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The Effect of Caffeic Acid on Wound Healing in Skin-incised Mice

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This study was carried out to investigate the wound healing effect of caffeic acid in skin-incised mice. Caffeic acid showed significant effects on anti-inflammatory activity and wound healing, such as myeloperoxidase activity, lipid peroxidation, phospholipase A_2 activity and collagen-like polymer synthesis, in incised-wound tissue. On the other hand, it significantly stimulated collagen-like polymer synthesis in NIH 3T3 fibroblast cells, while inhibited both silica-induced reactive oxygen species generation and melittin-induced arachidonic acid release and PGE_2 production in Raw 264.7 cells, and histamine release in RBL 2H3 cells stimulated by melittin or arachidonic acid. Therefore, caffeic acid appears to have a potent antioxidant and anti-inflammatory effect in cell culture system, which may be related to wound healing in skin-incised mice.

Key Words: Caffeic acid, Histamine, Arachidonic acid, Collagen, Wound healing

INTRODUCTION

Wound healing involves a variety of processes such as acute inflammation, cell proliferation and contraction of the collagen lattice formed (Bodeker & Hughes, 1996). Several types of cells are recruited to the site of injury to carry out the processes of repair. Following neutrophils and monocytes, fibroblasts are attracted to the site to initiate the proliferative phase of repair. Fibroblasts secrete collagens and glycosaminoglycans of new granulation tissue, subsequently affecting the remodeling of granulation tissue into mature dermis (Clark, 1993). It has been shown that increasing the number of fibroblasts in an artificial dermal substitutes leads to improved healing in experimental wounds (Lamme et al, 2000).

However, the healing process may be prevented by the presence of ROS or microbial infection, since the type of cells to be first recruited to the site of injury is the neutrophil which has a role in anti-microbial defense and the production of ROS. ROS (Reactive oxygen species) are produced in response to cutaneous injury (Gupta et al, 2002) and may cause cellular damage by peroxidation of membrane lipids, inactivation of sulphydryl enzymes, cross-linking of proteins and breakdown of DNA (Russo et al, 2002). These observations suggest that improving local antioxidant activity might be beneficial to healing outcome (James et al, 2001).

Many antioxidants such as vitamin E and plant extracts are used to eliminate the negative effects of ROS on wound healing (Suguna et al, 2002). Caffeic acid is an active component of honeybee propolis (Sudina et al, 1993), and has been shown to have anti-inflammatory, immunomodulatory and antioxidant effects and also to suppress lipid peroxidation (Pascual et al, 1994; Natarajan et al, 1996). Although caffeic acid has antioxidant effects, its effect on cutaneous wound healing has not yet been investigated. Therefore, the aim of the present study was to investigate the effect of caffeic acid on dermal wound healing in mice.

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METHODS

Materials

[^{3}H]arachidonic acid and enzyme-linked immunosorbent assay (ELISA) kit for the determination of prostaglandin E_2 (PGE_2) was provided by Amersham Pharmacia (Piscataway, NJ, USA), and 1-palmytoyl-2-(10-pyrendecanoyl) - sn-glycero-3-phosphorylcholine (10-pyren PC) was from Molecular Probes (Eugene, OR, USA). Caffeic acid and other reagents were purchased from Sigma Chemical Co. (USA).

Animals

Six-week-old BALB/c male mice, weighting 24 to 31 g, were purchased from HanLim Animal (Hwasung-gun, Gyunggi-do, Korea), and were housed in a normal environmentally controlled animal room (temperature 24±2°C, humidity 50±5%, illumination 300～500 Lux). Animal protocols were approved by the ethics committee for care and use of laboratory animals at Chung-Ang University. The animals were anaesthetized with xylazine hydrochloride (2

ABBREVIATIONS: AA, arachidonic acid; CLP, collagen-like polymer; MDA, malondialdehyde; PGE_2, prostaglandin E_2; ROS, reactive oxygen species.

