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Apoptosis resistance in tumor cells

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Abstract

Various antitumor agents induce apoptotic cell death in tumor cells. Since the apoptosis program in tumor cells plays a critical role in the chemotherapy-induced tumor cell killing, it is suggested that the defect in the signaling pathway of apoptosis could cause a new form of multidrug resistance in tumor cells. This article describes the recent findings concerning the mechanisms of chemotherapy-induced apoptosis and discusses the implication of apoptosis resistance in cancer chemotherapy.

Abbreviations: JNK/SAPK – c-Jun N-terminal kinase/stress-activated protein kinase; PARP – poly(ADP-ribose) polymerase

Introduction

Emergence of drug-resistant tumor cells is one of the serious obstacles to cancer chemotherapy. Resistance to such agents as viaca alkaloids and anthracyclines is associated with a variety of phonotypic alterations. The elevated expression of a Mr 170 000 membane protein termed P-glycoprotein is often associated with multidrug resistance (MDR). P-glycoprotein acts as an energy-dependent efflux pump of antitumor agents in MDR cells [1,2]. Meanwhile, another alterations in cytosolic and nuclear enzymes or components are associated with resistance to antitumor agents in tumor cells. For example, an increase in glutathione S-transferase was reported for an adriamycin-resistant tumor cells. A decrease in DNA topoisomerase II (Topo II) activity was reported to be involved in the resistance to Topo II inhibitors such as etoposide and adriamycin [3]. In clinical situations, however, many tumor cell lines have been reported to show resistance to chomotherapy without these changes, suggesting that the mechanism of drug resistance is composed of a multicomponent system and other clitical determinants of drug resistance could exist.

The recent progress in the field of immunology and developmental biology has indicated that programmed cell death (apoptosis) plays an important role in many biological processes such as embryogenesis [4] and metamorphosis [5]. Apoptosis differs from necrosis in that its early stages show chromatin condensation, DNA fragmentation, and cytoplasmic blebbing, whereas other cytoplasmic organelles remain intact [6,7]. The dying cells fragment into small, membrane-enclosed apoptotic bodies, which are rapidly taken up by resident phagocytic cells. The process of apoptosis often depends on RNA and protein synthesis in the dying cells, indicating positive participation of cells in the death processes [8,9]. Several previous works have revealed that various antitumor agents also induce apoptotic cell death in cancer cells [10–15]. The findings indicate that apoptosis program in tumor cells plays a critical role in chemotherapy-induced tumor cell killing and also suggest that blockade of the apoptosis-inducing pathway could be another

mechanisms for multidrug resistance. In this chapter, we summarize the recent findings concerning the mechanisms of chemotherapy-induced apoptosis and the relationship between apoptosis resistance and the resistance to chemotherapy in tumor cells.

Apoptosis as a Determinant of Resistance to Chemotherapeutic Agents

A number of tumor cells have been reported to undergo apoptotic cell death when treated with such chemotherapeutic agents as etoposide, camptothecin, cisplatin, 1-β-D-arabinofuranosyl cytosine (Ara-C), mitomycin C, adriamycin, and vincristine. In the case of Topo II inhibitors, most drugs induced DNA strand breaks through stabilization of cleavable complexes, although the frequency of DNA strand breaks did not always correlate with their cytotoxicity [10]. Etoposide-induced strand breaks rapidly resealed after drug removal, but such resealing could not always prevent apoptosis [16]. These observations suggest that although the initial cellular damages induced by anticancer drugs is important in the cell death process, there could be an additional and crucial mechanisms whereby cells sense the initial damages and then promote apoptosis. To study the mechanisms of chemotherapy-induced apoptosis and the relationship between apoptosis resistance and the resistance to chemotherapy, we developed a mutant from human monocytic leukemia U937 cells that showed resistance to chemotherapeutic agent-induced apoptosis.

U937 cells were reported to undergo apoptosis by several antitumor agents including etoposide, camptothecin, and Ara-C [11,17,18]. After the treatment of U937 cells with ethyl methanesulfonate, a strong mutagen [19], the mutant cells resistant to apoptosis inducers were selected and cloned. Finally, we isolated a mutant clone designated UK711, that was resistant to apoptosis induced by several antitumor agents [20]. When U937 cells were treated with etoposide, the apoptotic morphological changes and DNA fragmentation occurred in a large number of cells. Consistant with these observations, flow-cytometric analysis revealed that the majority of U937 cells in S phase underwent apoptosis within 2 h of the end of etoposide treatment. UK711 cells, however, did not show such morphological changes or DNA fragmentation with the same treatment. The levels of protein-DNA covalent links and DNA double-strand breaks caused by etoposide were comparable, whereas the following cellular responses that resulted in apoptosis differed between U937 and UK711 cells. UK711 cells, however, were sensitive to apoptosis induced by tumor necrosis factor (TNF), as were U937 cells, suggesting that UK711 could not have a defect in the final execution mechanisms of apoptosis. Moreover, in UK711 cells, the expression of Bcl-2 which suppresses the common pathway of apoptosis [21] was comparable to that in U937 cells. These observations imply that U937 cells may possess a mechanism which senses cellular DNA damage and promote apoptosis, while UK711 cells may have a defect in this mechanism.

