mySCIENCE 1(1), 2006, 79–92

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Research Article

Curcuma aromatica extract induces apoptosis and inhibits angiogenesis in Ehrlich Ascites Tumor cells *in vivo*

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(*Received November 2004; Accepted February 2005*)

Abstract

Formation of new blood vessels is critical for the growth of the tumor. Angiogenic factor, VEGF, secreted by the tumor cells induces neoangiogenesis. Inhibition of tumor angiogenesis and/or activation of tumor cell apoptosis will inhibit the growth of the tumor. In the present study, we have identified that, an ethanolic extract from the dried rhizome of the plant *Curcuma aromatica* (CAE), has potent antiangiogenic and pro-apoptotic activity in *in vivo* conditions. The growth of Ehrlich ascites tumor (EAT) cells and formation of ascites in the peritoneum of EAT bearing mice was inhibited by CAE. Our results on nuclear staining, formation of the characteristic DNA fragmentation ladder in agarose gel electrophoresis and downstream activation of an endonuclease by the CAE *in vivo* clearly demonstrate the pro-apoptotic activity induced by CAE. Further, results on decrease in the peritoneal angiogenesis and microvessel density shows the antiangiogenic potential of the CAE *in vivo*. The molecular mechanism of antiangiogenic effect of CAE involves inhibition of VEGF in CAE-treated mice. Taken together we conclude that the ethanolic extract of *Curcuma aromatica* has potent antiangiogenic and pro-apoptotic properties that can further be developed into potential anticancer drug.

Keywords: *Curcuma aromatica*, Ehrlich ascites tumor cells, antiproliferation, antiangiogenesis, proapoptosis.

Introduction

Angiogenesis is a tightly regulated process involving the formation of new blood vessels from the pre-existing ones and is critical in many physiological conditions such as pregnancy and wound healing. However, in pathological conditions such as inflammatory diseases and cancer, a chronic unregulated angiogenic state often exacerbates the disease (Folkman 1971). Cancer cells

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are known to secrete potent angiogenic growth factors. The levels of these factors in the serum and urine of cancer patients have been shown to correlate with disease status (Yabushita et al 2003). Among those angiogenic factors VEGF is one of the best characterized and studied (Ferrara et al 2003). VEGF is an endothelial cell-specific mitogen that promotes many other events necessary for angiogenesis and plays an important role in the growth and metastasis of cancer by promoting neovascularization (Terman and Stoletov 2001). VEGF exerts its actions by binding to two tyrosine kinase receptors, fms-like tyrosine kinase (Flt-1) and fetal liver kinase-1/kinase insert domain-containing receptor (Flk-1/KDR). Both have been shown to be almost exclusively expressed in endothelial cells (Griffioen and Molema 2000). It is known that tumors are angiogenesis dependent. Administration of an angiogenesis inhibitor, which is not directly cytotoxic to tumor cells, can increase tumor cell apoptosis, decrease VEGF production, and regress tumor growth either by inhibiting endothelial cell proliferation and formation of new capillaries feeding the growing tumor or by inducing tumor and/or endothelial cell apoptosis (Folkman 2003).

The understanding of the mechanisms that regulate angiogenesis and the discovery and successful use of several angiogenesis inhibitors in animal models, have led to clinical applications of antiangiogenic therapy. This approach, in principle, has several advantages over traditional chemotherapy, including less toxicity and side effects, a general broad spectrum of activity and reduced risk for development of resistance (Bisacchi et al. 2003).

Today, the number and type of antiangiogenic drugs are continuously growing. Anticancer properties have been associated with the components of various natural products including green tea polyphenols, *resveratrol, capsaicin*, taxol, berberine and curcumin. (Wu et al. 1999; Benelli et al. 2002; Gururaj et al. 2002; Niles et al. 2003; Min et al. 2004). The induction of apoptosis in tumor cells and inhibition of angiogenesis has been shown to be the most common anticancer mechanism conjoint by many cancer therapies. Thus to find the potential therapeutic anticancer drugs with potent and selective pro-apoptotic and antiangiogenic properties would be valuable. *Curcuma aromatica*, one of the aromatic and pretty ginger belonging to the genus *Curcuma* (Zingiberaceae) is a wild plant of the forests of western ghats and Bengal in India. It has long been used in traditional medicine for the treatment of cancer and lung diseases. It also promotes secretion of bile and has strong antibiotic properties (Behura et al. 2002). Oil extracted from *C. aromatica* has been shown to exert antitumor activity on various cancer cells *in vitro* and *in vivo* (Shi et al. 1981; Wu et al. 2000). Anticancer molecule A- elemene has also been isolated from volatile oil of *C. aromatica* (Dong et al. 1997). However, the mechanism responsible for the anticancer activity is still not clear and needs further investigation. In a screen for naturally

