Kaempferol Effects on Human Gingival Fibroblasts

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Abstract

Objective: Kaempferol is a natural flavonol found in many dietary fruits, vegetables, and medicinal herbs. It reportedly has multiple beneficial properties as analyzed in vivo and in vitro. The objective of this study was to determine cytoprotective, anti-inflammatory, and wound healing characteristics of kaempferol in cultured human gingival fibroblasts.

Materials & Methods: To determine cytotoxicity and dose-response, commercially available human gingival fibroblasts were cultured in 0, 5, 10, 25, 50, 100, 250 or 500 µM kaempferol for 24 hours prior to a colorimetric MTT assay. To determine anti-inflammatory effects, gingival fibroblasts were pre-treated with one of several concentrations of kaempferol and challenged with 10 ng/ml tumor necrosis factor alpha (TNFα). Culture supernatants were assayed for levels of secreted interleukin-8 by ELISA. Kaempferol effects on the cell migration phase of wound healing in confluent nicotine-treated fibroblasts were determined using an in vitro scratch assay.

Results: By MTT assay kaempferol at 5, 10, 25, and 50 µM was not detrimental to gingival fibroblast survival, in contrast 100, 250, and 500 µM kaempferol proved to be highly cytotoxic to gingival fibroblasts. Kaempferol at 5, 10, 25, and 50 µM significantly suppressed TNFα-induced IL8 secretion. Of concentrations assayed, 5 µM kaempferol significantly stimulated the migration of gingival fibroblasts from a scratch wound edge 24 hours post-wound. In nicotine-treated gingival fibroblasts, 5 µM kaempferol significantly increased the migration of gingival fibroblasts from the wound edge. The cells migrating from the wound edge retained the characteristic elongated spindle morphology.

Conclusion: In cultured human gingival fibroblasts, kaempferol demonstrates cytoprotective, anti-inflammatory, and pro-migratory properties in a concentration-dependent manner. These properties of kaempferol have implications for oral cavity mucosal wound healing.

Keywords: Cytoprotection; Gingival fibroblasts; Interleukin; Kaempferol; Migration; Wound healing

Introduction

More than 5000 polyphenol antioxidants called flavonoids have been isolated and identified. Structural differences categorize dietary flavonoids as flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavonoids [1,2]. Kaempferol (3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one, C₁₅H₁₀O₆) (Figure 1) is a naturally occurring flavonol found in many fruits and vegetables, just a few examples are peaches, squash, kale, and broccoli [3]. The content of kaempferol and two additional flavonols, quercetin and myricetin, was measured in 25 different types of edible berries [4]. The total average daily consumption of flavonols and flavones is estimated at 23 mg, with 17% (approximately 4 mg) as kaempferol [5]. Kaempferol is therefore a common component of a human diet that is varied in fruits and vegetables. Kaempferol is also found in many botanicals and Chinese herbs used in traditional medicine such as Ginkgo biloba, Moringa oleifera, Sophora japonica, Nelumbo nucifera, and propolis.

The reported biological effects of kaempferol are many and varied. As a polyphenol, kaempferol functions as an antioxidant and protects against oxidative stress and apoptosis [6-8]. It inhibited proliferation of human cervical cancer cells [9], and significantly reduced H₂O₂-induced cytotoxicity in lung and liver cells [10]. The ability of kaempferol to function as an anti-cancer agent is due to its anti-proliferation, cell-cycle arrest, and anti-metastasis/anti-angiogenesis activities in tumor cells [11]. Although kaempferol reportedly suppresses migration in cancer cells [12,13], it appears to stimulate migration in non-cancerous cells [14,15].

A significant amount of literature exists regarding the anti-inflammatory properties of kaempferol [11,16]. It has a beneficial modulatory effect on many inflammatory-mediated conditions including cancers, cardiovascular problems, and neurodegenerative diseases. This is hypothetically through inhibition of inflammatory cell function and expression of pro-inflammatory cytokines and chemokines [16]. For example, kaempferol inhibits interleukin (IL)-4 (17) and IL-8 [18] and downregulates nuclear factor-kB (NF-kB) [19].

There are relatively few studies that focus on the biological effects of kaempferol in the oral cavity. Many systemic health and disease conditions are also relevant to the oral mucosa, providing a rationale for determining the potential effects of kaempferol in the oral cavity. Therefore in order to add to the existing body of knowledge of kaempferol, in this study the effects of this flavonol on human gingival fibroblast survival, inflammatory response, and wound healing were examined.

Materials & Methods

Study design

This report used an in vitro study design utilizing human gingival fibroblasts in culture.