UK711 cells also showed resistance to apoptosis induced by such antitumor agents as Ara-C, adriamycin, mitomycin C, camptothecin, and by cytotoxic stimuli such as staurosporine, cycloheximide, and UV irradiation. These results suggest that these cytotoxic drugs could induce apoptosis via a common signaling pathway.

In U937 cells, a large population of cells in S phase underwent apoptosis within several hours when cells were treated with 10 μg mL^{-1} etoposide for 1 h. U937 cells in G1 phase did not die due to such a rapid process (rapid apoptosis). However, most of the U937 cells in G1 phase could not survive, and only 0.004% of U937 cells could form colonies after the treatment. These results indicate that etoposide caused two types of cell death in U937 cells; one was rapid apoptosis in S phase and the other was an uncharacterized slower cell death. Meanwhile, UK711 cells showed significant resistance to S phase-specific rapid apoptosis. The population of the dead UK711 cells, however, gradually increased with the time, and finally the majority of etoposide-treated cells died by an uncharacterized slow death mechanism. Then, could the defect of apoptosis signaling pathway in UK711 cells cause drug-resistance? Although the majority of the UK711 cells died at relatively high concentrations of etoposide, the survival fraction of UK711 cells was much higher than that of U937 cells as determined by colony formation assay (Table I). Similarly, the survival fractions of drug-treated UK711 cells were higher than those of U937 cells when cells were treated with other apoptosis-inducing drugs, Ara-C, adriamycin, and camptothecin. These results indicate that the apoptosis-resistant phenotype of UK711 cells obviously contributes to this higher cell survival and also suggest that the defect in the signaling pathway of apoptosis could cause multidrug resistance in tumor cells.

Table 1. Colony-formation assay. Cells were treated with the indicated concentrations of drugs for 1 h and colonies were counted after 10 days of incubation

Drugs	Concentration (μg mL^{-1})	Survival fraction (%)		
		U937	UK711	UK711/U937
Etoposide	1	15	40	2.7
	3	0.63	4.5	7.2
Ara-C	3	11	29	2.6
	10	3.5	24	6.8
Adriamycin	0.1	16	50	3.2
	0.3	0.67	8.9	13
Camptothecin	3	28	31	1.1
	10	2.4	5.4	2.3

Signaling Molecules of Chemotherapy-Induced Apoptosis

Although the molecular mechanisms of chemotherapeutic agent-induced apoptosis have not been well-difined yet, some molecules were reported to be positively or negatively involved in the process. Since the defect in the signaling pathway of apoptosis causes resistance to chemotherapy, the alterations of these molecules are possibly associated with resistance to antitumor agents in tumor cells.

ICE/ced-3 family proteases (Caspase)

Interleukin-1β-converting enzyme (ICE) was originally identified as a novel type of cysteine protease responsible for the conversion of precursor interleukin-1β (33 kDa) to 17.5 kDa mature form [22]. ICE was also identified as a mammalian homolog of CED-3, a positive regulator of apoptosis in the nematode *Caenorhabditis elegans* [23]. Several ICE/ced-3 family proteases have been isolated [24] and were recently renamed 'Caspase' [25]. The overexpression of those proteases induce apoptosis in various cells [26,27]. Moreover, it has been reported that specific inhibitors of caspase prevent several physiological cell death such as vertebrate neuronal death, TNF/Fas-induced apoptosis [28–30], cell killing by cytotoxic T lymphocytes (CTL) [31] and the apoptosis of epithelial cells detached from extracellular matrix [32]. These observations strongly suggest that caspase proteases could play a critical role in various physiological cell death.

Involvement of caspase in chemotherapy-induced apoptosis

We have tested whether these proteases could also be involved in chemotherapeutic agent-induced tumor cell apoptosis. To study the role of caspase in drug-induced apoptosis, we first examined the effect of a selective inhibitor of caspase, benzyloxycarbonyl-Asp-CH_2OC(O)-2,6,-dichloroben zene (Z-Asp-CH_2-DCB) [33], on apoptotic cell death of U937 cells caused by chemotherapeutic drugs [34]. This compound is an analogue of amino acid, based on L-aspartic acid residues, and it can preferentially inhibit ICE and ICE-like protease(s) [33–35]. As shown in Figure 1, Z-Asp-CH_2-DCB completely prevented apoptotic cell death induced by etoposide. Z-Asp-CH_2-DCB suppressed the fragmentation of cellular DNA into oligonucleosomal ladder, nuclear condensation, phosphatidylserine externalization and the morphological changes typical of apoptosis. Another caspase inhibitor, Z-VAD-CH_2-DCB [33] also inhibited the etoposide-induced U937 apoptosis. Moreover, ara-C, camptothecin and Adriamycin, which are the antitumor agents with different mechanisms of action, induce apoptosis in U937 cells, and the induction of apoptosis caused by these drugs were completely suppressed by the treatment with Z-Asp-CH_2-DCB (Figure 1). From these results, caspase proteases could be involved in the multiple antitumor agents-induced apoptosis. Caspase proteases are thought to be processed from a precursor form to an active form for their activation. After the treatment of U937 cells with anticancer drugs, caspase-3 (CPP32/YAMA/Apopain), a member of caspase, was

