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# Effect of green tea polyphenols on angiogenesis induced by an angiogenin-like protein

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**Abstract**

Angiogenesis is a fundamental process by which new blood vessels are formed. The angiogenesis process is induced by several growth factors. Among them angiogenin is the most potent blood vessel inducer known. In this paper, we have investigated the effect of green tea polyphenols, mainly the catechins, on an angiogenin-like protein induced angiogenesis process. The angiogenin-like protein was isolated from goat serum and the effect of green tea components was tested by the chicken chorioallantoic membrane (CAM) assay. The results show that green tea components are capable of reducing the vascularization on CAM that is induced by the angiogenin-like protein.


*Keywords:* Angiogenesis; Angiogenin-like protein; Tea polyphenols; Catechins; Chorioallantoic membrane assay

Angiogenesis, the process of developing a hemovascular network, is essential for the growth of solid tumors and is a component of normal wound healing and growth processes [1,2]. Angiogenesis happens in the body all the time. It occurs through a so-called angiogenesis "cascade" which involves a series of biochemical steps by which cells make and secrete molecules that initiate the growth of capillaries. After the process is over, certain other molecular "factors" turn off the angiogenesis process. Cancer cells use this normal process for another purpose—creating an imbalance of angiogenesis activators that overrides the inhibitors and gives the nearby tumor ready access to a blood supply. Angiogenin, the protein to be investigated, aids in such vascular proliferation and as such is a stimulator of angiogenesis [3].

Human angiogenin, a 14 kDa protein homologous to pancreatic ribonuclease, is one of the most potent stimulators of blood vessel formation known [1]. There are certain aspects of its structure that are critical for its biological function and the therapeutic potential of angiogenin inhibition. Even though angiogenin has the same arrangement of catalytic residues as ribonuclease A (RNase A), it has very low but characteristic ribonucleolytic activity which is essential for its angiogenic activity [4]. Angiogenin was first isolated from human tumor conditioned media [3] and subsequently from human serum [4], bovine serum [5], bovine milk [6], pig, rabbit, and mouse sera [7], and from goat serum in this laboratory [8]. The new blood vessels formed provide nutrients to proliferating cancer cells thus favoring tumor growth [2]. A major goal of angiogenin research has been the development of antagonists that may inhibit undesirable vascular proliferation such as in growth of solid tumors[1]. Neomycin, an aminoglycoside antibiotic, has been shown to inhibit angiogenin-induced angiogenesis [9].

A recent study relating angiogenesis and green tea catechins (GTC) focussed on the inhibition of the growth of new blood vessels [10]. Green tea has shown the presence of active anti-cancer constituents [10–13]. The leaves of green tea contain a special class of bioflavonoids—catechin polyphenols—with antioxidant properties [14]. It has been reported that green tea protects against cancer by causing cell cycle arrest including apoptosis [15,16]. The polyphenols increase the level of antioxidants in the body that neutralize free

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radicals before they cause cell damage. They are thought to block the attachment of carcinogens and bacteria to cells, and support healthy liver function [16]. Plant phenolic compounds are multifunctional antioxidants. They can act as metal chelators, singlet oxygen quenchers or reducing agents (free-radical terminators).

There are several catechins in green tea with antioxidant activity [14]. These extracts of green tea with antioxidant effects have been characterized as (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG). The study on the effect of angiogenesis with respect to the components of green tea particularly (−)-epigallocatechin gallate (EGCG) suppressed the formation of new blood vessels [17]. Another report mentioned that green tea appears to be emerging as a very important chemopreventive agent against cancer by virtue of its content of EGCG [18]. It has been reported that drinking of green tea could inhibit VEGF-induced angiogenesis in vivo [19]. The number of reports on whether green tea catechins can inhibit tumor induced angiogenesis is limited and the mechanism of such inhibition is still not clear. In the present study, we have investigated the effect of GTC on the angiogenesis process induced by a potent angiogenin-like protein isolated from goat serum.

## Materials and methods

*Materials.* Goat blood was collected from the local slaughterhouse and CM cellulose (CM-52 grade) and 0.45 microfiber filter paper were from Whatman. Yeast tRNA, bovine pancreatic ribonuclease A (RNaseA), and Placental Ribonuclease Inhibitor (PRI) were from Fluka. Human serum albumin (HSA) and diethyl pyrocarbonate were from Sigma. All other reagents were local analytical-grade reagents and used without further purification. Green tea leaves were obtained from the tea plantation at STEP, IIT Kharagpur.

