博士論文要旨

論文題目：**Characterisation of cell death mechanism induced by -(-)epigallocatechin gallate (EGCG) in chronic myelogenous leukemia cells**

研究分野：病態医療科学分野

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< Abstract >

Management strategies of chronic phase chronic myelogenous leukemia (CML) have drastically improved due to the development of selective tyrosine kinase inhibitor, imatinib mesylate (Glivec). However, there is the continuously arising problem of imatinib resistance mainly due to certain point mutations within the ATP-binding pocket of BCR/ABL suggesting for the necessity of alternative drugs.

Epigallocatechin-gallate (EGCG) is the major catechin component and chemopreventive polyphenol that is found in green tea, one of the most consumed beverages in the world. Anti-tumour activity of EGCG has been gaining much attention due to their selective cytotoxic effects towards various tumour cell lines and not to their normal counterparts. Anti-tumour activity of EGCG has been reported to result from inhibition of multiple signaling pathways such as cell cycle arrest, inhibition of telomerase activity, and inhibition of metastasis via binding to laminin receptor, LR67. Although several mechanisms for EGCG-induced anti-tumour activity have been proposed, they generally shared a common final phase of apoptosis. However, our preliminary data on Wright-Giemsa-stained CML cells after EGCG treatment showed atypical cell death morphology where apoptotic morphology was absent. This prompted us to specify the mode of EGCG-induced cell death in CML cell lines based on biochemical and morphological approaches not only to clarify its anti-tumour activity, but also on the hypothesis that if EGCG induced nonapoptotic cell death, it may be clinically beneficial in overcoming both imatinib-sensitive and -resistant CML cells, often with apoptosis-resistant characteristics due to constitutively active BCR/ABL suppressing the apoptotic pathway.

< Methods and Results >

EGCG induces cell death distinct from imatinib-induced apoptosis

In order to verify the effect of EGCG and imatinib on the mitochondrial transmembrane potential (MMP) and plasma membrane, K562 and C2F8 CML cell lines were stained with DiOC(6), mitochondria-specific marker, and PI, after treatment with increasing concentrations of EGCG and imatinib. As a result, both EGCG and imatinib induced the reduction of MMP and increasing of PI-positive cells (Fig 1), however, distinct cell death population pattern resulted between EGCG and imatinib treatments, suggesting for their distinct modes of cell death.

Caspase-independent necrosis-like cell death in EGCG-induced cell death

To ascertain whether EGCG induced a non-apoptotic cell death, activation of caspases, key executioners in apoptotic pathway, were examined in EGCG-induced cell death by Western blotting. Although imatinib-treated K562 and C2F8 cells showed activation of all caspases tested and PARP cleavage at 24 h (Fig 2A), EGCG-treated K562 and C2F8 cells only showed a partial activation of caspases-9 and caspase-8, but

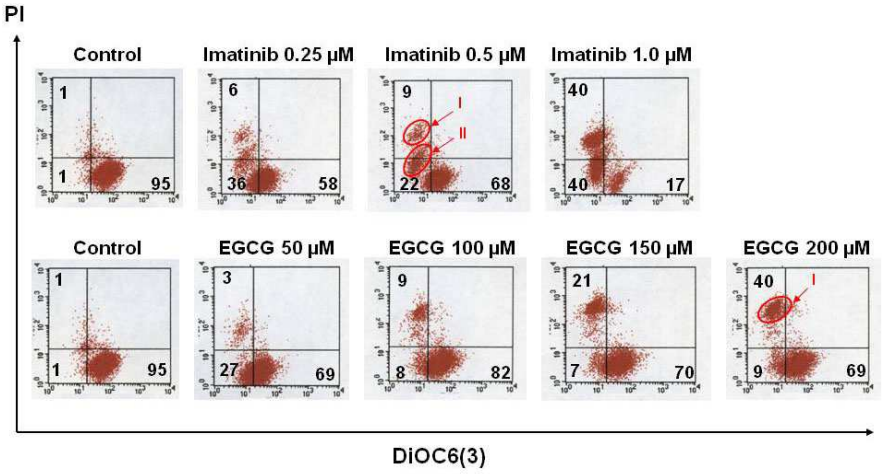

Fig 1. EGCG and imatinib showed distinct cell death patterns. K562 cells were treated with 0.1% ethanol (control), EGCG or imatinib for 48 h, harvested and incubated with $DiOC_6(3)$ and PI. Mitochondrial transmembrane potential ($\Delta\psi m$) and cell death were determined by dual-parameter flow cytometry. Results are representative of three independent experiments.

caspase-3 and PARP were not activated with exposure to EGCG at 24 h (Fig 2A). Also, to confirm this result, K562 cells were treated with either EGCG or imatinib alone or after pretreatment with Z-VAD-FMK, broad caspase inhibitor, for 48h. Imatinib-induced apoptosis was prevented by Z-VAD-FMK dose-dependently ($p < 0.01$) (Fig 2B), however, EGCG-induced cell death was not prevented at all by any concentrations of Z-VAD-FMK ($p < 0.01$) (Fig 2B), suggesting that EGCG induced predominantly caspase-independent cell death. Also, reduction of anti-apoptosis factors such as inhibitor of apoptosis proteins (IAP) and Bcl-2 could not be observed in EGCG-induced cell death, suggesting that EGCG did not interfere either with the safeguard apoptotic pathway (Data not shown).