~5 mg/kg) and ketamine hydrochloride (40~50 mg/kg). Their backs were shaved and cleaned with polyvidone-iodine. A linear full-thickness incision of 20 mm in length was made on the back of each mouse and sutured with 4/0 atraumatic silk suture under sterile conditions. The animals were randomly divided into two groups of equal number. Caffeic acid was suspended in 1% carboxymethyl cellulose, and further dilutions were made with saline. After incision, caffeic acid (10 mg/kg) was orally administered to the treatment group (n=15), while 1% carboxymethyl cellulose in equal volume was orally administered to the control group (n=15). Caffeic acid and saline were administered once daily during the study period. On days 5, 10 and 15, five animals in each group were randomly selected and killed with the same anaesthetic solution. Zones of about 1mm in thickness were taken from the edges of the wounds for biochemical analysis.

Malondialdehyde assay

Malondialdehyde (MDA) level was assessed as an index of lipid peroxidation according to the slightly modified method of Gan et al. (2006). Briefly, reaction substrate consisting of 0.1 ml of 8.1% sodium dodecylsulfate, 0.8 ml of acetic acid buffer, 0.8 ml of 0.8% thiobarbituric acid and 0.2 ml of distilled water was added to 0.1 ml of each sample and tetraethoxypropane as standard. Then, all reaction mixtures were incubated at 100°C for 1 h. After cooling on ice-water, 2 ml of n-butyl alcohol was added into the reaction mixtures, and samples were centrifuged for 10 min at 3,000×g. The absorbance of organic layer was measured at 532 nm and results were expressed as μg/mg protein. Protein concentration was measured by the bicinchoninic acid (BCA) method to correct the differences between preparations (Smith et al, 1987).

Myeloperoxidase assay

Tissue samples were assessed biochemically with the neutrophil marker enzyme, myeloperoxidase (MPO), using the method of Bradley et al. (1982). Dorsum tissues were thawed, weighted (50 mg) and homogenized in 10 volumes of ice-cold 80 mM sodium phosphate buffer (SPB), pH 5.4, containing 0.5% hexadecyl trimethyl ammonium bromide with a Polytron homogenizer (Art-Miccra D-8, Moderne ART Lab., Germany) 3 times for 30 sec each on ice. The supernatant (30 μl) was added to a 200 μl of reaction mixture containing 100 μl of 80 mM SPB (pH 5.4), 85 μl of 0.22 M SPB (pH 5.4), and 15 μl of 0.017% H_2O_2. The reaction started by the addition of 20 μl of 18.4 mM 3,3',5,5'-tetramethyl benzidine in 8% aqueous N,N-dimethyl formamide. The mixture was incubated for 3 min at 37℃ and then placed on ice. The reaction was stopped by addition of 30 μl of 1.46 M sodium acetate (pH 3.0). Enzyme activity was determined by measuring absorbance at 620 nm using a spectrophotometer (FL600 Microplate Reader, Bio-Tek, USA).

Collagen-like polymer assay

Collagen-like polymer (CLP) was assessed using the method of Yin et al (2002). Tissues or NIH 3T3 cells were homogenized in buffer (5 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.5) and tissue debris was then removed by centrifugation at 12,000 rpm for 20 min. The supernatant (crude tissue lysate) was collected. Crude tissue lysate was then treated at 80℃ for 10 min and centrifuged at 12,000 rpm for 20 min to remove the precipitated non-CLP proteins. The absorbance was increased by the amino acids and peptides liberated from CLP when hydrolyzed by 300 U/ml collagenase type III (Sigma C 0255). Absorbance was spectrophotometrically measured at 570 nm. The content of CLP in tissue was calculated as a μg/mg protein.

PLA_2 assay with 10-pyren PC

PLA_2 activity was measured using pyrene-labeled phospholipids (10-pyrene PC) (Radvanyi et al, 1989). Tissue homogenate (20 μl) was incubated in the reaction mixture containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA , and 10 μl of 0.2 mM 10-pyren PC (2 μM final concentration, 10 μl of 10% bovine serum albumin solution and 6 μl of 1 M $CaCl_2$ for 20 min. The fluorescence was measured using excitation and emission wavelengths of 345 and 398 nm, respectively (FL600, Microplate Fluorescene Reader, Bio-Tek).

Cell culture

Raw 264.7 cells, RBL 2H3 cells and NIH 3T3 cells obtained from the Korean Cell Line Bank (Seoul, Korea) were cultured in DMEM supplemented with 10% heat-inactivated FBS and antibiotic-antimycotic (100 IU/ml penicillin G, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B) at 37°C with 5% CO_2.

