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Impact of apigenin and kaempferol on human head and neck squamous cell carcinoma

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Abstract

Objective—Apigenin and kaempferol are plant flavonoids with reported chemopreventive activities. This study aimed to determine the effect of apigenin and kaempferol on cell viability in cultured cells derived from the pharynx (FaDu cell line), an oral cavity carcinoma (PCI-13 cell line), and a metastatic lymph node (PCI-15B cell line) and in explanted FaDu cells.

Study Design—The in vitro viability of FaDu, PCI-13, and PCI-15B cells treated with apigenin and kaempferol was determined. Tumor growth of FaDu explants was evaluated in athymic mice that were gavaged with either apigenin or kaempferol.

Results—Although apigenin and kaempferol treatment decreased viability of cells in vitro, cell-type-dependent differences in responsiveness were observed. In vivo apigenin treatment significantly increased the tumor size of FaDu explants. Results obtained using kaempferol were similar.

Conclusions—The in vitro decrease in FaDu cell viability by apigenin and kaempferol was not observed in in vivo tumor explants using the conditions described in this study.

Apigenin and kaempferol are flavonoids that have been widely investigated for their chemopreventive properties.^{1,2} Foods that contain significant amounts of apigenin include grapefruit, oranges, and onions, and those with significant amounts of kaempferol are grapefruit, edible berries, and ginkgo biloba.^{3,4} The chemopreventive properties of apigenin and kaempferol are largely attributed to their ability to induce apoptosis, which has been found using both cultured tumor cells and in vivo explants of a variety of tumor types.^{5–10} In addition to inducing apoptosis, apigenin and kaempferol have also been found to enhance the ability of chemotherapeutic agents to induce cell death, which has led to suggestions that these flavonoids may be useful as adjunct chemotherapeutics that “sensitize” the tumor cells to the tumoricidal actions of the primary chemotherapeutic.^{11,12} With respect to underlying mechanisms, the pathways proposed to mediate the pro-apoptotic actions of apigenin and kaempferol include induction of oxidative stress, p53, the MEK-MAPK (mitogen-activated

protein kinase) signaling cascade activation/inactivation of nuclear receptors and other transcription factors, and inhibition of growth factor signaling pathways.^{1,5,7,11,13–16}

Squamous cell carcinoma of the upper aerodigestive tract is a stepwise carcinogenic process in which normal epithelial cells sequentially form hyperplastic, dysplastic, and finally invasive lesions.¹⁷ Current chemoprevention strategies are focused on either preventing or reversing this process. In oral cancers, the chemopreventive agent would be applied to premalignant lesions (leukoplakia or erythroplakia) with the purpose of inhibiting malignant transformation or preventing the development of a second primary. Dietary flavonoids such as apigenin and kaempferol may have many of the desired characteristics of an ideal substance to be used for preventing the development of squamous cell carcinoma, because they appear to target many of the appropriate signaling pathways in cultured oral tumor cells^{10,11} yet exhibit low toxicity in the normal cells.^{11,18} Additional applications include the aforementioned potential as chemosensitizing agents that can enhance the tumoricidal activities of chemotherapeutic agents.^{11–13}

Previous work has used a panel of prostate tumor cells to demonstrate that apigenin inhibits cell growth in a cell-type-selective manner¹⁸ and, when administered in vivo, inhibits the growth of implanted prostate tumor cells (PC-3 cell line).^{9,16,19,20} In this study, we used a similar experimental design and tested the effects of apigenin and kaempferol on cultured HHNSCC (human head and neck squamous cell carcinoma) cells derived from the pharynx (FaDu cell line), a poorly differentiated oral cavity carcinoma (PCI-13 cell line), and a metastatic lymph node (PCI-15B cell line) to determine whether the effect of apigenin as well as kaempferol on cell viability was similar in these different cell lines. Given that the FaDu cells responded to the growth effects of apigenin and kaempferol in a sensitive and dose-responsive manner, we then selected the FaDu cell line to determine whether administration of apigenin and kaempferol could alter the in vivo growth of these HHNSCC cells.

MATERIALS AND METHODS

Chemicals

Unless otherwise mentioned, all chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

Cell culture

FaDu cells (obtained from ATCC) and PCI-13 and PCI-15B cells (obtained from Dr Theresa Whiteside, University of Pittsburgh, PA, USA)^{21,22} were cultured in DMEM/F12 media (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 1mM sodium pyruvate, and MEM nonessential amino acids (Invitrogen Life Technologies Corp, Carlsbad, CA, USA) at 37°C and 5% CO₂. The cell lines were authenticated by Genetica DNA Laboratories (Burlington, NC, USA). Apigenin and kaempferol were dissolved in dimethyl sulfoxide (DMSO). We have not observed an effect of DMSO on cell viability at the concentrations used in this study (0.1%). For analyses of viability, the cells were treated with either DMSO (vehicle control) or increasing concentrations (1×10^{-7} M to 2×10^{-4} M) of either kaempferol or apigenin (purchased from Sigma-Aldrich). After 24 and 48 hours of treatment, cell viability was determined using the WST-1 assay (Roche Applied Science, Indianapolis, IN, USA).