Since a clearer delineation of the mode of cell death was based on morphological studies, we next examined the morphological differences in EGCG-treated and imatinib-treated cell deaths in K562 and C2F8 cells using an electron microscope. As a result, imatinib-induced cell death exhibited a typical apoptotic feature with smoothing of cell surface, nuclear fragmentation with compaction of chromatin to the crescents adjacent to the nuclear envelope and rather structurally intact mitochondria (Fig 3A). EGCG-induced cell death resembled significantly the necrotic morphology induced by ATP-depletion without nuclear fragmentation but with significant cytoplasmic vacuolations with severe disruption of cell plasma membrane, mitochondrial membrane, and even detachment of nuclear envelope developing a large vacuole (Fig 3A).

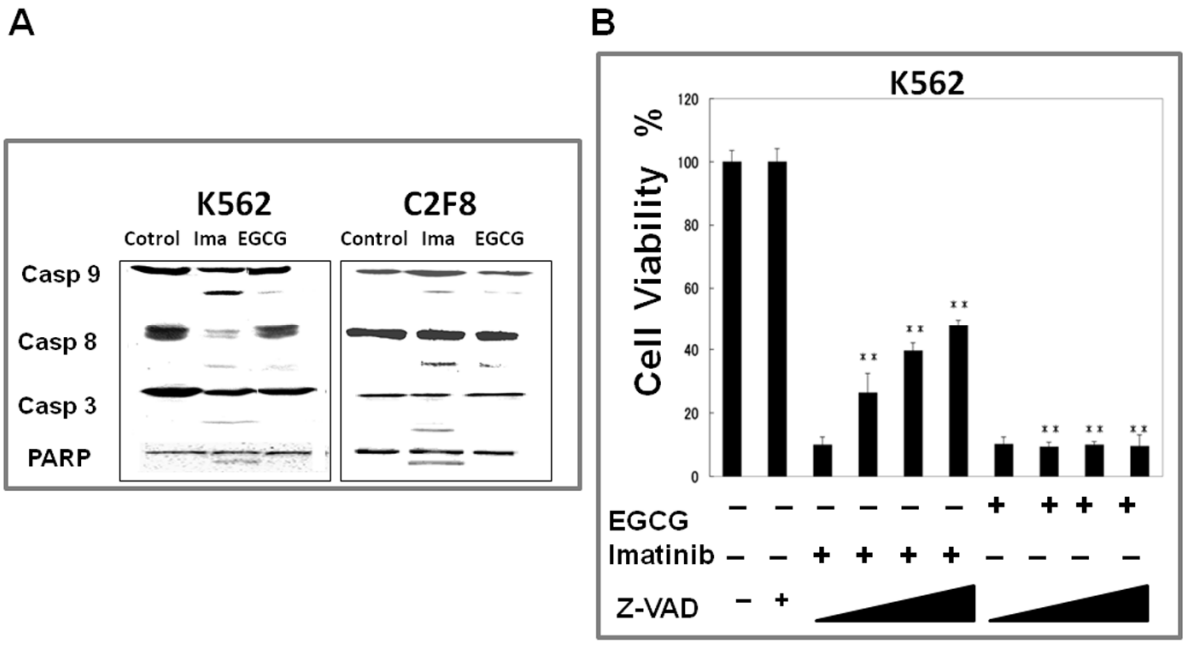

Fig 2. (A) EGCG-induced cell death is caspase-independent. K562 cells were treated with 0.1% ethanol (control), EGCG or imatinib for 24 h, and subjected to Western blotting. **(B) Z-VAD-FMK fails to prevent EGCG-induced cell death.** K562 cells were preincubated with 0.1% ethanol (control) or increasing doses of Z-VAD-FMK for 1 h and subsequently treated with or without 200 μM EGCG or 1 μM imatinb for 48 h. The cell viability was measured based on intracellular ATP content. Asterisks indicate significant difference from single imatinib treatment and significant indifference for EGCG-treated cells ($P < 0.01$).

Quantification analysis of these distinct types of cell death by counting toluidine blue stained cells showed that EGCG predominantly induced cell death with necrotic characteristics such as cytoplasmic vacuolated cells (65 ± 5%) with lumpy chromatin with almost no apoptptic bodies (0.6 ± 5%) (Fig 3B).

