ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science 2016 Vol. 26. No. 3. 376~386 DOI : http://dx.doi.org/10.5352/JLS.2016.26.3.376

- Review -

Microtubule-damaging Chemotherapeutic Agent-mediated Mitotic Arrest and Apoptosis Induction in Tumor Cells

Do Youn Jun and Young Ho Kim*

Laboratory of Immunobiology, School of Life Science and Biotechnology, College of Natural Sciences, Kyungpook National University, Daegu 41566, Korea

Received March 18, 2016 / Revised March 25, 2016 / Accepted March 29, 2016

Apoptosis induction has been proposed as an efficient mechanism by which malignant tumor cells can be removed following chemotherapy. The intrinsic mitochondria-dependent apoptotic pathway is frequently implicated in chemotherapy-induced tumor cell apoptosis. Since DNA-damaging agent (DDA)-induced apoptosis is mainly regulated by the tumor suppressor protein p53, and since more than half of clinical cancers possess inactive p53 mutants, microtubule-damaging agents (MDAs), of which apoptotic effect is mainly exerted via p53-independent routes, can be promising choice for cancer chemotherapy. Recently, we found that the apoptotic signaling pathway induced by MDAs (nocodazole, 17α-estradiol, or 2-methoxyestradiol) commonly proceeded through mitotic spindle defect-mediated prometaphase arrest, prolonged Cdk1 activation, and subsequent phosphorylation of Bcl-2, Mcl-1, and Bim in human acute leukemia Jurkat T cells. These microtubule damage-mediated alterations could render the cellular context susceptible to the onset of mitochondria-dependent apoptosis by triggering Bak activation, $\Delta\psi$m loss, and resultant caspase cascade activation. In contrast, when the MDA-induced Bak activation was inhibited by overexpression of anti-apoptotic Bcl-2 family proteins (Bcl-2 or Bcl-xL), the cells in prometaphase arrest failed to induce apoptosis, and instead underwent mitotic slippage and endoreduplication cycle, leading to formation of populations with 8N and 16N DNA content. These data indicate that cellular apoptogenic mechanism is critical for preventing polyploid formation following MDA treatment. Since the formation of polyploid cells, which are genetically unstable, may cause acquisition of therapy resistance and disease relapse, there is a growing interest in developing new combination chemotherapies to prevent polyploidization in tumors after MDA treatment.

Key words : Apoptotic cell death, endoreduplication, microtubule-damaging agents, polyploid Formation, prometaphase arrest

Introduction

Significant advances have recently been made in our understanding on cancer biology, techniques for early diagnosis of cancer, and treatment modalities such as surgery, radiation, and chemotherapy. Cancer, however, is still a leading cause of mortality and morbidity of worldwide, accounting for 8.2 million deaths or 14.6% of all human death in 2012 [79]. Since cancer arises from one single cell, the final goal of the treatment regimen for the cancer patient should be to remove all cancer cells throughout the whole body. Treatment for cancer patients comprises, in general, one or more modalities including radiation therapy, surgery, and chemotherapy. While chemotherapy is employed either alone or in combination with radiotherapy and/or surgery depending on the cancer status, the efficacy of chemotherapy in cancer patients is mainly exerted by the cytostatic and cytotoxic effects of chemotherapeutic drugs on tumor cells, which can systemically react with tumor cells.

Because one of the main characteristics of tumor cells is uncontrolled growth and proliferation resulting from defects in cell cycle regulation, a number of anticancer drugs have been developed to target cell cycle control mechanism [10, 23, 29, 44, 63]. Mammalian cells are known to duplicate their contents and then divide into two cells via completing the cell cycle, which is proceeded by four successive stages such as the periods of DNA synthesis (S phase) and mitosis (M phase) separated by gaps called G_1 and G_2 phases. Progression through each phase in dividing cells is controlled by the sequential activation and inactivation of a series of cyclin-dependent kinases (Cdks) [33]. To ensure the fidelity of

***Corresponding author**
Tel : +82-53-950-5378, Fax : +82-53-955-5522
E-mail : ykim@knu.ac.kr

cell division, the passage through a series of cell cycle checkpoints that act as molecular breaks to verify the accuracy of cell cycle progression is required [54]. Three major checkpoints such as the G_1 checkpoint, the G_2 checkpoint, and the mitotic spindle assembly checkpoint are involved in the cell cycle control (Fig. 1). The G_1 checkpoint functions in G_1 phase to confirm that the environment is favorable for committing to S phase, the G_2 checkpoint functions in G_2 phase and prevents entry into mitosis until DNA damage is repaired and DNA replication is completed, and the mitotic spindle assembly checkpoint functions during mitosis and ensures proper attachment of the replicated chromosomes to the mitotic spindles [7, 8, 19, 39]. Although the existence of tetraploid checkpoint remains controversial, several studies have shown that mammalian cells have a p53-dependent tetraploid checkpoint that blocks cell cycle progression in the G_1 phase in response to failure of cytokinesis during cell division. During G_1 or G_2 checkpoint activation, a combination of several distinct molecular mechanisms operates to negatively regulate Cdk activity. These include increased cyclin destruction, decreased cyclin gene expression, Cdk inhibition by Cdk inhibitors (CKIs; $p21^{CIP1/WAF1}$, $p27^{KIP1}$, and $p16^{INK4A}$) that bind and inactivate Cdk/cyclin complexes, and Cdc25 inactivation. Additionally, when the mitotic spindle assembly checkpoint becomes activated, Cdk1/cyclin B is kept in an active state by inhibiting the role of the anaphase-promoting complex (APC) in order to prevent the onset of anaphase. While checkpoint activation delays or halts cell cycle progression, the repair mechanisms operate to recover cellular integrity. However, if the damage is beyond repair, the cells generate a signal to undergo apoptosis [1, 32].

The efficacy of chemotherapy that targets cell cycle regulation depends on the status of cell cycle progression of tumor cells, because chemotherapeutic agents mainly impact on actively proliferating cells that are in the S phase or M phase rather than cells in the resting (G_0) phase. However, many chemotherapeutic drugs fail to distinguish the difference between proliferating cells of normal tissues and tumor cells, and thus may cause normal cell damage which results in side-effects. With respect to the efficacy of chemotherapy, which induces cancer cell death, several biochemical mechanisms including apoptosis, necrosis, and autophagy are implicated [3, 47, 61]. Autophagy is known to be a catabolic degradation process that proceeds by sequestering unnecessary or dysfunctional cellular components via the formation of double-membrane vesicles (autophagosomes), and targeting them for degradation via the fusion of autophagosomes with lysosomes to generate single-membrane autolysosomes. A number of studies have reported that autophagic response is enhanced in tumor cells than in normal cells, which allows tumor cells to survive under inadequate conditions such as nutritional deprivation and chemotherapy [17]. In addition, since tumor cells can activate autophagy in response to cellular stress associated with chemotherapeutic treatment, it has been suggested that inhibition of autophagy is an effective chemotherapeutic approach to accelerate tumor cell death [36]. The mechanism of chemotherapeutic drug-induced cell death needs to be studied further in order to clarify whether the antitumor effect of the drug is confined to tumor cells rather than normal cells. These efforts will improve the survival rates of cancer patients receiving chemotherapy [53].