*Isolation of the angiogenin-like protein.* The angiogenin-like protein was isolated from goat serum as described in Maiti and Dasgupta [8]. In brief, goat blood was centrifuged at 6000 rpm at 4 °C within 24 h of collection. The plasma was loaded onto CM-52 cation exchange column and eluted with 1 M NaCl. The column-eluted fraction was dialyzed extensively vs. water and lyophilized. This lyophilized protein was applied to a Mono S cation exchange column (0.5 cm × 5 cm Pharmacia) and eluted at 1 ml/min flow rate with 40 min linear gradient of 0.15–0.55 M NaCl in 10 mM Tris, pH 8.0. The fractions were assayed for RNase activity and PRI binding. Low RNase and high PRI binding activity indicate the presence of angiogenin [20].

*Isolation of green tea catechins.* Fresh green tea leaves (250 g) were refluxed with 1.5 liters of methanol at 80 °C for 3 h. Methanol was removed using a rotary evaporator. The residue was dissolved in 500 ml of water at 50 °C and washed three times with hexane once with chloroform and once with ethyl acetate. Ethyl acetate was removed and the residue was dissolved in 50 °C water and freeze dried. The light brown solid matter primarily contains the green tea polyphenols. This solid residue contains the four major polyphenolic compounds epigallocatechin-3-gallate, epicatechin-3-gallate, epigallocatechin, and epicatechin [15]. This was confirmed by HPLC (not shown). The aqueous solution of GTC (0.05 mg/ml) was scanned from 200 to 450 nm to check for degradation. The characteristic maxima at 212 and 272 nm with absorbance ⩾1.0 indicated that the polyphenolic catechins were present in proper form (i.e, not oxidized) [14]. This was further confirmed when the characteristic peaks gradually disappeared on addition of increasing concentrations of hydrogen peroxide ($H_2O_2$) to a 0.001% GTC solution.

*Ribonucleolytic assay with green tea catechins.* The effect of GTC on the ribonucleolytic activity of the angiogenin-like protein was examined with yeast tRNA as a substrate as described by Bond [21]. The angiogenin-like protein or its mixture with GTC was added to the assay mixture containing 0.6 mg of yeast tRNA, 40 μl of 0.5 M Tris, pH 7.5, 5 mM EDTA, and 0.1 mg HSA in a final volume of 200 μl. After incubation for 30 min at 25 °C, 300 μl of 1.14 N perchloric acid was added and centrifuged at 10,000 rpm for 10 min at 4 °C. The absorbance of the supernatants was measured at 260 nm.

*Angiogenic activity.* Angiogenesis was assessed by using the chick embryo chorioallantoic membrane (CAM) assay method of Knighton et al. [22] as described in Fett et al. [3]. In brief, the method is as follows. The CAM of the chicken egg is exposed by carefully cutting a small hole through the shell on day 2. Ten microliter aliquots of the protein sample and protein mixed with 0.1% GTC are placed on transparent plastic disks and dried under laminar flow. The disks are inverted over the CAM on day 10 and the response is assayed microscopically after 48 h. The density of blood vessel formation in each case is assessed by comparing with that of a control set.

## Results and discussion

The chromatogram for the isolation of the angiogenin-like protein is given in Fig. 1. Fractions with low RNase activity and high PRI binding indicate the presence of an angiogenin-like protein. These fractions were dialyzed, lyophilized, and then used for subsequent experiments. The angiogenin-like fractions show extended vascularization compared to a control set [8].

The absorbance spectra of green tea extract are indicative of the presence of catechins which is characterized by the peaks at 212 and 272 nm (Fig. 2). The peaks remain unchanged on addition of 20 μl of 4 μM $H_2O_2$ to 10 μl of 0.1% GTC but on addition of 5, 10, and 20 μl of 30% $H_2O_2$ the characteristic peaks at 212 and