EGCG effect on imatinib-resistant CML cells

In addition, we evaluated the effectiveness of EGCG towards imatinib-resistant CML cells, K562/sti, and EGCG effectively induced cell death in imatnib-resistant CML cell lines with similar IC50 value as for imatinib-sensitive cell line, whereas this cell line showed strong resistance to imatinib (Fig 4). Moreover, combination treatment of EGCG and imatinib induced enhanced (additive) cell death in K562 cells (Data not shown), which probably reflects the fact that EGCG and imatinib trigger distinct cell death pathways. Meanwhile, EGCG is expected to have minimal side effects to the patients as the anti-cancer drug, because EGCG did not show significant cytotoxicity to healthy peripheral blood mononuclear (PBMC) cells. These results suggest for the possibility of EGCG as a potential anticancer agent that may overcome the imatinib-resistance, and worthy of further pre-clinical experiments using mouse models.

< Summary >

We have demonstrated for the first time that EGCG predominantly induced necrosis-like cell death in CML cell lines via a caspase-independent mechanism, distinct from imatinib-induced typical apoptosis. We have also

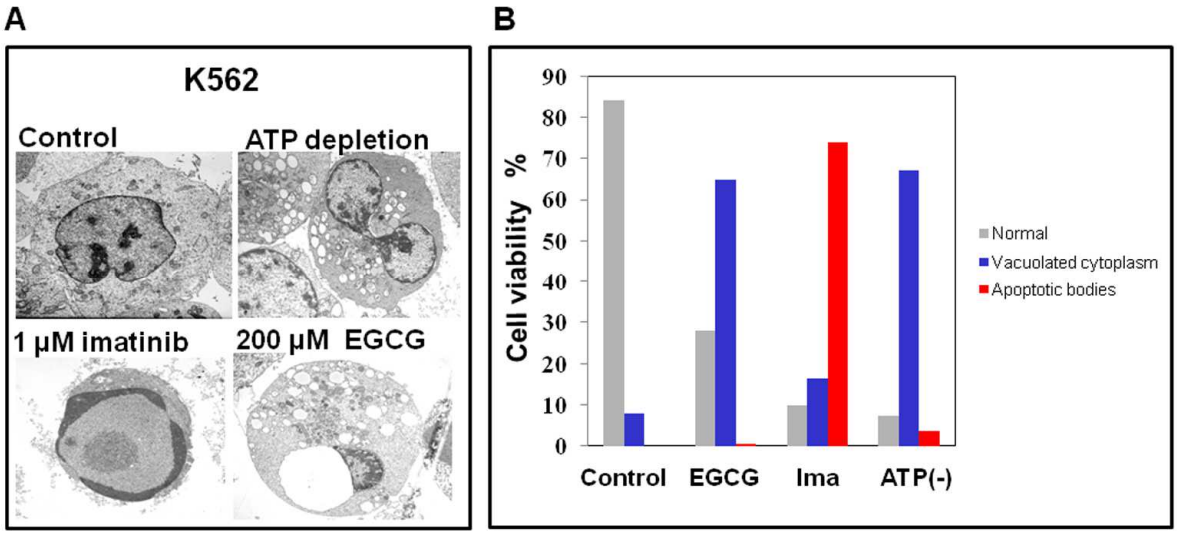

Fig 3. (A) EGCG treatment caused necrotic cell death morphology resembling ATP-depleted condition. K562 cells were grown in glucose-free medium with 5 μg oligomycin for ATP-depleted condition for 12 h; treated with 1 μM imatinib or 200 μM EGCG for 48 h and compared with the 0.1% ethanol-treated control K562. **(B) Quantification analysis of necrotic (cytoplasmic vacuolation) and apoptotic cells induced by EGCG, imatinib or ATP-depletion.**

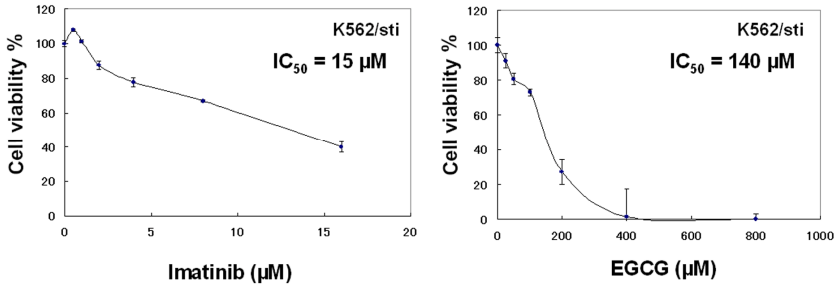

Fig 4. EGCG was equally effective to induce cell death in imatinib-resistant CML cells. Imatinib-resistant K562 (K562/sti) cells were seeded at a density of 5 x 10^3 cells per well in 96-well plates, and treated with increasing concentrations of EGCG or imatinib for 48 h, and the number of viable cells were measured based on intracellular ATP content.

shown that activating this alternative pathway has been advantageous in overcoming the imatinib-resistant CML cells. Since failure in apoptotic machinery is one of the features of chemoresistant tumors, this pro-necrotic effect of EGCG can possibly be extrapolated to other multidrug resistant leukemia or other types of tumors. It should also be of interests to oncologists and immunologists to further investigate the impact of the necrotic cell death in surrounding tissues and our immune response against cancer.

References

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2) Khan N, Afaq F., Mukhtar H. (2006) Cancer Research, 66: 2500-5.