Anti-neoplastic effect of chicken anemia virus VP3 protein (apoptin) in Rous sarcoma virus-induced tumours in chicken

Senthilkumar Natesan,† J. M. Kataria, K. Dhama, N. Bhardwaj and A. Sylvester

Molecular Biology Laboratory, Division of Avian Diseases, Indian Veterinary Research Institute, Izatnagar, Bareilly 243122 (UP), India

The anti-neoplastic effect of chicken anemia virus VP3 protein (apoptin) was investigated in vitro in Rous sarcoma virus (RSV)-transformed chicken embryo fibroblast (CEF) cells and in RSV-induced tumours of specific-pathogen-free (SPF) chicks in vivo. The apoptin gene was cloned in the pVAX expression vector and in vitro expression of the recombinant vector pVAX-CAV-VP3 was confirmed. Two groups of SPF chicks, each containing ten chicks, were used. Chicks in groups I and II were inoculated with RSV at 1 day old. Group I served as the control, receiving pVAX vector without insert, and group II received recombinant vector pVAX-CAV-VP3 containing the apoptin gene, on day 10. An in vitro study confirmed that apoptin induced apoptosis in RSV-transformed CEF cells, which was demonstrated by observation of the characteristic changes of apoptosis using the indirect immunofluorescence technique and acridine orange/ethidium bromide staining. In vivo study also indicated that apoptin induced apoptosis and caused tumour regression by an intratumoral-delivery method. Apoptotic changes, such as nuclear condensation, fragmentation of the chromatin and formation of apoptotic bodies in the tumour cells, were demonstrated by histopathology and acridine orange/ethidium bromide staining. No apoptotic changes were seen in the tumours of the control group. The results of the present study showed that apoptin had an anti-neoplastic effect in vivo and in vitro in RSV-induced tumours. The anti-neoplastic effect is due to apoptin-induced apoptosis. Further improvements in the dose, delivery method and delivery frequency of the apoptin-expressing recombinant vector could help to develop apoptin as an anti-neoplastic drug.

INTRODUCTION

Chicken anemia virus (CAV) belongs to the genus Gyrovirus of the family Circoviridae. It is the causative agent of chicken infectious anaemia, primarily an immunosuppressive disease of young chickens, but which also affects all age groups. Chicken is the only recognized natural host, but serological survey has revealed the prevalence of CAV in domestic and wild birds (Farkas et al., 1998). Clinical disease is noticed mainly in young chicks, which usually acquire the infection vertically, at 10–14 days of age. The disease is characterized by increased mortality, reduced weight gain, anaemia, aplasia of bone marrow and atrophy of thymus (McNulty, 1991; Pope, 1991; von Bulow & Schat, 1997; Coombes & Crawford, 1998; Rosenberger & Cloud, 1998; Todd, 2000; Dhama et al., 2002). The major economic loss caused by this virus is because of severe immunosuppression, which leads to increased mortality due to secondary bacterial or virus infections.

CAV is one of the smallest avian viruses; it is 23–25 nm in size, icosahedral in shape and non-enveloped, having a 2·3 kb, circular, single-stranded DNA genome. The genome encodes three viral proteins (VP1, VP2 and VP3) that are transcribed from a single major transcript of 2·0 kb. It is believed that the CAV genome replicates through the rolling-circle model (Meehan et al., 1992). The virus does not grow in commonly used primary cells and cell lines. Only Marek’s disease virus- or avian leukosis virus-transformed lymphoblastoid cell lines are susceptible to this virus and the virus usually multiplies to low titre. Among the viral proteins, VP1 is the major capsid protein (52 kDa) and VP2 is probably a non-structural protein found in the cells in the early stages of the virus replication cycle (Noteborn & Koch, 1995). VP1 and VP2 are the protective proteins that induce neutralizing antibodies (Koch et al., 1995).
CAV-VP3 is the smallest protein (13 kDa) and is called apoptin, having a unique apoptosis-inducing property (Noteborn et al., 1994; Noteborn, 1999). Its unique property of killing neoplastic cells and not normal cells by inducing apoptosis makes it a potential anti-neoplastic agent (Pietersen et al., 1999; Maddika et al., 2006). The p53 protein is the intracellular mediator of apoptosis, used by most anti-cancer chemotherapeutic agents to produce their anti-cancer effect. Cancer cells develop resistance to the therapy by inducing mutations in the p53 gene. However, it has been shown that apoptin can induce apoptosis independently without the p53 protein (Zhuang has been shown that apoptin can induce apoptosis most anti-cancer chemotherapeutic agents to produce their 1997; Maddika et al., 2006). The p53 protein is the intracellular mediator of apoptosis, used by most anti-cancer chemotherapeutic agents to produce their anti-cancer effect. Cancer cells develop resistance to the therapy by inducing mutations in the p53 gene. However, it has been shown that apoptin can induce apoptosis independently without the p53 protein (Zhuang et al., 1995). Pietersen & Noteborn (2000) suggested that it could be used as a potential agent against cancer cells that are resistant to current chemotherapeutic agents. However, the anti-neoplastic effect of this protein in virus-induced tumours has not yet been studied. The present study investigates the role of apoptin in the acutely transformed tumour induced by Rous sarcoma virus (RSV) in chicken.