Measurement of ROS generation

The level of intracellular ROS was quantified by fluorescence with DCF-DA. Raw 264.7 cells were suspended in 20 ml of Krebs buffer [137 mM NaCl, 2.7 mM KCl, 0.4 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 1.8 mM $CaCl_2$, 5 mM glucose] and incubated with 20 μM DCF-DA for 1 h at 37°C. The cells were washed twice with Krebs buffer and were suspended in Krebs buffer at the density of 10^6 cells/ml. The cells were incubated with caffeic acid for 10 min, and ROS generation was then induced by 1 mg/ml silica for 30 min at 37°C. The fluorescence intensity was measured using excitation and emission wavelengths of 485 and 535 nm, respectively. (Boland et al, 2000). Values were expressed as percentage of fluorescence in control.

Measurement of [^{3}H]AA release

Raw 264.7 cells were harvested with Krebs buffer and labeled with [^{3}H]AA (0.4 μCi/ml) at 37°C for 2 h. The cells were washed with Krebs buffer containing 0.5 mg/ml BSA to trap the [^{3}H]AA liberated. The release of AA was induced by 0.5 μM melittin in the presence or absence of caffeic acid for 30 min. After centrifugation, the supernatant and pellet were transferred to liquid scintillation vial for radioactivity measurement by liquid scintillation counter (Balboa et al, 1999). Percent release of [^{3}H]AA = supernatant/(supernatant+pellet)×100

Measurement of prostaglandin E_2

Total amount of cellular prostaglandin E_2 in Raw 264.7 cells which were stimulated with melittin was assayed us-

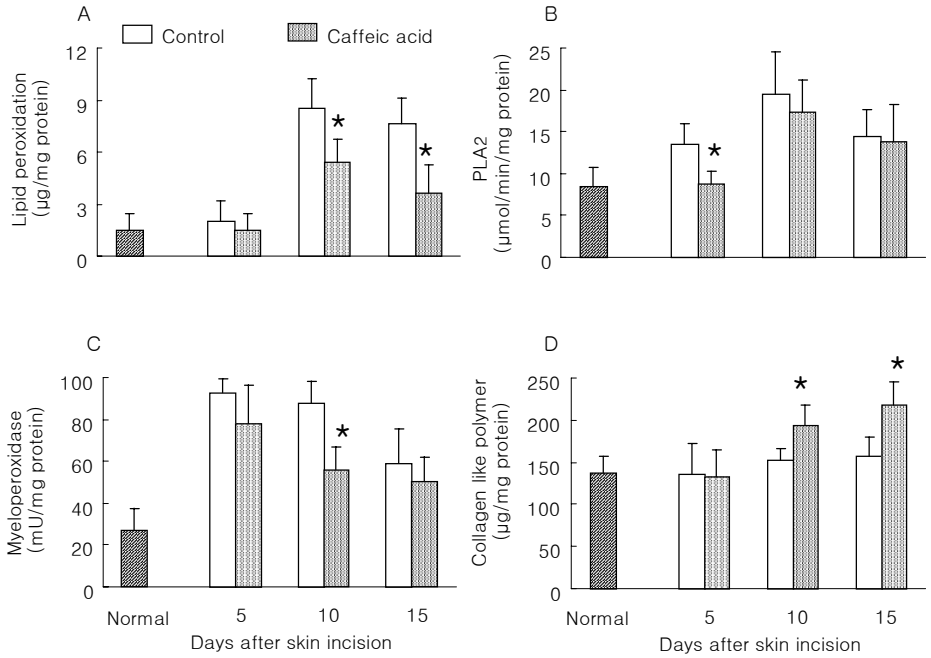

Fig. 1. Effect of caffeic acid on wound healing in skin-incised mice. Two cm long incision wound perforating the skin was made on the dorsal skin of mice. Mice were orally treated daily with 1% carboxymethyl cellulose solution (CMC; Control) and 10 mg/kg caffeic acid in 1% CMC solution during the study period. On days 5, 10 and 15, zones of about 1mm in thickness were taken from the edges of the wounds for biochemical analysis, such as lipid peroxidation (A), phospholipase A_2 (B), myeloperoxidase (C) and collagen-like polymer (D). Results are means±SD from 5 mice. *Significantly different from control ($p < 0.05$).

ing an enzyme immunoassay protocol provided by Amersham Pharmacia Biotech and expressed as ng/mg protein. Protein concentration was measured by the BCA method to correct for differences between preparations.

