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Propolis Suppresses Tumor Angiogenesis by Inducing Apoptosis in Tube-Forming Endothelial Cells

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We have reported that propolis suppresses tumor-induced angiogenesis in vivo and in vitro, but antiangiogenic mechanism of propolis at cellular level remains unclear. In this study, we observed that propolis not only inhibited tube formation but also induced apoptosis of endothelial cells. These results suggest that propolis exerts its antiangiogenic effects at least in part through induction of apoptosis.

Key words: propolis; angiogenesis; apoptosis; endothelial cell; tube formation

Propolis is a resinous substance collected by honeybees from buds and exudates of certain trees and plants. It is used in folk medicine to treat various ailments. It is also used in foods and beverages to improve health and prevent disease. Since propolis is generally used as an alcohol or water extract when applied to humans, it is very important to evaluate its biological activities in extracted form.

In this study, the antiangiogenic effects of propolis were evaluated using in vivo and in vitro angiogenesis models. Angiogenesis, or new blood vessel growth, is defined as a process in which a network of new blood vessels emerges from pre-existing vessels. It has been found that angiogenesis is essential for tumor growth and metastasis, and that tumor angiogenesis can be a very effective target in cancer prevention and treatment.

We investigated to determine how propolis exerts its antiangiogenic effects at the cellular level. Propolis and all the other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. Mouse dorsal air sac assay was performed as previously described, with slight modifications. Female ICR mice were purchased at 5–8 weeks old from Japan SLC (Shizuoka, Japan). A chamber covered with Millipore filters (Millipore, Billerica, MA) of 0.45 µm pore size on both sides was filled with either saline or S180 tumor cells (3.0 × 10^6 cells) and implanted into a subcutaneous dorsal air sac, which was created by injecting air into the back of each mouse. The mice were fed a control diet (20% casein) with and without 5% propolis (calculated by dry weight) for 12 d. They were implanted with the chamber on day 8. On day 13, the implanted chambers were removed from the subcutaneous area, and the angiogenic response was assessed by the angiogenesis index by determining the number of newly formed blood vessels greater than 3 mm in length and 0.075 mm in diameter. In cellular analysis, capillary tube-like structures formed by HUVECs were prepared and quantified as previously described, with slight modifications.

Briefly, HUVECs (6.0 × 10^4 cells/cm^2) were seeded between two layers of collagen gels and incubated in MCDB-104 with 0.5% FBS supplemented with 10 ng/ml of bFGF, 8 nM PMA, and 25 µg/ml of ascorbic acid with various concentrations of propolis or vehicle (dimethylsulfoxide) for up to 48 h. Observation and quantification of apoptosis were carried out as previously described. Briefly, cells were fixed with 1% glutaraldehyde overnight at 4°C and stained with 500 ng/ml of DAPI overnight at room temperature. Cells exhibiting chromatin condensation and/or nuclear fragmentation were counted as apoptotic cells. Results were expressed as means ± SE. Differences were ascertained by analysis of variance (ANOVA). Multiple comparisons for angiogenesis and tube formation experiments were checked by the Fisher-PLSD test. A comparison of the two treatments for the apoptosis experiment was performed using Student’s unpaired t-test (*P < 0.05, **P < 0.01).

We observed that oral administration of the propolis purchased from Sigma suppressed tumor-induced angiogenesis (Fig. 1A). In the negative control group, little or no indication of neovessel formation was observed. In the positive control group, a drastic induction of new blood vessel formation, characterized by zigzagging...
lines of vessels, was observed. In the 5% propolis group,
substantial reduction in such new blood vessel formation
was observed. None of the treatments exhibited any
notable effect on pre-existing vasculature. The angiogenesis
indexes were 0.75, 6.5, and 2.0 for the negative
control, the positive control, and 5% propolis respectively (Fig. 1B). The number of newly formed blood
vessels in the mice treated with the diet containing 5.0%
propolis was suppressed to 22% as compared to the
positive control group. In addition, no signs of toxicity
were observed in any ICR mice (body weight change
during the feeding period, data not shown). Thus it was
found that the propolis from Sigma suppressed tumor-
induced angiogenesis \textit{in vivo}. Normal (pre-existing)
vessels are known to be stable, since they are protected
by pericytes and smooth muscle cells, as compared to
newly formed vessels, which lack such protecting cells.
Such differences might be the reason propolis failed to
affect normal vessels while it significantly suppressed
neovessel formation.

**Fig. 1.** Suppressive Effect of Propolis on Tumor Cell-Induced Angiogenesis in a Mouse Dorsal Air Sac Assay.
A, Chambers filled with S180 cells or saline were inoculated subcutaneously into ICR mice. Arrowheads point to newly formed vessels of a
zigzagging character. S180-treated positive control mice induced strong angiogenic responses as compared to the saline-treated negative control.
Oral administration of 5.0% propolis reduced the number of newly formed blood vessels. Representative photographs are shown. The bar
indicates 3 mm. B, The angiogenesis index was defined as the number of newly formed blood vessels above 3 mm in length and 0.075 mm in
diameter. Propolis significantly reduced the angiogenesis index. Values are expressed as means ± SE (n = 4 or 5). **P < 0.01 (Fisher-PLSD test) vs.
the positive control group.
We then observed that propolis inhibited tube formation of HUVECs cultured in a 2-D system in a concentration-dependent manner (Fig. 2). It slightly reduced the width of the tubes at 12.5 μg/ml, and completely inhibited elongation of the tubes at 50 μg/ml. Inhibition of tube formation by propolis was also accompanied by partial fragmentation of endothelial cells, an indication of cell-death induction. Thus, it was found that propolis inhibited tube formation of endothelial cells and induced cell death at the same time.

We then observed the morphology of the cell nuclei to determine whether the cell death induced by propolis was apoptosis. Propolis induced chromatin condensation, a morphological marker of apoptosis (Fig. 3A). The rates for apoptotic cells were 10.8 and 57.9% for control and propolis (50 μg/ml) respectively, calculated to be a 5.4-fold increase for propolis as compared to the control group (Fig. 3B). Thus it was confirmed that propolis caused cell death by inducing apoptosis in tube-forming HUVECs.

Fig. 2. Inhibitory Effect of Propolis on Tube Formation of Endothelial Cells.

A, HUVECs were sandwiched between two layers of collagen gel and induced to form blood vessel-like tubes. The cells were treated with the indicated concentrations of propolis for 48 h. Representative photographs are shown. The bar indicates 100 μm. B, The areas of formed tubes (area ratios of the tubes per pictured field) were quantified. Values are expressed as means ± SE (n = 4). *P < 0.05 and **P < 0.01 (Fisher-PLSD test) vs. control group.
In this study, we found for the first time that propolis can induce apoptosis in tube-forming endothelial cells. Such induction of apoptosis appears to be a likely mechanism of angiogenesis suppression by propolis. Apoptosis induction in endothelial cells is also known to be an important mechanism of antiangiogenic drugs.\(^{10}\) Several propolis components have been detected in the plasma after oral administration to rats.\(^{11}\) Among these, kaempferol and galangin have been reported to possess antiangiogenic activities.\(^{12}\) We intend to further investigate how propolis induces apoptosis in endothelial cells at the molecular level, and to determine which propolis constituents are responsible for induction. Since propolis inhibited elongation of HUVECs during tube formation, it is likely that it is capable of affecting endothelial cell migration. We also observed that propolis inhibited proliferation of HUVECs (data not shown). Such antiangiogenic aspects of propolis should also be further investigated. We hope our findings on the antiangiogenic effects of propolis will help the research community improve medical prevention and treatment of human cancer in the near future.

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