New Era of Polyphenol Research

Review

Stiffening of Cancer Cell Membranes Is a Key Biophysical Mechanism of Primary and Tertiary Cancer Prevention with Green Tea Polyphenols

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Over the past 30 years, research of green tea polyphenols, especially (–)-epigallocatechin gallate (EGCG), has revealed that consumption of green tea is a practical and effective primary cancer prevention method for the general population. More recently, we believe that green tea polyphenols are beneficial for tertiary cancer prevention using green tea alone or combined with anticancer drugs because EGCG has the potential to inhibit metastatic progression and stemness, and enhance antitumor immunity. In an effort to identify a common underlying mechanism responsible for EGCG's multifunctional effects on various molecular targets, we studied the biophysical effects of EGCG on cell stiffness using atomic force microscopy. We found that EGCG acts to stiffen the membranes of cancer cells, leading to inhibition of signaling pathways of various receptors. Stiffening of membranes with EGCG inhibited AXL receptor tyrosine kinase, a stimulator of cell softening, motility and stemness, and expression of programmed cell death-ligand 1. This review covers the following: i) primary cancer prevention using EGCG or green tea, ii) tertiary cancer prevention by combining EGCG and anticancer drugs, iii) inhibition of metastasis with EGCG by stiffening the cell membrane, iv) inhibition of AXL receptor tyrosine kinase, a stimulator of cell softening and motility, with EGCG, v) inhibition of stemness properties with EGCG, and vi) EGCG as an alternative chemical immune checkpoint inhibitor. Development of new drugs that enhance stiffening of cancer cell membranes may be an effective strategy for tertiary cancer prevention and treatment.

Key words AXL; cancer stem cell; (–)-epigallocatechin gallate; motility; Young's modulus

1. Introduction

Interest in the health benefits of polyphenols has dramatically increased over the last 30 years. The most studied polyphenols are green tea polyphenols or green tea catechins of which the main constituent is (–)-epigallocatechin gallate (EGCG) (Fig. 1). When we started screening Japanese original cancer chemopreventive agents in 1983 using the inhibition of receptor binding of the tumor promoter ³H-12-*O*-tetradecanoylphorbol-13-acetate (TPA), we were fortunate to receive 30 polyphenols isolated from medicinal plants, including EGCG, from Takuo Okuda, Faculty of Pharmaceutical Sciences, Okayama University.¹⁾ From this screening, EGCG was identified as one of the five polyphenols that most potently inhibited the receptor binding. Our studies focused on EGCG because green tea is widely consumed in Japan and it is known to be non-toxic to humans.²⁾ In 1987, we reported that EGCG inhibited tumor development with the TPA-type tumor promoter teleocidin in mice initiated with 7,12-dimethylbenz[*a*]anthracene (DMBA).³⁾ After this report, numerous researchers around the world initiated studies on cancer prevention with green tea catechins.^{4,5)}

Cancer prevention is composed of primary, secondary and tertiary prevention⁶⁾ (Fig. 2). Primary cancer prevention aims to reduce cancer incidence in the general population including high-risk people or patients with preneoplastic diseases. Secondary cancer prevention works to reduce cancer death in the general population using early diagnosis and treatment. An increased number of cancer patients require tertiary prevention, which targets people with a history of cancer. Tertiary cancer prevention covers cancer patients following treatment who need prevention of metastatic progression. Today, green tea and EGCG are accepted as effective agents for primary cancer prevention, and the significance of EGCG in tertiary cancer prevention has attracted attention around the world.^{7,8)} Numerous biochemical and biological studies with EGCG and green tea have revealed multifunctional effects *in vitro* and *in vivo* that include inhibition of receptor binding, cancer cell growth, invasion, migration, angiogenesis, proinflammatory cytokine expression, signaling pathways, epithelial–mesenchymal transition (EMT), as well as spheroid formation of cancer stem cells (CSCs) and induction of apoptosis and cell-cycle arrest.^{4,9–11)} Recently, we found EGCG stimulated anticancer immunity by inhibition of expression of the immune checkpoint molecule, programmed cell death-ligand 1 (PD-L1).¹²⁾ How-