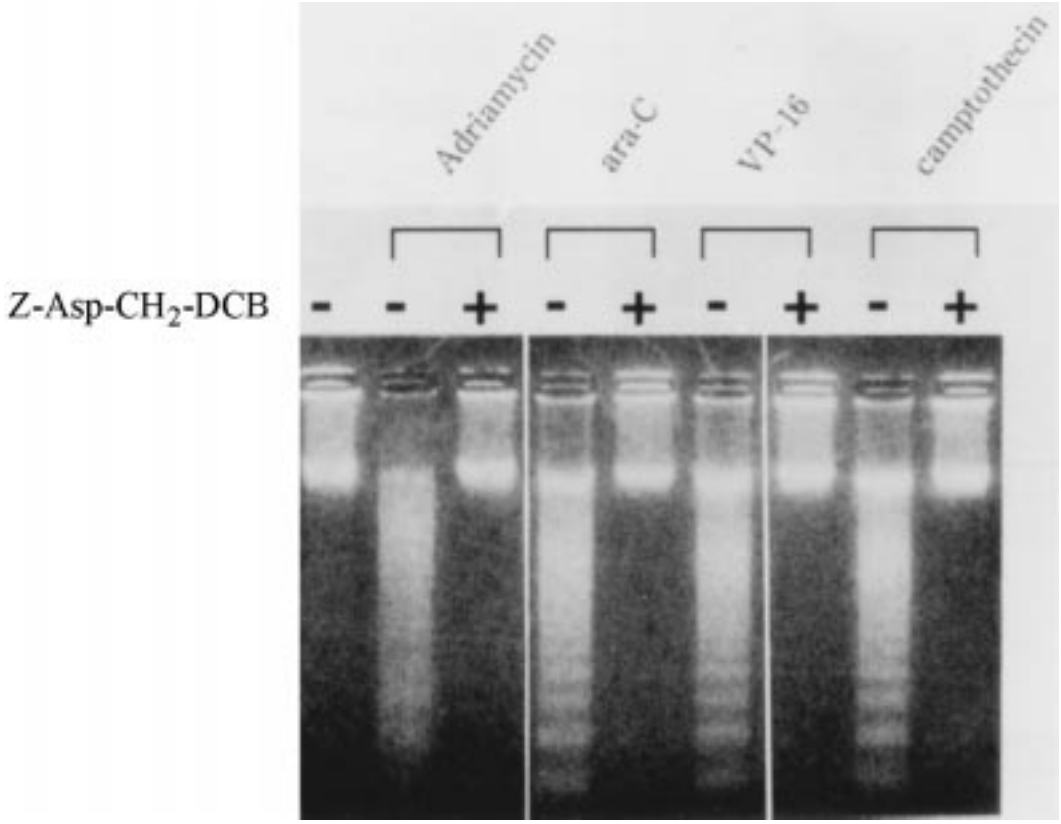

Figure 1. Prevention of various drug-induced apoptotic DNA degradation in U937 cells by a caspase inhibitor. Cells were continuously treated with 3 μm Adriamycin for 18 h, with 10 μg mL^{-1} of ara-C for 6 h, with 10 μg mL^{-1} of etoposide (VP-16) for 4 h, with 10 μg mL^{-1} of camptothecin for 4 h in the presence (+) or absence (–) of 100 μg mL^{-1} of Z-Asp-CH_2-DCB.

processed from 32 kDa precursor form to an active 17 kDa fragment [36]. Consistant with the observation, the cleavage activity for DEVD-MCA, a fluorogenic substrate for caspase-3, was elevated in the drug-treated U937 cells (Figure 2) [37]. Similarly, caspase-2 (Ich-1L) was also processed from 45 kDa precursor form to an active fragment [37]. These results indicate that the activation of several caspase proteases are critical steps in chemotherapeutic agent- induced tumor cell apoptosis. In U937 cells, caspase proteases were markedly activated at 3 h after the treatment with 10 μg mL^{-1} etoposide, while DNA-Topo II cleavable complexes were formed and concomitant DNA double strand breaks were induced within 1 h after the drug treatment [20]. Accordingly, caspase inhibitor, Z-Asp-CH_2-DCB, prevented etoposide-induced apoptosis and caspase activation, without affecting the formation of DNA-Topo II crosslinks (unpublished observation). Moreover, although 1 h exposure of 10 μg mL^{-1} etoposide to U937 cells caused marked increase in DNA double strand breaks, the caspase inhibitor was still effective in preventing apoptosis even after the induction of the DNA breaks [34]. These results indicate that caspase proteases are involved in chemotherapy-induced apoptosis pathway following the initial cellular damages in U937 cells. By contrast, in UK711 cells, which show resistance to etoposide-induced apoptosis, etoposide treatment caused DNA damage but not the proteolitic activation of caspase [20,38]. More recently, it was reported that selective inhibition of caspase proteases protects human T-leukaemic Jurkat cells from apoptosis dramatically increasing their survival in a colony formation assay [39]. These observations suggest that the loss of caspase activation could cause resistance to cytotoxic agent-induced tumor cell killing.

