways [12]. The levels of systemic bile acids in liver cirrhosis and hepatocellular dysfunction are high and are close to those that are normally present in enterohepatic portal circulation [13]. High levels of bile acid are linked with an impaired immune cell function which increases patient morbidity and even mortality [14]. Therefore, high levels of bile acids are considered immune suppressors. Among all of the bile acids that are present in liver cirrhosis, taurocholic acid (TCA) is the one that is the most changed [15]. Serum TCA is significantly increased in hepatitis B-induced cirrhotic patients [16], in alcoholic liver disease [17] and drug-induced liver injury [18]. There is little evidence of TCA detected in periodontal tissue; however, it is transported via the bloodstream and may therefore reach the periodontium.

TCA promotes hormonal activity and can therefore interfere with the innate immunity, in addition to its main function as a surfactant to support fat emulsion. The hormonal activity of bile acids requires the famesoid X receptor (FXR) and Takeda G protein-coupled receptor 5 (TGR5; also called GPBAR1) [19]. FXR regulates gene expression via a FXR-depenent mechanism [20] and is a potent TGR5 ligand [21]. A deficiency of both receptors not only increased the bile acid pool, but also caused liver fibrosis and inflammation in mice [22]. TGR5 gene ablation enhanced the recruitment of macrophages in the colonic lamina propria and worsened the severity of inflammation, whereas TGR5 activation reversed the colitis [23]. It is widely accepted that TGR5 is expressed in macrophages and its activation mediates potent anti-inflammatory effects [24] which it also does in vitro with LPS-stimulated macrophages [25]. In screening assays, TCA suppressed inflammatory cytokines and various chemokines that were significantly up regulated by lipopolysaccharide (LPS) in macrophages, [26] which supports the role of bile acids in controlling innate immunity.

With respect to the possible anti-inflammatory effects of bile acids, FXR and TGR5 have been identified in bone where they prevent ovariectomy-induced bone loss [27]. No data are available for their effects in the periodontium. However, liver fibrosis and inflammation are the clinicai hallmark of liver cirrhosis and hepatocellular dysfunction, and high levels of bile acids over a long time period exert detrimental effects on the process of periodontitis. However, liver fibrosis and inflammation are the clinical hallmarks of liver cirrhosis and hepatocellular dysfunction, and high levels of bile acids over a long time period exert detrimental effects on the periodontium. The aim of the present study was to extend the current knowledge about the behavior of macrophages in liver disease and to evaluate if TCA modulates the inflammatory response in vitro.

Materials and Methods

Human gingival fibroblasts and human oral squamous cell carcinoma cell line

Human gingival fibroblasts (GF) were harvested from gingiva obtained during wisdom tooth extraction from patients who had given written informed consent. Approval was obtained from the Ethics Committee of the Medical University of Vienna EK NR 631/2007. All of the methods were performed in accordance with the relevant guidelines and regulations. Two strains of fibroblasts were established and fewer than 10 passages were used for the experiments. The human oral squamous cell carcinoma cell line (HSC-2) was kindly provided by Professor Rauch-Fan from the Department of Periodontology at the Medical University of Vienna, Austria. The cells were seeded at the concentration of 30,000 cells/cm² onto culture dishes one day prior to stimulation. The cells were exposed to...
interleukin 1β (IL1β) and tumor necrosis factor (TNFα) at a concentration of 5 ng/mL for 1 h followed by the addition of 100 μM TCA (Sigma, St. Louis, MO, USA) in serum-free medium for 3 h prior to RNA isolation. In order to harvest the supernatant, the experiment was extended to 24 h. The concentration of TCA that was used was based on previous studies with primary hepatocytes [29-31] and viability testing.

**Viability experiments**

For viability, GF were exposed to TCA in a serum-free medium for 24 h before a MTT solution at a final concentration of 0.5 mg/mL was added to each well of a microtiter plate for 2 h at 37°C. The medium was removed, and formazan crystals were solubilized with dimethyl sulfoxide. The optical density was measured at 570 nm. The data were expressed as percentage of optical density in the treatment groups normalized to unstimulated controls.

**Mouse macrophages and saliva preparation**

The mouse macrophages (RAW 264.7) were from the American Type Culture Collection Health Science Research Resources Bank (ATCCCTIB-71). For the inflammatory experiments, the cells were exposed to 5% fresh sterile saliva for 1 h followed by the addition of 100 μM TCA for 3 h before preparing the total RNA. Saliva serves as an agonist of TLR-signaling, and consequently provokes a robust inflammatory response in macrophages [32]. To collect the supernatant, the experiment was prolonged for 24 h. Whole human saliva was collected from the two authors (R.T, R.G) who are non-smokers and gave their informed consent. Saliva flow was measured at 570 nm. The data were expressed as percentage of optical density in the treatment groups normalized to unstimulated controls.