Chemotherapy-induced apoptosis in tumor cells results in their own destruction into apoptotic bodies that can be cleared by phagocytic cells without accompanying a local inflammatory response, whereas necrosis in tumor cells leads to release of their intracellular contents that possibly provoke a damaging inflammatory response. In this context, apoptotic cell death has been proposed as an efficient mecha-

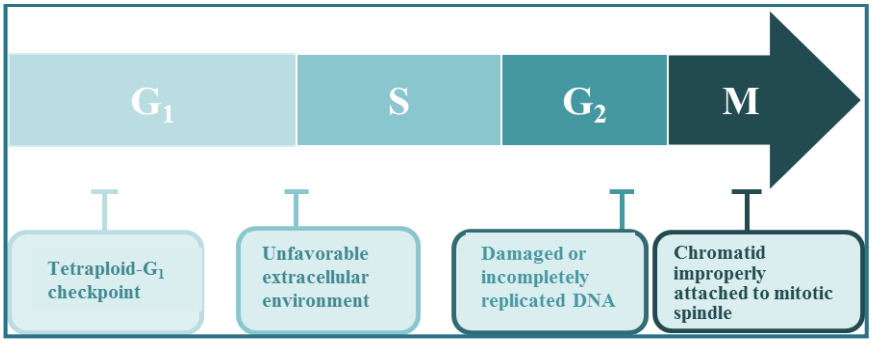

Fig. 1. Mammalian cell cycle progression and checkpoints.

nism by which malignant tumor cells can be removed following chemotherapeutic treatments [30, 40]. In chemotherapeutic drug-induced apoptosis of tumor cells, three different death signaling pathways, such as the extrinsic death receptor-dependent pathway [74], the intrinsic mitochondria-dependent pathway [18] and the intrinsic endoplasmic reticulum (ER) stress-mediated pathway [55], are likely to be implicated. The intrinsic mitochondria-dependent pathway is most frequently associated with tumor cell apoptosis caused by chemotherapeutic drugs that are DNA-damaging and microtubule-damaging agents [49].

Since numerous studies have reported that the apoptosis induction of tumor cells following treatment with DNA-damaging agents (DDAs) is associated with the regulatory role of tumor suppressor protein p53, and since more than half of clinical cancers possess mutations in the p53 gene so that p53 cannot be functional as tumor suppressor protein, it is reasonable to assume that microtubule-damaging agents (MDAs), of which cytotoxic effect can be exerted in a p53-independent manner, are better choice for chemotherapeutic treatment of cancers with p53 mutations [26, 35, 62].

In this review, the molecular and cellular mechanisms underlying MDA-induced apoptosis of tumor cells are discussed. These understandings will provide new insights and opportunities for better treatment of cancer patients as well as for drug development.

MDA-induced cell cycle arrest is the prometaphase arrest

The segregation of the replicated chromosomes during M phase of the cell cycle is governed by a complex cytoskeletal structure of microtubules and associated proteins, which is known as the mitotic spindle [24, 76]. These microtubules are highly dynamic polymers, composed of α- and β-tubulin heterodimers, and they tend to switch between states of rapid growth and rapid shrinkage in a situation of dynamic instability [20, 71]. Microtubule dynamics increases significantly during mitosis, compared with interphase, to achieve the proper alignment of the duplicated chromosomes at the metaphase plate and accurate segregation of the chromosomes to the daughter cells. Based on the important role of microtubule dynamics in controlling mitosis, microtubules have been considered a critical target for the development of anticancer drugs [51, 52, 64]. Among the MDAs, the microtubule-polymerizing drugs such as paclitaxel and docetaxel promote microtubule assembly and stabilize microtubules, whereas the microtubule-depolymerizing drugs including vinblastine, colchicine and nocodazole inhibit microtubule assembly and promote depolymerization. Tumor cells treated with these MDAs are known to commonly exhibit loss of microtubule function, leading to the disruption of mitotic spindle, mitotic arrest of the cell cycle, and mitotic catastrophe-mediated apoptotic cell death [38, 52, 64].

The M phase of the cell cycle can be subdivided into the prophase, the prometaphase, the metaphase, the anaphase, and the telophase, based on the microscopic characteristics of the microtubule organization, chromosomes, and nuclear envelop [26, 58]. The sophisticated regulation of Cdk1 activity is known to be critical for the G_2/M transition and M phase-progression of the cell cycle. When cells enter into the prophase from the G_2 phase, the Cdk1/cyclin B complex, which is held in an inactive state by phosphorylation at Thr-14 and Tyr-15 mediated by the kinases Myt1 and Wee1, becomes activated as a result of phosphorylation at Thr-161 by the Cdk-activating kinase (Cak) as well as dephosphorylation at Thr-14 and Tyr-15 by the phosphatase Cdc25 [21, 75]. The activated Cdk1 phosphorylates a large number of substrates that are associated with nuclear envelope breakdown, centrosome separation, spindle assembly, and chromosome condensation [34]. Subsequently, the activated Cdk1 should be inactivated through the degradation of cyclin B by anaphase promoting complex (APC) to progress from the metaphase to the anaphase.

In relation to the chemotherapy-mediated G_2/M arrest accompanying apoptosis in tumor cells, two different molecular mechanisms, the G_2 checkpoint pathway and the mitotic spindle assembly checkpoint pathway, have been implicated [8, 39, 43]. Following exposure to DDAs, the cells can be arrested at the G_2 checkpoint by inactivating Cdk1/cyclin B through the action of Wee1 and Cdc25, and by p53-mediated transcriptional repression of cyclin B. When the cells being treated with MDAs fail to align all the chromosomes on the metaphase plate, the mitotic spindle assembly checkpoint becomes activated to prevent the onset of anaphase and Cdk1/cyclin B is kept in an active state by inhibiting the role of APC. As the mechanism underlying MDA-induced apoptotic cell death, it was reported that a prolonged activation of Cdk1, resulting from an enforced arrest at the mitotic spindle assembly checkpoint by MDAs, could act as an apoptotic mediator [46, 68, 78].

Although numerous studies reported that MDAs induce

G_2/M-arrest of the cell cycle and apoptotic cell death in tumor cells, the link between mitotic spindle assembly checkpoint activation-mediated mitotic arrest and apoptotic death signaling pathway was not fully understood. Furthermore, little information was known regarding the sub-stage of the M phase, at which cells were arrested due to the mitotic spindle assembly checkpoint activation following treatment with MDAs. Recently, we have investigated the effects of MDAs such as nocodazole (NOC), 17α-estradiol (17α-E_2), and 2-methoxyestradiol (2-MeO-E_2) on microtubule network, mitotic spindle assembly checkpoint activation, and subsequent induction of apoptosis by employing human Jurkat T cell clone stably transfected with an empty expression vector (JT/Neo) or a *Bcl-2* expression construct (JT/Bcl-2). The analysis of the immunofluorescence microscopy of NOC-, 17α-E_2-, or 2-MeO-E_2-induced mitotic arrest in Jurkat T cells showed that the chromosomes failed to congress at the metaphase plate; however, aberrant bipolar array of microtubules and nuclear envelop breakdown were provoked [27, 28, 45]. These hallmarks of prometaphase arrest were more apparently observed in JT/Bcl-2 cells compared to JT/Neo cells, because the prometaphase arrest was induced without accompanying apoptotic cell death in JT/Bcl-2 cells.