Substrate proteins for caspase

Since the activation of caspase proteases plays a key role in apoptosis, the target substrate proteins of caspase are thought to be critical in the control of apoptosis. Cleavage of poly(ADP-ribose) polymerase

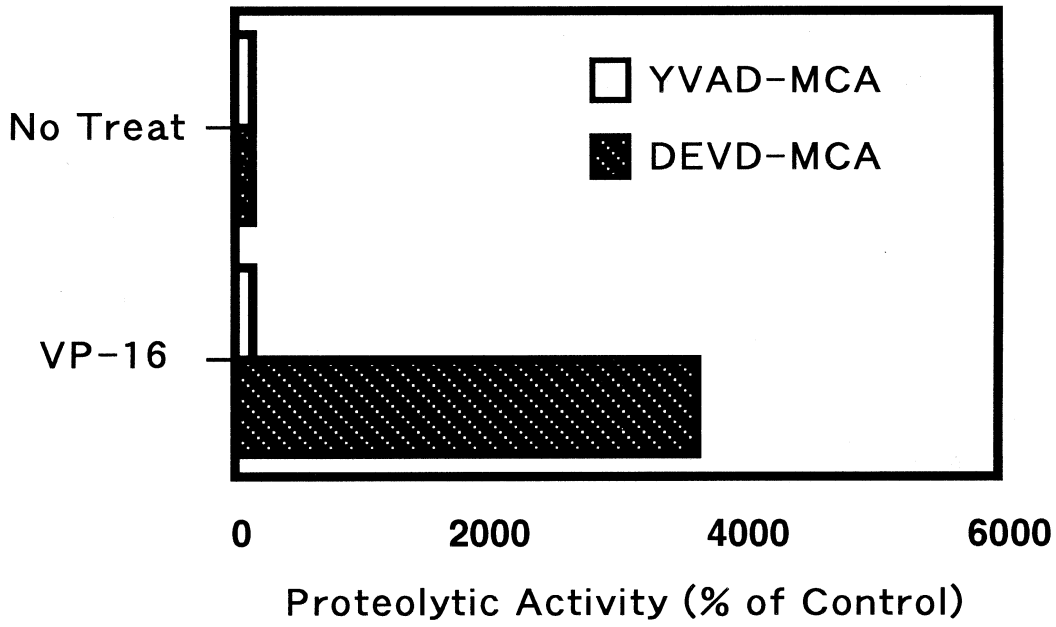

Figure 2. Activation of caspase protease in U937 cells by the treatment with etoposide. U937 cells were left untreated (No Treat) or treated with etoposide (VP-16) and the proteolytic activity of caspase-3(-like) protease and caspase-1(-like) protease were measured with fluologenic substrate peptide DEVD-MCA for caspase-3(-like) protease and YVAD-MCA for caspase-1(-like) protease.

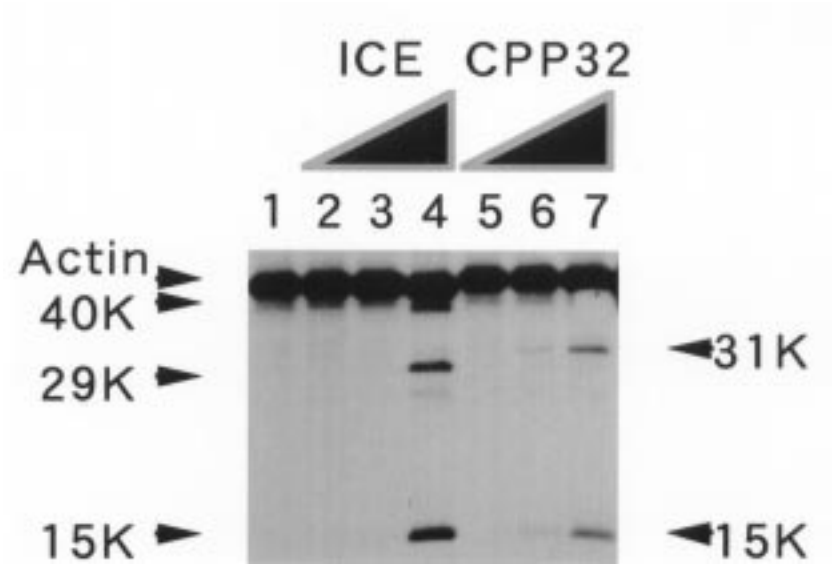

Figure 3. Cleavage of actin by caspase-3 (CPP32) and caspase-1 (ICE). Biotinylated actin was incubated with either caspase-3 or caspase-1. The cleavage of actin was detected. Lane1: no protease, lane2: caspase-1 0.02unit, lane3: caspase-1 0.1unit, lane4: caspase-1 0.5unit, lane5: caspase-3 0.5unit, lane6: caspase-3 2.5unit, lane7: caspase-3 12.5unit.