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Fig. 1. Structures of (-)-Epigallocatechin Gallate (EGCG) and Three Other Green Tea Polyphenols

Fig. 2. Strategies for Cancer Prevention and Proposed Use of Green Tea

(Color figure can be accessed in the online version.)

ever, it is still not known how EGCG induces numerous beneficial effects on cancer, including inhibition of metastasis and CSCs. In an effort to understand this, we studied the biophysical effects of EGCG on cell stiffness or elasticity using atomic force microscopy (AFM), and found that EGCG increased cell stiffness or elasticity of cancer cells, that is, it stiffened cells.⁹⁾

This review summarizes recent topics on green tea polyphenols relative to tertiary cancer prevention with a focus on their biophysical effects, specifically the stiffening of the cell membrane in relation to inhibition of metastasis and cancer stemness, and our recent finding of inhibition of PD-L1 expression. The topics include i) primary cancer prevention with EGCG and green tea, ii) tertiary cancer prevention by combining EGCG and anticancer drugs, iii) inhibition of metastasis with EGCG by stiffening the cell membrane, iv) inhibition of AXL receptor tyrosine kinase, a stimulator of cell softening and motility, with EGCG, v) inhibition of stemness properties with EGCG, and vi) EGCG as an alternative chemical immune checkpoint inhibitor.

2. Primary Cancer Prevention with EGCG and Green Tea

As the historical development of EGCG and green tea as primary cancer preventives was summarized in several review articles,^{2,7)} we briefly highlight the important results of EGCG and green tea as follows. 1) Numerous scientists have shown that oral administration of EGCG and green tea extract (GTE) inhibited carcinogenesis in many organs induced by various

carcinogens in rodents, indicating that EGCG and green tea have a wide range of target organs.²⁾ 2) A prospective cohort study in Saitama prefecture first revealed that consumption of green tea had cancer preventive activity in humans. Specifically, consumption of green tea over 10 cups per day delayed cancer onset by 7.3 years in women and 3.2 years in men and was effective in the target organs lung, liver, colon, and stomach.¹³⁾ The results led us to determine the cancer preventive amounts of green tea to be 10 cups/d or 2.5 g GTE/d. 3) A significant preventive activity of green tea in humans was proven by randomized phase II clinical trials. Consumption of 10 cups of green tea supplemented with green tea tablets showed 50% preventive activity for recurrence of colorectal adenomas in collaboration with the Moriawaki group at Gifu University in Japan.¹⁴⁾ Later, Shin *et al.* reported similar preventive results using green tea tablets made in Korea.¹⁵⁾ A large randomized controlled trial for prevention of colorectal adenomas with green tea extract, named “Minimizing the Risk of metachronous Adenomas of the CoLorectum with green tea Extract (MIRACLE),” was started in 2011 in Germany.¹⁶⁾ Interim results from MIRACLE, reported in 2019 at the European Society for Medical Oncology Congress, showed that green tea extract displayed a preventive tendency with no adverse effects.¹⁷⁾ Furthermore, several phase II clinical trials with GTE indicated its preventive effects toward cancer development for patients with preneoplastic diseases in the prostate and oral cavity.^{18–20)} All results indicated that “Primary cancer prevention” for the general population can be achieved by

drinking 10 cups of green tea/d to delay cancer onset (Fig. 2).