**Real time polymer nucleotide chain reaction (RT-PCR) and immunoassay**

Reverse transcription (RT) was performed with the SensiFast cDNA synthesis kit (Bioline Reagents Ltd., London, UK). RT-PCR was done with the SensiFast SYBR kit following the manufacturer’s instructions (Bioline Reagents Ltd.). Amplification was monitored on the CFX connect real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). The primers sequences are shown in Table 1. Relative gene expression was calculated based on GAPDH and β-actin using the ΔΔCT method. The reactions were run in duplicates. The supernatant was analyzed for IL6 using an immunoassay according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA).

**Immunofluorescence analysis**

Immunofluorescence analysis was performed on GF plated onto Millicell EZ slides (Merckmillipore, Darmstadt, Germany) that were pretreated with IL1β and TNFα at a concentration of 5 ng/mL for 20 min before being exposed to 100 μM TCA for 1 h. Cells were fixed in paraformaldehyde and blocked in 1% BSA and 0.3% Triton in PBS for 1 h at room temperature. The cells were subsequently incubated with a NFKB p65 primary antibody (Cell Signaling Technology, Frankfurt am Main, Germany). An Alexa 488 secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) was applied for 1 h. The cells were washed and mounted onto glass slides. Images were captured at 100× in an oil immersion using a fluorescence microscope (Zeiss Axiosvert 200M, Oberkochen, Germany).

**Statistical analysis**

All of the experiments were repeated up to four times. GF were isolated from two donors. The statistical analysis was based on the Wilcoxon matched-pairs signed rank test for the results of RT-PCR and on the Friedman test followed by Dunn’s multiple comparisons for the results of the immunoassay with GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA).

**Results**

TCA decreased the inflammatory response of gingival fibroblasts to IL1β and TNFα

In order to confirm the lack of toxicity of TCA in GF, a viability test was performed. The formazan formation was not significantly changed by the addition of 100 μM TCA, but decreased at 1 mM TCA (Table 2). TCA at 100 μM failed to change the expression of the anti-apoptotic Bcl2, while etoposide caused a robust decrease (data not shown). In order to understand the possible modulation of TCA on the inflammatory response, GF were exposed to pro-inflammatory cytokines IL1β and TNFα in the presence and absence of 100 μM TCA, and then the gene expression of IL1, IL6, and IL8 was measured. The GF showed the expected increase in expression of inflammatory cytokines after exposure to pro-inflammatory cytokines, while the presence of TCA caused a significant decrease in the expression of IL1 (P = 0.0156), IL6 (P = 0.0137), and IL8 (P = 0.0243) as indicated in Fig. 1. The decrease in IL6 production was confirmed by the protein level with an immunoassay (Table 3). TCA also prevented the nuclear translocation of p65 after incubation of the cells stimulated with IL1β and TNFα (Fig. 2).

TCA diminished the inflammatory response of HSC-2 cells to IL1β and TNFα

In order to extend this research to other cell types, human oral squamous cell carcinoma cells (HSC-2) were exposed to the pro-inflammatory cytokines with and without TCA. Then, the expression of the inflammatory cytokines was determined. RT-PCR revealed that TCA causes a significant reduction in the gene expression of IL1 (P = 0.0313), IL6 (P = 0.0313), as well as IL8 (P = 0.0313) (Fig. 3). An immunoassay established the capacity of TCA to decrease IL6 production with the protein levels (Table 3).

| Gene          | Forward primer | Reverse primer |
|---------------|----------------|----------------|
| Human IL1β    | CTGTGGGCTTAAACAGATGAAT | AGCCCTTGTGTAAGTGGT |
| Human IL6     | GAAAAGACAGGTTACGACACTG | GATTTTACACGGGCAAGTCT |
| Human IL8     | AACTTCCTCCAAACCTCTCG | TGGCAGCCCTCTCAGATTTC |
| Human GAPDH   | GGCTACTGAGGACACCCAG | GACAAATCAGGAAAGT |
| Mouse IL1α    | TTTGTTAATTGACTGTCACA | GAAGCTTCACAGAAGTTCG |
| Mouse IL1β    | AAGGCGTCTCCTCAACACTTGTGAC | ATACGTGCTGCTGAAGCTTGTG |
| Mouse IL6     | GCTACAAACTTGGATATAATCAGGAA | CCAGTGTTCTATGTGATCAGAA |
| Mouse GAPDH   | AACTTGGACATTGTCGAAAGG | GGATTGACGAGATGATTTGCT |

**Table 1** Primers sequences

**Table 2** Impact of TCA on formazan production in gingival fibroblasts

|        | 10 μM | 100 μM | 1000 μM |
|--------|-------|--------|---------|
| Experiment 1 | 116.0 | 107.6  | 52.8    |
| Experiment 2 | 109.4 | 103.3  | 63.7    |
| Experiment 3 | 96.8  | 102    | 53.6    |