(PARP) is a generally observed event during apoptosis [40], and recently it was showed that caspase proteases are responsible for the cleavage of PARP [41–43]. However, the fact that PARP-deficient mice have only a mild phenotype [44] suggests that PARP cleavage could not be indispensable for apoptotic cell death. Another substrates cleaved by caspase proteases could be involved in apoptosis. Recently several proteins such as U1-70K, DNA-PK, lamin A/B, Rb, Gas2, fodrin, D4-GDI, MDM2 and SREBP have been reported to be cleaved by caspase [45]. The cleavage of these proteins could have some roles in the induction of apoptosis, although their significance in apoptosis is not entirely elucidated. We have also searched for the substrate proteins for caspase and identified actin as a selective substrate of caspase proteases [35,36]. As shown in Figure 3, when purified actin was incubated with recombinant caspase-3 or caspase-1, actin was efficiently cleaved to 15 kDa and 30 kDa fragments. Similarly, the treatment of U937 cells with etoposide induced the actin-cleavage activity in the cytosolic fraction during the induction of apoptosis. The actin-cleavage activity in apoptotic U937 cell extract was almost completely immunoadsorbed with anti-caspase-3 antibody. Moreover, the actin-cleavage in U937 cells was actually detected with an antibody raised against Gly-Gln-Val-Ile-Thr peptide, the N-terminal sequence of the cleaved 15 kDa actin fragment, and the actin-cleavage *in vivo* was completely suppressed by Z-EVD-CH2-DCB, a selective inhibitor of caspase-3 [36]. These results indicate that actin is cleaved mainly by caspase-3 during the development of apoptosis. At present, the role of actin-cleavage during apoptosis is not clear. The actin-cleavage by caspase could cause damage to the cells, since actin is one of the major proteins in the cells and has various roles in cellular activities and morphogenesis. It is possible that actin cleavage is involved in morphologic changes characteristic of apoptosis. More recently, PAK2 was identified as another substrate for caspase which could be responsible for the apoptotic morphological changes [46]. The analysis with cell free apoptosis system showed that DFF (DNA fragmentation factor), which is also cleaved by caspase, is responsible for the induction of DNA fragmentation during apoptosis [47]. These proteins could modulate antitumor agents-induced tumor cell apoptosis by interacting with caspase proteases.

c-Jun N-terminal kinase/stress-activated protein kinase

Recent studies, including ours, have demonstrated that some kinds of apoptotic signaling are mediated by c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), characterized as a member of mitogen-activated protein kinase (MAPK) family [48–55]. For its enzymatic activation, JNK/SAPK requires dual phosphorylation at Thr and Tyr within the motif Thr-Xaa-Tyr located in kinase subdomain VIII [48,56]. Like other members of MAPK family, JNK/SAPK and further upstream protein kinases constitute a protein kinase cascade, in which MAPKKK (MEKK, MUK, MLK3, ASK1) phosphorylates and activates MAPKK (SEK1/MKK4/JNKK) that subsequently phosphorylates and activates MAPK (JNK/SAPK) (Figure 4) [57]. The activated JNK/SAPK, in turn, phosphorylates the c-Jun transcription factor at Ser-63 and Ser-73 within its N-terminal transactivation domain, which induces the expression of c-Jun-responsive genes, such as c-*jun* itself [58]. JNK/SAPK also phosphorylates other transcription factors, such as ATF2, TCF/Elk-1, and p53 [59–64].

JNK/SAPK activation during anticancer drug-induced apoptosis

JNK/SAPK is activated by a wide array of stresses, such as UV irradiation, heat shock, and inflammatory cytokines [48,49]. In addition, various chemicals that elicit an apoptosis program also activate the JNK/SAPK cascade. Among them are many kinds of anticancer drugs, including cisplatin, mitomycin C, Adriamycin, etoposide, camptothecin, Ara-C and vinblastine [54,65–67]. This means that efficacy of cancer chemotherapy could be in part determined by the JNK/SAPK activating potential of the target cancer cells.

To elucidate the role of JNK/SAPK signaling in anticancer drug-induced apoptosis, we monitored JNK1 activity during the course of etoposide- and camptothecin-induced apoptosis of U937 cells. As shown in Figure 5A, JNK1 is transient activated in U937 cells by treatment with 10 μg mL^{-1} etoposide or camptothecin. This JNK1 activation preceded the chromosomal DNA fragmentation, one of characteristic properties observed in apoptotic cells. Meanwhile, UT16, a TPA-resistant variant of U937 cells [68], exhibits decreased susceptibility to apoptosis induced by these agents [54]. UT16 cells did not express P-glycoprotein nor downregulate topoisomerase