Animals

Female athymic mice (*nu/nu*), 4 to 6 weeks old, were purchased from Harlan Sprague–Dawley (Indianapolis, IN, USA). The mice were housed in a specific pathogen-free room

within the animal facilities at the University of Kentucky, Lexington, KY, USA, and were allowed to acclimate for 1 week before treatment. Animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Kentucky and were conducted in compliance with the NIH guide for the care and use of laboratory animals.

Tumor implantation and treatment

The effect of apigenin and kaempferol on *in vivo* growth of HHNSCC was examined in athymic mice that were implanted with FaDu cells using conditions similar to those used previously to demonstrate the growth inhibitory effects of apigenin on implanted prostate tumor cells.^{9,16,19,20} Three groups (10 to 12 mice per group) of 4- to 6-week-old athymic nude mice were treated by gavage for 1 week before inoculation. Group 1 was treated with kaempferol (50 µg per mouse per day, suspended in corn oil) via gavage for 5 days per week for at least 1 week before tumor cell inoculation, and these treatments were continued until humane killing. Group 2 was treated in the same fashion with apigenin (50 µg per mouse per day) via gavage. Group 3, the control group, was treated with corn oil in the same fashion. After 1 week of gavage, the left flank of each mouse was injected subcutaneously with 0.1 mL (1×10^5 cells) of FaDu cell suspension. The right flank of each mouse served as a control and was injected with 0.1 mL of Matrigel mixed with cell-free culture medium. Once the tumor became palpable, it was serially measured in 3 dimensions with a caliper. All animals were humanely killed 28 days after inoculation with tumor cells. Tumor tissues were immediately harvested, measured, and weighed after euthanasia. Animals were carefully observed for signs of toxicity throughout the experiment, and all animals were weighed on a weekly basis.

Statistical analyses

The data were analyzed using Prism software (Irvine, CA, USA). The *in vitro* results are representative of at least 3 independent experiments that were subjected to 2-way analysis of variance and Bonferroni post hoc test analyses.

RESULTS

The effect of apigenin and kaempferol on the *in vitro* viability of HHNSCC cells was first examined using the FaDu cell line. The FaDu cell line has been used previously to examine the ability of apigenin to inhibit survival of HHNSCC cells *in vitro*¹⁵ and to compare the *in vitro* versus *in vivo* effects of other pharmacologic agents with respect to tumor cell growth.^{23,24} As shown in Figure 1, both apigenin and kaempferol treatments resulted in a dose-dependent decrease in cell viability. At the lower doses (0.1 µM and 1 µM), kaempferol was more effective than apigenin at reducing cell viability. However, at the higher doses (25 µM, 50 µM, and 100 µM) and with 48 hours of treatment (see Figure 1, *B*), apigenin (half maximal inhibitory concentration [IC₅₀] = 19.5 µM ± 1.2 µM) was more effective than kaempferol (IC₅₀ = 45.9 µM ± 5.4 µM) in reducing cell viability.

To determine whether apigenin and kaempferol would similarly alter cell viability from HHNSCC cells derived from either an oral cavity carcinoma or a metastatic lymph node,^{21,22} we performed similar experiments using PCI-13 and PCI-15B cells, respectively. As shown in Figure 2, the effects of apigenin and kaempferol were more pronounced after 48 hours of treatment. With respect to apigenin treatment, the viability of both the PCI-13 and PCI-15B cells was significantly decreased at concentrations of 25 µM and greater. Interestingly, treatment of the PCI-15B cells with kaempferol resulted in a biphasic response in which lower concentrations (i.e., 10 µM and 25 µM; see Figure 2, *B, D*) resulted in an increase in cell numbers, but at higher concentrations (100 µM and 200 µM), a decrease in

cell viability was apparent. TI shows a direct comparison of the response of the different cell lines to apigenin and kaempferol treatments (at 25 μ M).