Human gingival fibroblasts were exposed to TCA at the indicated concentration for 24 h. The data shows the formation of formazan crystals that are expressed as percentages of unstimulated controls.
Table 3  TCA decreased production of IL6 in GF, HSC-2, and RAW247.6

|        | Basal                  | IL1β + TNFα       | IL1β + TNFα + TCA       |
|--------|------------------------|-------------------|-------------------------|
| GF     | 32.6 (19.3; 43.6)      | 112.6 (58.3; 316.8)** | 77.5 (29.7; 183.0)      |
| HSC-2  | 20.9 (14.5; 29.1)      | 63.1 (50.8; 115.7)* | 34.0 (16.5; 72.7)       |
| RAW    | 7.9 (7.9; 7.9)         | 23.3 (23.3; 23.3)  | 16.3 (15.1; 17.5)       |

Human gingival fibroblasts (GF), human oral squamous cell carcinoma cells (HSC-2) and mouse macrophages (RAW247.6) were exposed to IL1β + TNFα or saliva for 1 h followed by the addition of 100 µM TCA for 24 h. The supernatant was collected and analyzed for IL6 using an immunoassay. ** and * represent significant differences to the basal levels based on a Friedman test followed by Dunn’s multiple comparisons. The data from the RAW247.6 experiment remain descriptive. The data are presented as the median (min; max).
TCA reduced the inflammatory response of mouse macrophages to saliva

In order to further investigate the possible anti-inflammatory response of hematopoietic cell lineage to TCA, mouse RAW 247.6 macrophages were exposed to saliva [32]. As expected, the saliva increased the gene expression of the inflammatory cytokines IL1α, IL1β, and IL6. Also with RAW 247.6 cells, TCA significantly reduced the expression of IL1α (P = 0.0078), IL1β (P = 0.0200), and IL6 (P = 0.0025) in the presence of 5% saliva (Fig. 4). TCA also reduced the production of the IL6 protein which was evidenced by an immunoassay that was consistent with the changes in gene expression (Table 3).

Discussion

Epidemiological studies have discovered an association between periodontal disease and liver disease [4,5] and preclinical models support this observation [7-11]. Apart from some associated factors such as alcohol abuse, obesity and viral hepatitis [6], the chronic elevated levels of bile acids in liver cirrhosis and hepato cellular dysfunction may modulate the initiation and progression of periodontal disease was proposed here. This assumption is supported by previous observations that show that bile acids promote hormonal activity and may therefore affect innate immunity, for example, by lowering the stimulated expression of cytokines and chemokines in macrophages [23-26]. If cells that are characteristic of periodontium respond to TCA, the highly elevated levels of bile acids in liver cirrhosis and other hepatocellular dysfunctions [15-18] is unclear.

The main finding of the present research is that GF and oral epithelial cells, even though they are from oral squamous cell carcinoma, respond to TCA with a robust decrease in stimulated inflammation. The finding that RAW 247.6 cells exert a similar response is in line with previous observations that show that TGR5 is expressed in monocyes and macrophages and its activation mediates anti-inflammatory effects in LPS-stimulated macrophages in vitro [23-26]. The findings further support the previous research on ursodeoxycholic acid (UDCA) that is considered a weak agonist of TGR5 and used as a pharmacological treatment for a wide range of hepatobiliary disorders [33,34]. UDCA also shows anti-inflammatory activity in a similar setting with mouse macrophages in vitro [35] and as GF and human oral squamous cell carcinoma cells (HSC-2) [36]. TCA should be considered as a bile acid, similar to UDCA, with anti-inflammatory effects on oral cell lines.

The clinical relevance of the findings is not easy to interpret considering that periodontitis is an inflammatory disease and bile acids are supposed to have an anti-inflammatory activity for macrophages [23-26], which is in support of the in vitro observations. In vivo, however, a continuous suppression of the innate immunity due to the high levels of bile acids could be a pathological factor that provides an environment facilitating an impaired defense against periodontal pathogens. Therefore, a major pathological factor might be that bile acids impair the early immune response by down regulating inflammatory cytokines in macrophages and other cell types. Also, the current in vitro setting was optimized to show anti-inflammatory activity, therefore higher concentrations of TCA and even other bile acids might promote pro-inflammatory activity [28]. The present study is an introduction that promotes further evaluation about how elevated bile acids are linked with periodontal disease.

There are a few limitations of this study. First, this study was restricted to pro-inflammatory cytokines, as the expression of potential anti-inflammatory cytokines such as IL-10 was not determined. Second, even though the anti-inflammatory effects of TCA are likely mediated via FXR and inflammatory cytokines such as IL-10 was not determined. Second, even though the cellular and molecular reasons of the association of periodontal disease with liver disease.

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Conflict of interest

The authors of this manuscript certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or companies that are discussed in this article.

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