occurring angiogenic inhibitors, we have identified that an ethonolic extract from the dried rhizome of the plant *C. aromatica* has potent antiangiogenic and pro-apoptotic activity in Ehrlich ascites tumor (EAT) cells model *in vivo*.

Materials and Methods

Plant Material

Rhizomes of *Curcuma aromatica* were collected from Western Ghats of Karnataka, India. The rhizomes were air dried in shade and powdered using commercial blender.

Animals and Chemicals

Swiss albino mice were obtained from animal house, Department of Zoology, University of Mysore. Mysore, India. Ehrlich Ascites Tumor (EAT) cells were obtained from American type cell culture, Rockville, USA. Trypan blue and agarose were procured from Hi- Media, India. Giemsa stain was obtained from Qualigens, Mumbai; India. Proteolytic inhibitors from Boehringer, Manhiem, Germany. Alkaline phosphatase- tagged goat anti-rabbit IgG was procured from Genei, Bangalore, India. Recombinant VEGF165 was used as an antigen for producing rabbit anti-VEGF polyclonal antibodies. All chemicals and reagents were of highest grade commercially available.

Preparation of *Curcuma aromatica* Extract (CAE)

Powdered *C.aromatica* was extracted (1:1 weight/volume) using 50% ethanol overnight at room temperature. The residue was removed by filtration and the extract was allowed to evaporate at 30°C under reduced pressure in a rotary evaporator. The extract was then centrifuged at 5,000 rpm for 10 min and the supernatant was dialyzed against distilled water and the pH was adjusted to 7.0 before storing at 4°C for all further experiments.

Culture and isolation of Ehrlich ascites tumor (EAT) cells and ascites fluid

EAT cells are mouse mammary carcinoma cells that grow as ascites tumor in peritoneal cavity of mice. EAT cells were grown in the peritoneal cavity of eight to ten weeks old Swiss albino mice by peritoneal transplantation of 0.5 ml of cell suspension (5×10^6 cells/ml) in sterile citrated saline (0.9%). CAE (50 μl) was injected into the EAT bearing mice every alternate day after 5 days of tumor transplantation. The weight of the animals was recorded

from the day of transplantation to sacrifice. The rationale for injecting crude extract on the 6^{th} day is to stimulate the natural state where a partially or wholly grown tumor has to be treated. The animals were sacrificed on 13^{th} day. The cells were harvested in saline after 10-12 days of transplantation and separated from ascitic fluid by centrifugation at 400 g for 8 minutes. Contaminating RBCs (if any) during isolation of EAT cells were lysed using 0.4% ammonium chloride. After sacrifice of treated and control EAT bearing animals, an incision was made in the abdominal region and EAT cells along with the ascites fluid were harvested into a beaker containing 2 ml of saline and centrifuged at 3000 rpm for 3 min. The pelleted cells were resuspended in saline and placed on ice. Cells were counted and the viability was determined using trypan blue dye exclusion method. The ascites volume was measured by subtracting 2 ml of volume of saline used for collecting the cells and ascites.

Giemsa and Acridine orange/ethidium bromide staining

Both treated (*in vivo*) and control cells were fixed in a solution of methanol: acetic acid (3:1). The fixed cells were smeared on clean microscopic glass slides and slides were stained with 0.1% Giemsa stain and observed under Nikon compound microscope.

Nuclear staining (Srinivas et al. 2003) was performed using control as well as CAE treated EAT cells after washing the cells once with phosphate-buffered saline (PBS), the cells were stained with 100 μl of a mixture (1:1) of acridine orange/ethidium bromide. The cells were immediately washed once with PBS and viewed under Leitz-DIAPLAN florescent microscope and photographed.