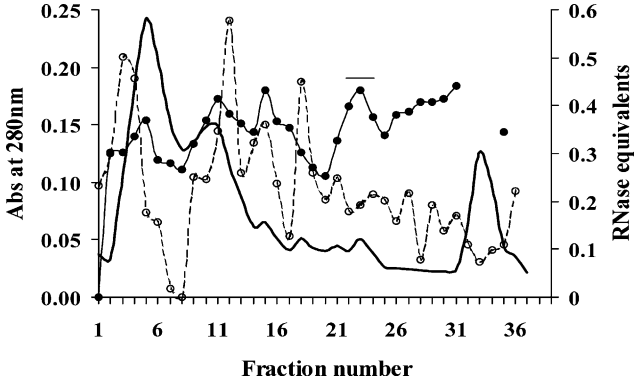


Fig. 1. Mono S cation exchange FPLC of pooled fractions from CM cellulose chromatography. (———) Mono S profile; PRI binding (—●—) and RNase activity (– – ○ – –) plotted as RNase equivalents. Fractions containing the angiogenin-like protein (indicated by the horizontal bar) were pooled and subjected to further purification.

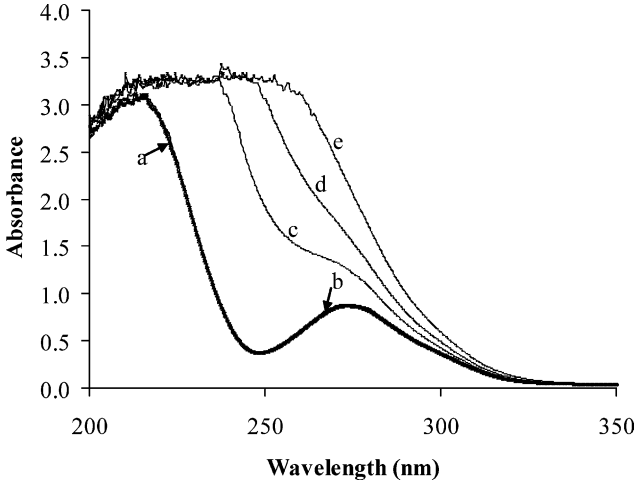


Fig. 2. (a) Ten microliters of 0.1% GTC; (b) 10 μl of 0.1% GTC + 20 μl of 4 μM $H_2O_2$; (c) 10 μl of 0.1% GTC + 5 μl of 30% $H_2O_2$; (d) 10 μl of 0.1% GTC + 10 μl of 30% $H_2O_2$; and (e) 10 μl of 0.1% GTC + 20 μl of 30% $H_2O_2$. The characteristic peaks at 212 and 272 nm are lost on addition of $H_2O_2$ which is indicative of oxidative destruction of GTC.

272 nm are lost. This is suggestive of oxidative degradation of GTC. The aqueous solution of GTC appears to be quite stable to long-term storage with no oxidative change. The effect of GTC on the ribonucleolytic activity of the angiogenin-like protein was studied with yeast tRNA as a substrate. The protein is found to retain 94% of its ribonucleolytic activity in the presence of GTC compared to that of the control. This indicates that GTC do not inhibit the cleavage of tRNA by the angiogenin-like protein. At higher concentration of catechins a precipitate is formed with tRNA. These data are further confirmed from agarose gel electrophoresis.

The CAM assay indicated that green tea catechins (GTC) reduce the angiogenin-like protein induced angiogenesis. Fig. 3A shows the normal growth of blood vessels which gets enhanced due to the presence of the angiogenin-like protein as indicated in Fig. 3B. The presence of GTC results in relatively thinner and sparse blood vessel formation (Fig. 3C). With the angiogenin-like protein in the presence of GTC shown in Fig. 3D blood vessel growth is distinctly different from the one in which only the protein is present, indicating that an inhibitory effect is induced by the presence of GTC. These results suggest that the reduction of blood vessel formation due to the presence of GTC is not associated with the ribonucleolytic activity of the angiogenic protein.