Cell culture

Non-transformed primary human gingival fibroblasts (HGF, ScienCell, Carlsbad, CA, USA) cryopreserved at passage one and delivered frozen, were cultured in medium as provided by the manufacturer. The basal medium was supplemented with 2% fetal bovine serum, 1% fibroblast growth supplement, and 1% penicillin/streptomycin solution. Cells were maintained at 37°C in 95% air and 5% carbon dioxide and were used within two to three passages. HGF maintained the characteristic spindle morphology throughout all passages.

MTT assay

Kaempferol (Sigma Aldrich Chemical Co., St. Louis, MO, USA)

![Figure 1: Chemical structure of kaempferol.](image-url)
solutions were prepared in the medium described above but also containing 0.05% (v/v) DMSO to final concentrations of 5, 10, 25, 50, 125, 250, and 500 µM. HGF were seeded into wells of a 24-well tissue culture plate, and at approximately 100% confluence in 3 wells of the plate were treated with 2 ml of one of the kaempferol solutions for 24 hours. Three wells of HGF were incubated in the culture medium (without DMSO) only as negative control.

After 24 hours of treatment with the kaempferol solutions, a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was performed according to the protocol of the Sigma Cell Growth Determination Kit MTT Based. HGF were incubated with MTT reagent for 3 hours. The resulting formazan crystals were dissolved with MTT solubilization solution, and the optical density of samples (n = 6, 2 per each of the 3 treatments) was determined at 570 nm using a SpectraMax M3 (Molecular Devices, Sunnyvale, CA, USA) microplate reader.

The assay was repeated three times. Data reported are representative of one assay, and is shown in Figure 2 as the relative percent cell survival, setting the negative control to 100%. Relative percent cell survival was calculated as the absorbance of the treatment/absorbance of negative control x 100%. Statistical analysis was completed using one-way ANOVA with Bonferroni post-test comparing all treatments to negative control and probability set at p < 0.05.

ELISA analysis of IL-8 secretion

Confluent HGF in wells of a 24-well tissue culture plate were pre-treated with 0, 5, 10, 25, or 50 µM kaempferol for 1 hour to allow cells to respond to the presence of the compound. Kaempferol-adapted cells were then challenged with 10 ng/ml of the pro-inflammatory agent tumor necrosis factor alpha (TNFα) for an additional hour. Culture medium samples were assayed for secreted IL-8 using the protocol provided with the ELISA kit (RnD Systems Quantikine ELISA, Minneapolis, MN, USA). Absorbance values were read at 450 nm and IL-8 concentration was calculated using a standard curve. All concentrations of IL-8 were within the detectable range of the assay.

Prior to concentration calculations, absorbance values were standardized to total cell number in individual tissue culture wells using crystal violet blue staining methodology. Adherent cells were fixed, stained with 2% crystal violet blue, thoroughly rinsed, and eluted with 1% SDS. Optical density was read at 595 nm. Corrections were calculated by dividing the optical density at 450 nm for a given well by the optical density at 595 nm of the same well.

The ELISA was repeated twice with two replicates per sample. Data is reported as the mean concentration of IL-8 in pg/ml ± SEM. Statistical analysis was completed using one-way ANOVA with Bonferroni post-test comparing all treatments to negative control (0 µM kaempferol) and probability set at p < 0.05.

Cell migration assay

HGF were cultured in wells of a 24-well plate until confluent. Scratch wounds were created in each well by dragging a sterile tip for an automatic pipetter down the middle of a well several times. After rinsing twice with medium to remove cell debris, the remaining adherent HGF were treated with 0, 5, 10, 25, or 50 µM kaempferol diluted in culture medium. At 24 hours post-wounding, phase contrast light micrographs were captured, and the extent of cell migration from the wound edge was measured and calculated in mm using the NIH Imagej image free software processing program. Experiments were repeated three times with three replicates of each treatment. Data were analyzed using one way ANOVA with Bonferroni post-test comparing all treatments to negative control (0 µM kaempferol) and probability set at p < 0.05.

In a separate study HGF were cultured in wells of a 24-well plate until confluent and then treated with 0, 10, 25, 50, 125, 250, 500, or 1000 µg/ml nicotine, an agent known to affect cell migration, for 24 hours. Scratch wounds were created and after rinsing twice with medium, HGF were cultured in 5 µM kaempferol. The culture medium did not contain nicotine during this incubation. As one control HGF cultured in nicotine were wounded, rinsed in medium, and cultured in medium only. As a second control HGF were wounded, rinsed in medium, and cultured in medium only. After 24 hours the extent of cell migration from the wound edge was analyzed for all treatments and controls. Experiments were repeated two times with three replicates per treatment. Data were analyzed using one way ANOVA with Tukey post-test comparing treatments with probability set at p < 0.05.