Induction of apoptosis following treatment with MDAs is mediated by Cdk1-dependent phosphorylation of Bcl-2 family proteins (Bcl-2, Bim, and Mcl-1) and subsequent activation of mitochondrial apoptotic pathway

In our studies, comparative analysis of NOC-induced apoptotic events between JT/Neo and JT/Bcl-2 cells showed that Bak activation, mitochondrial membrane potential (Δψm) loss, mitochondrial cytochrome *c* release and resultant activation of caspase-9 and -3, which could be abrogated in the presence of Bcl-2 overexpression, were crucial for NOC-induced apoptosis [28]. Additionally, the results showed that NOC-induced prometaphase arrest, Cdk1 activation, and phosphorylation of Bcl-2, Mcl-1, and Bim were upstream of the Bcl-2-sensitive Bak activation and mitochondria-dependent caspase cascade activation. Similar prometaphase arrest, Cdk1 activation, phosphorylation of Bcl-2 family proteins (Bcl-2, Mcl-1 and Bim), and mitochondria-dependent apoptotic events were observed in Jurkat T cells treated with 17α-E_2 or 2-MeO-E_2 [27, 45]. In Jurkat T cells treated with MDAs (NOC, 17α-E_2, or 2-MeO-E_2), Bcl-2 phosphorylation at Thr-56 and Ser-70, Mcl-1 phosphorylation at Ser-159/Thr-163, and Bim phosphorylation were commonly detected along with prometaphase arrest and prolonged Cdk1 activation (Fig. 2). However, Bcl-xL phosphorylation at Ser-62 was detected only in Jurkat T cell clone overexpressing Bcl-xL (J/Bcl-xL). Under these conditions, 2-MeO-E_2-induced Mcl-1 phosphorylation was accompanied by a significant reduction in the level of Mcl-1; however,

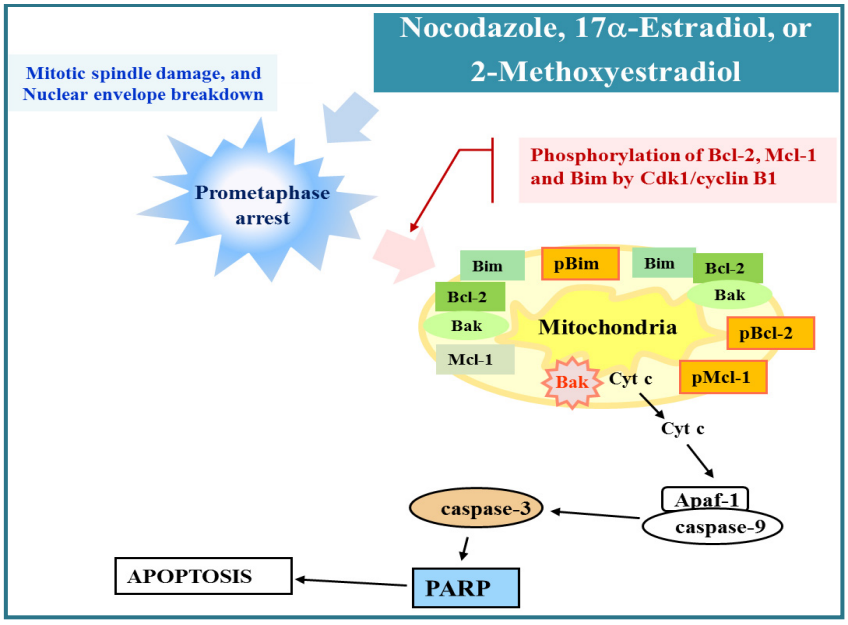

Fig. 2. The apoptogenic activity of NOC, 17α-E_2, or 2-MeO-E_2 was attributable to mitotic spindle damage, prometaphase arrest, Cdk1 activation, phosphorylation of Bcl-2, Mcl-1, and Bim, and activation of Bak and mitochondria-dependent caspase cascade.

17α-E_2-induced phosphorylation of Mcl-1 failed to cause a remarkable down-regulation of Mcl-1 level. These results suggest that the phosphorylation of Bcl-2, Mcl-1, and Bim rather than that of Bcl-xL might be the more critical apoptotic signals for the MDA-induced apoptosis.

In the literature, the phosphorylation of anti-apoptotic Bcl-2 proteins such as Bcl-2, Bcl-xL, and Mcl-1 by Cdk1 is required for coupling prolonged mitotic arrest to microtubule inhibitor-induced apoptosis [12, 13, 31, 68]. The phosphorylation of Bcl-2 is known to cause its conformational changes that inactivate the anti-apoptotic function of Bcl-2, leading to the activation of the pro-apoptotic multidomain Bcl-2 family members Bax and/or Bak to provoke mitochondrial cytochrome *c* release [12, 16, 60, 64]. The BH3-only pro-apoptotic proteins, such as Bim or tBid, can trigger the activation of Bax and Bak either directly or indirectly by antagonizing the anti-apoptotic Bcl-2 proteins [11, 15]. When we performed a co-immunoprecipitation assay to examine if prometaphase arrest-mediated phosphorylation of Bcl-2 and Bim can alter the protein-protein associations among the Bcl-2 family proteins to allow Bak activation, a reduction in the association of Bcl-2 with Bak or Bim following the prometaphase arrest-dependent phosphorylation of Bcl-2 and Bim was easily detected in JT/Bcl-2 cells overexpressing Bcl-2 [28]. Under these conditions, however, the association of Bcl-2 with Bax or p53 was relatively constant regardless of NOC treatment causing the prometaphase arrest. Since the mitochondrial Bax level increased by ~2.6-fold after NOC treatment, it raised the possibility that Bax could contribute indirectly to NOC-induced Bak activation by binding with Bcl-2. It is noteworthy that the association of Bcl-2 with Bak or Bim was barely detected in JT/Neo cells that underwent apoptosis after NOC treatment. Consequently, these results suggest that a reduction in the association of Bcl-2 with Bak or Bim following the prometaphase arrest-dependent phosphorylation of Bcl-2 and Bim might be underlying factors responsible for the Bak activation by MDAs.