DNA extraction and agarose gel electrophoresis

DNA from control and CAE treated cells was isolated. In brief, cells were washed twice in PBS at 4°C and pellets were lysed using SDS (10%) for 30 min. Potassium acetate (8M) was added to the supernatant and incubated for 1h at 4°C. After spinning at 7000 rpm, an equal volume of distilled phenol, chloroform and isoamyl alcohol (25:24:1) mixture was added. The mixture was subjected to centrifugation for 30 min at 3000 rpm. The above step was repeated twice using the supernatant obtained and to the final supernatant, an equal volume of chloroform was added. After centrifugation at 2000 rpm for 30 min at room temperature, 20 μl/ml of RNase was added and incubated for 30 min at 37°C. Later, 2 volumes of chilled ethanol was added and allowed to stand at 20°C overnight. The solution was centrifuged (10, 000 rpm for 1h at 4°C) and the pellet obtained was dissolved in Tris-EDTA (TE) buffer. DNA was estimated by recording the absorbance at 260 nm and 280 nm. DNA was electrophoresed on 2% agarose gel at 50V using TBE buffer. DNA was

visualized by incorporation of ethidium bromide (1mg/ml) in the gel during casting and viewed under UV illumination (302 nm). Documentation was done using BIO-DOC IT^{TM} system.

Endonuclease assay

Endonuclease assay was performed by the method previously described (Stein et al. 1999) with slight modification. In brief, 1% agarose was prepared in distilled water to which 10 mg/ml of ethidium bromide and 50 mg/ml of heat denatured salmon sperm DNA was incorporated. Agarose was allowed to solidify in petriplate and wells were made by sterile cork borer. Cytosolic extracts were prepared using CAE treated or control EAT cells in a buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EDTA, 0.25 M sucrose, 1 mM PMSF, 2 mg/ml leupeptin and 10 mg/ml aprotenin. Equal concentration of protein was loaded to each well and incubated for 12 h at 37°C in a humidified atmosphere. Petriplates were visualized using transilluminator and the activity of endonuclease was observed as lysis zone around the well and photographed.

Peritoneal angiogenesis and micro vessel density (MVD)

After harvesting the EAT cells from control or CAE treated animals, the peritoneum was cut open and the inner lining of the peritoneal cavity was examined for extent of neovasculature and photographed. Formaldehyde fixed and paraffin embedded tissues of peritoneum from EAT bearing mice either treated or untreated with CAE was taken and 5 micron sections were prepared using automatic microtome (SLEE Cryostat) and stained with Hematoxylin and Eosin. The blood vessels were counted.

Quantification of VEGF

Indirect ELISA was carried out in 96 well microplates. Each well was coated with 10μl of ascites fluid collected during the sacrifice of EAT bearing animals treated and untreated with CAE in coating buffer (50 mM carbonate buffer pH 9.6) and plates were incubated overnight at 4°C. Subsequently, the wells were washed and blocked using 5% skimmed milk in PBS for 2 h at 37°C.

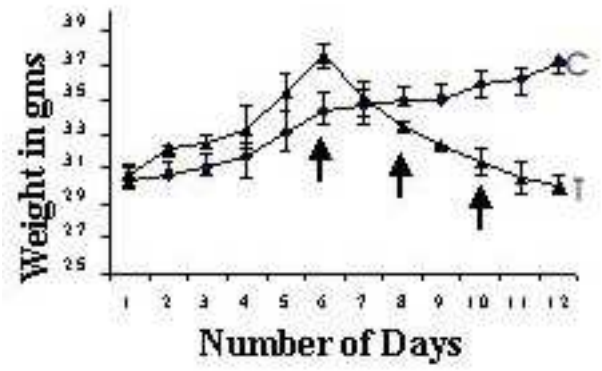

Figure 1: Effect of CAE on *in vivo* growth of Ehrlich ascites tumor. Arrows indicate *in vivo* treatment A. Control, T. CAE treated.