**METHODS**

**Viruses.** An Indian field isolate of CAV (CAV-A) and RSV, maintained in the division of Avian Diseases, IVRI, Izatnagar, India, were used in the present study.

**Propagation of virus.** CAV was propagated in MDCC-MSB-1 cells according to the method described by Goryo et al. (1987). Virus inoculum (0-5 ml) was mixed with a cell pellet, suspended in 0-5 ml medium and incubated at 39 °C for 1 h in a 15 ml centrifuge tube. Then, 5 ml RPMI medium was added to the tube and cells were suspended in the medium, seeded into a culture flask and incubated at 39 °C with 5% CO2 in a humidified atmosphere for 72 h. After 72 h incubation, 1 ml cell suspension was transferred into 4 ml fresh medium and incubated. The process was repeated five to seven times to appreciate the cytopathic effect and virus was harvested at every passage.

Chicken embryo fibroblast (CEF) cell cultures were prepared from 10-11-day-old chicken embryos as described previously (Kataria et al., 1997) with modifications. The embryos were removed under aseptic conditions and washed three times with Hank’s balanced salt solution (HBSS). Using sterile scissors, the viscera, head and appendages of the embryos were removed. The tissue was minced and washed twice with sterile HBSS. The minced tissue was trypsinized by using 0.5% trypsin solution (which was kept warm at 37 °C) in a trypsinnization flask with gentle stirring on a magnetic stirrer for 15 min at room temperature. The cell suspension was then filtered through a gauge 25 filter. The cell concentration was adjusted to 2 x 10^6 cells/ml (growth medium). Freeze-dried RSV prepared from the tumour suspension was dissolved in 2 ml HBSS and filtered through a 0.4 μm syringe filter. The filtrate (0.5 ml) was mixed with CEF cells (2 ml per well), seeded in a six-well culture plate and incubated in a CO2 incubator.

**PCR amplification of the apoptin gene.** PCR amplification of the apoptin gene was carried out by using the primer set VP3F (5'-ATGAAACCCTCTCCAAGAAG-3') and VP3R (5'-CTTACAGCTTATACACTT-3'). The expected product size is 367 bp, containing the complete apoptin open reading frame. The reaction mixture was prepared in PCR buffer containing 1.5 mM MgCl2, 200 μM each dNTP and 10 pmol each primer. Taq polymerase (1.0 unit; Promega) and 1 ng template DNA were added to a total reaction volume of 25 μl. The reaction was carried out in an automated thermal cycler (PTC 200; MJ Research) with an initial denaturation for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min and the final extension was carried out at 72 °C for 5 min. A specific 367 bp PCR-amplified product was purified from the gel by using a QIAex II gel-extraction kit (Qiagen). It was ligated into EcoRI-digested and dephosphorylated pvXV expression vector (Invitrogen). The ligation mixture was used to transform One-Shot Top-10 competent cells (Invitrogen). The bacterial colonies were picked and grown in 3 ml Luria–Bertani broth (LB) medium. The clones were screened for the right orientation by PCR using the T7 promoter forward primer of the vector and reverse primer of the insert. Clones that gave positive amplification by PCR were further confirmed by nucleotide sequencing using T7 forward and BGH reverse primers. Transformed bacteria of an individual positive clone were grown in 200 ml LB medium overnight at 37 °C in a shaking incubator. The plasmid was purified by using a plasmid midi-prep kit (Qiagen). The purified plasmid was used for in vitro expression studies.