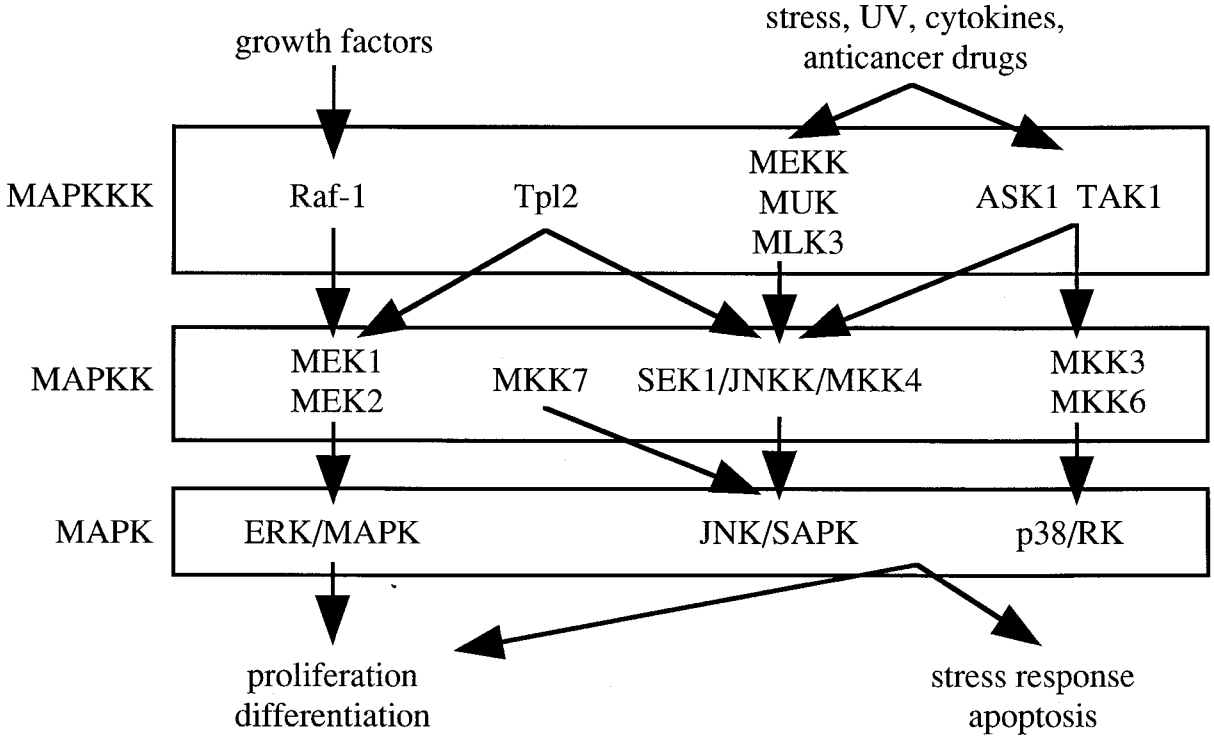

Figure 4. The JNK/SAPK cascade. Like other members of MAPK family, JNK/SAPK and further upstream protein kinases constitute a protein kinase cascade, in which MAPKKK phosphorylates and activates MAPKK that subsequently phosphorylates and activates MAPK.

II, a target of etoposide. In fact, DNA-protein covalent cross-links mediated by etoposide [69] occurred equally in both cell lines. These observations indicate that the decreased susceptibility to apoptosis of UT16 cells is due to an abnormality in a cellular response subsequent to initial DNA damage caused by anticancer drugs. In other words, the cells do not die from disarray caused by DNA damage itself but rather by a triggered apoptosis program. In UT16 cells, neither etoposide nor camptothecin significantly activated JNK1, suggesting a failure in signaling linkage between the damaged DNA and JNK/SAPK cascade [54]. These situations; DNA damage comparable to that in parental cells, failure in JNK1 activation, and decreased susceptibility to apoptosis; were also the case in another apoptosis-resistant variant, UK711 [20].

Signal transducers surrounding the JNK/SAPK cascade

Many anticancer drugs are genotoxic; they target the cellular DNA with alkylation, adduct formation and subsequent strand breakage, for example. The damaged DNA triggers various intracellular responses, including arrest of the cell cycle, recruitment of a repair system, or activation of an apoptotic program. So far, several candidates have been reported for the signaling mediators between the damaged DNA and the JNK/SAPK cascade. For example, genotoxic stress-induced activation of c-Abl tyrosine kinase leads to activation of MKK4/SEK1/JNKK, that directly phosphorylates and activates JNK/SAPK [65,70]. Since c-Abl-deficient cells fail to activate the JNK/SAPK cascade and that is rescued by introduction of an intact c-*abl* gene into the cells [65], c-Abl would be one of the upstream regulators of JNK/SAPK cascade. On the other hand, SHP-1 protein tyrosine phosphatase could be a negative feedback regulator for JNK/SAPK signaling since this phosphatase blocks the radiation-induced activation of JNK/SAPK in a c-Abl-dependent manner [71]. However, precise interaction between the damaged DNA and the JNK/SAPK cascade still remains to be questioned.

