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ABSTRACT

The objective of the present study was to evaluate the combined effects of dietary supplements consisting of *Ganoderma lucidum* (GL) and green tea (GT) extracts on human breast cancer cells MDA-MB-231. The effect on growth was evaluated by the inhibition of cell proliferation (anchorage-dependent growth) and colony formation (anchorage-independent growth), whereas the effect on invasive behavior was evaluated by the inhibition of cell adhesion to vitronectin, cell migration and cell invasion through matrigel. GL as well as GT inhibited proliferation and colony formation of MDA-MB-231 cells in a dose-dependent manner, and these effects were profoundly enhanced by the combination of GL/GT. In addition, the combination of GL/GT demonstrated synergism against invasive behavior of breast cancer cells. The inhibition of cell invasiveness (adhesion, migration invasion) is mediated through the urokinase-plasminogen activator (uPA), since GT, GL as well as GT/GL suppressed secretion of uPA. In summary, combination of *G. lucidum* and green tea extracts could be considered in the prevention/therapy of breast cancer.

INTRODUCTION

Breast cancer is the leading cause of cancer death among women 20-59 years old and contributed to one third of newly diagnosed cases of breast cancer in the United States in the year 2005. On the other hand, the low incidence of breast cancers among Asian women was associated with the dietary behavior, suggesting the preventative effect of nutrition against cancer. Although inconclusive a variety of epidemiological studies suggested the importance of different nutritional/natural products for cancer prevention. However, while important, these studies are highly correlative because they are usually focused only on one nutritional product. For example, a consumption of green tea was correlated to the prevention of a variety of cancers, and an inverse correlation between mushroom intake and the risk of gastric cancer was described. Extracts from an oriental medicinal mushroom *Ganoderma lucidum* has been used in traditional Chinese medicine for the prevention or treatment of a variety of diseases. *G. lucidum* is currently consumed in the form of tea, powder or extract as a dietary supplement. Furthermore, *in vivo* animal studies demonstrated inhibition of hepatoma, sarcoma, and lung and colon cancers in mice by *G. lucidum* extracts containing a variety of biologically active compounds, including β -glucan-based polysaccharides and lanostane-type triterpenes. In addition, we have recently demonstrated *in vitro* that an extract from *G. lucidum* inhibits proliferation and invasive behavior of highly metastatic breast cancer cells through the suppression of Akt/NF- κ B signaling, which resulted in the down-regulation of expression of cyclin D1, Cdk4 and urokinase-plasminogen activator (uPA) in breast cancer cells. Tea, from the plant *Camellia sinensis*, belongs to the most globally consumed beverages. Moreover, green tea and especially its major biologically active compound (-)epigallocatechin-3-gallate (EGCG) also demonstrated cancer chemopreventive effects in different cancer models. The biological mechanism of the chemopreventive effects of EGCG was linked to the modulation of multiple signaling pathways finally resulting in the down-regulation of expression of AP-1 and NF- κ B regulated genes. In addition, we have recently demonstrated that green tea extract containing 50% of EGCG induced cell cycle arrest and suppressed invasive behavior of breast cancer cells through the inhibition of AP-1 and NF- κ B signaling. The present study was undertaken to evaluate the effect of combination of green tea and *G. lucidum* extracts on the growth and invasive behavior of breast cancer cells.

MATERIALS AND METHODS

Materials.

G. lucidum extract (GLE, ReishiMax[®]), containing 13.5% polysaccharides and 6% triterpenes, and green tea leaf extract (GTE, Tegreen[®]), containing 97% polyphenols (38% EGCG) were obtained from Pharnanex LLC (Provo, UT). These extracts were dissolved in sterile water at a concentration of 50 mg/ml and stored at 4°C.

Cell culture.

The human breast cancer cells MDA-MB-231 were obtained from ATCC (Manassas, VA) and were maintained in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (50 U/ml), streptomycin (50 U/ml), and 10% fetal bovine serum (FBS). Media and supplements were from Invitrogen (Grand Island, NY). FBS was obtained from Hyclone (Logan, UT).

Cell proliferation (anchorage-dependent growth).

MDA-MB-231 cells were cultured in a 96-well plate and treated for 24, 48, and 72 hours with GLE, GTE or the combination of GLE and GTE. Cell proliferation was determined by the tetrazolium salt method. Data points represent mean \pm SD in one experiment repeated at least twice.

Colony formation (anchorage-independent growth).