**In vitro expression studies.** Human hepatoma cell line Hep-2 showing 50% monolayer at 20 h in a six-well culture plate was used for expression studies of the pVAX-CAV-VP3 plasmid. The monolayer cells were transfected using Lipofectamine reagent (Invitrogen). Six micrograms of the plasmid and 12 μl Lipofectamine Plus reagent were mixed in 100 μl OptiMEM medium (Invitrogen) in a 1:5 sl sterile microcentrifuge tube (tube 1) and were allowed to stand at room temperature for 15 min. Simultaneously, 8 μl Lipofectamine was mixed with 100 μl OptiMEM medium in tube 2 and kept at room temperature for 15 min. The contents of tube 1 and 2 were then mixed together and kept at room temperature for 15 min. The monolayer in the six-well plate was washed with sterile PBS and 2 ml growth medium containing 5% fetal bovine serum was added. Eight hundred microlitres of OptiMEM medium was added drop-wise to the Lipofectamine/DNA mixture and mixed well. The content was added to the wells containing the cell monolayer drop-wise and incubated at 37 °C with 5% CO2. After 4 h incubation, the medium over the cells was removed and fresh medium was added. At the end of 48 h, medium was removed completely, cells were washed with PBS (pH 7.5), fixed using methanol for 20 min and stained.

Immunostaining of the pVAX-CAV-VP3-transfected Hep-2 cells showed clear intranuclear immunofluorescence, which confirmed the in vitro expression of apoptin and its intranuclear localization in transformed cells (Fig. 1). It also showed characteristic changes of apoptosis, i.e. nuclear condensation, fragmentation of chromatin and formation of apoptotic bodies.

**Indirect immunofluorescent staining.** The methanol-fixed monolayer was washed three times using PBS and 1:100-diluted CAV-specific polyclonal serum was added to the apoptin plasmid-transfected cells. The plate was incubated at 4 °C overnight. The wells were washed three times with PBS and 1 ml 1:30-diluted anti-chicken–fluorescein isothiocyanate (FITC) was added to the apoptin plasmid-transfected wells. The plate was incubated for 1 h at room temperature. The wells were washed three times with PBS and 1 ml 50% (v/v) buffered glycerol saline (pH 7.2) was added per well. The plate was visualized under an inverted fluorescence microscope.
Anti-neoplastic effect of apoptin in RSV-induced tumours

Bulk purification of pVAX-CAV-VP3. The expression plasmid pVAX-CAV-VP3 was bulk-purified by using a Mega/Giga plasmid-purification kit as described in the manufacturer’s protocol (Qiagen).

In vitro study of the anti-neoplastic effect of apoptin. The in vitro anti-neoplastic effect of apoptin was studied in CEF cells transformed by using RSV and transfected with recombinant plasmid pVAX-CAV-VP3; cytomorphological alterations revealed apoptosis. CEF cells (1 x 10^5) were mixed with RSV inoculum (1 x 10^6 focus-forming units ml^-1) and incubated for 1 h at 37 °C. The cells were washed once with PBS and seeded in four wells (2 - 5 x 10^5 cells per well) of a six-well culture plate. Similarly, mock-infected control cells were seeded in two wells of the same six-well culture plate. After 24 h, the monolayer CEF cells were transfected with apoptin recombinant plasmid pVAX-CAV-VP3 and mock transfection was performed using the pVAX plasmid. Apoptotic changes in the transfected cells were studied by the indirect immunofluorescence technique and acridine orange/ethidium bromide staining.

In vivo study of the anti-neoplastic effect of apoptin

Specific-pathogen-free (SPF) chicks were divided into two groups with ten chicks in each and were used to study the anti-neoplastic effect of apoptin. The experiment was carried out according to the following schedule.

**Group I.** RSV (freeze-dried tumour suspension dissolved in sterile PBS) was inoculated subcutaneously on day 1 into one of the wings and pVAX plasmid was injected intratumorally at a dose rate of 100 µg per bird on day 10.

