Catechin's anti-angiogenic effects in epithelial ovarian cancer

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Background

Epithelial ovarian cancer (EOC) is a common and lethal malignancy of the female reproductive tract (2). Often detected at later stages of the disease, tumours frequently display angiogenesis (the growth of new blood vessels) and abnormal vasculature, which contributes to metastatic spread of cancer cells throughout the body, poor drug diffusion and hypoxia (3). The growth of new blood vessels is regulated in the ovary primarily by the pro-angiogenic factor VEGF (vascular endothelial growth factor) and anti-angiogenic factor TSP-1 (thrombospondin-1) and their respective receptors, VEGFR-2 and CD36 (3). Catechin, a polyphenol from the plant *Camellia sinensis*, has numerous anti-tumour effects, including induction of apoptosis and cell cycle arrest in some types of cancers (10). Guruvayoorappan and Kuttan, 2008, report that catechin prevents angiogenesis in melanoma *in vivo*, by suppressing VEGF, inflammatory cytokines and endothelial cell (EC) proliferation (4). Green tea extracts and catechin derivatives, most notably epigallocatechin-3-gallate, have considerable anti-angiogenic effects in vitro, including prevention of EC growth and VEGF/VEGFR-2 inhibition (4-6). However, there is little *in vivo* evidence of the therapeutic effects of catechin on angiogenesis-dependent tumours.

Purpose, Hypothesis

It is hypothesized that catechin can effectively prevent ovarian tumour progression, inhibit angiogenesis and simultaneously normalize tumour vasculature. The purpose of this investigation is to define catechin's effect on angiogenesis, the mechanics of its effect and the result of this on EOC.

Methods

Animal Model of EOC

Tumour angiogenesis was studied in an orthotopic, syngeneic model of epithelial ovarian cancer. Under anesthesia, $1.0x10^6$ cells of a tumourigenic mouse epithelial cell line (ID8) were injected into the ovarian bursa of C57BL6 mice. After 30 days post tumour induction, primary tumours begin to form on the surface of the ovary, and angiogenesis is induced to facilitate tumour development (3). By 60 days post tumour induction, tumours are generally limited to the ovary but extensively vascularized (3). +(-)Catechin from Spectrum Chemicals was dissolved in 12% ethanol to maintain the solution and mixed with D5W dextrose to improve absorption. At 27 days post tumour induction, mice were divided into 4 groups and catechin was administered into the intraperitoneal cavity of mice daily via 200 μl injection at doses of 20, 50 and 100 milligrams per kilogram of body weight, with control animals given D5W and 12% ethanol, for 26 days. Following 30 days of treatment, mice were euthanized (CO_2 asphyxiation) and ovarian tissues were collected, weighed and frozen or processed and embedded in paraffin for analysis and sliced into 5μm sections. Immunohistochemistry and immunofluorescence was performed as described in (3). The following antibodies were implemented to detect specific proteins in the tumour sections; CD31 (EC marker), Ki67 (proliferation), TSP-1, CD36, VEGF, VEGFR-2 and the immunofluorescence antibodies IB4 (EC marker) with αSmA (alpha smooth muscle actin), indicating mature blood vessels. After application of reactive chromogen or fluorescent reagent, pictures were taken at 100 times magnification and the images were analyzed using Aperio Imagescope pixel counting software to determine the

amount of tissue expression specific proteins in tumour sections. Frozen tissues were homogenized, the protein was extracted and analyzed using immunoblotting as described in (3). The proteins probed for in the samples were VEGF, VEGFR-2 and bcl-2, an anti-apoptotic protein utilized by tumours and fas-ligand, a pro-apoptotic signaling protein.

Cell Viability and Proliferation

An MTT assay as described in (11) was performed to test the effect of catechin on mouse endothelial cell (MEC) viability. Catechin was dissolved in ethanol and administered to cell cultures at 5, 10 and 25 micrograms per milliliter. The scratch assay as described in (9) was performed to determine the effect of catechin on MEC migration. Cells cultured to 90% confluence were treated and scratched with a pipette tip. Pictures were taken at the beginning of the experiment and periodically until the scratch filled in with migrating cells. The width of the scratch throughout the experiment of catechin treated cell cultures was compared to the control. Statistical analysis was performed on the data obtained from image analysis and densitometry from immunoblotting. The student's t test was performed and the significance of a population was assigned based upon the p value less than 0.05 being significant (*) and the p value being less then 0.01 being very significant (**).