After washing, anti-VEGF165 antibody was added and the plates were incubated for 2 h at 37°C. The plates were washed 100 μl/well of goat anti-rabbit IgG conjugated to alkaline phosphatase (1:5000) was added. After 1h at 37°C, plates were washed and developed using 100 μl of p-nitro-phenyl phosphate (PNPP). The reaction was terminated by addition of 0.1 N NaOH and absorbance was read at 405 nm in microplate ELISA reader. The experiment was carried out in triplicates.

Results

Effect of CAE on growth of EAT cells *in vivo*

Upon treatment of CAE to mice transplanted with EAT cells; there was a decrease of nearly 50% of the body weight of the animals when compared to the weight of the control animals (figure 1). This effect of CAE extract on *in vivo* growth and proliferation of EAT cells is more evident in the results on EAT cell number and ascites volume obtained from both control and CAE treated animals. The results in figure 2 indicate that 2.4×10^9 cells/animal were obtained from control mice, where as in CAE treated animals the cell number is 0.4×10^9 cells/animal. The results clearly indicate that CAE inhibits proliferation of EAT cells. CAE is not cytotoxic to the EAT cells as verified by trypan blue exclusion method. In EAT bearing mice; a total volume of 6 to 7 ml of ascites accumulates during the tumor growth period. The volume of ascites fluid was reduced to 1.5 ml in CAE-treated mice resulting in a 3-fold decrease in ascites volume burden (figure 3).

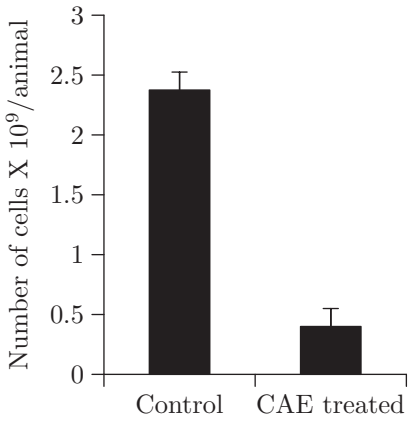

Figure 2: Effect of CAE on Ehrlich ascites tumor cell Number.

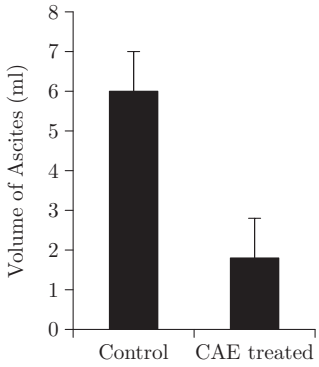

Figure 3: CAE inhibits formation of ascites fluid in Ehrlich ascites tumor bearing mice.

Effect of CAE on apoptosis of EAT cells *in vivo*

The result on inhibition of growth of EAT cells *in vivo* clearly indicate the involvement of the process of apoptosis. EAT cells from CAE treated mice *in vivo* or untreated were stained with Giemsa (1%) or ethidium bromide/acridine orange stain (1:1). The results indicated the typical apoptotic morphology including condensed kidney-shaped nucleus and formation of apoptotic bodies in CAE treated

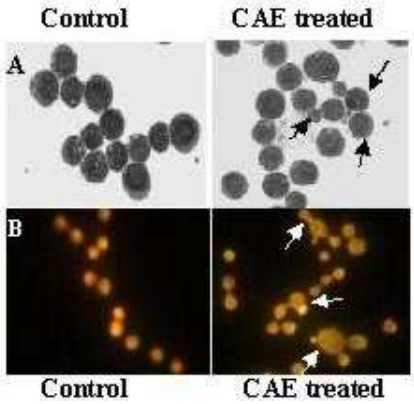

Figure 4: Effect of CAE on EAT cells showing characteristic apoptotic morphology when stained with giemsa ethidium bromide and acridine orange stain. A (c): Control, A (t): CAE treated, B (c): Control, B(t): CAE treated. Arrows indicate membrane blebbing, apoptotic bodies and condensed chromatin.

Figure 5: *In vivo* effect of CAE on DNA fragmentation of Ehrlich ascites tumor cells. DNA run and detected on 2% agarose gel electrophoresis. C: DNA from control EAT cells, T: DNA from CAE treated EAT cells (DNA fragmentation detected from CAE treated EAT cells).

cells (figure 4A and 4B). DNA fragmentation was also evident as seen in agarose gel electrophoresis of DNA extracted from CAE treated cells The DNA from control EAT cells was found to be intact as seen in figure 5.