The activated JNK/SAPK phosphorylates c-Jun on its transactivation domain [58], that results in activation of AP-1 transcription factor, a heterodimer of c-Jun and c-Fos [72]. Consistent with etoposide- and camptothecin-induced activation of JNK1, c-*jun* gene

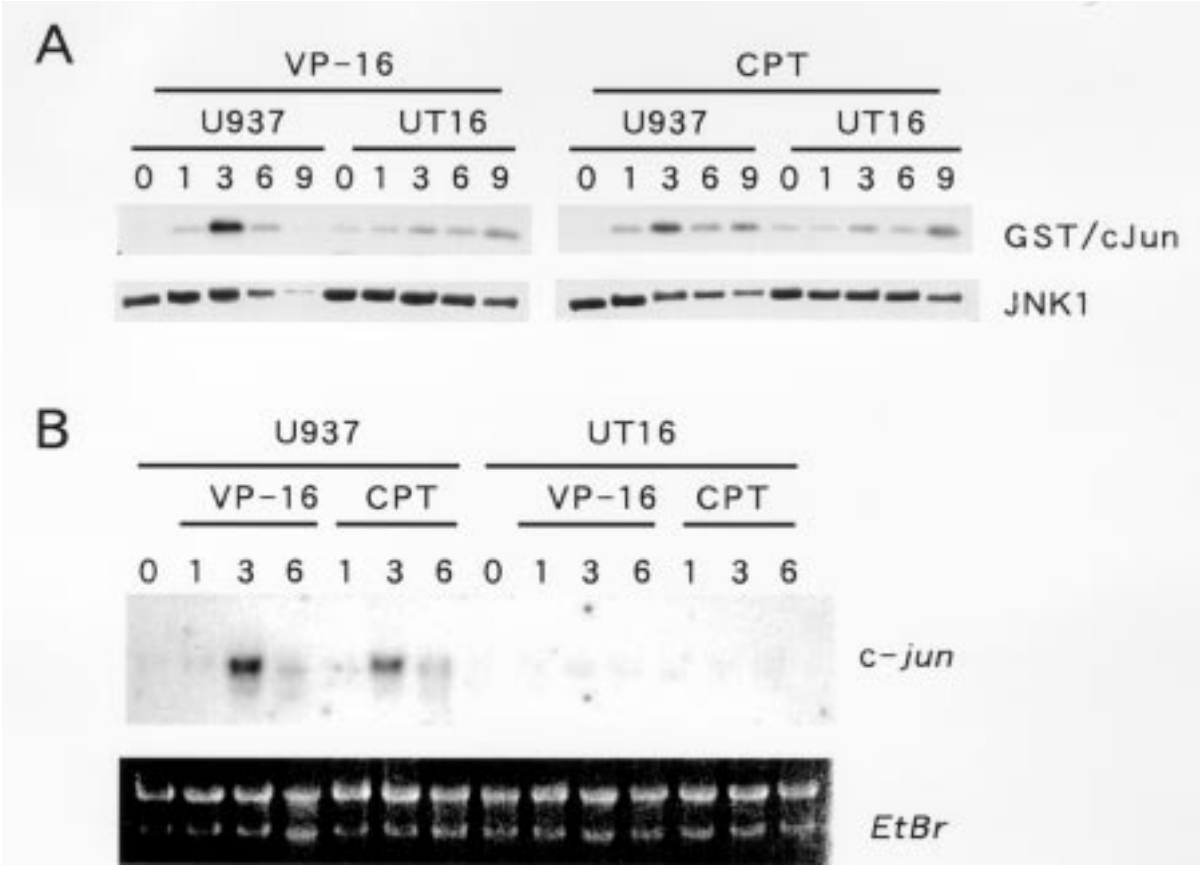

Figure 5. JNK1 activation during apoptosis of U937 but not of UT16 cells. Cells were treated with 10 mg mL^{-1} of either VP-16 or CPT for the indicated periods of time. *A*, effects of VP-16 and CPT on JNK1 activity. Cellular extract was prepared, and JNK1 activity was monitored by phosphorylation of bacterially expressed GST/c-Jun(1-92) as a specific substrate of the kinase (*upper panels*). The amounts of JNK1 protein were also monitored by Western blot analysis (*lower panels*). *B*, effects of VP-16 and CPT on c-*jun* expression. Total RNA was resolved on agarose gel and transferred to a nylon membrane. The blot was hybridized with c-*jun* probe (*upper panel*). Also shown are the RNAs loaded and stained with ethidium bromide (*EtBr*).

expression was transiently induced in U937 but not in UT16 cells by treatment with those agents (Figure 5B). On the other hand, c-*jun* gene expression is also induced through the ERK/MAPK pathway, which is activated during phorbol ester-induced monocytic differentiation of U937 cells [68,73]. In U937 cells, however, ERK/MAPK was not activated by either etoposide or camptothecin [54]. These observations indicate that c-*jun* induction by etoposide or camptothecin is mediated by the JNK/SAPK rather than the ERK/MAPK pathway and is correlated with the drug-induced apoptosis of U937 cells. In fact, c-Jun regulates various types of apoptosis [50,51,74–77], and some of them are elicited by the JNK/SAPK but not the ERK/MAPK pathway [50,51,74,75].

Meanwhile, p53 tumor suppresser protein, which regulates genotoxic stress-induced growth arrest and apoptosis [78], is another substrate for JNK/SAPK *in vivo* [64]. However, U937 cells are p53-negative and, at least in these cells, interaction between JNK/SAPK and p53 could not be associated with the anticancer drug-induced apoptosis. This also means that JNK/SAPK-dependent and p53-dependent signaling pathways [79] might be in part shared by each other and JNK/SAPK signaling could elicit an apoptosis program even in the absence of the wild-type p53.

