Artepillin C Suppresses Angiogenesis by Inhibiting Tube-Formation and Inducing Apoptosis of Endothelial Cells

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Abstract Artepillin C (ARC) is a major active ingredient of Brazilian propolis which is a resinous mixed compound collected by honeybees from various plant sources. Previously, we reported that the ethanol extract of Brazilian propolis possesses antiangiogenic activity both in vitro and in vivo. However, the mechanism of angiogenesis inhibition by purified ARC has not been well-clarified. In this study, we investigated the effects of ARC on tube-forming human umbilical vein endothelial cells (HUVECs). We found that inhibition of tube formation by ARC was accompanied by partial fragmentation of endothelial cells, indicating that it induced cell death. Western blotting revealed that ARC induced the reduction of vascular endothelial cadherin and platelet endothelial cell adhesion molecule-1. ARC also induced chromatin condensation and cell nuclear fragmentation, morphological markers of apoptosis, in tube-forming HUVECs. Furthermore, ARC suppressed phosphorylation of extracellular signal-regulated kinase 1/2, but upregulated phosphorylation of p38. It was also shown that ARC induced apoptosis via activation of proapoptotic signaling, activation of caspase-3 and cleavage of poly ADP-ribose polymerase and lamin A/C. In conclusion, ARC exerts its antiangiogenic effects through induction of endothelial apoptosis.

Keywords: angiogenesis, apoptosis, artepillin C, endothelial cell, tube formation


1. Introduction

Propolis, a folk medicine employed in treating various ailments, is a resinous substance collected by honeybees from the bud and bark of certain trees and plants, and stored inside their hives. It has been used in folk medicine from ancient times and is extensively used in foods and beverages to improve health and prevent diseases such as inflammation, diabetes, heart disease, and cancer [1,2]. Artepillin C (3,5-diprenyl-4-hydroxycinnamic acid, ARC) is one of the principal phenolic acids found specifically in Brazilian propolis, which possesses antitumor and chemopreventive activities [3]. Although we and other investigators have reported that ARC can suppress tumor growth both in vitro and in vivo [3,4,5], the actual mechanisms of these effects are not yet fully understood.

Angiogenesis is defined as a process in which a network of new blood vessels emerges from preexisting vessels, which is essential for tumor growth and metastasis. Judah Folkman first found in the early 1970s that new blood vessel growth was required for a tumor to grow over a few mm³ in size, supplying the tumor with nutrition and oxygen for its exponential growth [6]. Endothelial cells play a key role in the formation of such neovessels [7]. In addition, the induction of apoptosis in endothelial cells is known to be one of the major antiangiogenic mechanisms by many angiogenesis inhibitors [8]. Apoptosis is a genetically programmed form of cell death, which is strictly regulated by a balance between apoptotic signals and survival signals. Dhanabal et al. have demonstrated that endostatin, a specific inhibitor of angiogenesis, causes apoptosis of endothelial cells in vitro, but not nonendothelial cells [9]. Thus, angiogenesis through induction of apotosisin endothelial cells can be an effective strategy in cancer prevention and treatment [8,10].

In this study, we explored the antiangiogenic and apoptotic effects of ARC on human umbilical vein endothelial cells (HUVECs) and investigated its mechanisms of action at the molecular level. We also examined whether ARC-induced angiogenesis inhibition involved apoptosis or not and analyzed changes in survival signals and the apoptotic pathway using Western blotting.

2. Materials and Methods

2.1. Cell Culture and Reagents

HUVECs were isolated from human umbilical cord and were cultured as previously reported [3]. Briefly, cells were
grown in MCDB-104 medium (Nihon Pharmaceutical Co., Tokyo, Japan) supplemented with 10 ng/mL epidermal growth factor (EGF) (BD Biosciences, Bedford, MA, USA), 100 µg/mL heparin, 100 ng/mL Lendothelial cell growth factor (ECGF), and 10% fetal bovine serum (FBS) (Moregate, Brisbane, Australia). ARC was purchased from Wako Pure Chemicals Industries (Osaka, Japan). Medium 199 and all other chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. All antibodies used in this experiment were from Cell Signaling Technology (Beverly, MA, USA).

