Cellular Mechanism of Newly Synthesized Indoledione Derivative-induced Immunological Death of Tumor Cell

Su-Jin Oh¹, Chung-Kyu Ryu², So-Young Baek¹ and Hyunah Lee¹*
¹Office of Biomedical Science, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, ²College of Pharmacy & Division of Life and Pharmaceutical Sciences, Ewha Womans University, Seoul 120-750, Korea

Background: EY-6 is one of the newly synthesized indoledione derivatives to induce tumor cell-specific cell death. In this study, we investigated the mechanism of immunological death induced by EY-6 at mouse colon cancer cell as well as at the normal immune cell represented by dendritic cell.

Methods: C57BL/6 mouse syngeneic colon cancer cell MC38 was treated with EY-6, and analyzed by MTT for viability test, flow cytometry for confirming surface expressing molecules and ELISA for detection of cytokine secretion. Normal myeloid-dendritic cell (DC) was ex vivo cultured from bone marrow hematopoietic stem cells of C57BL/6 mice with GM-CSF and IL-4 to analyze the DC uptake of dead tumor cells and to observe the effect of EY-6 on the normal DC. Results: EY-6 killed the MC38 tumor cells in a dose dependent manner (25, 50 and 100 μM) with carleticulin induction. And EY-6 induced the secretion of IFN-γ but not of TNF-α from the MC38 tumor cells. EY-6 did not kill the ex-vivo cultured DCs at the dose killing tumor cells and did slightly but not significantly induced the DC maturation. The OVA-specific cross-presentation ability of DC was not induced by chemical treatment (both MHC II and MHC I-restricted antigen presentation).

Conclusion: Data indicate that the EY-6 induced tumor cell specific and immunological cell death by modulation of tumor cell phenotype and cytokine secretion favoring induction of specific immunity eliminating tumor cells.

INTRODUCTION

The toxicity to the normal cell is the biggest limitation of anti-tumor therapy modules including chemotherapy and radiotherapy. Also as a systemic disease, tumor can’t be treated or protected from metastasis by surgery which removes the local burden. To achieve the complete elimination of disease, one should consider the systemic minimal residual tumors (1). Thus, inducing specific immunity to remove the tumor is considered as the promising choice of therapy (2). Recent reports address the immunological aspects of certain chemotherapeutics (3-6). Generally, chemotherapeutics kill the rapid proliferating cells including tumor cells as well as bone marrow stem cells which are the cause of immune-suppression in treated patients. High dose cyclophosphamide, a chemotherapeutics, inhibits T cell function and anthracyclines affect the macrophages (7-10). On the other hand, low dose cyclophosphamide induces the immunity. Unlike other anthracyclines, doxorubicin (10) did not inhibit but induce macrophage-related anti-tumor activity in vivo. Limited number of recent paper reports the immunological death of tumor cells killed by certain type of chemotherapeutics. Surface expression of carleticulin (CRT) or heat-shock proteins on the killed tumor cells leads to the induction of tumor-specific immune responses (1,11-13). Especially, translocation of cytosolic CRT onto the dead tumor cell surface makes the cell more attractive for uptake by antigen-presenting cell, DC. These findings allow us to make the hypothesis that a chemical inducing tumor cell specific and immunological killing may increases the...
tumor-specific immunity thus be a safe and effective anti-tumor agent.

The compound EY-6 is the newly synthesized indoleidione derivatives with transposition of heterocyclic ring (QIDs). Early study of related QIDs reveals that the compounds induce the tumor cell apoptosis by cell cycle control, angiogenesis control or topoisomerase II inhibition (14-17). In this study, the induction of immunological death of colon cancer cells by EY-6 is observed to learn the scientific basis to develop the candidate materials for efficacious and safe anti-cancer drug.

**MATERIALS AND METHODS**

**Animals**
Specific pathogen-free female C57BL/6 mice (H2k), 5~6 weeks old, were purchased from the Dae-Han Biolink (Eum-Sung, Korea). The mice were provided with water and food, ad libitum and quarantined under 12 h light: 12 h dark photoperiod in the animal care facility of the Samsung Biomedical Research Institute, Samsung Medical Center, Seoul, Korea. Animal care was performed following the ILAR guideline. The mice were acclimated for at least one week before any experiments were conducted.