**Group II.** RSV was inoculated subcutaneously on day 1 into the wing and pVAX-CAV-VP3 plasmid was injected intratumorally at a dose rate of 100 µg per bird on day 10.

In groups I and II, all of the birds were sacrificed at 20 days of age to study the tumour mass : body mass ratio and tumour tissues were collected. The collected tissues were analysed for cytomorphic changes of apoptosis by the following methods.

**Acridine orange/ethidium bromide staining.** Fine cryosections of 2–3 μm tumour tissue were cut in a cryotome (International Equipment Company). The sections were fixed on clean microscope slides by using chilled acetone for 15 min. The fixed slides were used for acridine orange/ethidium bromide staining and indirect immunofluorescent staining as described previously (Lam & Vasconcelos, 1994; Dham et al., 2002). The fixed section was washed three times in PBS (pH 7.5) and an acridine orange/ethidium bromide stain mixture was added to cover the entire tissue section. After 30 min incubation, the slide was washed three times in PBS and air-dried. A drop of PBS was added to cover the stained section and the slide was viewed under a UV microscope (Nikon).

**Indirect immunofluorescence technique.** Sections, made as described above, were washed three times in PBS and a 1:50-diluted anti-CAV chicken serum was added. The slide was incubated for 1 h at 37 °C in a moist chamber. Then, it was washed three times with PBS (pH 7.5) and 1:3-diluted anti-chicken–FITC conjugate was added and incubated for 1 h at 37 °C. After three washes, the slide was air-dried and 20 μl 50 % (v/v) glycerol/saline was added. A cover-slip was placed on the glass slide and it was visualized under a UV microscope (Nikon).

**Histopathology.** The tumour tissues were collected in 10 % formalin and fine sections were made with a microtome. The sections were stained by using haematoxylin/eosin (H&E) stain and studied for apoptotic changes under the light microscope as described by Lee (1993).

**Tumour mass : body mass ratio.** The total body mass of each chick was determined and the tumour tissue separated from the wing was weighed in an electronic weighing balance. The tumour mass : body mass ratio was calculated by using the formula (tumour mass / body mass) × 100. The data obtained were analysed statistically.

**RESULTS**

**In vitro anti-neoplastic effect of apoptin**

The in vitro anti-neoplastic effect was studied by analysing the cytomorphological changes using acridine orange/ethidium bromide staining and fluorescent antibody staining of CEF cells transformed with RSV, which were also transfected with pVAX-CAV-VP3. Indirect immunofluorescent staining using the CAV-specific polyclonal antiserum in apoptin-transfected RSV-transformed CEF cells showed expression of apoptin inside the nuclei of the transformed cells and cytomorphological changes of apoptosis (Fig. 2). Acridine orange/ethidium bromide staining revealed apoptotic changes of nuclear condensation, fragmentation of nuclei and formation of small,
rounded apoptotic bodies (Fig. 3). The study revealed clearly that apoptin was expressed in the pVAX-CAV-VP3-transfected RSV-transformed CEF cells to a high level and induced cell death via apoptosis, as confirmed by indirect immunofluorescent staining and acridine orange/ethidium bromide staining. No such changes were observed in control CEF cells transformed with RSV.

**In vivo anti-neoplastic effect**

The anti-neoplastic effect of the recombinant pVAX-CAV-VP3 plasmid was studied in tumours induced with RSV in SPF chicks. The study was carried out in two groups as described in Methods. Group II, in which the tumour was induced on day 1 and recombinant plasmid was injected intratumorally, showed tumour suppression due to apoptin-induced apoptosis. Grossly, the site of injection of pVAX-CAV-VP3 in the tumour showed contraction and formation of scar tissue in the chicks of group II (Fig. 4). No gross or microscopic apoptotic changes were seen in the tumours of control group I.

Analysis by the indirect immunofluorescence technique of tumour tissues collected from chicks of group II revealed expression of apoptin in the cells of the tumours (data not shown). Analysis of the tissue by using acridine orange/ethidium bromide staining also revealed the characteristic sequential changes of apoptosis in nuclear material, i.e. condensation, fragmentation and formation of apoptotic bodies (Fig. 5) in the chicks of group II. No nuclear damage could be identified in the chicks of control group I.