MDA-MB-231 cells were harvested and seeded in 6-well plates coated with 1% agarose. Colony formation was assessed after incubation for 10-14 days with culture media with GLE, GTE or the combination of GLE and GTE, which was replaced every 4 days. Plates were stained with 0.005% Crystal Violet, and the colonies were counted manually under a microscope and photographed.

Cell adhesion, migration, and invasion assays.

MDA-MB-231 cells were treated with GLE, GTE or the combination of GLE and GTE. Cell adhesion was performed with Costar's Adhesion Strips coated with human vitronectin. Cell migration of MDA-MB-231 cells was assessed in Transwell chambers in the DMEM medium containing 10% FBS. Invasion of MDA-MB-231 cells was assessed in Transwell chambers coated with 100 μ l of Matrigel[™], after 72 hr of incubation.

uPA Secretion.

DMEM media from MDA-MB-231 cells treated with GLE, GTE or their combination for 24 hr were collected and concentrated, and the secretion of uPA was detected by Western blot analysis with anti-uPA antibody.

Western blot analysis.

Whole cell extracts were prepared from MDA-MB-231 cells treated with GLE, GTE or their combination. Equal amounts of proteins were separated on 4-12 % Bis-Tris gel and transferred to a PVDF membrane. The protein expression was detected with anti-c-myc antibody and anti- β -actin antibody.

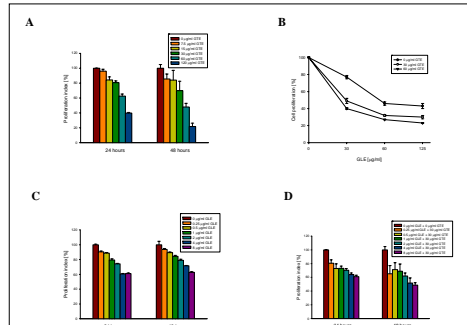


Figure 1. Green tea extract and *G. lucidum* extract inhibit proliferation of breast cancer cells. MDA-MB-231 cells were treated with (A) GTE (0-120 mg/ml) for 24 and 48 hours, (B) GLE (0 - 125 mg/ml) and GTE (0 - 60 mg/ml) for 24 hours, (C) GLE (0 - 8 mg/ml) for 24 and 48 hours, and (D) GLE (0 - 8 mg/ml) and 30 mg/ml GTE for 24 and 48 hours. Proliferation was assessed, as described in *Materials and Methods*. Each bar represents the mean \pm SD of three experiments.

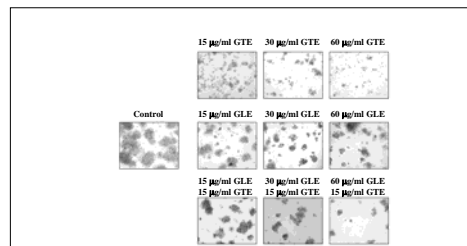


Figure 2. Green tea extract and *G. lucidum* extract suppress colony formation of MDA-MB-231 cells. MDA-MB-231 cells were harvested and seeded in 6 well plates coated with agarose in the presence of GTE (0 - 60 μ g/ml), GLE (0 - 60 μ g/ml), or the combination of GLE (0 - 60 μ g/ml) and 15 μ g/ml GTE. Anchorage-independent growth was assessed as described in *Materials and Methods*.

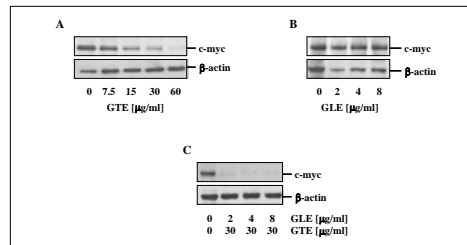


Figure 3. Effect of GTE and GLE on the expression of c-myc in breast cancer cells. MDA-MB-231 cells were treated with (A) GTE (0 - 60 μ g/ml), (B) GLE (0 - 8 μ g/ml), and (C) the combination of GLE (0 - 8 μ g/ml) and 30 μ g/ml GTE for 24 hours. Whole cell extracts were subjected to Western blot analysis with anti-c-myc antibody. The equal protein loading was verified with anti- β -actin antibody and the expression of c-myc quantified by densitometry as described in *Materials and Methods*. The results are representative of three separate experiments.