3. Tertiary Cancer Prevention by Combining EGCG and Anticancer Drugs

Today, many “healthy” cancer patients who complete treatment (cancer survivors) still have a long life expectancy because of recent advancements in cancer diagnoses and treatments. The latest cancer statistics in Japan showed that the 5-year survival rate of cancer patients was about 62.1% for all cancers.²¹⁾ A number of cancer survivors require drugs and preventives with potent efficacy that can prevent recurrence, second primary tumors and metastasis. We first demonstrated that the combination of sulindac or tamoxifen with EGCG induced synergistic or additive effects on the induction of apoptosis in human PC-9 lung cancer cells, and inhibition of tumor necrosis factor- α (TNF- α) release from BALB/3T3 cells stimulated by the tumor promoter okadaic acid.²²⁾ Because TNF- α is an endogenous tumor promoter, inhibition of TNF- α release and its gene expression are a crucial mechanism functioning in cancer prevention.^{23,24)} We have also reported synergistic inhibition of intestinal tumor formation in Min mice, a mouse model of Familial Adenomatous Polyposis (FAP), by combining GTE and sulindac, and strong inhibition of lung tumor formation in A/J mice by combining GTE and celecoxib.⁸⁾ The molecular mechanisms involved in synergistic enhancement by the combination with EGCG and cyclooxygenase inhibitors (sulindac and celecoxib) or a synthetic retinoid Am80 were demonstrated to involve strong induction of *growth arrest and DNA damage-inducible 153 (GADD153, CHOP)* gene expression, resulting in induction of the death receptor and the TNF-related apoptosis-inducing ligand (TRAIL)-apoptotic pathway.^{25,26)} Numerous reports have revealed synergistic effects of the combination with EGCG and various anticancer agents, which we summarized in a review article.²⁷⁾ Briefly, EGCG and other green tea catechins were shown to act in synergy toward 46 anticancer drugs *in vitro* using 58 human cancer cell lines. A strong reduction of tumor volume was shown in 13 *in vivo* experiments in xenograft models in mice with an average reduction by 70.3% after treatment with a combination of EGCG and various anticancer agents.²⁷⁾ Based on these results, we have proposed tertiary

cancer prevention should include using green tea or a combination of green tea plus anticancer drugs (Fig. 2).

4. Inhibition of Metastasis with EGCG by Stiffening the Cell Membrane

Cell stiffness, cell motility and EMT are closely related to the metastatic potential of cancer cells.²⁸⁾ The stiffness of living cells can be quantitatively measured as Young's modulus (Pa) using AFM by direct attachment to the cells.^{29,30)} The cell stiffness of a single cell is usually measured over the center of the nuclei. A low Young's modulus value indicates softness and is a common biophysical phenotype of various human cancer cells, such as breast, cervix, ovary, bladder, pancreas, stomach, lung and the oral cavity, compared with the normal corresponding cells.³¹⁾ Cross *et al.* reported that metastatic cancer cells obtained from the body fluids of patients with lung, breast, and pancreas cancers have significantly lower Young's modulus than normal mesothelial cells from the same body fluids.³²⁾ We also reported that Young's modulus could show discrete metastatic potentials among three mouse B16 melanoma subclones, B16-F1, B16-BL6 and B16-F10 with similar morphologies and growth rates. The highly metastatic B16-F10 cells with the highest motility showed a 0.48-fold lower Young's modulus compared with the less metastatic B16-F1 cells.³³⁾ The concept that stiffness is a good indicator for cancer progression is strongly supported by a study of cell stiffness and motility using human normal bronchial epithelial cells (NBE) established from the bronchi of four lung cancer patients and six human lung cancer cell lines. The Young's moduli of the four normal NBEs were significantly higher than those of all six human lung cancer cell lines. Additionally, the three cancer cell lines with low motility showed significantly higher Young's moduli than those of the other three cancer cell lines with high motility (Table 1).

It is important to note that treatment with green tea extract increased cell stiffness about 6.2-fold in cancer cells obtained from the pleural effusion of patients with pancreas, lung, ovary, and breast cancers, while EGCG did not affect the cell stiffness of normal mesothelial cells in a pleural effusion (1.1-fold)³⁴⁾ (Table 2). We also found that treatment with EGCG increased the stiffness of B16-F10 cells about 1.8-fold

Table 1. Distinct Stiffness Measured with Young's Moduli among Human Normal Bronchial Epithelial Cells and Human Lung Cancer Cell Lines with Different Motilities