Histopathological analysis of the tumour tissues obtained from group II showed progressive changes of cell death due to apoptosis. The cells showed condensation of nuclei, fragmentation of chromatin and formation of apoptotic bodies (Fig. 6). The apoptotic changes were progressive towards the healthy portion of the tumour tissue, spreading from a central zone towards the periphery. Cells that underwent apoptosis showed loss of nuclear material and dissolution of cell structure adjacent to the normal cells in the tumour tissue sections. Between the healthy cells and degenerated cells with dissolved nuclei, a layer of cells showing all characteristic changes of apoptosis was observed.

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**Fig. 2.** RSV-transformed CEF cells showing intranuclear immunofluorescence, indicating apoptin expression and apoptotic changes, at 48 h post-transfection with pVAX-CAV-VP3 plasmid (b) or control pVAX plasmid (a). Viewed by the indirect immunofluorescence technique. Magnification, ×400.

**Fig. 3.** Acridine orange/ethidium bromide staining showing apoptotic changes, i.e. nuclear condensation, fragmentation and formation of small, rounded bodies, in RSV-transformed CEF cells transfected with pVAX-CAV-VP3 plasmid (b, c), along with control, pVAX plasmid-transfected CEF cells (a). Magnification, ×400.
This indicated that the apoptosis was progressive towards the healthy tumour cells.

Tumour mass and body mass of chicks sacrificed at 20 days of age were measured. The mean tumour masses of the chicks of groups I and II were $6.05 \pm 4.47$ and $3.96 \pm 2.47$ g, respectively. The mean body masses of the chicks of groups I and II were $122.60 \pm 26.32$ and $124.00 \pm 6.05$ g, respectively. The tumour mass : body mass ratios of groups I and II were $4.97 \pm 3.29$ and $3.26 \pm 2.09$, respectively. Statistical analysis indicated that groups I and II were not significantly different in the level of tumour suppression; however, the apoptin-injected group showed a lower tumour mass : body mass ratio than the control groups, which indicated that regression due to the apoptin plasmid occurred to a mild extent. The apoptin plasmid-injected group also showed gross regression around the site of the apoptin-plasmid injection. Microscopic analysis of the tissue section also revealed that the anti-neoplastic effect of apoptin in tumour tissue is by induction of apoptosis. The in vitro observations also revealed that apoptin induced apoptosis in RSV-transformed CEF cells.

**DISCUSSION**

The present study was undertaken to observe the effect of apoptin in RSV-induced tumours in vivo and in vitro and, in the event of tumour suppression, whether this is due to apoptosis induced by apoptin. We cloned the apoptin gene in the pVAX expression vector and confirmed in vitro expression in Hep-2 cells. The recombinant plasmid pVAX-CAV-VP3 was used to study the anti-neoplastic effect of apoptin in an RSV-induced in vivo and in vitro transformation system. The in vitro study was carried out in RSV-transformed CEF cells by transfecting pVAX-CAV-VP3 and the in vivo study was carried out by intratumoral injection of pVAX-CAV-VP3 plasmid into RSV-induced tumours of young chicks. Expression of apoptin in transformed cells was observed by employing the indirect immunofluorescence technique and induction of apoptosis was observed by employing acridine orange/ethidium bromide staining and histopathology. In RSV-transformed CEF cells, expression of apoptin could be seen at 48 h post-transfection and induction of apoptosis by the apoptin in these cells was confirmed by the characteristic cytological changes of nuclear condensation, fragmentation and formation of small, rounded apoptotic bodies. Similar characteristic morphological changes of apoptosis have been reported by using the same techniques (Lee, 1993; Lam & Vasconcelos, 1994; Vasconcelos & Lam, 1994). The in vivo
study in chicks was done by first inducing tumour in the wings by injecting RSV at 1 day of age and direct intratumoral delivery of pVAX-CAV-VP3 on day 10, with appropriate controls. The chicks were sacrificed humanely on day 20 and tumour mass and body mass were measured. The tumour tissue was collected for making cryosections and histopathological sections. We found that histopathological analysis of RSV-induced tumour tissue stained with H&E was very useful to study the changes of apoptosis. It revealed the distinct characteristics of cells undergoing apoptosis, showing red-coloured cytoplasm with purple-coloured, condensed, spherical nuclear chromatin structures inside the cells undergoing apoptosis. At lower magnification under a light microscope, an opaque zone in the centre, containing cells at different stages of apoptosis, surrounded by a peripheral zone containing healthy cells with normal nuclei, was observed. Between these two zones of healthy and apoptotic tumour cells, a thin layer of cells containing both apoptotic and healthy cells was noticed. It

