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BRIEF COMMUNICATION

Chemotherapeutic Candidate Inducing Immunological Death of Human Tumor Cell Lines

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The immunological death induction by EY-6 on the human tumor cell lines was screened. Human colon carcinoma (HCT15, HCT116), gastric carcinoma (MKN74, SNU668), and myeloma (KMS20, KMS26, KMS34) cells were died by EY-6 treatment with dose-dependent manner. CRT expression, a typical marker for the immunological death, was increased on the EY-6-treated colorectal and gastric cancer cells. Interestingly, the effects on the myeloma cell lines were complicated showing cell line dependent differential modulation. Cytokine secretion from the EY-6 treated tumor cells were dose and cell-dependent. IFN-γ and IL-12 secretion was increased in the treated cells (200% to over 1000% of non-treated control), except HCT116, SNU668 and KMS26 cells which their secretion was declined by EY-6. Data suggest the potential of EY-6 as a new type of immuno-chemotherapeutics inducing tumor-specific cell death. Further studies are planned to confirm the efficacy of EY-6 including *in vivo* study.

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In the previous study (1), our laboratory defined the EY-6 (a newly synthesized indoledione derivative, methyl 6-oxo-3, 6-dihydro-2H-naphtho[2,1-b][1,4]thiazine-2-carboxylate) as a possible candidate of chemotherapeutic agent killing tumor cell specifically by inducing immunological death in mouse colon cancer (MC38 cells in C57BL/6 mouse) model. Data revealed that EY-6 could kill the tumor cell without harming the normal immune cells. The killed-tumor cells expressed significantly up-regulated calreticulin (CRT), heat-shock proteins (HSPs) on their surface inducing increased DC uptake of these cells. Also EY-6 treated tumor cells secreted the IFN-γ which may manipulate the tumor-microenvironment favorable to eliminate tumor cells. Thus the study was expanded with human tumor cell lines to confirm EY-6 as a possible candidate as immuo-chemotherapeutics. Human colon carcinoma (HCT15, HCT116), gastric carcinoma (MKN74, SNU668), and myeloma (KMS20, KMS26, KMS34) cell lines were treated with EY-6 at different doses (0, 25 and 50 uM) for 24, 48 and 72 hrs to observe the cytotoxicity by MTT assay. All the tumor cells were died by EY-6 with dose-dependent manner. However, differential intensity of cytotoxicity among tumor cells was observed. Cell death was peaked on 48 hr after EY-6 incubation. Thus the data from 48 hr incubation with EY-6 were presented as representative cytotoxic effect of chemicals on the human tumor cells (Fig. 1). It has been noted that the surface expression of CRT or heat-shock proteins on the anthracyclines-killed tumor cells leads to the induction of tumor-specific immune responses (2-5). Especially, tumor cell surface translocation of CRT, a Ca_2^+-binding chaperone protein that is located in the lumen of the endoplasmic reticulum (ER), was reported as a representative event defining immunological death of tumor cells. Translocating cytosolic CRT onto the dead tumor cell surface functions as an "eat-me" signal, making the cell more attractive for uptake by antigen presenting cell and thus inducing tumor-specific immunity (6). Treating tumor cells with EY-6 for 48 hr in-

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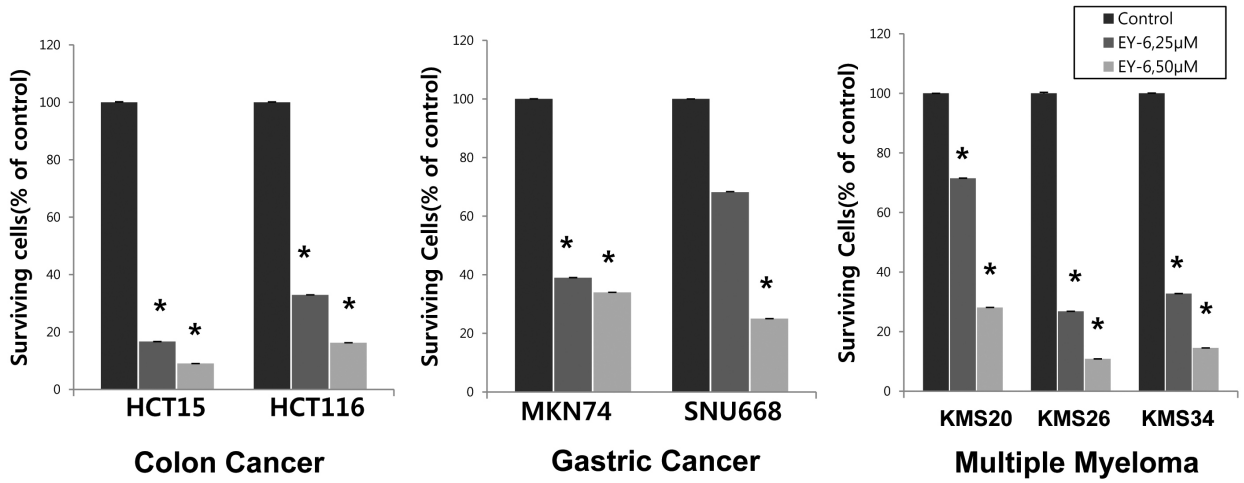