Because Jurkat T cells treated with NOC were arrested at prometaphase, prior to metaphase-anaphase transition, it was likely that NOC treatment could cause prolonged Cdk1 activation. Along with NOC-induced prometaphase arrest, the upregulation of the cyclin B1 levels, the decrease in the Tyr-15-phosphorylation, and the increase in the Thr-161-phosphorylation of Cdk1, all of which were previously shown to be required for the activation of Cdk1 kinase at the G_2/M boundary [21, 75], were detected. An *in vitro* kinase assay using histone H1 as the substrate revealed that the Cdk1 kinase activity in mitotic-arrested cells by NOC appeared to be 4.8-fold higher than that in exponentially growing cells. Western blot analysis also showed NOC-induced increase in the phosphorylation of cellular histone H1, which is known to be catalyzed by Cdk1 during G_2/M phase [14]. NOC-induced phosphorylation of cellular histone H3 at Ser-10 further reflected the activation of Cdk1 during NOC-induced mitotic prometaphase arrest, because the Aurora B kinase activation responsible for the histone H3 phosphorylation at Ser-10 during G_2/M phase is known to be dictated by Cdk1 [50]. Previously, taxol and several other MDAs were reported to induce Cdk1 kinase-mediated serine phosphorylation of Bcl-2, resulting in abrogation of its anti-apoptotic function, suggesting a pro-apoptotic role of Cdk1 kinase as the Bcl-2 kinase [37, 59]. However, there was much controversy over the protein kinases responsible for the Bcl-2 phosphorylation because a number of protein kinases, in addition to Cdk1 kinase, including Raf-1, PKA, JNK, and p38 MAPK, were also reported to mediate the phosphorylation of Bcl-2 during the mitotic arrest caused by various MDAs [6, 16, 22, 59, 65, 80]. In our recent studies, we investigated the effects of a Cdk1 kinase inhibitor (roscovitine), a JNK inhibitor (SP600125), and a p38 MAPK inhibitor (SB203580) on MDA (NOC, 17α-E_2, or 2-MeO-E_2)-induced phosphorylation of Bcl-2 by western blot analysis using specific antibodies for phospho-Bcl-2 (Thr-56) and phospho-Bcl-2 (Ser-70) [28]. At the same time, to obtain evidence for Bim phosphorylation, the phosphorylation-induced retardation of the SDS-polyacrylamide gel electrophoretic mobility of Bim isoforms (BimEL and BimL) was measured in JT/Neo and JT/Bcl-2 cells following NOC treatment. As the results, roscovitine, but not SP600125 or SB203580, could prevent the MDA-induced phosphorylation of Bcl-2 and Bim, $\Delta\psi$m loss, and apoptotic cell death. These our data demonstrate that Cdk1 kinase is the protein kinase responsible for the MDA-induced phosphorylation of Bcl-2 (Thr-56 and Ser-70) and Bim, and the resultant $\Delta\psi$m loss and caspase cascade activation.

Prometaphase arrest is the casual event of MDA-induced apoptosis

To examine whether MDA-induced prometaphase arrest of the cell cycle is a prerequisite for the phosphorylation of Bcl-2 and Bim, Bak activation, and mitochondria-dependent caspase cascade activation in Jurkat T cells, we inves-

tigated if forced arrest of cell cycle progression at the G_1/S boundary by hydroxyl urea (HU) can protect cells from NOC-induced apoptotic processes. HU inhibits the ribonucleotide reductase that converts ribonucleotides to deoxyribonucleotides required for DNA synthesis, thereby inhibiting entry into S phase in a variety of cells [2, 41]. While Jurkat T cells in the concomitant presence of NOC and HU exhibited G_1/S arrest rather than prometaphase arrest, none of the apoptotic events including Cdk1 activation and the phosphorylation of Bcl-2 and Bim were induced, demonstrating the dependency of MDA-induced apoptotic events on prometaphase arrest of the cell cycle [28]. In addition, when Jurkat T cells concomitantly treated with 2-MeO-E_2 and aphidicolin were arrested at the G_1/S boundary by the action of aphidicolin, which blocks the cell cycle at the G_1/S boundary by inhibiting DNA polymerase α [42, 67], 2-MeO-E_2-induced apoptotic events were completely abrogated, confirming that the prometaphase arrest is the casual event of MDA-induced apoptosis [45].

Our results demonstrated that the MDA-induced apoptotic signaling pathway, which leads to apoptotic DNA fragmentation in Jurkat T cells, was provoked by mitotic prometaphase arrest of the cell cycle and resultant Cdk1 kinase-mediated phosphorylation of Bcl-2 family members (Bcl -2, Bim, and Mcl-1). These effects rendered the cell susceptible to the onset of Bak activation, leading to mitochondrial cytochrome *c* release and subsequent activation of the caspase cascade via a reduction of the association of Bcl-2 with Bak or Bim [28]. Consequently, these results provide an insight into the molecular and cellular mechanism that underlies the pro-apoptotic role of prolonged Cdk1 activation in the prometaphase-arrested tumor cells by MDAs including NOC, 17α-E_2, and 2-MeO-E_2.

Endoreduplication induction can be a fate of tumor cells possessing the failure in apoptotic mechanism when treated with MDAs

Previously, it was reported that following treatment with MDAs, tumor cells deficient of p53 [48], pRb [72], APC [69], Lats2 [5], Bax [9], $p21^{Waf1/Cip1}$ [73], or $p16^{Ink4}$ [72] were able to undergo mitotic slippage and endoreduplication, and thus generate polyploid such as tetraploid (4N), and octaploid (8N), and hexadecaploid (16N). Although these results initially suggested that negative regulatory mechanism of cell cycle progression and apoptosis-inducing mechanism might be associated with preventing endoreduplication and resultant polyploid generation in tumor cells treated with MDAs, the precise mechanisms responsible for the generation of polyploid cells remain to be elucidated. Furthermore, because the polyploid cells being generated after chemotherapy are apt to cause genetic instability that leads to anticancer drug resistance and to become a factor for cancer recurrence following chemotherapy [56, 57], further investigation are urgently required for a better understanding of endoreduplication, which occurs and causes genetically unstable polyploids in tumor cells following treatment with MDAs.

Several studies reported the existence of a cytokinesis checkpoint (tetraploid checkpoint) which functions to prevent polyploid generation in cells with chromosome segregation errors [4, 48, 66]. In this regard, it has been indicated that if the binucleate cells exit mitosis without completing the cytokinesis, the cytokinesis checkpoint is activated to arrest cells in the following G_1 phase in a p53-dependent manner. By contrast, Wong and Stearns have reported that there might be no tetraploid checkpoint in mammalian cells, as evidenced by that tetraploid cells created from primary human diploid fibroblasts after treatment with 2 μM cytochalasin could traverse the G_1/S transition point and complete S phase [70, 77]. Additionally, it has been suggested that all previous reports of a tetraploid checkpoint might be due to side effects of the drug treatments used to observe them [77].