Results

Protein	Control	20 mg/kg	50 mg/kg	100 mg/kg
CD31	1.00 SD=0.154	0.625* SD=0.093	0.742 SD=0.074	0.855 SD=0.116
VEGF	1.00 SD=0.141	0.857 SD=0.162	0.949 SD=0.086	1.45* SD=0.172
VEGFR-2	1.00 SD=0.127	0.531 SD=0.050	0.561 SD=0.060	1.00 SD=0.106

TSP-1	1.00 SD=0.120	1.04 SD=0.045	1.29* SD=0.074	1.12 SD=0.089
CD36	1.00 SD=0.127	1.04 SD=0.131	1.27* SD=0.096	1.17 SD=0.097

Table 1. Protein levels relative to control from immunohistochemistry

Protein	Control	20 mg/kg	50 mg/kg	100 mg/kg
Bcl-2	1.00 SD=0.254	0.497* SD=0.194	0.770 SD=0.091	0.743 SD=0.153

Table 2. Densitometry of Bcl-2 relative to control from immunoblotting

Cell line	Control	5 μg/mL	10 μg/mL	25 μg/mL
MEC	1.00 SD=0.00	1.22* SD=0.0647	1.44** SD=0.0211	2.00** SD=0.142

Table 3. Cell viability from the MTT assay

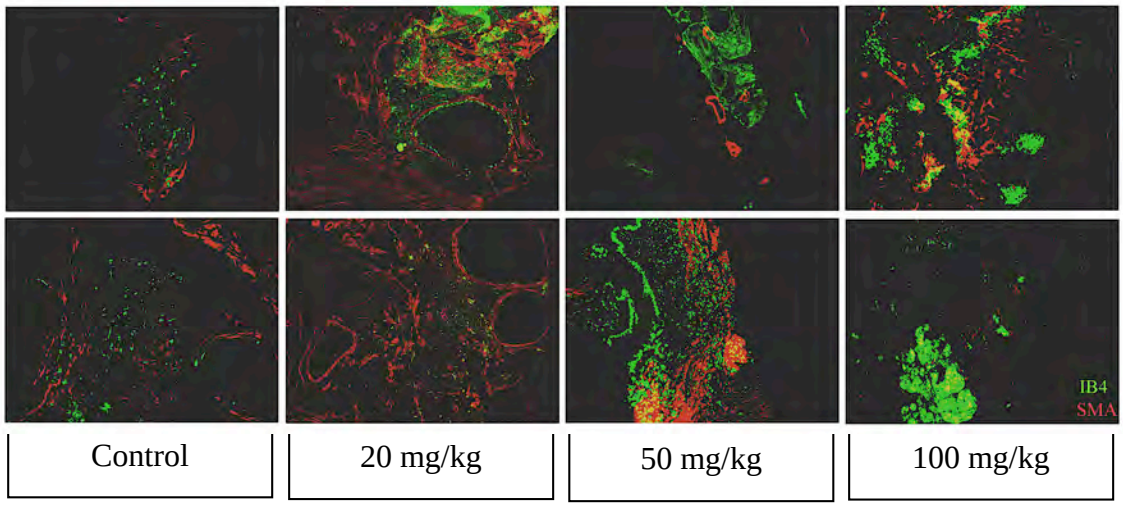

Figure 1. Endothelial IB4 (green) and SMA (red) from immunofluorescence

Discussion

The results obtained from immunohistochemistry demonstrate a 37% decrease in CD31 expression in ovarian tumour section treated with 20 mg/kg of catechin, which was a significant response. Other significant differences were 100 mg/kg doses increasing VEGF by approximately 40%, and doses of 50 mg/kg increasing TSP-1 and CD36 by approximately 30%. EC staining (IB4) demonstrated significant proliferation and erratic areas of EC density proportional to the dose (Figure 1). Catechin did not affect