**Reagents**
EY-6 was synthesized and supplied by Dr. Chung-Kyu Ryu (Ewha Women’s University, Seoul, Korea). RPMI-1640 medium, fetal bovine serum and penicillin-streptomycin were obtained from GIBCO laboratories (Grand Island, NY, USA). Following antibodies for flow cytometric phenotyping were purchased from eBioscience (SanDiego, CA, USA); fluorescein isothiocyanate (FITC)-or phycoerythrin (PE)-labeled monoclonal Abs for FAS, HSP60, HSP90, HSP70, MHC class I (H2k), CD8a, CD11c, CD80, CD11b (Mac1), and Gr-1. Antibody against CRT was obtained from ABCAM (Cambridge, UK). ELISA sets for cytokines including TNF-α and IFN-γ was purchased from eBioscience (SanDiego, CA, USA).

**Cell lines**
C57BL/6 syngeneic MC38, a colon carcinoma cell line was purchased from American type culture collection (ATCC) (Rockville, MD, USA), OVA-specific T cell hybridomas, B3Z86/90.14 (B3Z, MHC-I) and DOBW (MHC-II), were kindly provided by Dr. Kyungjae Kim (Sahn Yook University, Seoul, Korea). All the cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium) unless otherwise specified.

**Ex vivo culture of bone marrow derived DC**
Mononuclear cells (MNCs) from bone marrow were obtained from the tibia and femur of cervical dislocated C57BL/6 mouse. Viability of red blood cell (RBC) free-MNCs was routinely over 90% by trypan blue exclusion. Plastic-adhered purified monocytes (1×10⁶/ml) were incubated with GM-CSF and IL-4 (1×10⁵ units/ml each) at 37°C for 7 days in humidified CO₂ incubator. Harvested DCs were used in following experiments: 1) EY-6 treated tumor cell uptake by DC, 2) analysis of the effects of EY-6 on cultured DC (cytotoxicity, phenotype, antigen-specific cross presentation).

**MTT assay**
The cells were cultured in the presence of EY-6 (25, 50 and 100 μM) for 24 h, 48 h, or 72 h in 96-well plates (1×10⁴/well). After incubation at 37°C, modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT solution, 5 mg/ml, 20 μl) 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).

**Flow cytometric analysis**

**Phenotype observation**
EY-6 treated MC38 colon cancer cells stained with fluorescein isothiocyanate (FITC)-or phycoerythrin (PE)-labeled monoclonal Abs against FAS, HSP60, HSP90, HSP70, MHC class I (H2k), CD8a, CD11c, CD80, CD11b (Mac1), and Gr-1. Antibody against CRT was obtained from ABCAM (Cambridge, UK). ELISA sets for cytokines including TNF-α and IFN-γ was purchased from eBioscience (SanDiego, CA, USA).

**DC uptake of tumor cells**
EY-6 treated tumor cell (MC38) was labeled with CRT-FITC then co-cultured with CD11c-PE labeled DC for 6 hr. Flow cytometric analysis was performed by FACS Calibur (BD Biosciences, SanJose, CA, USA) within 3 hrs after the staining.
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Figure 1. EY-6 induced tumor-specific killing. MC38 cell (A) or cultured BM-DC (B) was treated with different doses of EY-6 (25, 50 and 100 μM) for 48 hr at 37°C. At the end of the incubation, supernatants were removed and MTT solution was added to analyze the cell death. Asterisks indicate the statistical significance at p<0.05.

OVA-specific cross-presentation assay
MHC class I-restricted presentation assay
Cultured BM-DCs were treated with different concentrations of EY-6 for overnight (1×10^5/well). DCs were added with OVA-peptide (257-264, SIINFEKL) (Peptron, Daejeon, Korea) for 2 h incubation at 37°C. After the washing twice with PBS, cells were fixed with 100 μl/well of ice-cold 1% paraformaldehyde for 5 min at room temperature. MHC I-restricted OVA-specific B3Z cells were (2×10^5/well) co-cultured with above DCs for 4 h at 37°C. After incubation, luc2 activity was measured by colorimetric analysis of freeze-thaw lysed cells with β-galactosidase substrate, chlorophenol red β-D-galactopyranoside (Calbiochem, Darmstadt, Germany) with ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

MHC class II-restricted presentation assay
EY-6 treated and OVA peptide (323-339, ISQAVHAAHAEINEAGR) (Peptron, Daejeon, Korea)-introduced-DCs were co-cultured with MHC II-restricted DOBW cells (1×10^5/well). After 24 h incubation at 37°C, the plate was centrifuged at 1,800 rpm to collect the supernatant. OVA-specific secretion of IL-2 was measured in the supernatant by ELISA.