In our recent studies, to obtain direct evidence for contribution of apoptosis-inducing system to prevention of polyploid formation in tumor cells treated with MDAs, we decided to take advantage of Bcl-2 overexpression, which protects cells from the drug-induced apoptosis without affecting cell cycle progression. When Jurkat T cell clone stably transfected with a *Bcl-2* expression vector (JT/Bcl-2) were treated with MDAs (NOC or 2-MeO-E_2) for 6 days, 5～10% cells were <2N, 1～3% cells were 2N, 10～20% cells were 4N, 40～50% cells were 8N and 10～20% cells were 16N, respectively (Fig. 3). At the same time, the control Jurkat T cell clone stably transfected with an empty expression vector (JT/Neo) showed that most cells (～90%) were <2N resulting from accumulation of apoptotic sub-G_1 cells, and significantly lower level of cells were 2N (6.3%), 4N (1.0%), 8N (0.5%), and 16N (0.3%). These results demonstrate that cellular apoptogenic mechanism, which can be blocked by anti-apoptotic Bcl-2 or Bcl-xL, plays a key role in preventing polyploid formation following MDA treatment (Fig. 4).

Based on these results, it can be speculated that chemotherapeutic treatment of tumor cells, which possess defect(s) in the apoptogenic pathway, should be performed using MDA plus an S phase inhibitor, in order to abrogate the occurrence of genetically unstable polyploid cells.

Conclusions

Our recent results show that the apoptotic cell death provoked in Jurkat T cells treated with MDAs such as NOC, 17α-E_2, and 2-MeO-E_2 was attributable to activation of the mitotic spindle assembly checkpoint, which causes prometaphase arrest of the cell cycle, prolonged activation of

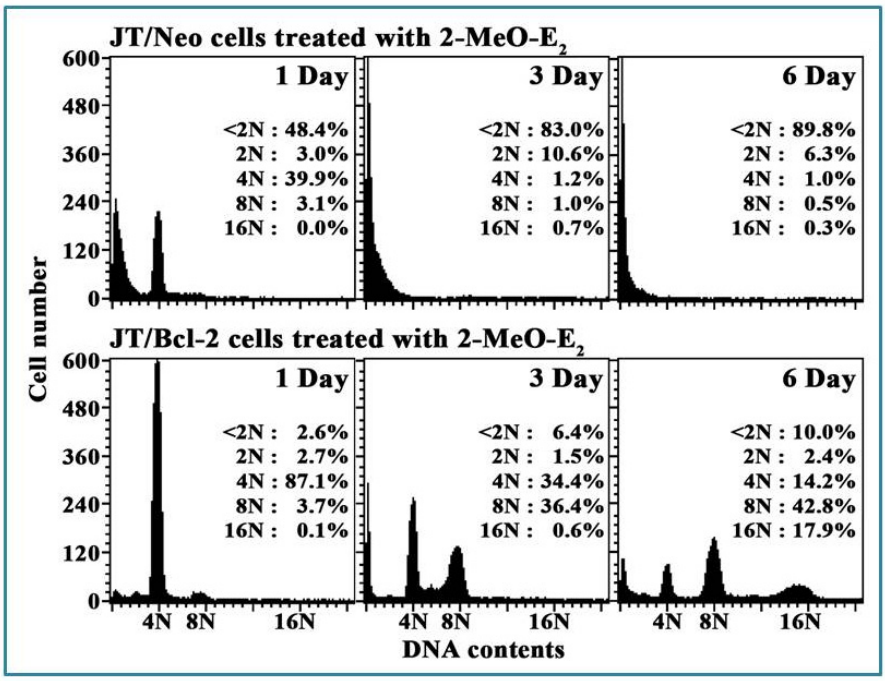

Fig. 3. Time kinetics of polyploid formation and apoptosis induction in Jurkat T cell clone stably transfected with an empty expression vector (JT/NeO) and Jurkat T cell clone stably transfected with a *Bcl-2*-expression vector (JT/Bcl-2) following treatment with 2-methoxyestradiol (2-MeO-E_2) at a concentration of 5 μM.

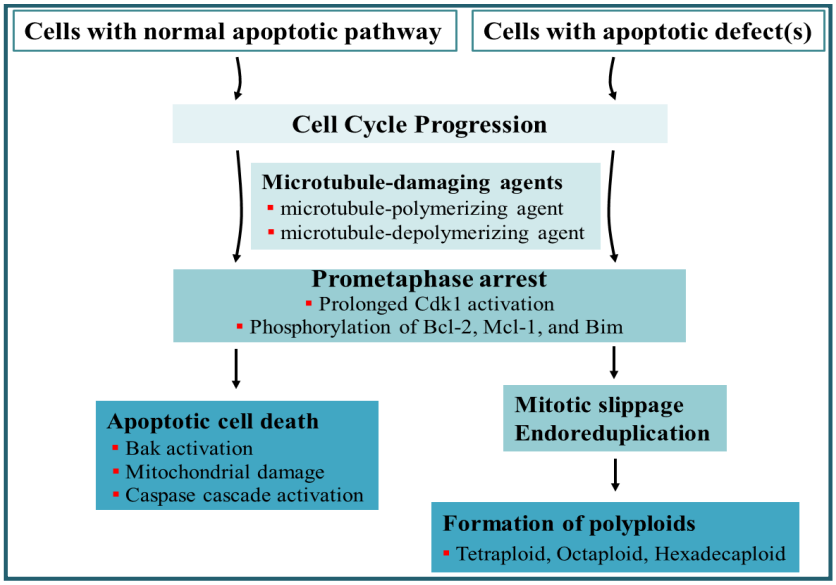

Fig. 4. Apoptotic cell death is the key regulator in preventing tetraploid formation in Jurkat T cells following MDA treatment.

Cdk1, and subsequent phosphorylation of Bcl-2, Mcl-1, and Bim. The Cdk1-dependent phosphorylation of Bcl-2 resulted in a reduction of the association of Bcl-2 with Bak or Bim. These microtubule damage-mediated alterations in prometaphase-arrested cells could render the cellular context susceptible to the onset of mitochondria-dependent apoptosis by triggering Bak activation, $\Delta\psi$m loss, and resultant caspase cascade activation. These MDA-induced apoptotic events including Cdk1 activation and phosphorylation of Bcl-2, Mcl-1 and Bim were completely abrogated, when Jurkat T cells were concomitantly treated with MDAs and the G_1/S blocking agent (hydroxyurea or aphidicolin) and thus arrested at the G_1/S boundary of the cell cycle, confirming that the prometaphase arrest is the initial causal event of MDA-induced apoptosis. In contrast, when the MDA-induced apoptosis was blocked in Jurkat T cells by overexpression of anti-apoptotic Bcl-2 family members such as Bcl-2 or Bcl-xL, the prometaphase-arrested cells with mitotic spindle defect appeared to eventually undergo mitotic slippage to enter the G_1 phase. Under these conditions, however, the cells failed to induce tetraploid-G_1 arrest and instead underwent endoreduplication cycle. This allowed generation of populations with 8N and 16N DNA content following incubation with MDA for 2-6 days. These findings suggest that elimination of prometaphase-arrested cells by inducing apoptotic cell death is the key regulator rather than the 4N-G_1 checkpoint causing tetraploid-G_1 arrest in preventing polyploid formation in Jurkat T cells treated with MDAs. Since the formation of polyploid cells following chemotherapeutic MDA treatment of tumor cells is known to be connected with cancer recurrence in cancer patients undergoing chemotherapy, and since the final goal of chemotherapy for the cancer patient is to eliminate all cancer cells throughout the whole body, microtubule-targeting chemotherapy for tumors possessing apoptotic defect(s) needs to be combined with new therapeutic approaches to prevent polyploidization rather than a single-agent MDA therapy.