	Cell stiffness, Young's modulus (kPa, mean $\pm$ S.D.)	Motility, No. of migrated cells (mean $\pm$ S.D.)	Phospho-AXL/AXL relative amount
Normal bronchial epithelial cells			
NBE-1	7.23 $\pm$ 0.19	—	—
NBE-2	5.10 $\pm$ 0.15	—	—
NBE-3	3.78 $\pm$ 0.07	—	—
NBE-4	4.76 $\pm$ 0.12	—	—
Low motility lung cancer cells			
A549	2.71 $\pm$ 0.14	83.5 $\pm$ 24.3	0.05 $\pm$ 0.02
H322	3.01 $\pm$ 0.31	10.5 $\pm$ 4.4	0
H1703	3.18 $\pm$ 0.38	85.3 $\pm$ 25.3	0
High motility lung cancer cells			
LC-AI	1.44 $\pm$ 0.06	312.8 $\pm$ 111.6	0.34 $\pm$ 0.12
H1299	1.75 $\pm$ 0.04	229.7 $\pm$ 70.9	1.23 $\pm$ 0.67
Lu99	1.66 $\pm$ 0.08	257.9 $\pm$ 71.2	0.31 $\pm$ 0.14

Normal bronchial epithelial cells (NBEs); standard deviation (S.D.); * $p < 0.01$.

Table 2. Increased Stiffness and Inhibition of Motility with Green Tea Extract, (–)-Epigallocatechin Gallate and Methyl- β -cyclodextrin

Treatments	Cells	Stiffness (fold)	Motility (fold)	Ref.
GTE	Tumor cells in pleural effusion from pancreatic, lung, ovarian and breast cancer patients	6.2	—	33
GTE	Normal mesothelial cells in pleural effusion	1.1	—	33
	Mouse melanoma cells			
EGCG	B16-F10	1.8	0.6	32
EC	B16-F10	0.8	1.1	32
	Human lung cancer cells			
EGCG	H1299	1.8	0.4	36
	Lu99	1.8	0.3	
M β CD	H1299	2.2	0.4	36

Green tea extract (GTE); (–)-epigallocatechin gallate (EGCG); (–)-epicatechin (EC); methyl- β -cyclodextrin (M β CD).

and inhibited motility by 0.6-fold, but (–)-epicatechin (EC), an inactive catechin, showed no significant effects in B16-F10 cells³³⁾ (Table 2). These results were supported by our previous results that oral administration of EGCG in drinking water significantly inhibited spontaneous lung metastasis of mouse melanoma B16-BL6 cells inoculated into the right foot pad, and artificial lung metastasis of B16-F10 cells injected intravenously in male C57BL/6 mice.³⁵⁾ EGCG also increased the stiffness of H1299 and Lu99 human lung cancer cells by about 1.8-fold, and was associated with inhibition of motility (Table 2), and significant reduction of the high expression of vimentin and Slug, two mesenchymal marker proteins at the leading edge of the scratch.³⁶⁾ Stiffening of cancer cells with EGCG is a key biophysical mechanism for inhibition of motility, EMT and metastasis.

We have proposed that EGCG acts through a “sealing effects” that it interrupts the interaction of ligands with their receptors on the cell membrane. This proposal was based on our previous results that topical application of EGCG inhibited tumor promotion induced by two different tumor promoters acting *via* distinct mechanisms, teleocidin (TPA-type) activating protein kinase C and okadaic acid, which inhibits protein phosphatases 1 and 2A. EGCG inhibited the specific binding of both ³H-TPA and ³H-okadaic acid to a particulate fraction of mouse skin containing their receptors.²⁾ Furthermore, the “sealing effects” of EGCG were observed by inhibition of binding of estrogen and epidermal growth factor (EGF) to their receptors. The Weinstein group also reported that EGCG caused a reduction in the detergent-insoluble membrane domain, *i.e.*, a decrease in the lipid raft in the cellular membrane, resulting in inhibition of EGF receptor signaling.³⁷⁾ Therefore, we hypothesize that the cell membrane is biomechanically affected by EGCG. Treatment with methyl- β -cyclodextrin (M β CD), a compound that disrupts the lipid raft in the cell membrane, induced effects similar to EGCG with a 2.2-fold increase of Young’s moduli and 0.4-fold decrease of motility in H1299 cells³⁶⁾ (Table 2). Thus, EGCG induces alteration of membrane organization, resulting in an increase of stiffness and inhibition of motility.