Statistical analysis
The analyzed values were reported as the mean±standard error. Statistical significance was determined by two-tailed Student’s t-test. All p values <0.05 were interpreted to represent statistically significant differences.

RESULTS
EY-6 induced tumor-specific killing
MTT assay was performed to observe the differential cytotoxic effect of EY-6 against the tumor cell and normal immune cells, MC38, a mouse colon cancer cell line was killed by EY-6 in dose-dependent manner (37.5% vs. 30.1% vs. 21.3% of non-treated control viability, for 48 hr exposure to 25, 50 and 100 μM EY-6, respectively) (Fig. 1A). Chemical could induce the MC38 apoptosis even with short time (18 hr) of lower dose (15 μM) exposure as determined by 26.3% annexin V+PI+ apoptotic MC38 cells. However the cultured normal BM-DC viability (136.6% vs. 127.0% vs. 157.6% of non-treated control viability, for 48 hr exposure to 25, 50 and 100 μM EY-6, respectively) was not affected or slightly proliferated by EY-6 (Fig. 1B). Following studies to define the EY-6 induced-immunological death were performed with 25 μM dose.

EY-6 induced immunological death of MC38 colon cancer cells
Surface expression of immunogenicity-inducing molecules on the tumor cells killed by EY-6
To observe the ability of EY-6 inducing immunological death of tumor cells, chemical treated tumor cell surface molecule expression was analyzed by flow cytometry. Death receptor Fas (CD95) expression was increased on the chemical-treated tumor cells (28.9% vs. 79.2% for the non-treated control vs. EY-6 treated MC38, respectively) (Fig. 2). Also the expression of natural adjuvant for immune response, heat shock proteins (HSPs), was induced on the tumor cell surface (7.5% vs. 80.6% for Hsp60; 2.5% vs. 28.4% for Hsp70; 16.1% vs. 40.6% for Hsp90 as control vs. EY-6 treated MC38, respectively) (Fig. 2). The most significant molecule speaks for the increased immunogenicity, CRT expression (20.7% vs. 50.4% for control vs. EY-6 treated MC38, respectively) was induced by EY-6 (Fig. 2). Thus chemical-treated tumor cells may be uptaken and presented to the immune system by DC, more easily than non-treated cells.
Increased DC uptake of EY-6 treated MC38 cells
MC38 cell surface expression of Fas, Hsps, and CRT molecules, which are known to be responsible for the increased immunogenicity of dead cells, was significantly induced by EY-6 (Fig. 2). Then to observe the DC uptake of MC38 cells, it was observed that the CRT^{+}CD11c^{+} double positive proportion in the co-culture of EY-6 treated MC38 cell and CD11c^{+} cultured-DC, EY-6 treatment significantly induced DC uptake of MC38 tumor cells (9.4% vs. 22.4% for non-treated control vs. EY-6 treated MC38, respectively) (Fig. 3). EY-6 induced CRT expression on the MC38 surface and DC uptake of these cells were proportionally co-related.

Cytokine secretion from the EY-6 treated MC 38 cells
EY-6 treatment induced the secretion of IFN-γ from the MC38 cells (Fig. 4). Secretion of an inflammatory cytokine TNF-α was also increased but the absolute level was very low (78.3±7.2 vs. 17.1±2.1 pg/ml at 6 h exposure to 25 μM EY-6 for IFN-γ vs. TNF-α) (Fig. 4). Data indicating that EY-6 treatment altered the tumor cell microenvironment favorable to anti-tumor immune responses.