Results

To determine a dose-response curve in a human gingival fibroblast system, kaempferol at seven concentrations ranging from 5 to 500 µM was assayed for cytotoxicity using a colorimetric assay. The concentrations of kaempferol assayed in this study were similar to those investigated in other studies [18,20]. Compared to negative control, no cytotoxicity was observed at concentrations of 5, 10, or 25 µM kaempferol (Figure 2). Upon statistical analysis, cell survival was significantly inhibited at 50 µM, dropping to 79% (p < 0.01 compared to control). This percentage however is still slightly above the International Standard Organization guidelines which delineate 70% as minimum in vitro cell viability compared to control for a material to be considered non-cytotoxic and biocompatible with oral cells and tissues [21]. Concentrations beyond 50 µM resulted in a significant and massive amount of cell death; therefore kaempferol at 125, 250, and 500 µM was not used for subsequent assays.

There are a large number of studies addressing the anti-inflammatory effects of kaempferol in a number of cell types, however the oral cavity is not well studied. To determine the effects of kaempferol on inflammation specifically in HGF, cells were challenged with TNFα, a pro-inflammatory cytokine that can upregulate other cytokines, and subsequently assayed for IL-8 secretion [22]. HGF were initially pre-treated with kaempferol as a standard protocol used to determine the anti-inflammatory properties of a compound. Although the scope of this study was not to determine cellular pathways, it is an assumption that the presence of kaempferol may inhibit subsequent TNFα-mediated cell signaling.

Compared to untreated control, TNFα significantly upregulated the secretion of IL-8 (p < 0.01, Figure 3). Kaempferol at all concentrations assayed significantly inhibited TNFα-induced IL-8 secretion (p < 0.01 for all comparisons). Kaempferol functioned equally to or better than negative control.
Migration is an essential component of wound healing and it is documented that tobacco use correlates to poor wound healing [23]. A major compound that contributes to the observed effects of tobacco is nicotine. Because kaempferol appeared to stimulate cell migration (Figure 4), the potential effects of kaempferol on stimulating nicotine-affected migration was analyzed. Therefore the scratch assay was used to determine the effects of kaempferol in stimulating nicotine-dampened HGF migration. Because kaempferol at 5 µM most effectively stimulated migration, this concentration alone was used along with a range of nicotine concentrations (Figure 5).

HGF were initially treated with nicotine ranging from 10 to 1000 µg/ml for 24 hours, the scratch wound was initiated, then the wounded cells were treated or not with kaempferol for an additional 24 hours. Nicotine alone resulted in a dose-dependent response with respect to cell migration, with inhibition occurring at the relatively larger scale of dosage (500 and 1000 µg/ml). However at all concentrations of nicotine, treatment with kaempferol significantly stimulated the migration of HGF from the wound edge (p < 0.01 for all treatments). Increased migration was especially prevalent at nicotine concentrations greater than 125 µg/ml.

Phase contrast microscopy revealed no significant difference in the morphology of HGF that are migrating from the wound edge (Figure 6). Cells maintained the characteristic elongated spindle morphology and the majority of the cells were oriented perpendicular to the wound edge.

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### Conclusion

Many flavonoids have diverse biological activities that include anti-oxidant function, anti-inflammatory effects, and inhibition of cell-signaling proteins. As a type of flavonoid called a flavanol, kaempferol exhibits many of these same biological properties. Since many of the...
flavonoid compounds are under consideration for therapeutic use in inflammation-mediated disease, it is rational to investigate the effects of the family of flavonoids in diverse biological systems. To this end, this study focused on one aspect of the oral cavity, and analyzed the effects of kaempferol on human gingival fibroblast cytotoxicity, inflammation, and wound healing.

There is stated concern regarding the high concentrations of flavonoids that may be necessary to cause beneficial results in humans [24]. The in vitro cytotoxicity assay accomplished in this study demonstrated that kaempferol concentrations greater than 50 µM caused significant cell death in gingival fibroblasts. The wide range of biological activities reported for kaempferol has been determined using micromolar concentrations [18,20]. However Zabela et al. [25] found plasma concentrations of free kaempferol that were one to two orders of magnitude lower than the micromolar concentrations using micromolar concentrations [18,20]. Kaempferol protects against oxidative stress and apoptotic damage in experimental model of isoproterenol-induced cardiac toxicity in rats. Phytomed. 2016; 23:1401-1408.


References