Our final experiments were designed to determine whether in vivo administration of either kaempferol or apigenin would similarly affect the growth of HHNSCC cells. Given that the response of the FaDu cells to treatment with either apigenin or kaempferol was relatively sensitive and dose dependent (see Figure 1), we chose this cell line for our in vivo analyses. Previous in vivo apigenin studies that have used a dose of 50 μ g per mouse delivered daily by gavage found that these conditions were similar to those observed in vitro and effectively inhibited the growth of explanted human prostate tumor cells.^{9,16,19,20} Based on these previous studies, we chose to use the same dosing regimen to administer either kaempferol, apigenin, or the corn oil (control) for 1 week before inoculation with the FaDu cells and until humane killing. After 1 week of gavage, the left flank of each mouse was injected subcutaneously with 0.1 mL (1×10^5 cells) of a FaDu cell suspension. The right flank of each mouse served as a control. Daily weighing revealed that each group of test mice had similar weight gains throughout the experiment (Figure 3, A). The tumor volume was statistically higher for the apigenin treatment group as compared with that of the control group at 28 days (see Figure 3, B) (i.e., the average volume of the apigenin group was 3024 mm³, versus 1858 mm³ for the control group). Similarly, treatment with apigenin resulted in a significant increase in tumor weight when compared with that of the control (see Figure 3, C). The tumor weights obtained from the kaempferol-treated mice versus those of the control were not statistically different. These data indicate that using these conditions, in vivo administration of apigenin increased the growth of explanted human FaDu cells.

DISCUSSION

Apigenin and kaempferol, like many flavonoids, have provoked considerable interest, owing to their putative chemopreventive properties, which are thought to be due, in part, to their ability to induce apoptosis (as observed in cultured tumor cells) and to modulate a variety of signaling pathways.^{1,2,5,7,11,13–16} Although clinical trials to test their effects in human subjects have been proposed, it is not yet clear whether the activities of apigenin and kaempferol that are often monitored in vitro and using tumor explant models are predictive of clinical success. Furthermore, the extent to which different tumor cells may vary with respect to their response to either apigenin or kaempferol is poorly understood.

Our results indicate that treatment with apigenin and kaempferol resulted in a dose-dependent loss of cell viability in FaDu cells (see Figure 1). The responses of the FaDu, PCI-13, and PCI-15B cells to apigenin treatment (see Figures 1 and 2) were similar to those found in studies using CAL-27, SCC-15, and FaDu as representative HHNSCC cells.¹⁵ Although high concentrations (>100 μ M) of apigenin and kaempferol significantly decreased cell viability in all cell lines examined (FaDu, PCI-13, and PCI-15B), cell-type differences in responses were observed (see Figures 1 and 2). For example, at some concentrations of kaempferol (i.e., 10 μ M and 25 μ M; see Figure 2), cell numbers were increased in the metastatic PCI-15B cell line. It remains to be determined whether kaempferol treatment would similarly increase the growth of other metastatic HHNSCC cells. It has previously been reported that flavonoids induce apoptosis in a cell-type-selective manner.^{18,25} Here, apoptosis-inducing concentrations in the tumor cells failed to exert adverse effects in their normal-cell counterparts. The mechanisms by which apigenin and kaempferol affect cell viability are thought to involve formation of reactive oxygen species and induction of apoptosis.^{26,27} However, they are capable of exerting both pro- and antioxidant activities, depending on the concentrations used and the specific milieu of the cell.

Our results also indicate that despite the ability of apigenin and kaempferol to dose-dependently decrease the viability of FaDu cells in vitro (see Figure 1), in vivo administration of apigenin significantly increased the tumor size of the FaDu explants (see Figure 3). These results were surprising given that using similar in vivo conditions (i.e., daily gavage of 50 µg per mouse per day), apigenin was found to significantly decrease tumor growth of explanted prostate tumor cells.^{16,19,20} Other dosing strategies have also been used to examine the effects of apigenin on tumor growth. For example, daily intraperitoneal injections of apigenin (50 mg/kg) were found to significantly reduce the growth of explanted progestin-induced breast tumors.⁸ However, administration of higher concentrations of apigenin (75 mg/kg and 150 mg/kg administered via gavage) reduced metastatic, but not primary, ovarian tumor growth,²⁸ and when administered in the diet (approximately 300 and 75 mg/kg per mouse per day), it failed to alter the formation of colorectal adenomas.²⁹ With respect to oral cancers, apigenin treatment (administered via oral gavage, 2.5 mg/kg/d) significantly reduced tumors formed by the carcinogen 7,12-dimethyl(a)anthracene via mechanisms thought to involve antioxidant activities and modulation of phase I and phase II metabolism.³⁰

In comparing the results obtained from these different studies, the dose and delivery of apigenin should be noted, because its bioavailability is poorly understood and is thought to be quite low.³¹ For example, the dosing regimen used in the current study has been previously found to result in serum apigenin concentrations of approximately 1 µM and tumor apigenin concentrations of approximately 0.6 µmol/g.¹⁹ Dietary administration of 300 mg/kg apigenin for 5 days has been found to result in plasma concentrations of only 0.08 µM to 0.11 µM. An additional concern is whether metabolites of apigenin exert pharmacologic effects that are similar to those of the parent compounds. For example, a considerable amount of apigenin in the plasma is present as a glucuronide, but it is not known whether the glucuronide form of apigenin is capable of altering tumor growth.