From the dual site model for the organogenic activity of the angiogenin it is evident that angiogenin has both a

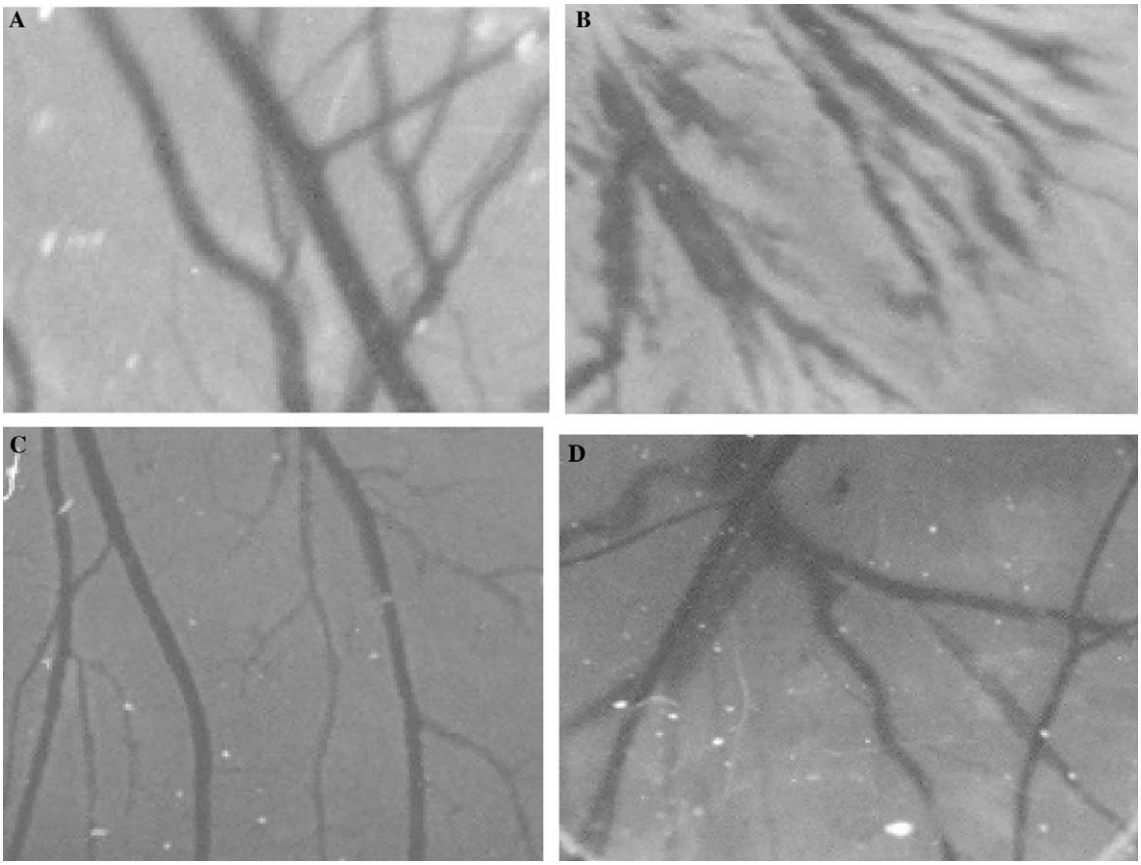


Fig. 3. (A) Control set. (B) With angiogenin-like protein. (C) With 0.1% GTC. (D) Angiogenin-like protein + 0.1% GTC.

catalytic site and a cell binding site [23,24]. These two sites are required for its biological activity. The ribonucleolytic activity of angiogenin is necessary [25] but not sufficient for angiogenic activity [23]. It has been reported that the biological effects of the protein are mediated through a receptor [20] and further delineate a specific region of angiogenin, separate from the active site, as binding to the putative receptor. Disruption of either one prevents angiogenesis. It may possible that the GTC block the cell binding region of the angiogenin-like protein disrupting the nuclear translocation of the protein to the endothelial cell. Further investigation is underway with regard to the specific components of green tea and their effect on angiogenin induced vascularization.