Acknowledgement

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2010-0006341).

References

1. Abraham, R. T. 2001. Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev.* **15,** 2177-2196.
2. Adams, R. L. and Lindsay, J. G. 1967. Hydroxyurea: reversal of inhibition and use as a cell synchronizing agent. *J. Biol. Chem.* **242,** 1314-1317.
3. Amaravadi, R. K. and Thompson, C. B. 2007. The roles of therapy-induced autophagy and necrosis in cancer treatment. *Clin. Cancer Res.* **13,** 7271-7279.
4. Andreassen, P. R., Lohez, O. D., Lacroix, F. B. and Margolis, R. L. 2001. Tetraploid state induces p53-dependent arrest of nontransformed mammalian cells in G_1. *Mol. Biol. Cell* **12,** 1315-1328.
5. Aylon, Y., Michael, D., Shmueli, A., Yabuta, N., Nojima, H. and Oren, M. 2006. A positive feedback loop between the p53 and Lats2 tumor suppressors prevents tetraploidization. *Genes Dev.* **20,** 2687-2700.
6. Blagosklommy, M. V., Schulte, T., Nguyen, P., Trepel, J. and Neckers, L. M. 1996. Taxol-induced apoptosis and phosphorylation of Bcl-2 protein involves c-Raf-1 and represents a novel c-Raf-1 signal transduction pathway. *Cancer Res.* **56,** 1851-1854.
7. Brito, D. A. and Rieder, C. L. 2006. Mitotic checkpoint slippage in humans occurs via cyclin B destruction in the presence of an active checkpoint. *Curr. Biol.* **16,** 1194-1200.
8. Burke, D. J. and Stukenberg, P. T. 2008. Linking kinetochore-microtubule binding to the spindle checkpoint. *Developmental Cell* **14,** 474-479.
9. Castedo, M., Coquelle, A., Vivet, S., Vitale, I., Kauffmann, A., Dessen, P., Pequignot, M. O., Casares, N., Valent, A., Mouhamad, S., Schmitt, E., Modjtahedi, N., Vainchenker, W., Zitvogel, L., Lazar, V., Garrido, C. and Kroemer, G. 2006. Apoptosis regulation in tetraploid cancer cells. *EMBO J.* **25,** 2584-2595.
10. Cavenee, W. K. and White, R. L. 1995. The genetic basis of cancer. *Sci. Am.* **272,** 72-79.
11. Chipuk, J. E. and Green, D. R. 2008. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends Cell Biol.* **18,** 157-164.
12. Chipuk, J. E., Moldoveanu, T., Llambi, F., Parsons, M. J. and Green, D. R. 2010. The BCL-2 family reunion. *Mol. Cell* **37,** 299-310.
13. Chu, R., Terrano, D. T. and Chambers, T. C. 2010. Cdk1/cyclin B plays a key role in mitotic arrest-induced apoptosis by phosphorylation of Mcl-1, promoting its degradation and freeing Bak from sequestration. *Biochem. Pharmacol.* **83,** 199-206.
14. Contreras, A., Hale, T. K., Stenoien, D. L., Rosen, J. M., Mancini, M. A. and Herrera, R. E. 2003. The dynamic mobility of histone H1 is regulated by cyclin/CDK phosphorylation. *Mol. Cell. Biol.* **23,** 8626-8636.
15. Czabotar, P. E., Colman, P. M. and Huang, D. C. 2009. Bax activation by Bim? *Cell Death Differ.* **16,** 1187-1191.
16. De Chiara, G., Marcocci, M. E., Torcia, M., Lucibello, M., Rosini, P., Bonini, P., Higashimoto, Y., Damonte, G.,