Comparison of the in vitro results (see Figure 1) versus the in vivo results (see Figure 3) should consider not only the bioavailability of apigenin and kaempferol but also factors that may offer insights into their underlying mechanisms of action. For example, given our previous findings that apigenin can block the ability of the aryl hydrocarbon receptor (AHR) to induce phase I metabolizing enzymes (e.g., CYP1A1),³² the AHR may play a role in the ability of apigenin to inhibit 7,12-dimethyl(a)anthracene-induced oral carcinogenesis via inhibition of the metabolic activation of the procarcinogen, but this mechanism likely would not be relevant in the apigenin-induced increase in growth of tumor explants (see Figure 3). Instead, the apigenin-induced increase in tumor size of the explanted FaDu and other tumor cells may arise from its anti- and pro-proliferative effects mediated by other signaling pathways such as the estrogen receptor.

Both apigenin and kaempferol can activate estrogen receptors α and β ,³³ and the pro-proliferative effects of apigenin in breast cancer cells have been correlated with its ability to activate estrogen receptor α .³⁴ Estrogen receptor α is highly expressed in FaDu and other HHNSCC cells³⁵ and is implicated in the growth and poor prognosis of HNCC.³⁶ Although additional events, including host/tumor interactions, cannot be excluded at this time, the possibility that the apigenin-induced tumor growth observed herein (see Figure 3) is mediated via estrogen receptor α is an attractive speculation that requires further study. A related question is whether the chemopreventive activities of apigenin and other flavonoids may be gender dependent.

Our work indicates that clinicians considering the use of apigenin and kaempferol as either chemopreventive or chemotherapeutic agents should proceed with caution when considering their use for treating head and neck cancers. Premalignant conditions frequently exist in

human mucosa with undetected frank malignancy at adjacent mucosal sites. Under certain conditions, tumor growth may be favored rather than impaired by these agents, and further study is required to understand the mechanisms that underlie tumor-, host-, or cell-type-specific responses to these agents.

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Statement of Clinical Relevance

This work indicates that the in vitro effects of apigenin and kaempferol are not predictive of effects observed with respect to in vivo tumor growth. Thus, additional analyses of the in vivo effects of apigenin and kaempferol should be undertaken before further studies involving human subjects at risk of or with head and neck cancer.

Fig. 1.

Effect of apigenin and kaempferol on viability of FaDu cells. FaDu cells were incubated with increasing concentrations of either apigenin or kaempferol. After either 24 hours (A) or 48 hours (B), the cells were harvested, and cell viability was determined using the WST assay. *** $P < .001$, ** $P < .01$, and * $P < .05$ comparing apigenin- versus kaempferol-treated groups; ### $P < .0001$ comparing either apigenin- or kaempferol-treated groups to the DMSO vehicle control. (FaDu, a cell line cultured from a squamous cell carcinoma of a human pharynx; DMSO, dimethyl sulfoxide; WST, a proprietary assay.)

Fig. 2.

Effect of apigenin and kaempferol on viability of PCI-13 and PCI-15B cells. The indicated cells were incubated with increasing concentrations of either apigenin (A, C) or kaempferol (B, D). After either 24 hours (A, B) or 48 hours (C, D), the cells were harvested, and cell viability was determined using the WST assay. *** $P < .001$, ** $P < .01$, and * $P < .05$ comparing either the apigenin- or kaempferol-treated groups to the DMSO vehicle control. (DMSO, dimethyl sulfoxide; WST, a proprietary assay; PCI-13, a cell line cultured from an oral cavity carcinoma; PCI-15B, a cell line cultured from a metastatic lymph node.)

Fig. 3.

In vivo administration of kaempferol or apigenin results in increased tumor growth. Nude mice were administered kaempferol, apigenin, or the corn-oil-only control via gavage for 1 week before injection of FaDu cells (1×10^6) and for the duration of the experiment. The mice were weighed weekly (A), and tumor volumes were calculated from 3-dimensional tumor measurements (B). At 28 days after inoculation with the FaDu cells, the tumor tissues were removed and weighed (C).

Table I

Percentage of cell viability after 48-hour treatment with 25 μ M solution of either apigenin or kaempferol

	Apigenin	Kaempferol
FaDu	35.6 $\pm$ 18.5 ^{a,b}	49.7 $\pm$ 19.5
PCI-13	46.2 $\pm$ 2.9	81.4 $\pm$ 2.2 ^b
PCI-15B	58.2 $\pm$ 3.0 ^a	118.78 $\pm$ 2.0 ^b

FaDu, *PCI-13*, and *PCI-15B*, cell lines.

^{ab} Values with the same letters are statistically different (^a*P* < .05 and ^b*P* < .0001) and represent mean $\pm$ standard deviation.