Recently, Matsuzaki *et al.* reported that incubation of cell-sized lipid vesicles (the giant liposome) with EGCG increased the bending stiffness of the lipid membrane by approximately 60-fold, while EC had no detectable effect.³⁸⁾ They discussed that the membrane stiffening was mainly attributed to the physical adsorption of catechins with a galloyl moiety, such

as EGCG and (–)-epicatechin gallate (ECG), to the membrane surface (Fig. 1). Tsuchiya *et al.* also reported using fluorescence polarization that EGCG reduced the fluidity of the lipid membranes.³⁹⁾ The galloyl moiety of green tea polyphenols is likely more strongly adsorbed to phospholipids *via* either hydrophobic interactions and/or cation- π interactions, while catechins without a galloyl moiety do not strongly adsorb to phospholipids. We believe this difference is because of higher numbers of flexible conformations of catechins with a galloyl moiety than catechins without a galloyl moiety.⁹⁾ Changes of the cell membrane with EGCG treatment were also observed in reduction of the contact area to substrate in B16-F10 cells, indicating inhibition of adhesion.⁴⁰⁾ Interaction of green tea polyphenols with lipids and proteins in the cell membrane causes stiffening of the cell membrane resulting in inhibition of receptor signaling including “sealing effects,” leading to inhibition of motility and metastasis, and eventually resulting in primary and tertiary prevention of cancer.

5. Inhibition of AXL Receptor Tyrosine Kinase, a Stimulator of Cell Softening and Motility, with EGCG

Reduction of cell stiffness or cell softening plays an important role in malignant progression, and activation of AXL receptor tyrosine kinase is involved in cell softening and an increase of motility and probably metastasis. AXL is a member of the TAM (Tyro3, AXL and Mer) receptor tyrosine kinase family.⁴¹⁾ AXL induces EMT and malignant phenotypes of various cancer cells derived from the lung, breast and pancreas.⁴²⁾ Overexpression of AXL and highly phosphorylated AXL are frequently found in human lung cancer cell lines and lung cancer tissues, but not in normal lung tissues.⁴²⁾ High expression of AXL and growth arrest specific 6 (Gas6), a ligand of AXL, have been associated with poor survival of patients along with metastasis in lung, breast and pancreas cancers.⁴³⁾ Biophysical studies of six lung cancer cell lines revealed that those with high motility and low stiffness have high phospho-AXL (an active form of AXL) levels compared with other cell lines with low motility (Table 1). Knockdown of AXL with AXL-targeted small interfering RNA (siRNA) enhanced the Young’s modulus value with 236.4% and inhibited motility to 48% in H1299 cells with high motility and low stiffness⁴⁴⁾ (Table 3). Conversely, exogenous expression of the *AXL* gene in human H1703 lung cancer cells with low motility and high stiffness reduced the Young’s modulus value to about 50%, and stimulated motility by 250%. Activation of AXL reduces

actin stress fiber formation mediated through the Ras/Rac pathway.⁴⁴⁾ Thus, AXL is a stimulator of cell softening (reduces Young's modulus value) resulting in high motility and metastatic potential. Pretreatment with EGCG significantly inhibited phosphorylation of AXL, an active form of AXL, stimulated by Gas6; the phospho-AXL/AXL ratio was reduced to 0.42 and 0.55 by treatment with EGCG and ECG, respectively⁴⁵⁾ (Fig. 3, Table 3). Therefore, we conclude that stiffening of the cell membrane with green tea polyphenols inhibits AXL activation, leading to a further increase of cell stiffness by stimulation of actin stress fiber formation, and eventually inhibiting malignant progression.