Fig. 6. Histopathological examination of RSV-induced tumour tissue section, stained with H&E and showing apoptotic changes, i.e. nuclear condensation, fragmentation and formation of small, rounded apoptotic bodies, at different stages in tumours that received intratumoral delivery of pVAX-CAV-VP3 (b, c), along with a control that received pVAX plasmid intratumorally (a). Magnification, ×200.

Fig. 7. Histopathological examination of apoptin plasmid pVAX-CAV-VP3-injected tumour tissue section stained with H&E, showing a zone of cells with completely dissolved nuclei and another zone of cells with apparently normal nuclei. Cells containing partially dissolved nuclei and cells with apparently normal nuclei can be seen between these two zones. Magnification, ×100 (a); ×200 (b). Control plasmid pVAX-injected tumour tissue showed normal nuclei (magnification, ×200) (c).
indicated that the zone of apoptosis was progressing from the central zone towards the peripheral healthy tissue. The central zone might be the site of pVAX-CAV-VP3 plasmid DNA deposition by intratumoral delivery. Acidine orange/ethidium bromide staining also revealed characteristic changes of apoptosis in cells of tumours that received pVAX-CAV-VP3 plasmid. No such changes could be seen in tumours of control chicks.

Intranuclear localization of apoptin was noticed both in RSV-transformed CEF cells in vitro and in RSV-induced tumour cells in vivo by the indirect immunofluorescence technique. It has been reported previously by many researchers that intranuclear localization of apoptin is vital to induce apoptosis (Danen-van Oorschot et al., 2003; Leliveld et al., 2003a, b). Leliveld et al. (2003b) reported that apoptin predominantly co-localized with heterochromatin and nucleoli within tumour cells and formed distinct superstructures, containing around 20 multimeric apoptin complexes with DNA and approximately 3 kb in size. Danen-van Oorschot et al. (2003) reported that the C-terminus of apoptin contains a bipartite-type nuclear-localization signal and two domains that induce apoptosis independently. Both domains have a strong correlation between nuclear localization and killing activity. They concluded that nuclear localization alone was not sufficient for the apoptin to become active and to induce apoptosis leading to cell death.

Tumour suppression was noticed in RSV-induced tumours at the site of injection; however, the suppression level was not significantly different from the control group, as determined quantitatively by tumour mass:body mass ratio. The reason could be the faster growth rate of RSV-induced tumours compared with the rate of apoptin-induced cell death. The dose, frequency, method and site of delivery of the apoptin-expressing plasmid could also influence the rate of tumour suppression by apoptin. In a previous study, the anti-tumour efficiency of apoptin was evaluated by injecting a mixture of pcDNA-VP3 and murine liver carcinoma cell line H22 subcutaneously into BALB/c mice. The results suggested that injection of the pcDNA-VP3/H22 mixture results in a significant reduction of tumour growth in mice due to apoptosis, compared with the rate of apoptin-induced tumours in nude mice treated with multiple injections over a period of 10 days and showed complete regression of the tumours. Similarly, administration of therapeutic DNA of apoptin and E4orf4 genes by electroporation into murine B16 (F10) tumours showed distinct tumour-growth inhibition only during the treatment. Cessation of therapy caused tumour regrowth. Obviously, the efficiency of gene transfer using electroporation was low and did not induce a permanent therapeutic effect (Mitrus et al., 2005).

The results of the present study showed that apoptin has an anti-neoplastic effect in vivo and RSV-induced tumours. The anti-neoplastic effect is due to apoptin-induced apoptosis. When the apoptin-expression plasmid was delivered intratumorally, induction of apoptosis was observed in tumour cells by employing different techniques. Further improvements in dose and delivery method of the apoptin-expressing plasmid by either the local or systemic route could help to develop apoptin as an anti-neoplastic drug. Further knowledge on how apoptin differentiates normal cells from tumour cells and specific induction of apoptosis in tumour cells will help to design an effective delivery method targeting specific tumour cells.

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