Armirotti, A., Amodei, S., Palamara, A. T., Russo, T., Garaci, E. and Cozzolino, F. 2006. Bcl-2 phosphorylation by p38 MAPK: identification of target sites and biologic consequences. *J. Biol. Chem.* **281,** 21353-21361.
17. Degenhardt, K., Mathew, R., Beaudoin, B., Bray, K., Anderson, D., Chen, G., Mukherjee, C., Shi, Y., Gelinas, C., Fan, Y., Nelson, D. A. and Jin, S. 2006. White E. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell* **10,** 51-64.
18. Desagher, S. and Martinou, J. C. 2000. Mitochondria as the central control point of apoptosis. *Trends Cell Biol.* **10,** 369-377.
19. Donzelli, M. and Draetta, G. F. 2003. Regulating mammalian checkpoints through Cdc25 inactivation. *EMBO Rep.* **4,** 671-677.
20. Etienne-Manneville, S. 2010. From signaling pathways to microtubule dynamics: the key players. *Curr. Opin. Cell Biol.* **22,** 104-111.
21. Fesquet, D., Labbe, J. C., Derancourt, J., Capony, J. P., Galas, S., Girard, F., Lorca, T., Shuttleworth, J., Doree, M. and Cavadore, J. C. 1993. The MO15 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues. *EMBO J.* **12,** 3111-3121.
22. Furukawa, Y., Iwase, S., Kikuchi, J., Terui, Y., Nakamura, M., Yamada, H., Kano, Y. and Matsuda, M. 2000. Phosphorylation of Bcl-2 protein by CDC2 kinase during G_2/M phases and its role in cell cycle regulation. *J. Biol. Chem.* **275,** 21661-21667.
23. Gabrielli, B., Brooks, K. and Pavey, S. 2012. Defective cell cycle checkpoints as targets for anti-cancer therapies. *Front. Pharmacol.* **3,** 9.
24. Gadde, S. and Heald, R. 2004. Mechanisms and molecules of the mitotic spindle. *Curr. Biol.* **14,** R797-805.
25. Goshima, G. and Scholey, J. M. 2010. Control of mitotic spindle length. *Annu. Rev. Cell Dev. Biol.* **26,** 21-57.
26. Hainaut, P. and Hollstein, M. 2000. p53 and human cancer: the first ten thousand mutations. *Adv. Cancer Res.* **77,** 81-137.
27. Han, C. R., Jun, D. Y., Kim, Y. H., Lee, J. Y. and Kim, Y. H. 2013. Prometaphase arrest-dependent phosphorylation of Bcl-2 family proteins and activation of mitochondrial apoptotic pathway are associated with 17α-estradiol-induced apoptosis in human Jurkat T cells. *Biochim. Biophys. Acta* **1833,** 2220-2232.
28. Han, C. R., Jun, D. Y., Lee, J. Y. and Kim, Y. H. 2014. Prometaphase arrest-dependent phosphorylation of Bcl-2 and Bim reduces the association of Bcl-2 with Bak or Bim, provoking Bak activation and mitochondrial apoptosis in nocodazole-treated Jurkat T cells. *Apoptosis* **19,** 224-240.
29. Hanahan, D. and Weinberg, R. A. 2000. The hallmarks of cancer. *Cell* **100,** 57-70.
30. Hannun, Y. A. 1997. Apoptosis and dilemma of cancer chemotheraphy. *Blood* **89,** 1845-1853.
31. Harley, M. E., Allan, L. A., Sanderson, H. S. and Clarke, P. R. 2010. Phosphorylation of Mcl-1 by CDK1-cyclin B1 initiates its Cdc20-dependent destruction during mitotic arrest. *EMBO J.* **29,** 2407-2420.
32. Hartwell, L. H. and Weinert, T. A. 1989. Checkpoints: controls that ensure the order of cell cycle events. *Science* **246,** 629 - 634.
33. Hochegger, H., Takeda, S. and Hunt, T. 2008. Cyclin-dependent kinases and cell-cycle transitions: does one fit all? *Nat. Rev. Mol. Cell Biol.* **9,** 910-916.
34. Holloway, S. L., Glotzer, M., King, R. W. and Murray, A. W. 1993. Anaphase is initiated by proteolysis rather than by the inactivation of maturation-promoting factor. *Cell* **73,** 1393-1402.
35. Hollstein, M., Rice, K., Greenblatt, M. S., Soussi, T., Fuchs, R., Sorlie, T., Hovig, E., Smith-Sorensen, B., Montesano, R. and Harris, C. C. 1994. Database of p53 gene somatic mutations in human tumors and cell lines. *Nucleic Acids Res.* **22,** 3551-3555.
36. Huang, J. and Klionsky, D. J. 2007. Autophagy and human disease. *Cell Cycle* **6,** 1837-1849.
37. Ibrado, A. M., Kim, C. N. and Bhalla, K. 1998. Temporal relationship of CDK1 activation and mitotic arrest to cytosolic accumulation of cytochrome c and caspase-3 activity during Taxol-induced apoptosis of human AML HL-60 cells. *Leukemia* **12,** 1930-1936.
38. Jordan, M. A. and Wilson, L. 2004. Microtubules as a target for anticancer drugs. *Nat. Rev. Cancer* **4,** 253-265.
39. Kastan, M. B. and Bartek, J. 2004. Cell-cycle checkpoints and cancer. *Nature* **432,** 316-323.
40. Kaufman, S. H. and Earnshaw, W. C. 2000. Induction of apoptosis by cancer chemotherapy. *Exp. Cell Res.* **256,** 42-49.
41. Kim, Y. H., Proust, J. J., Buchholz, M. J., Chrest, F. J. and Nordin, A. A. 1992. Expression of the murine homologue of the cell cycle control protein $p34^{cdc2}$ in T lymphocytes. *J. Immunol.* **149,** 17-23.
42. Krokan, H., Wist, E. and Krokan, R. H. 1981. Aphidicolin inhibits DNA synthesis by DNA polymerase alpha and isolated nuclei by a similar mechanism. *Nucleic Acids Res.* **9,** 4709-4719.
43. Kuntz, K. and O'Connell, M. J. 2009. The G_2 DNA damage checkpoint: could this ancient regulator be the Achilles heel of cancer? *Cancer Biol. Ther.* **8,** 1433-1439.
44. Lapenna, S. and Giordano, A. 2009. Cell cycle kinases as therapeutic targets for cancer. *Nat. Rev. Drug Discov.* **8,** 547-566.
45. Lee, S. T., Lee, J. Y., Han, C. R., Kim, Y. H., Jun, D. Y., Taub, D. and Kim, Y. H. 2015. Dependency of 2-methoxyestradiol-induced mitochondrial apoptosis on mitotic spindle network impairment and prometaphase arrest in human Jurkat T cells. *Biochem. Pharmacol.* **94,** 257-269.
46. Li, Y. M. and Broome, J. 1999. Arsenic targets tubulins to induce apoptosis in myeloid leukemia cells, *Cancer Res.* **59,** 776-780.
47. Mansilla, S., Llovera, L. and Portugal, J. 2012. Chemotherapeutic targeting of cell death pathways. *Anticancer Agents Med. Chem.* **12,** 226-238.
48. Margolis, R. L. 2005. Tetraploidy and tumor development. *Cancer Cell* **8,** 353-354.