6. Inhibition of Stemness Property with EGCG

Recently, CSCs have been attracting attention as a key target for inhibiting cancer recurrence and metastasis. CSCs were defined in 2006 by the American Association for Cancer Research Workshop on Cancer Stem Cells as “a cell within a tumor that possesses the capacity to self-renew and cause the heterogeneous lineages of cancer cells that comprise the tumor.”⁴⁶⁾ CSCs are a small minority of cells that have a high ability to form tumors in xenograft models in immunodeficient mice. Theoretically, one CSC can produce a tumor. Furthermore, CSCs are resistant to chemotherapy and radiotherapy, and thus, CSCs regenerate tumors even after the bulk tumor cells are killed by treatment. Thus, the development of inhibitors of CSCs is desired to improve cancer treatment and tertiary cancer prevention.

Numerous investigators reported that EGCG inhibited the expression of stemness marker genes and EMT-related

genes in human CSCs enriched by tumor sphere formation from breast, lung, prostate and liver cancer cell lines.^{10,11)} Herein, we describe our investigation of CSCs from lung cancer cells. Tumor spheres of H1299 cells obtained by culture in low attached and serum free conditions showed 2- to 67-fold higher expression of stemness marker genes (*CD133*, *ALDH1A1*, *NANOG*, *SOX2*, and *Oct4*) and EMT-related genes (*N-cadherin*, *Vimentin*, *Snail*, *Slug*, and *ZEB1*), compared with the parental H1299 cells. Treatment with EGCG, (–)-epigallocatechin (EGC) and ECG reduced the numbers of tumor spheres from 35.0 to 16.0 (45.7%), 27.6 (78.8%), and 23.7 (67.7%), respectively, while EC did not reduce tumor sphere formation.⁴⁵⁾ Further, EGCG significantly reduced *ALDH1A1* and *Slug* mRNA expression in tumor spheres.

Recently, we succeeded in measuring the stiffness of CSCs isolated from tumor spheres of H1299 cells (named H1299-sdCSCs) using AFM, and found that the Young's modulus values of the CSCs were 0.68-fold (*i.e.*, 1.52/2.24 kPa) lower than the H1299-parental cells, indicating CSCs possessed less stiffness and softer elasticity than parental cells⁴⁵⁾ (Fig. 4). Similar results were reported for the human MHCC97H hepatoma stem cell line, which showed 0.7-fold lower stiffness than the parental cells.⁴⁷⁾ A study using a microfluidic device showed that aldehyde dehydrogenase (ALDH)⁺ CSCs isolated from the inflammatory breast cancer cell line SUM149 revealed higher deformability than ALDH[–] cells.⁴⁸⁾ These results coincided with those from several studies showing that embryonic stem cells are considerably softer before undergoing the cellular differentiation process. Treatment of CSCs derived from tumor spheres with EGCG resulted in a 1.8-fold increase of Young's modulus values⁴⁵⁾ (Fig. 4). Thus, EGCG reversed the biophysical property of CSCs to that of the parental cells. It is important to note that salinomycin, one of the most potent anti-CSC compounds screened by the Weinburg group, increased the stiffness of liver CSCs in a manner similar to that of EGCG.^{49,50)} The study of whether salinomycin increases the stiffness of the CSCs would be a topic of interest for future work.

AXL receptor tyrosine kinase, a stimulator of cell softening, also contributes to the stemness of H1299-sdCSCs. Knockdown of AXL with siRNA reduced the number of tumor spheres of H1299 by 13.3% (Table 3), along with reduction of *ALDH1A1* and *Slug* mRNA expression.⁴⁵⁾ Furthermore, transplantation of 1×10^6 cells of an AXL-high clone, which expressed AXL protein >2-fold higher than bulk H1299 cells, produced larger tumors more rapidly than bulk H1299 cells in immunodeficient mice (SCID/Beige). The AXL-high clones

Table 3. Comparison of the Biophysical and Biological Effects of Treatment with (–)-Epigallocatechin Gallate and Knockdown of AXL in H1299 Cells