2.2. Tube Formation Assay

Capillary tube-like structures formed by HUVECs were prepared as previously described with slight modifications [3]. Briefly, HUVECs (6.0 × 10^4 cells/cm²) were seeded between two layers of collagen gel and then incubated in MCDB-104 medium with 0.5% FBS supplemented with 10 ng/mL of basic fibroblast growth factor, 8 nM phorbol 12-myristate 13-acetate, and 25 µg/mL ascorbic acid along with various concentrations of ARC or vehicle (ethanol). For morphological observation, the tube-forming HUVECs were fixed with 2% glutaraldehyde in PBS and then stained with 0.1% toluidine blue in 30% methanol.

For Western blot analysis, cells (2.4 × 10^5 cells/48-well plate) were suspended three-dimensionally in collagen gel for 6, 12, 24, and 36 h.

2.3. Apoptosis

The observation and quantification of apoptosis were carried out as previously described [11]. Briefly, fixed cells were stained with 500 ng/mL of 4',6-diamidino-2-phenylindole (DAPI). Cells exhibiting chromatin condensation and/or nuclear fragmentation were counted as apoptotic cells. A total of more than 500 cells from six fields were counted for each treatment.

2.4. Western Blot Analysis

Western blotting was performed as previously described [11]. In brief, the cellular proteins from tube-forming HUVECs in the 3-D culture model were separated in a 6–12% SDS-polyacrylamide gel and then transferred to a Hybond-ECL nitrocellulose membrane (GE Healthcare, Chalfont St. Giles, UK). Immunoreactive protein bands were visualized using an ECL or ECL plus detection system (GE Healthcare). The values were obtained from at least three independent experiments.

2.5. Statistical Analysis

Each result is expressed as the mean ± SE. Differences were ascertained by analysis of variance (ANOVA). Control and treatment outcomes were compared using Student’s unpaired t-test (*P < 0.05, **P < 0.01).

Figure 1. ARC inhibits tube formation and induces reduction of angiogenesis-related adhesion molecules in tube-forming HUVECs. A: HUVECs were sandwiched between two layers of collagen gel and induced to form blood vessel-like tubes. The cells were treated with various concentrations of ARC (3.13, 12.5, and 50 µg/mL). Tube-forming HUVECs in a 2-D culture model were observed at 6, 12, and 24 h. Each experiment was repeated at least three times and representative data are shown. Bar indicates 100 µm. B: ARC blocked the tube formation of HUVECs in a 2-D culture model. The tube-forming cells were treated with various concentrations of ARC for 24 h and then fixed. The fixed cells were stained with 0.1% toluidine blue in PBS and then stained with 0.1% toluidine blue in 30% methanol. Representative photos are shown. Bar indicates 200 µm. Values are expressed as means ± SE (n = 3). *P < 0.05 vs. the control group. C: Cellular proteins were collected from tube-forming HUVECs that were treated with various concentrations of ARC (3.13, 12.5, and 50 µg/mL) for 12 and 36 h. Changes in VE-cadherin and PECAM-1 were analyzed by Western blotting. Each experiment was repeated at least three times and representative data are shown.
3. Results

3.1. Inhibition of HUVEC Tube Formation and Reduction of Angiogenesis-Related Adhesion Molecules by ARC

To clarify the mechanism of angiogenesis inhibition by ARC, we first investigated the effect of ARC on the early stages of capillary tube formation using an in vitro tube formation model of HUVECs cultured in a 2-D system. After induction of tube formation, the endothelial cells formed a network of capillary-like tubes composed of multiple cells that gathered together and adhered to each other. As we previously reported, ARC significantly inhibited the tube formation of HUVECs in a concentration-dependent manner (3.13–50 µg/mL) (Figure 1A). Area ratios of the tubes per pictured field were 41.5%, 30.1%, 20.5%, and 9.8% at concentrations of 0, 3.13, 12.5, and 50 µg/mL, respectively, and were calculated to be 72.2%, 49.3%, and 23.5% (% of control) at concentrations of 3.13, 12.5, and 50 µg/mL, respectively (Figure 1B). To further acquire clues about the inhibitory mechanisms, we next examined whether ARC modulates the protein levels of angiogenesis-related cell adhesion molecules. There were significant concentration-dependent decreases in vascular endothelial cadherin (VE-cadherin) and platelet endothelial cell adhesion molecule-1 (PECAM-1) protein levels after ARC treatment (3.13–50 µg/mL) (Figure 1C).