49. Mathias, S. and Holger, B. 2007. Mitotic drug targets and the development of novel anti-mitotic anticancer drugs. *Drug Resist. Update* **10,** 162-181.
50. Maton, G., Thibier, C., Castro, A., Lorca, T., Prigent, C. and Jessus, C. 2003. Cdc2-cyclin B triggers H3 kinase activation of Aurora-A in Xenopus oocytes. *J. Biol. Chem.* **278,** 21439-21449.
51. McGrogan, B. T., Gilmartin, B., Carney, D. N. and McCann, A. 2008. Taxanes, microtubules and chemoresistant breast cancer. *Biochim. Biophys. Acta.* **1785,** 96-132.
52. Mollinedo, F. and Gajate, C. 2003. Microtubules, microtubule-interfering agents and apoptosis. *Apoptosis* **8,** 413-450.
53. Morgan, G., Wardy, R. and Bartonz, M. 2004. The contribution of cytotoxic chemotherapy to 5-year survival in adult malignancies. *Clin. Oncol.* **16,** 549-560.
54. Murray, A. 1994. Cell cycle checkpoints. *Curr. Opin. Cell Biol.* **6,** 872-876.
55. Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A. and Yuan, J. 2000. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* **403,** 98-103.
56. Nitta, M., Tsuiki, H., Arima, Y., Harada, K., Nishizaki, T., Sasaki, K., Mimori, T., Ushio, Y. and Saya, H. 2002. Hyperploidy induced by drugs that inhibit formation of microtubule promotes chromosome instability. *Genes Cells* **7,** 151-162.
57. Ogden, A., Rida, P. C., Knudsen, B. S., Kucuk, O. and Aneja, R. 2015. Docetaxel-induced polyploidization may underlie chemoresistance and disease relapse. *Cancer Lett.* **367,** 89-92.
58. Ohi, R. and Gould, K. L. 1999. Regulating the onset of mitosis. *Curr. Opin. Cell Biol.* **11,** 267-273.
59. Pathan, N., Aime-Sempe, C., Kitada, S., Haldar, S. and Reed, J. C. 2001. Microtubule-targeting drugs induce Bcl-2 phosphorylation and association with Pin1. *Neoplasia* **3,** 70-79.
60. Rathinasamy, K. and Panda, D. 2006. Suppression of microtubule dynamics by benomyl decreases tension across kinetochore pairs and induces apoptosis in cancer cells. *FEBS J.* **273,** 4114-4128.
61. Ricci, M. S. and Zong, W. X. 2006. Chemotherapeutic approaches for targeting cell death pathways. *Oncologist* **11,** 342-357.
62. Robles, A., Linke, S. P. and Harris, C. C. 2002. The p53 network in lung carcinogenesis. *Oncogene* **21,** 6898-6907.
63. Sherr, C. J. 1996. Cancer cell cycles. *Science* **274,** 1672-1677.
64. Singh, P., Rathinasamy, K., Mohan, R. and Panda, D. 2008. Microtubule assembly dynamic: An attractive target for anticancer drugs. *IUBMB Life* **60,** 368-375.
65. Srivastava, R. K., Srivastava, A. R., Korsmeyer, S. J., Nesterova, M., Cho-Chung, Y. S. and Longo, D. L. 1998. Involvement of microtubules in the regulation of Bcl2 phosphorylation and apoptosis through cyclic AMP-dependent protein kinase. *Mol. Cell Biol.* **18,** 3509-3517.
66. Steigemann, P., Wurzenberger, C., Schmitz, M. H., Held, M., Guizetti, J., Maar, S. and Gerlich, D. W. 2009. Aurora B-mediated abscission checkpoint protects against tetraploidization. *Cell* **136,** 473-484.
67. Takahashi, Y., Ogra, Y. and Suzuki, K. T. 2004. Synchronized generation of reactive oxygen species with the cell cycle. *Life Sci.* **75,** 301-311.
68. Terrano, D. T., Upreti, M. and Chambers, T. C. 2010. Cyclin-dependent kinase 1-mediated Bcl-xL/Bcl-2 phosphorylation acts as a functional link coupling mitotic arrest and apoptosis. *Mol. Cell Biol.* **30,** 640-656.
69. Tighe, A., Johnson, V. L. and Taylor, S. S. 2004. Truncating APC mutations have dominant effects on proliferation, spindle checkpoint control, survival and chromosome stability. *J. Cell Sci.* **117,** 6339-6353.
70. Uetake, Y. and Sluder, G. 2004. Cell cycle progression after cleavage failure: mammalian somatic cells do not possess a tetraploidy checkpoint. *J Cell Biol* **165,** 609-615.
71. van der Vaart, B., Akhmanova, A. and Straube, A. 2009. Regulation of microtubule dynamic instability. *Biochem. Soc. Trans.* **37,** 1007-10013.
72. Vogel, C., Kienitz, A., Hofmann, I., Muller, R. and Bastians, H. 2004. Crosstalk of the mitotic spindle assembly checkpoint with p53 to prevent polyploidy. *Oncogene* **23,** 6845-6853.
73. Waldman, T., Lengauer, C., Kinzler, K. W. and Vogelstein, B. 1996. Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. *Nature* **381,** 713-716.
74. Wallach, D., Boldin, M., Varfolomeev, E., Beyaert, R., Vandenabeele, P. and Fiers, W. 1997. Cell death induction by receptors of the THF family: towards a molecular understanding. *FEBS Lett.* **410,** 96-106.
75. Watanabe, N., Broome, M. and Hunter, T. 1995. Regulation of the human WEE1Hu CDK tyrosine 15-kinase during the cell cycle. *EMBO J.* **14,** 1878-1891.
76. Wittmann, T., Hyman, A. and Desai, A. 2001. The spindle: a dynamic assembly of microtubules and motors. *Nat. Cell Biol.* **3,** E28-34.
77. Wong, C. and Stearns, T. 2005. Mammalian cells lack checkpoints for tetraploidy, aberrant centrosome number, and cytokinesis failure. *BMC Cell Biol.* **6,** 6.
78. Woods, C. M., Zhu, J., McQuenet, P. A., Bollag, D. and Lazarides, E. 1995. Taxol-induced mitotic block triggers rapid onset of a p53-independent apoptotic pathway. *Mol. Med.* **1,** 506-526.
79. World Health Organization. World cancer report, 2014. In: WHO, eds. WHO Report. Geneva: WHO; 2014.
80. Yamamoto, K., Ichijo, H. and Korsmeyer, S. J. 1999. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G_2/M. *Mol. Cell Biol.* **19,** 8469-8478.

초록 : 미세소관-손상 항암제 처리에 의한 세포주기의 정지 및 에폽토시스 유도

전도연 · 김영호*
(경북대학교 자연과학대학 생명과학부 면역학연구실)

에폽토시스에 의한 세포자멸사는 암세포에 대한 항암제 효능의 핵심적 기전이다. 항암제의 대표적인 두 종류로 알려진 DNA-손상 약제(DNA-damaging agents, DDAs)와 미세소관-손상 약제(microtubule-damaging agents, MDAs)가 암세포에 야기하는 초기 항암신호전달 기전은 다르지만, 최종적으로는 대부분 미토콘드리아 의존-에폽토시스를 통해 암세포를 사멸시킨다. 한편, DDAs에 의한 에폽토시스 유도에는 wild-type 종양억제 단백질 p53의 역할이 매우 중요하다. 그러나 인체 암의 약 50% 이상이 p53유전자의 돌연변이 때문에 종양억제 단백질로서의 p53 기능이 불활성화 되어 있다. 따라서 p53과 무관하게 에폽토시스를 유도할 수 있는 MDAs를 이용한 항암치료는 돌연변이 p53을 지닌 암세포에 대해 유리한 화학요법으로 이해된다. 최근 본 연구진은 인체 급성 백혈병 세포주인 Jurkat T 세포를 모델로 하여, MDAs (nocodazole, 17-α-estradiol, 혹은 2-methoxyestradiol)의 항암작용과 관련된 세포주기 정지 및 에폽토시스 유도 기전을 구명하였다. 그 결과, Jurkat T 세포를 MDAs로 처리할 경우, 유사분열방추사의 결함에 의한 세포주기(전중기, prometaphase) 정지, 장시간에 걸친 Cdk1의 활성화, 활성화된 Cdk1에 의한 에폽토시스 조절인자들(Bcl-2, Bcl-xL, Mcl-1 및 Bim)의 인산화, 이에 따른 Bak 활성화, 미토콘드리아 막 손상 및 카스파아제 연쇄 활성화에 의해 에폽토시스가 유도됨을 밝혔다. 또한 동일한 MDA 처리 조건하에서 Bcl-2 혹은 Bcl-xL의 과발현시켜 에폽토시스 진행을 차단할 경우, Jurkat T 세포는 약제처리 후에 전중기 정지된 4N 상태에 도달하지만, 이어서 유사분열 불이행(mitotic slippage) 및 내재복제(endoreduplication)가 진행되어 다배수체들(polyploids; 8N, 16N)을 생성하게 됨을 확인하였다. 이러한 결과는 MDAs처리에 따른 다배수체들의 생성을 차단하는 세포 내 기전으로서, 전중기 정지된 4N 세포의 에폽토시스에 의한 제거가 매우 중요함을 보여준다. 특히, 다배수체는 유전적으로 매우 불안정하여 암세포의 항암제 내성 획득 및 암 재발과 직접 연관되는 것으로 알려져 있으므로, 에폽토시스 기전에 결함이 있는 암세포를 대상으로 MDAs를 이용한 항암 화학요법을 시행할 경우에는 다배수체 세포의 생성을 차단하기 위한 새로운 수단이 반드시 병행되어야 할 것으로 사료된다.