	EGCG (50 μ M) % of control	Knockdown of AXL % of control
Biophysical effect		
Cell stiffness	181.5	236.4
Biological effects		
Phospho-AXL/AXL	42	0
Motility	72	48
Tumor spheres of CSCs	45.7	13.3
Tumor volume	50.0 ^{a)}	38.7 ^{b)}

a) Mice were given 100 mg/kg (–)-epigallocatechin gallate (EGCG) and 0.2% green tea extract daily. b) H1299 cells treated with siRNA targeting AXL were inoculated into the mice.

Fig. 3. Inhibition of Phosphorylation of AXL Stimulated with Gas6 by Treatment with Green Tea Polyphenols

H1299 cells were pretreated with green tea polyphenols 1 h before treatment with Gas6, a ligand of AXL. Phosphorylation levels of AXL were compared with phospho-AXL/AXL ratios. Phospho-AXL/AXL ratios in Gas6-treated cells were expressed as 1.00. * $p < 0.05$.

Fig. 4. EGCG Increased the Stiffness of Cancer Stem Cells-Derived from Tumor Spheres

Histogram of Young's modulus values for cells separated from H1299 cancer stem cells-derived from tumor spheres (H1299-sdCSCs) compared with those from H1299-parental cells. The black line indicates the log of the normal fitting curve of each treatment and the dotted line indicates the mean Young's modulus values of the control H1299-sdCSCs. E values indicate the mean Young's modulus values $\pm$ standard deviation (S.D.). $**p < 0.01$.

produced tumors at 100% of injected sites by day 31, but the AXL-low clones with only a 0.5-fold AXL level developed no tumors by day 35. Additionally, the AXL-high clone produced tumors at 100% of the injected sites when $1/10$ of the cells (1×10^5 cells) were transplanted.⁴⁵⁾ Thus, AXL protein plays a key role in inducing high tumorigenicity and maintaining the CSCs in H1299 cells. Treatment with EGCG specifically reduced the AXL-high population in tumor spheres. Importantly, the effects of knockdown of AXL on H1299 cells are very similar to the biophysical and biological effects of EGCG: an increase of Young's modulus, reduction of phospho-AXL, motility, tumor sphere formation and tumorigenicity as shown in Table 3. Knockdown of AXL with siRNA inhibited tumor formation with reduction of tumor volumes to 38.7% in a mouse xenograft model. Oral administration of EGCG (100 mg/kg of body weight) and 0.2% GTE significantly inhibited tumor growth of H1299 cells *in vivo* using a mouse xenograft model. The EGCG + GTE group developed tumors at 75% of the injected sites, while the non-treated group developed tumors at 100% of the sites. The tumor weight of the EGCG + GTE group was significantly lower than that of the non-treated

group. Additionally, treatment with EGCG + GTE reduced phospho-AXL, ALDH1A1 and SLUG protein levels.⁴⁵⁾ Taken together, inhibition of CSCs and metastasis with EGCG mediated inhibition of the AXL/ALDH/SLUG axis by stiffening the cell membrane. Because CSCs are resistant to chemotherapy, inhibition of stemness with EGCG is anticipated to elicit synergistic effects when combined with anticancer agents.

7. EGCG as an Alternative Chemical Immune Checkpoint Inhibitor

Recently, immune checkpoint inhibitors are attracting attention around the world for their effective anticancer properties based on evidence that monoclonal antibodies of immune checkpoint molecules, cytotoxic T-lymphocyte antigen-4 (CTLA-4), programmed cell death 1 (PD-1) and PD-1-ligand 1 (PD-L1) have shown great success in cancer therapy against various cancer types.^{51,52)} Expression of PD-L1, the ligand of PD-1, in cancer cells leads to T cell dysfunction; binding of PD-L1 to PD-1 inhibits T cell effector function by inducing exhaustion and apoptosis of T cells. Immune checkpoint inhibitors directly inhibit the binding of PD-L1 to PD-1, leading

to restoration of T cell function and an increase of anti-tumor immunity.