3.2. Induction of Apoptosis in Tube-Forming HUVEC by ARC

We next investigated whether ARC, which exhibited antiangiogenic properties, could also induce apoptosis in tube-forming endothelial cells. ARC treatment induced chromatin condensation and cell nuclear fragmentation, which are widely accepted morphological markers of apoptosis, during inhibition of tube formation (Figure 2A and Figure 2B). After 24 h of treatment, the rates of apoptotic cells were 19.1%, 29.3%, 43.5% and 53.0% for control and ARC (3.13, 12.5, and 50 µg/mL) respectively, which were calculated to be a 1.5-, 2.3- and 2.8-fold increase for ARC (3.13, 12.5, and 50 µg/mL) compared to the control. Thus, it was shown that ARC caused cell death by inducing apoptosis in tube-forming HUVECs.

Figure 2. ARC induces apoptosis in tube-forming HUVECs. A: The tube-forming cells were treated with various concentrations of ARC (3.13, 12.5, and 50 µg/mL) for 24 h, fixed and stained with DAPI to observe cell nuclei. (a) 0 µg/mL, (b) 3.13 µg/mL, (c) 12.5 µg/mL, (d) 50 µg/mL. Representative photos are shown. Bar indicates 50 μm. B: Rates of apoptosis (percentage of condensed and fragmented cell nuclei against total cell nuclei) were quantified. A total of more than 500 cells from six fields were counted for each data point. Values are expressed as means ± SE (n = 6). *P<0.05, **P<0.01 vs. the control group

Figure 3. ARC inactivates ERK 1/2 and induces activation of caspase cascade. A, B: Cellular proteins were collected from tube-forming HUVECs that were treated with various concentrations of ARC (3.13, 12.5, and 50 µg/mL) for 6, 12, and 24 h. (A) Changes in phosphorylation state of ERK 1/2, p38, and Akt and (B) caspase-3, PARP, and lamin A/C were analyzed by Western blotting. Each experiment was repeated at least three times and representative data are shown.

3.3. Activation of Caspase Pathway in Tube-Forming HUVEC by ARC

We further analyzed whether ARC modulates survival and apoptotic signals using Western blotting. ARC inactivated extracellular signal-regulated kinase 1/2 (ERK1/2) in a concentration-dependent manner (Figure 3A). After treatment with ARC, dephosphorylation of ERK 1/2 was observed within 6 h and it gradually progressed as the time proceeded. Phosphorylation of p38 was also induced in a concentration-dependent manner. The induction was observed as early as 6 h, which was the same as the dephosphorylation of ERK1/2. On the other hand, treatment with ARC did not induce changes in phosphorylation of Akt within 6 h and only induced
relatively minor changes compared to ERK1/2. Therefore, the ARC-induced inhibition of angiogenesis may be primarily due to blockade of the ERK signaling pathway. These results suggest that ARC can selectively induce deactivation of ERK1/2 and activation of p38 before the morphological changes of apoptosis in HUVECs.

To confirm the induction of apoptosis by ARC in HUVECs, cells were treated with ARC at various concentrations for different time periods (12 and 24 h), and changes in the activation state of caspase-3 were evaluated. As shown in Figure 3B, treatment of HUVECs with ARC at concentrations of 0–50 μg/mL for 12 and 24 h significantly induced activation of caspase-3 in a time- and concentration-dependent manner. These results further confirmed that activation of caspase-3 by ARC may be involved in the induction of apoptosis in HUVECs. ARC also induced cleaved forms of caspase substrates, poly ADP-ribose polymerase (PARP) and lamin A/C, in a concentration-dependent manner. These results indicated that ARC can induce apoptosis in HUVECs through the activation of caspase-3.

4. Discussion

In our previous paper, we reported that the ethanol extract of Brazilian propolis possesses antiangiogenic activity which is exerted through the induction of apoptosis [3,12]. In addition, purified ARC showed antiangiogenic effects on HUVEC, but its mechanism, such as apoptosis signaling, was not clarified. In this study, we showed that ARC-induced antiangiogenesis also involved apoptosis and changes in intracellular signaling of HUVEC.