Histamine assay

RBL-2H3 cells were treated with caffeic acid for 10 min, and then histamine release was induced by melittin and arachidonic acid for 30 min at 37°C. The histamine released was assayed using the fluorometric method (Shore et al, 1959). After centrifugation, histamine contents in both supernatant and pellet were measured with 0.1 ml of 1% o-phthaldialdehyde in methanol. The fluorescence intensity was measured using excitation and emission wavelengths of 355 and 455 nm, respectively. Data are expressed as % release (histamine contents in supernatant / histamine contents in supernatant and pellet×100).

Data analysis

The results are represented as means±S.D. and analyzed statistically by analysis of variance (ANOVA), and differences between groups were determined with Newman-Keuls test. The level of significance was set at less than 5%.

RESULTS

Wound healing effect of caffeic acid

There was a progressive increase in the level of lipid peroxidation in skin-incised tissue on day 10 and showed a slight decrease on day 15 (Fig. 1A). Caffeic acid was shown to significantly reduce tissue lipid peroxidation levels on days 10 and 15 as compared with control. The change of phospholipase A_2 activity in the skin-incised tissue was similar to that of lipid peroxidation. PLA_2 activity of caffeic acid-treated group showed a significant difference on day 5 as compared with control (Fig. 1B).

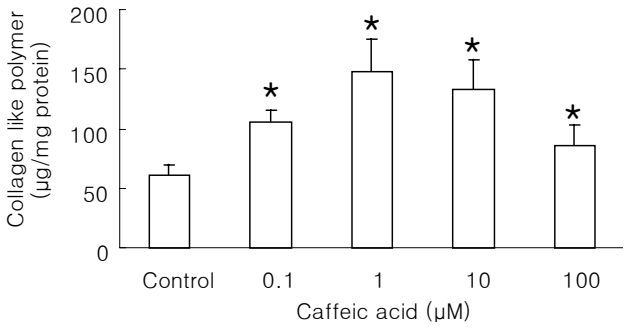

Fig. 2. Effect of caffeic acid on collagen-like polymer production in NIH 3T3 cells. NIH 3T3 cells were incubated with caffeic acid at 37°C for 48 h. Control was the cells treated with 1% DMSO. Results are means±SD from 5 separate experiments. *Significantly different from control ($p < 0.05$).

After wounding, several types of cells, such as neutrophils, monocytes and fibroblasts, are recruited into the site of injury to carry out the repair processes. Therefore, myeloperoxidase activity was used to determine the recruitment of neutrophils into injury site. Fig. 1C shows that myeloperoxidse activity in incised-skin showed a peak level on day 5 and gradually declined to the basal level on day 15. Caffeic acid significantly reduced myeloperoxidse activity on day 10 (Fig. 1C) and progressively increased collagen-like polymer levels with a significant difference on days 10 and 15 as compared with control (Fig. 1D).

In order to investigate the effect of caffeic acid on collagen-like polymer synthesis, we next measured the amount of collagen-like polymer in NIH 3T3 fibroblast cells. Caffeic acid stimulated collagen-like polymer production, the peak

stimulatory effect of which occurred at 1 μM concentration (Fig. 2). Fig. 3 shows that caffeic acid dose-dependently inhibited the silica-induced ROS generation in Raw 264.7 cells.

Anti-inflammatory effect of caffeic acid

To investigate the effect of caffeic acid on anti-inflammatory effect, we measured melittin-induced AA release and PGE_2 production in Raw 264.7 cells. As shown in Fig 4, melittin dose-dependently increased AA release in Raw 264.7 cells and 0.5 μM concentration caused AA release by 12.8%. However, caffeic acid at 30 μM and 100 μM concentrations inhibited 0.5 μM melittin-induced [^{3}H]AA release from 12.8% to 10.2% and 7.8%, respectively (Fig. 4). Also, it dose-dependently decreased the melittin-induced PGE_2 production from 16.2 ng/mg protein to 11.3 and 8.2 ng/mg protein at 30 μM and 100 μM concentrations, respectively (Fig. 4). Caffeic acid significantly inhibited histamine release which was stimulated by melittin and arachidonic acid in RBL 2H3 mast cells (Fig. 5).