Expression of PD-L1 in cancer cells is induced by various cytokines and growth factors in the inflammatory tumor microenvironment, such as interferon- γ (IFN- γ), EGF and TNF- α .⁵⁰ Based on our results of the inhibition of receptor signaling by stiffening of the cell membrane with EGCG treatment, we inferred that EGCG might inhibit PD-L1 expression in cancer cells stimulated by cytokines or growth factors or EGCG might inhibit PD-L1 and PD-1 binding, resulting in inhibition of the immune escape of cancer cells. Although we did not examine the effect of EGCG on PD-L1/PD-1 binding, we found that green tea polyphenols, including EGCG, ECG and EGC, reduced PD-L1 expression in human lung cancer cells induced by IFN- γ or EGF. Namely, IFN- γ enhanced *PD-L1* mRNA expression and cell-surface PD-L1 protein about 4-fold in A549 cells. However, pretreatment of A549 cells with GTE, EGCG, ECG or EGC reduced cell-surface PD-L1 protein by about 40 to 80% as examined by flow cytometry with an anti-PD-L1 antibody¹² (Fig. 5). EGF-induced PD-L1 expression was also downregulated by pretreatment of Lu99 cells with EGCG. To examine the relationship between the inhibition of PD-L1 expression and inhibition of tumor development, we used the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) to induce lung carcinogenesis in A/J mice. Oral administration of 0.3% GTE in drinking water reduced the average numbers of tumors per mouse from 4.1 to 2.6 (36.6% reduction). Immunohistochemical staining of PD-L1 in lung tumors revealed that GTE reduced PD-L1 positive cells from 9.6 to 2.9% (69.8% reduction).¹² Reduction of PD-L1 protein in lung cancer cells by EGCG restored *IL-2* mRNA expression in tumor specific CD3⁺ T cells co-cultured with ovalbumin-overexpressed B16-F10 cells. EGCG partially restored T cell activity that was suppressed by PD-L1/PD-1 signaling. Importantly, other

group reported that apigenin, which is a polyphenol found in fruits and vegetables, showed very similar effects to EGCG: Apigenin inhibited IFN- γ -induced PD-L1 expression in A375 melanoma cells and breast cancer cells MDA-MB-231.^{53,54} And apigenin increased T cell-mediated killing, resulting in strong inhibition A375 melanoma xenograft growth *in vivo*.⁵⁴ A combination of these chemical immune checkpoint inhibitors with monoclonal antibodies of immune checkpoint molecules may lead to a new immunotherapeutic strategy as shown by the synergistic effects achieved from the combination of EGCG and anticancer drugs.

8. Conclusion

Green tea polyphenols, especially EGCG, have been intensively studied as primary cancer preventive agents for healthy populations, and now we recommend their use for tertiary cancer prevention among people who have experienced cancer. Based on the observation that inhibition of motility, probably metastasis, stemness properties and immune escape by treatment with EGCG, we believe that green tea polyphenols have potential clinical implications for cancer therapy. When we studied the effects of several polyphenols including curcumin, diallyl sulfide, shogaol and resveratrol on the stiffness of cells, phosphorylated AXL and tumor sphere formation, we found that curcumin showed similar effects to EGCG. Curcumin enhanced the stiffness of lung cancer cells along with reducing phosphorylated AXL stimulated by Gas6 and the number of tumor spheres.

To summarize, cumulated evidence indicates that stiffening of cancer cell membrane is a commonly underlying mechanism that may explain diversified beneficial effects of green tea polyphenols. Examination of membrane stiffening should be an effective strategy to develop new drugs for tertiary cancer prevention and treatment.

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Fig. 5. Inhibition of IFN- γ -Induced Cell Surface Programmed Cell Death-Ligand 1 (PD-L1) Protein by Green Tea Extract and Green Tea Polyphenols in A549 Cells

** $p < 0.01$, *** $p < 0.001$. (Color figure can be accessed in the online version.)

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