ARC, one of the principal phenolic acids found in Brazilian propolis extract, exhibits various biological activities [3,4,5]. ARC exhibits direct anti-proliferative, cytotoxic, and apoptotic effects on cancer cells and inhibits the growth of transplanted solid human and mouse tumors [5]. Xuan et al. recently reported that Brazilian propolis may be an apoptosis-inducing agent associated with the intracellular signaling mediated by integrin [4], p53, reactive oxygen species and mitochondrial membrane potential [13]. However, there have been a limited number of reports concerning the antiangiogenic effects of purified ARC and only a few reports on the effects of ARC on angiogenesis at the molecular level.

ARC treatment inhibited tube formation and reduced the protein levels of angiogenesis-related adhesion molecules. Interendothelial junctions play a critical role in the regulation of endothelial functions, such as angiogenesis, vasculogenesis, and inflammation [14]. VE-cadherin is endothelial-specific and the major component of intercellular adherens junctions [14]. PECAM-1, the endothelial-specific marker, has been implicated in endothelial cell migration during angiogenesis [15]. Thus, the lowering effects of ARC on these adhesion molecules appear to be involved in its antiangiogenic effects.

In this study, we showed that ARC induces apoptosis in endothelial cells. There is a considerable amount of evidence indicating that several members of the mitogen-activated protein kinase (MAPK) superfamily, including ERK, c-Jun N-terminal protein kinases (JNK), and p38 MAPK, play crucial roles in the regulation of angiogenesis [16]. ERK 1/2 and Akt signals are also known as important molecules in endothelial cell survival [17]. Inactivation of one of these survival signals triggers apoptosis through the caspase pathway in endothelial cells [17]. We have previously shown the role of p38 as an apoptosis signal in tube-forming HUVECs undergoing hypoxia-induced apoptosis [17]. Consistent with our previous report, we have shown that apoptosis was induced by ARC via MAPK signaling, down regulation of ERK1/2 signaling and up regulation of p38 signaling, in a concentration-dependent manner in this study.

Furthermore, ARC activated the caspase cascade that leads to oligonucleosomal fragmentation of DNA and cleavage of protein substrates in the cell, thus jeopardizing the maintenance of cellular integrity [18]. Components of the apoptosis signaling cascade include caspases along with several other triggers and regulators [19]. In this paper, ARC increased the expression of cleaved caspase-3 as well as those of cleaved PARP and lamin A/C, which are targeted by caspase-3 and caspase-6, respectively [20,21,22]. Thus, these results suggested that ARC induced caspase-dependent apoptosis in endothelial cells through MAPK signaling.

Health-promoting food factors that are capable of inducing selective apoptosis in cancer cells have garnered much attention in the development of new approaches to cancer prevention. Many natural substances already used in cancer chemotherapy have also been found to possess apoptosis-inducing activity [23,24,25]. Anticancer properties of these substances are considered to be attributable to such apoptosis induction. Since various antiangiogenic stimuli are accompanied by endothelial cell apoptosis, apoptosis induction in endothelial cells should also be given appropriate consideration in cancer prevention [8,26,27]. Similarly, ARC has been studied to induce apoptosis in cancer cells as well as endothelial cells in the previous and current studies [4,9]. Thus, ARC is also a promising candidate as an antitumor agent.

In conclusion, in this study we confirmed that the antiangiogenic effects of ARC were exerted via induction of apoptosis in tube-forming endothelial cells. Especially, the results indicated that ARC was a potent apoptosis-inducing agent as well as an angiogenesis inhibitor; its action was accompanied by activation of p38 and caspase-3, and cleaved PARP and lamin A/C. Furthermore, the inhibitory effect of ARC on tubeformation was implicated as being mediated via inactivation of the survival signal ERK 1/2 and upregulation of apoptosis signaling such as caspase cascades. It implies that ARC might be one constituent of Brazilian propolis which is mainly responsible for its antiangiogenic activity in vivo. These results strongly support the possibility of ARC as an anticancer drug.

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References


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