	Adhesion to vitronectin [%]		
	GTE [0 μ g/ml]	GTE [30 μ g/ml]	GTE [60 μ g/ml]
GLE [0 μ g/ml]	100 \pm 4.8	24 \pm 1.1	7 \pm 1.7
GLE [125 μ g/ml]	55 \pm 4.8	20 \pm 3.7	2 \pm 1.1
GLE [250 μ g/ml]	45 \pm 2.4	13 \pm 9.2	1.5 \pm 0.6
GLE [500 μ g/ml]	27 \pm 9.8	10 \pm 6.2	0.3 \pm 0.2

Table 1. Combined effect of GLE and GTE on adhesion of MDA-MB-231 cells. MDA-MB-231 cells were treated with the combination of GTE (0 - 60 μ g/ml) and GLE (0 - 500 μ g/ml) for 24 hours and cell adhesion to vitronectin determined as described in *Materials and Methods*.

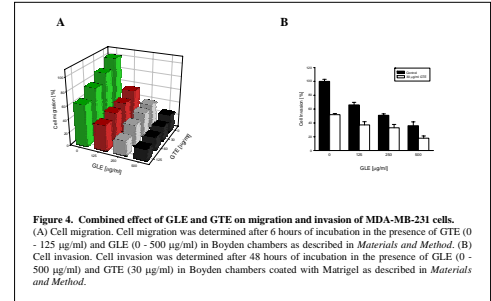


Figure 4. Combined effect of GLE and GTE on migration and invasion of MDA-MB-231 cells. (A) Cell migration. Cell migration was determined after 6 hours of incubation in the presence of GTE (0 - 125 μ g/ml) and GLE (0 - 500 μ g/ml) in Boyden chambers as described in *Materials and Method*. (B) Cell invasion. Cell invasion was determined after 48 hours of incubation in the presence of GLE (0 - 500 μ g/ml) and GTE (30 μ g/ml) in Boyden chambers coated with Matrigel as described in *Materials and Method*.

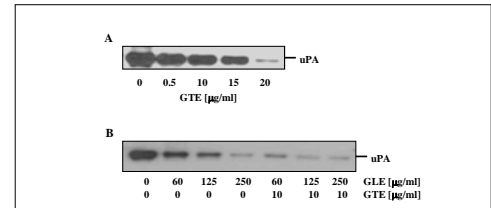


Figure 5. Combined effect of GLE and GTE on uPA secretion. Media from MDA-MB-231 cells treated with (A) GTE (0 - 20 μ g/ml), (B) GLE (0 - 250 μ g/ml) and GTE (0, 10 μ g/ml) for 24 hours were concentrated and secretion of uPA was detected by Western blot analysis with anti-uPA antibody as described in *Materials and Methods*.

SUMMARY

- GTE increases anti-cancer effect of GLE of anchorage-dependent (cell proliferation) as well as anchorage-independent (colony formation) of breast cancer cells through the down-regulation of expression of c-myc.
- The combination of GTE and GLE synergistically inhibits invasive behavior of breast cancer cells through the suppression of secretion of uPA from breast cancer cells.
- The combination of *G. lucidum* and green tea extracts as dietary supplements can be considered for breast cancer chemoprevention and/or treatment.

Acknowledgment



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References

1. D. Sliva, C. Labarere, V. Slivova, M. Sedlak, F.P. Lloyd Jr, N.W.Y. Ho, *Ganoderma lucidum* suppresses motility of highly invasive breast and prostate cancer cells. *Biochem. Biophys. Res. Commun.* 298 (2002) 603-612.
2. V. Slivova, T. Valachovicova, J. Jiang, D. Sliva, *Ganoderma lucidum* inhibits invasiveness of breast cancer cell. *Cancer Integr. Med.* 2 (2004) 25-30.
3. J. Jiang, V. Slivova, T. Valachovicova, K. Harvey, D. Sliva, *Ganoderma lucidum* inhibits proliferation and induces apoptosis in human prostate cancer cells PC-3. *Int. J. Oncol.* 24 (2004) 1093-1099.
4. J. Jiang, V. Slivova, K. Harvey, T. Valachovicova, D. Sliva, *Ganoderma lucidum* suppresses growth of breast cancer cells through the inhibition of Akt/NF- κ B signaling. *Nutr. Cancer* 49 (2004) 209-216.
5. V. Slivova, G. Zaloga, S.J. DeMichele, P. Mukerji, Y.S. Huang, R. Siddiqui, K. Harvey, T. Valachovicova, D. Sliva, Green tea polyphenols modulate secretion of urokinase plasminogen activator (uPA) and inhibit invasive behavior of breast cancer cells. *Nutr. Cancer* 52 (2005) 65-72.