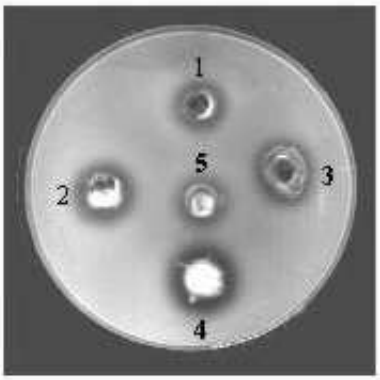

Figure 6: CAE treated cytosolic extract of EAT cells showing endonuclease activity on salman sperm DNA in a dose dependent manner. 1 : 200 mg of enzyme source; 2 : 400 mg of enzyme source; 3 : 600 mg of enzyme source; 4 : 800 mg of enzyme source; 5 : 800 mg of cytosolic enzyme source from control EAT cells.

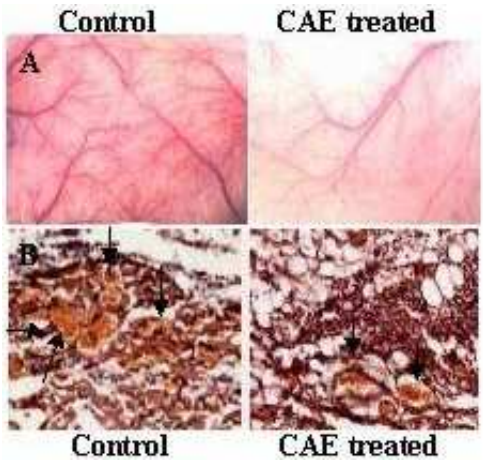

Figure 7: A) Suppression of *in vivo* angiogenesis by CAE. Peritineal lining of mice treated with saline (0.9%) or CAE was inspected for angiogenesis. Inhibition of angiogenesis in CAE-treated mice is evident. B) Formaldehyde fixed, paraffin embedded peritoneum of control as well as CAE treated mice was sectioned (3μ) and stained with H&E and observed for microvessel density. Decreased MVD in CAE treated mice peritoneum is evident.

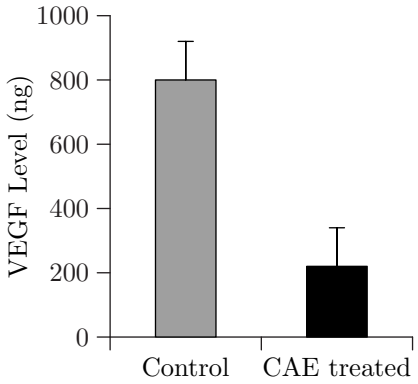

Figure 8: Effect of CAE on production of VEGF by EAT cells.

The degradation of DNA is by the activation of endonuclease in CAE treated cells as is evidenced by a clear lysis zone as seen in figure 6, when compared to the results of control, in which there is no clean lysis zone.

Angioinhibitory activity of CAE

The peritoneal cavity was observed in control as well as treated animals for the extent of vasculature. The results clearly indicated that there is significant inhibition of neovascularization in CAE treated animals as compared to the peritoneum of control animals that show extensive neovasculature as is seen in figure 7A. The H and E stained sections of the peritoneum further indicated that there is a reduction in micro vessel density (MVD) (figure 7B), in the peritoneum of CAE treated animals.

Quantitation of VEGF

The level of angiogenic growth factor, VEGF secreted into ascites during the growth of EAT cells *in vivo* either in presence or in the absence of CAE was measured by indirect ELISA. In the ascites of CAE treated animals, a significant reduction of VEGF level was observed (figure 8) when compared to that of VEGF levels in ascites fluid from control mice. While 830-ng/animal of VEGF was detected in control ascites (6.5 ml), 230 ng/animal of VEGF was detected in CAE treated ascites (1.5 ml).