Direct effect of EY-6 on the normal, cultured-DC
Effect of EY-6 on the DC maturation
Cultured-DC phenotype was observed by flow cytometry to see if EY-6 directly induced the cultured DC maturation. Unlike the killing effect on the MC38 tumor cells, cultured-DCs were not killed by EY-6 treatment rather, the cell proliferation induced slightly (Fig. 1B). Phenotype analysis of DC indicated that the EY-6 did not induce DC maturation as was observed by decreased surface expression of CD80 (53.3% to 41.1% after EY-6 treatment), and CD11c (48.8% to 37.1% after EY-6 treatment) (Fig. 5). However, myeloid derived suppressor cell (MDSC) proportion was clearly reduced by EY-6 treatment (26.1% to 2.1% in EY-6 treated cells), suggesting the possible role of EY-6 on the myeloid cell differentiation (Fig. 5).
Figure 5. Effect of EY-6 on the DC maturation. Cultured BM-DC by the methods described in the "Materials and Methods" section, was treated with 25 μM EY-6 for 18~24 hr at 37°C. At the end of the incubation, harvested and stained the cells with FITC or PE-tagged antibodies for the surface molecules to identify DCs.

DISCUSSION

EY-6 is newly synthesized indoledione derivatives with expectation of anti-fungal agent by an author (Dr. Chung-Kyu Ryu). Interestingly, in our screening test, tumor-specific killing by EY-6 without affecting normal immune cell viability was observed. Rather, the proliferation of normal splenocytes was increased by EY-6 in dose-dependent manner (data not shown). With an expectation for developing anti-tumor agent having immune-stimulatory effect, the influence of EY-6 on the MC38 mouse colon cancer cell line and normal mouse myeloid-DC was observed. MC38 colon cancer cell was killed by EY-6 in dose-dependent manner (Fig. 1A). It has been reported that some of the chemotherapeutics with certain doses induce the tumor cell apoptosis with increased surface expression of CRT. CRT is a cytosolic calcium-binding protein and expressed on the apoptotic tumor cell surface by certain type of chemotherapeutics such as anthracyclines. Surface expressed CRT work as an eat-me signal to DCs to induce tumor antigen-specific immune responses (11-13). Thus, this phenomenon called chemotherapeutics induced "immunological death" of tumor cell. In this study, tumor specific killing by chemical EY-6 was tested if it can induce the "immunogenic tumor cell death". EY-6 induced MC38 cell surface expression of not only CRT but also natural adjuvant Hsp 60s, 70s and 90s which are considered as immunogenicity-related molecules (Fig. 2). The CRT expression and the DC uptake of these dead cells were proportionally correlated (Fig. 3) meaning EY-6 can induce immunological death of MC38 tumor cells. Interestingly, EY-6 stimulated MC38 tumor cells to produce IFN-γ much more than TNF-α (Fig. 4). In general, IFN-γ is known to be produced by T cells or NK cells not...
by tumor cells. Immunotherapy with IFN-γ secreting tumor cells were proven to be effective cancer vaccine in animal model (18,19), suggesting that EY-6-manipulated IFN-γ secretion from the MC38 cells may be one of the important mechanism for inducing anti-tumor responses. Unlike the anti-tumor immune effect on the tumor cells, IFN-6 did not affect normal antigen presenting cell, DC maturation or cross presentation function (Fig. 5 and 6). Together with the fact that EY-6 do not kill the normal DC, data confirm the tumor cell specific response of the chemicals. Data observed in this study suggest the possibility of developing EY-6 as a chemotherapeutics to kill the tumor cells specifically without toxicity to normal cells and manipulate the host immunity favorable to eliminate the tumor cells by inducing immunological death.

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CONFLICTS OF INTEREST

The author have no financial conflict of interest.