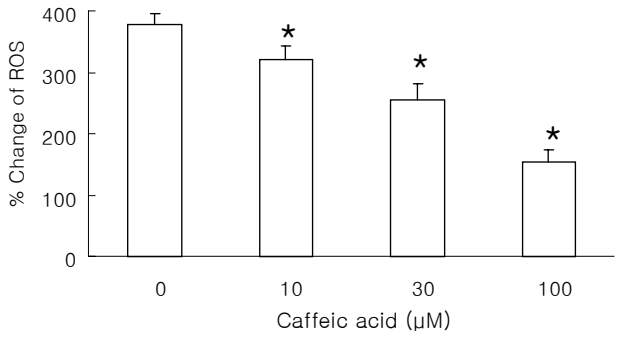

Fig. 3. Effect of caffeic acid on reactive oxygen species (ROS) generation in Raw 264.7 cells. DCF-loaded Raw 264.7 cells were preincubated with caffeic acid and stimulated with 1 mg/ml silica at 37°C for 30 min. Results are means±SD from 5 separate experiments. *Significantly different from control ($p<0.05$).

DISCUSSION

When wounding occurs, it is accompanied within quite a short period of time by pain, and reddening and edema of the surrounding tissue. They are all classical symptoms of inflammation due to the release of prostaglandins, leukotrienes, and ROS. This study was carried out to investigate the wound healing effect of caffeic acid in skin-incised mice. The significant effect of caffeic acid on anti-inflammatory activity and wound healing, such as myeloperoxidase activity, lipid peroxidation, PLA_2 activity and collagen-like polymer synthesis, was shown in incised-wound tissue. After wounding, several types of cells, such as neutrophils, monocytes and fibroblasts, are recruited to the site of injury to carry out the processes of repair. In the present study, mye-

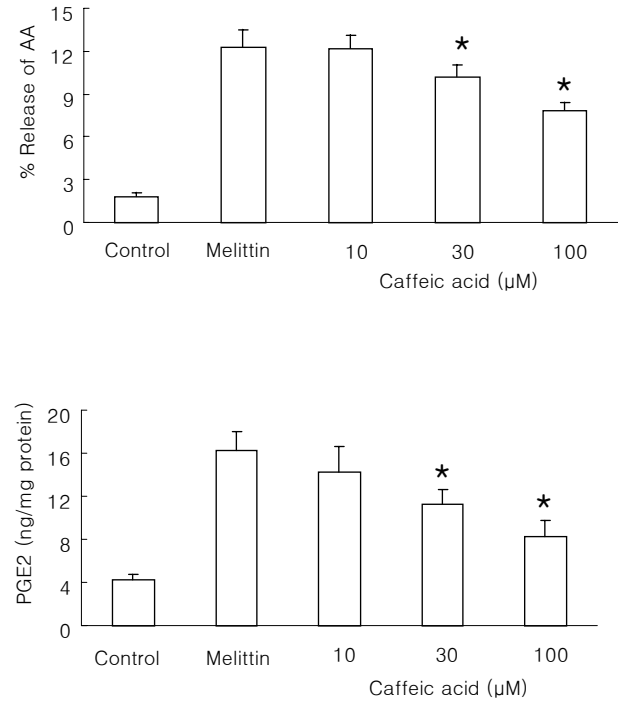

Fig. 4. Effect of caffeic acid on melittin-induced arachidonic acid release and PGE_2 production in Raw 264.7 cells. The cells were stimulated with 0.5 μM melittin in the presence or absence of caffeic acid at 37°C for 30 min. Control was treated with 1% DMSO in the absence of melittin. Intracellular amount of PGE_2 was measured using PGE_2 assay kit. Results are means±SD from 4 separate experiments. *Significantly different from control ($p<0.05$).