Discussion

Cancer chemoprevention utilizing chemical compounds or natural products revert or inhibit malignant cell transformation and prevent invasion and

metastasis would be less painful, more economical and rational approach for cancer control. The use of natural herbal medicines or dietary agents is being increasingly utilized as an effective way for the management of many cancer treatments (Wenzel et al. 2000; Miyoshi et al. 2003).

Recent advances in our understanding of the mechanisms of carcinogenesis have led to the synthesis of new drugs that can inhibit tumor development in experimental animals by selective action. Angioinhibition and induction of apoptosis in tumor cells are the newer strategies for cancer therapy. Thus the discovery of angiogenic inhibitors with proapoptotic activity would provide an important therapeutic value. With the goal of finding a potent proapoptotic and antiangiogenic plant components, we have initiated a screening program in our laboratory designed to test a wide variety of indigenous plant extracts for antiangiogenic and proapoptotic activity.

In the present study, we have shown that ethanolic extract of *C. aromatica* has potent antiangiogenic and proapoptotic effects in Ehrlich ascites tumor (EAT) cells *in vivo*. Various plant components and extracts from plants such as Taxol, curcumin, *Lindera strychifolia, Gleditsia sinensis, Livistona*, green tea are reported to inhibit angiogenesis and induce apoptosis in many tumor cell types (Lee et al. 2001; Pal et al. 2001; Sartippour et al. 2001; Chow et al. 2003; Li et al. 2003; Yuasa et al. 2003). We examined, in the present study, whether CAE can also inhibit angiogenesis and induce apoptosis in the EAT cell lines in a dose dependent manner. EAT cells treated with CAE acquired apoptotic morphological features. The extracted DNA showed a ladder pattern when analyzed by gel electrophoresis and this degradation is through the activation of endonuclease suggesting an association of the antineoplastic effect of CAE on EAT cells with the induction of apoptosis. The morphological changes in CAE treated EAT cells were similar to those found in the aforementioned plant extracts and which were shown to be a dose dependent process (data not shown).

There are not many studies focusing on the induction of apoptosis by C. aromatica. The apoptosis inducing effect of elemene isolated from *Curcuma aromatica* oil was first demonstrated in many cancer cell lines (Yang et al. 1996). We showed that administration of CAE inhibited tumor cell growth *in vivo* corresponding reduction in cell number in EAT bearing mice. The rate of tumor growth is dependent on the balance between the proliferative activity and death rate of the tumor cells. It was suggested that CAE induced high level of apoptotic activity, which may be linked to slower tumor growth.

Formation of ascites and pleural effusion is a common problem for patients with advanced stage of cancer (Verheul et al. 2000). These fluid accumulations cause severe symptoms such as abdominal distention, shortness of breath and fatigue. Our results have clearly shown that CAE inhibits formation of ascites probably as a consequence of inhibition of VEGF secretion. Preclinical

models have demonstrated that vascular endothelial growth factor (VEGF) plays a pivotal role in the accumulation of ascites because it is a potent permeability factor. The results obtained in the present study demonstrated that the expression of VEGF was reduced upon CAE treatment leading to less accumulation of ascites thereby reducing the tumor burden.

It has been proposed that antiangiogenic drugs may be useful in the prevention of cancer. Recently, antiangiogenic drugs have been shown to eradicate certain mouse tumors and induce long-term tumor dormancy and disease free survival (Bochm et al. 1997). Our results show that there is angiogenesis in mice bearing EAT cells, which is due to the presence of angiogenesis inducing factors in the ascites fluid. In EAT bearing animals treated with CAE, showed less peritoneal angiogenesis. Paraffin sections of the peritoneum stained with H and E showed reduction in microvesssel density count suggesting that CAE has an angioinhibitory activity.

In conclusion, we demonstrate that CAE may act as an angiogenesis inhibitor and is pro-apoptotic in nature. Further studies are necessary to identify the active principle responsible for pro-apoptotic and antiangiogenic actions of CAE. Consequently, the studies of these antiangiogenic and pro-apoptotic pathways will advance our knowledge and understanding of the efficacy of many chemo-preventive compounds some of which may become very potent therapeutic drugs of the future.

Acknowledgements

The authors acknowledge financial support from Department of Science and Technology, New Delhi, No. SP/SO/B38/99 and Indian Council of Medical Research, No.45/15/2002/BMS/TRM.

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