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## References

[1] J. Folkman, M. Klagsbrun, Angiogenic factors, Science 235 (1987) 442–447.
[2] J. Folkman, Y. Shing, Angiogenesis, J. Biol. Chem. 267 (1992) 10931–10934.
[3] J.W. Fett, D.J. Strydom, R.R. Lobb, E.M. Alderman, J.L. Bethune, J.F. Riordan, B.L. Vallee, Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells, Biochemistry 20 (1985) 5480–5486.
[4] R. Shapiro, D.J. Strydom, K.A. Olson, B.L. Vallee, Isolation of angiogenin from normal human plasma, Biochemistry 26 (1987) 5141–5146.
[5] M.D. Bond, B.L. Vallee, Isolation of bovine angiogenin using a placental ribonuclease inhibitor binding assay, Biochemistry 27 (1988) 6282–6287.
[6] P. Maes, D. Damart, C. Rommens, J. Montreuil, G. Spik, A. Tartar, The complete amino acid sequence of bovine milk angiogenin, FEBS Lett. 241 (1988) 41–45.
[7] M.D. Bond, D.J. Strydom, B.L. Vallee, Characterization and sequencing of rabbit, pig and mouse angiogenins discernment of functionally important residues and regions, Biochim. Biophys. Acta 1162 (1993) 177–186.
[8] T.K. Maiti, S. Dasgupta, Isolation and characterization of an angiogenin-like protein from goat plasma, Protein Peptide Lett. 9 (2002) 283–288.
[9] G.F. Hu, Neomycin inhibits angiogenin-induced angiogenesis, Proc. Natl. Acad. Sci. USA 95 (1998) 9791–9795.
[10] D.A. Balentine, Manufacturing and chemistry of tea, in: C.-T. Ho, M.-T. Huang, C.Y. Lee (Eds.), Phenolic Compounds in Food and their Effects on Health, Analysis, Occurrence, and Chemistry, vol. 8, American Chemical Society, Washington, DC, 1992, pp. 102–117.
[11] F. Tosetti, N. Ferrari, S. De Flora, A. Albini, 'Angioprevention': angiogenesis is a common and key target for cancer chemopreventive agents, FASEB J. 16 (2002) 2–14.
[12] J.D. Lambert, C.S. Yang, Cancer chemopreventive activity and bioavailability of tea and tea polyphenols, Mutat. Res. 523 (2003) 201–208.
[13] H. Fujiki, M. Suganuma, K. Imai, K. Nakachi, Green tea, cancer preventive beverage and/or drug, mini-review, Cancer Lett. 188 (2002) 9–13.
[14] H. Cutter, L.-Y. Wu, C. Kim, D.J. Morre, D.M. Morre, Is the cancer protective effect correlated with growth inhibitions by green tea (−)-epigallocatechin gallate mediated through an anti-oxidant mechanism? Cancer Lett. 162 (2001) 149–154.
[15] N. Ahmad, D.K. Feyes, A.L. Nieminen, R. Agarwal, H. Mukhtar, Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells, J. Natl. Cancer Inst. 89 (1997) 1881–1886.
[16] G.-Y. Yang, J. Liao, K. Kim, E.J. Yurkow, C.S. Yang, Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols, Carcinogenesis 19 (1998) 611–616.
[17] T. Kondo, T. Ohta, K. Igura, Y. Hara, K. Kaji, Tea catechins inhibit angiogenesis in vitro, measured by human endothelial cell growth, migration and tube formation, through inhibition of VEGF receptor binding, Cancer Lett. 180 (2002) 139–144.
[18] Z.P. Chen, J.B. Schell, C.-T. Ho, K.Y. Chen, Green tea epigallocatechin gallate shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts, Cancer Lett. 129 (1998) 173–179.
[19] Y. Cao, R. Cao, Angiogenesis inhibited by drinking tea, Nature 398 (1999) 381.
[20] R. Shapiro, B.L. Vallee, Human placental ribonuclease inhibitor abolishes both angiogenic and ribonucleolytic activities of angiogenin, Proc. Natl. Acad. Sci. USA 84 (1987) 2238–2241.
[21] M.D. Bond, An in vitro binding assay for angiogenin using placental ribonuclease inhibitor, Anal. Biochem. 173 (1988) 166–173.
[22] D. Knighton, D. Asuprunk, D. Tapper, J. Folkman, Br. J. Cancer 35 (1977) 347–356.
[23] G.F. Hu, S.I. Chang, J.F. Riordan, B.L. Vallee, An angiogenin-binding protein from endothelial cells, Proc. Natl. Acad. Sci. USA 88 (1991) 2227–2231.
[24] J. Badet, F. Soncin, J.D. Guitton, O. Lamare, T. Cartwright, D. Barritault, Specific binding of angiogenin to calf pulmonary artery endothelial cells, Proc. Natl. Acad. Sci. USA 86 (1989) 8427–8431.
[25] R. Shapiro, B.L. Vallee, Human placental ribonuclease inhibitor abolishes both angiogenic and ribonucleolytic activities of angiogenin, Proc. Natl. Acad. Sci. USA 84 (1987) 2238–2241.