REFERENCES

1. Obeid M, Panaretakis T, Tesniere A, Jora N, Tufi R, Apetoh L, Ghiringhelli F, Zitvogel L, Kroemer G: Leveraging the immune system during chemotherapy: moving calreticulin from the cell surface converts apoptotic death from "silent" to immunogenic, Cancer Res 67;7941-794, 2007.
2. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Page C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoué F, Bruyneel P, Cugnenc PH, Trajanoski Z, Fridman WH, Pagès F: Type, density, and location of immune cells within human colorectal tumors predict clinical outcome, Science 313;1900-1904, 2006.
3. Apetoh L, Mignot G, Panaretakis T, Kroemer G, Zitvogel L: Immunogenicity of anthracyclines: moving towards more personalized medicine, Trends Mol Med 14:141-151, 2008.
4. Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G: Immunological aspects of cancer chemotherapy, Nat Rev Immunol 8:59-73, 2008.
5. Chan OT, Yang LX: The immunological effects of taxanes, Cancer Immunol Immunother 49:181-185, 2000.
6. Spreafico F, Vecchi A, Golotta F, Montovani A: Cancer chemotherapy as immunomodulators, Springer Semin Immunopathol 8:361-379, 1985.
7. North RJ: Cyclophosphamide-facilitated adaptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells, J Exp Med 155:1063-1074, 1982.
8. Nowak AK, Robinson BW, Lake RA: Gemcitabine exerts a selective effect on the tumor immune response: implications for combination chemo-immunotherapy, Cancer Res 62:2353-2358, 2002.
9. Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM: Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity, Clin Cancer Res 11:5713-5721, 2005.
10. Machiels JP, Reilly RT, Emens LA, Ercolani AM, Lei RY, Weintraub D, Okoye Fl, Jaffer EM: Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice, Cancer Res 61:3069-3079, 2001.
11. Zitvogel L, Kepp O, Senovilla L, Mengen L, Chaput N, Kroemer G: Immunogenic tumor cell death for optimal anti-cancer therapy: the calreticulin expression pathway, Clin Cancer Res 16:3100-3104, 2010.
12. Panaretakis T, Kepp O, Brockmeier U, Tesniere A, Bjorklund AC, Chapman DG, Dirschlag M, Jora N, Pirron G, van Endert P, Yuan J, Zitvogel L, Madeo F, Williams DB, Kroemer G: Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death, EMBO J 28:578-590, 2009.
13. Obeid M, Tesniere A, Ghiringhelli F, Finia GM, Apetoh L, Perfettini JI, Castedo M, Mignot G, Panaretakis T, Casares N, Mittivier D, Larochette N, van Endert P, Ciocca F, Piacentini M, Zitvogel L, Kroemer G: Calreticulin exposure dictates the immunogenicity of cancer cell death, Nat Med 13;54-61, 2007.
14. Ko JH, Yeon SW, Ryu JS, Kim TY, Song EH, You HJ, Park RE, Ryu CK: Synthesis and biological evaluation of 5-arylamino-6-chloro-1H-indazole-4,7-diones as inhibitors of protein kinase B/Akt, Bioorg Med Chem Lett 16;6001-6005, 2006.
15. Chung KH, Hong SY, You HJ, Park RE, Ryu CK: Synthesis and biological evaluation of 5-arylamino-1H-benzo[d]imidazole-4,7-diones as inhibitor of endothelial cell proliferation, Bioorg Med Chem 14;5795-5801, 2006.
16. Kim JS, Rhee HK, Park HJ, Lee IK, Lee SK, Sub ME, Lee HJ, Ryu CK, Choo HY: Synthesis of 6-chloroisooquinoline-5,8-di-ones and pyrid-3,4-biphenazine-5,12-diones and evaluation of their cytotoxicity and DNA topoisomerase II inhibitory activity, Bioorg Med Chem 15;451-457, 2007.
17. Seo JM, Jin YR, Ryu CK, Kim TJ, Han XH, Hong JT, Yoo HS, Lee GK, Yun YP, JM91, a newly synthesized indoleidine derivative, inhibits rat aortic vascular smooth muscle cells proliferation and cell cycle progression through inhibition of ERK1/2 and Akt activations, Biochem Pharmacol 75;1331-1340, 2008.
18. Esumi N, Hunt B, Itaya T, Frost P: Reduced tumorigenicity
of murine tumor cells secreting gamma-interferon is due to nonspecific host responses and is unrelated to class I major histocompatibility complex expression, Cancer Res 51:1185-1189, 1991.

19. Janelidze S, Bexell D, Badn W, Durabi A, Smith KE, Fritzell S, Gunnarsson S, Milos P, Bengzon J, Saltford LG, Siesjö P, Visse E: Immunizations with IFNγ secreting tumor cells can eliminate fully established and invasive rat gliomas, J Immunother 32:593-601, 2009.