tumour cell proliferation from qualitative assessment of Ki67 staining. Catechin dose dependently increased EC viability in the MTT assay up to 2-fold, supporting observations made from IB4 staining, yet did not influence EC migration in the scratch assay. Several studies have found that green tea consumption (the primary dietary source of catechin) is correlated with reduced risks of ovarian cancer and lower mortality rates among patients with the disease (1)(7). Tumours treated with 20 mg/kg of catechin (comparable to human consumption) had 50% less Bcl-2, an anti-apoptotic regulatory factor important to tumour cell survival, and 37% less blood vessels. Therefore, prevention of ovarian cancer could be attributed to anti-angiogenic and pro-apoptotic effects. Catechin appears to regulate angiogenesis in ways dependent on the dose and tumour environment. This is suggestive that the anti-tumour effects of catechin at low doses will elicit therapeutic benefits in advanced late stage tumours.

Conclusions

Catechin dose-dependently regulated angiogenesis in early stage epithelial ovarian cancer of mice. At a dose of 20 mg/kg, catechin inhibited blood vessel density and Bcl-2, while higher doses of catechin increased EC proliferation *in vitro* and *in vivo* and induced VEGF and TSP-1/CD36.

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Appendix

References

1. Clement, Y. (2009). Can green tea do that? A literature review of the clinical evidence. *Preventative Medicine*, 49(2-3), 83-87.
2. Cramer, DW. (2005). Epidemiology and Statistics. In Berek JS, Hacker NF, Eds. Practical gynecologic oncology. 4th Ed. Philadelphia, Lippincott Williams and Wilkins; p. 243-262.
3. Greenaway, J, Henkin, J, Lawler, J, Moorehead, R, Petrik, J. (2009). ABT-510 induces tumor cell apoptosis and inhibits ovarian tumour growth in an orthotopic, syngeneic model of epithelial ovarian cancer. *Molecular Cancer Therapeutics*, 8(1), 64-74.
4. Guruvayoorappan, Chandrasekkharan & Kuttan, Girija. (2008). +(-)Catechin inhibits tumour angiogenesis and regulates the production of nitric oxide and TNF-α in LPS-stimulated macrophages. *Innate Immunity*, 14(3), 160-174.
5. Fassina G, et al. (2004). Mechanisms of inhibition of tumor angiogenesis and vascular tumor growth by epigallacatechin-3-gallate. *Clinical Cancer Research*, 10(14), 4865-73.
6. Kondo, T, Toshiro, O, Koichi, I, Yukihiki, H, Kazuhiku, K. (2002). Tea catechins inhibit angiogenesis in vitro, measured by human endothelial cell growth, migration and tube formation, through inhibition of VEGF receptor binding. *Cancer Letters*, 180(2), 139-144.
7. Lee, AH, et al. (2005). Possible role for green tea in ovarian cancer prevention. *Future Oncology*, 1(6), 771-777.
8. Leone, Marlisa, et al. (2003). Cancer prevention by tea polyphenols is linked to their direct inhibition of antiapoptotic Bcl-2-family proteins. *Cancer Research*, 63, 8118-8121.
9. Liang, Chun-Chi, et al. (2007). In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature Protocols*, 2(2), 329-333.
10. Park, Sung-Jin, et al. (2008). Anticancer effects of genistein, green tea catechins, and cordycepin on oral squamous cell carcinoma. *J Kor. Oral Maxillofac. Surg.*, 34(1), 1-10.
11. Proliferation Assay: MTT Protocol (2007). *Wallert and Provost Lab*. Retrieved February 2, 2010, from http://www.mnstate.edu/provost/MTT_Proliferation_Protocol.pdf

Bibliography

1. Boehm, K., Borrelli, F., Ernst E., et al. (2009). Green tea (Camillial sinensis) for the prevention of cancer. *Cochrane Database System Review*, 8(3).
2. Jain, R. K. (2008, January). Taming vessels to treat cancer. *Scientific American*, 298(1), 56-63.
3. Mickinnell, Robert, Parchment, Ralph, Perantoni, Alan, et al. (2006). The biological basis of cancer (2nd ed.) New York: Cambridge University Press.