Figure 1. Cytotoxicity induced by EY-6 was determined by MTT assay. Human tumor cell lines (colon cancer cells HCT15, HCT116; gastric cancer cells MKN74, SNU668; and myeloma cells KMS20, KMS26, KMS34) were incubated with different doses (0, 25 and 50 μM) of EY-6 for 48 hr at 37°C. After the incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT solution, 5 mg/ml) was added and incubated for 4 h at 37°C. At the end of the incubation, supernatant was removed and the color change induced by dimethyl sulfoxide (DMSO) was determined at 540 nm with ELISA reader (Molecular Devices, Sunnyvale, CA, USA). The analyzed values were reported as the mean±standard error. Statistical significance was determined by two-tailed Student's *t*-test. Asterisks represented the statistical significance ($p<0.05$) compared to 0 μM-control group.

Table I. EY-6-induced CRT expression on the tumor cell surface. Cells expressing CRT-FITC on their surface after 48 hr incubation with EY-6 was observed by flow cytometry. Alteration of CRT-FITC expression was reported by MFI in each group

EY-6 dosage	Colon cancer		Gastric cancer		Multiple myeloma		
	HCT15	HCT116	MKN47	SNU668	KMS20	KMS26	KMS34
0 μM (control)	16.0	18.8	15.3	11.9	58.1	12.5	50.1
25 μM	36.7	18.2	17.4	12.5	86.5	24.7	48.5
50 μM	59.7	30.5	22.2	22.5	57.9	48.6	44.7

duced the surface expression of CRT most significantly on the HCT15, a colon cancer cell and KMS26, a myeloma cell with dose-dependent manner (Table I). On the other hand, the expression of CRT on the HCT116, a colon cancer cell and the two gastric cell lines MKN47 and SNU668 was induced only with higher concentration (50 uM) of EY-6. Response in multiple myeloma cells was interesting that without EY-6 exposure, two cell lines KMS20 and KMS34 expressed CRT about 4 times more than all the other tumor cells tested. And EY-6 did not affect the CRT expression of these cells (Table I). In mouse colon cancer model (1), EY-6 induced the secretion of immune-stimulatory cytokine, IFN-γ from the tumor cells. It suggested the possible involvement of EY-6 in the improvement of immune-suppressive tumor microenvironment which may allow the tumor immunity induction. Thus, the secretion of immune-stimulatory cytokines from the EY-6 treated human tumor cells was observed. Cytokine that induces the Th1 responses, IL-12 has an important role in anti-tumor immunity like antigen-specific cytotoxic T cell induction. EY-6 exposed HCT15, a colon cancer cell, produced significant amount of IL-12 (Fig. 2). Same phenomenon was detected in MKN74, a gastric cell and KMS20, a myeloma cell but with lower level secretion (Fig. 2). However, EY-6 dose-dependent reduction of IL-12 secretion was also observed in tumor cells like HCT116, a colon cancer cell and KMS26, a myeloma cell (Fig. 2). IFN-γ secreted into the tumor-microenvironment by infiltrated cells was known to have an important role as immune-angiogenic switch (7) which can induce the anti-tumor effect. Immunotherapy with IFN-γ secreting tumor cells was proven to be effective cancer vaccine in animal model (8,9). However, in general, IFN-γ was known to be produced by T cell or NK cell but not by tumor