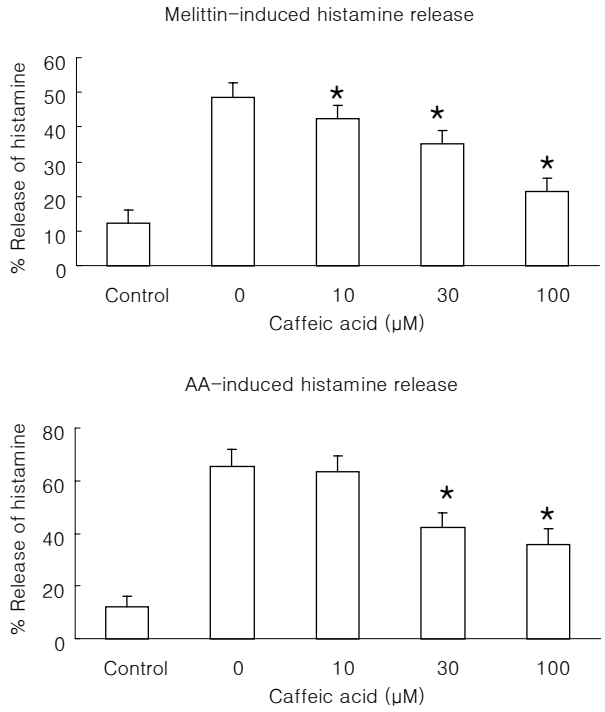

Fig. 5. Effect of caffeic acid on melittin or arachidonic acid-induced histamine release in RBL 2H3 cells. The cells were stimulated with 0.5 μM melittin (A) or 100 μM arachidonic acid (AA; B) in the presence or absence of caffeic acid at 37°C for 30 min. Control was treated with 1% DMSO in the absence of melittin or arachidonic acid. Results are means±SD from 5 separate experiments. *Significantly different from control ($p<0.05$).

loperoxidase activity was used to determine the recruitment of neutrophils into injury site. The results showed that myeloperoxidse activity in incised-skin showed a peak level on day 5 and gradually declined to the basal level on day 15, and that caffeic acid significantly reduced myeloperoxidse activity on day 10 as compared with control. Fibroblasts secrete collagens and glycosaminoglycans of new granulation tissue, subsequently affecting the remodeling of the granulation tissue into mature dermis (Clark, 1993). In the present study, caffeic acid was found to progressively increase collagen-like polymer levels with significant difference on days 10 and 15 as compared with control. It has previously been shown that increasing the number of fibroblasts leads to improved healing in experimental wounds (Lamme et al, 2000). Furthermore, skin fibroblast proliferation is considered to be extremely important in the initial stages of tissue repair and wound healing (Tran et al, 1997).

In NIH 3T3 fibroblast cells, caffeic acid significantly stimulated collagen-like polymer production, in support of the results that caffeic acid increased the collagen-like polymer synthesis in skin-incised mice. These data, it is suggest that caffeic acid has a wound healing effect in skin-incised mice. Also, caffeic acid dose-dependently inhibited silica-induced ROS generation in Raw 264.7 cells. Antioxidant activity of caffeic acid has been shown in cell free system and cell culture system (Pascual et al, 1994; Natarajan et al, 1996). ROS, produced especially during the inflammatory of wound healing process, delays wound healing (Yamasaki et al, 1994). Therefore, antioxidant activity of caffeic acid may contribute to wound healing (Mensah et al, 2001).

In the experiment to investigate anti-inflammatory effect of caffeic acid in cell culture system, caffeic acid was found to significantly inhibit both melittin-induced [^{3}H]AA release and PGE_2 production. An another inflammatory mediator, histamine, released from mast cells in initial inflammatory stages causes vasodilation and attenuates wound healing process (Mills et al, 2007). In the present study, caffeic acid significantly inhibited histamine release stimulated by melittin and arachidonic acid in RBL 2H3 mast cells. These results suggest that caffeic acid has a potent anti-inflammatory activity in cell culture system. In conclusion, therefore, caffeic acid appears to have antioxidant and anti-inflammatory activity in cell culture system, which may be related to wound healing in skin-incised mice.

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