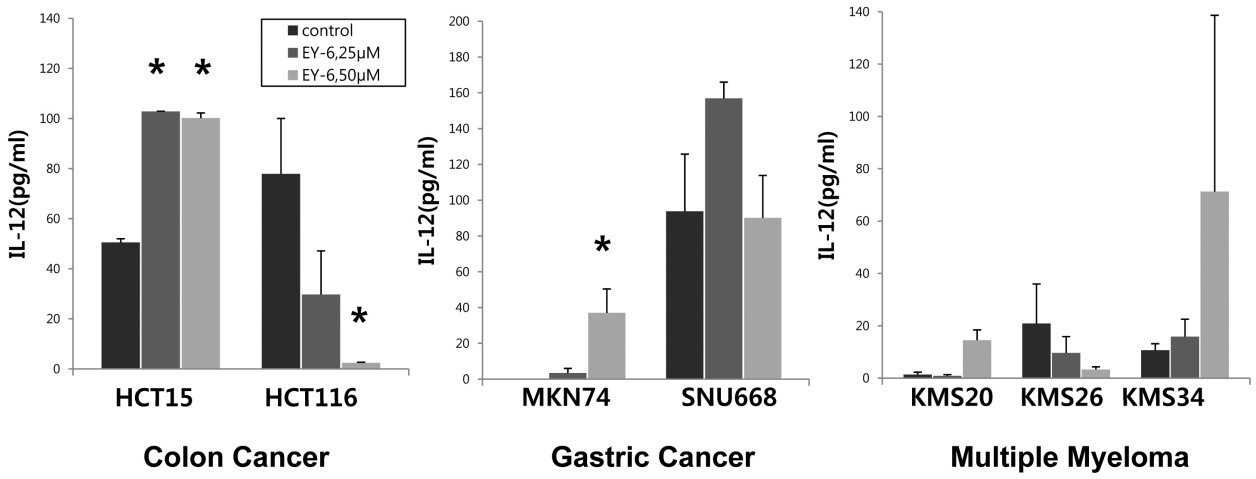

Figure 2. EY-6 induced IL-12 secretion from the tumor cells. Culture supernatants from tumor cells incubated with different doses of EY-6 for 24 hr were obtained to measure the secreted IL-12 by ELISA using commercially available assay kit (eBioscience, SanDiego, CA, USA). The amount of IL-12 (pg/ml) was reported as the mean±standard error. Statistical significance was determined by two-tailed Student's *t*-test. Asterisks represented the statistical significance ($p<0.05$) compared to control group.

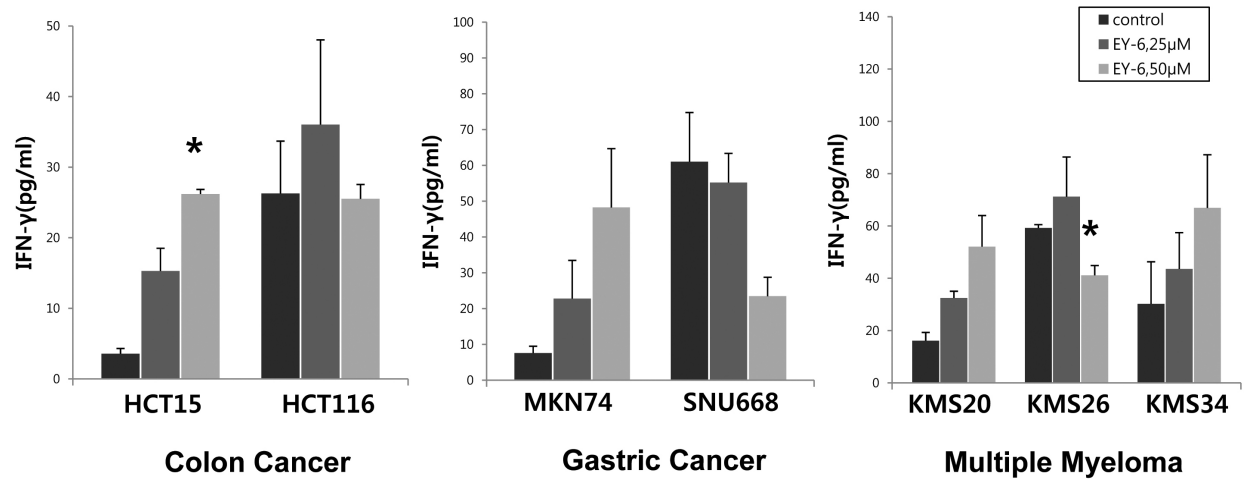

Figure 3. EY-6 induced IFN-γ secretion from the tumor cells. Culture supernatants from tumor cells incubated with different doses of EY-6 for 24 hr were obtained to measure the secreted IFN-γ by ELISA using commercially available assay kit (eBioscience, SanDiego, CA, USA). The amount of IFN-γ (pg/ml) was reported as the mean±standard error. Statistical significance was determined by two-tailed Student's *t*-test. Asterisks represented the statistical significance ($p<0.05$) compared to control group.

cells. Thus to achieve the anti-tumor effect with tumor cell vaccine, IFN-γ gene was transfected. However, interestingly enough, EY-6 treated mouse colon cancer cells produced a significantly elevated amount of IFN-γ (1). This might suggest one of the mechanisms that EY-6 induced the immunological anti-tumor effect. A significantly elevated IFN-γ secretion was observed in human tumor cells also, as demonstrated in EY-6 treated HCT15, a colon cancer cell and MKN74, a gastric cancer cell (Fig. 3). On the other hand, significant reduction of IFN-γ secretion was observed in the EY-6 treated SNU668, a gastric cancer cell and in KMS26, a myeloma cell (Fig. 3). The sensitivity to the EY-6 induced cytotoxicity correlated with CRT expression and immune-stimulatory cytokine secretion in HCT15 colon cancer cells. Data suggest the possibility of EY-6 as a candidate of immuno-chemotherapeuics for some cancer by which may kill the tumor

cells directly as well as induce the tumor antigen specific immunity to evoke complete tumor cell elimination. Possible mechanisms are increased "eat-me" signal for DC uptake of dead tumor cells by CRT expression and induction of stimulatory cytokine secretion such as IL-12 or IFN-γ, which may modulate the tumor microenvironment from immune-suppression to immune-induction. However, the EY-6 induced cytotoxicity and immune modulatory responses were differential among the tested tumor cells. Studies defining the differences are on-going in this laboratory.

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CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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