JNK/SAPK activation causes caspase activation and apoptosis

To determine whether the failure in JNK1 activation in UT16 cells could be associated with abnormality in caspases, we monitored caspase-3 activation and actin cleavage in U937 and UT16 cells [54]. After treat-

ing U937 cells with either etoposide or camptothecin, caspase-3 was activated and actin was cleaved as one of specific substrates for the activated caspase-3 [35,36], whereas that was not the case in UT16 cells [54]. These data indicate that failure in caspase activation is correlated with the loss of JNK1 activation and decreased susceptibility to the drug-induced apoptosis of UT16 cells. Interestingly, while Z-Asp-CH_2-DCB completely protected U937 cells from etoposide- and camptothecin-induced apoptosis [34], it did not inhibit JNK1 activation at all in these cells (Figure 6A). This result suggests that JNK/SAPK, as an upstream regulator, might control the caspase activity in a single cascading pathway. Alternatively, JNK/SAPK and caspases might participate in independent apoptotic pathways.

To examine the possibilities above, we employed an antisense trial [54]. When we diminished the cellular amounts of JNK1 protein by *JNK1* antisense oligonucleotide, etoposide or camptothecin-induced apoptosis of U937 cells was significantly blocked whereas control sense oligonucleotide did not affect the JNK1 content nor the apoptotic response of the cells. Furthermore, the inhibition of apoptosis was associated with the loss of caspase activation (Figure 6B). Taken together, these data indicate that JNK1 activates caspase-3 and facilitates apoptosis of U937 cells. Since caspase-3 does not have a proline-directed serine/threonine residue, which is preferentially phosphorylated by MAPK family [56], it is unlikely that JNK/SAPK directly phosphorylates and activates caspase-3. There must be additional transducers which connect two events, JNK/SAPK activation and caspase-3 activation.

Diverse functions of JNK/SAPK

While JNK/SAPK system is positively involved in the apoptosis induced by growth factor-withdrawal, UV-C, γ-radiation or DNA-damaging anticancer drugs [50–52,54,55], it has not been thought to be linked to the death domain-mediated cell death [80], such those through FasL/Fas or TNF/TNFR1 systems [81–83]. In fact, TNF activates JNK/SAPK through a noncytotoxic TRF2-dependent pathway [81,83]. Recently, however, Ichijo *et al*. have reported induction of apoptosis by a novel type of MAPKKK, ASK1, that activates JNK/SAPK (and another MAPK family, p38/RK) signaling [84]. Furthermore, Yang *et al*. have reported a novel type of Fas-associating factor, Daxx, that activates JNK/SAPK cascade and causes apoptosis of cells in a FADD-independent manner [85]. These findings still suggest a functional involvement of JNK/SAPK signaling in death receptor-mediated apoptosis, too.

Meanwhile, the JNK/SAPK system exhibits diverse functions, including activation and/or protection of lymphocytes [86,87], oncogenic transformation [88], and DNA repair [89]. JNK/SAPK is regulated by a variety of upstream kinase/phosphatase systems and the activated JNK/SAPK probably phosphorylates not only transcription factors but also other cytoplasmic substrates. Diverse functions of the JNK/SAPK system would reflect the multiple combination of these effectors. In fact, this is also the case in the ERK/MAPK system, which is parallel to but distinct from the JNK/SAPK system. For example, 12-*O*-tetradecanoyl-phorbol-13-acetate, a potent activator of the ERK/MAPK pathway, induces either growth, differentiation, or apoptosis of cells in each case.

Other molecules (Bcl-2 family proteins, p53)

bcl-2

bcl-2 was originally identified as the protooncogene involved in the t(14; 18) translocation in human malignant lymphomas [90]. It encodes a 26-kDa protein that suppresses apoptosis induced by various stimuli [91,92]. The expression of the Bcl-2 protein was reported to suppress apoptosis induced by antitumor agents [93]. Bcl-XL, a relative of Bcl-2, [94] can also inhibit chemotherapy-induced apoptosis [95]. The expression of Bcl-2/Bcl-XL was shown to cause resistance to chemotherapy *in vivo* [96], suggesting that these proteins could be other determinants of tumor cell responce to chemotherapy. Recently, it was reported that human breast cancer MCF-7 cells overexpressing Bcl-2/Bcl-XL was sensitized to etoposide and Taxol by expressing Bcl-XS, a dominant negaive inhibitor of Bcl-2/Bcl-XL [97]. These results indicate that suppression of apoptosis inhibitor proteins such as Bcl-2/Bcl-XL could be a new strategy for cancer chemotherapy.

p53

p53 is the tumor suppressor gene with transcriptional regulator activity [98]. Loss of p53 occurs in more than half of all human tumors, indicating that the defect in the gene is a critical step in the pathogenesis of cancer. p53 has also been shown to be closely related to the induction of apoptosis, since the overexpression of wild-type p53 in some p53-deficient tumors resulted in rapid loss of cell viability (apop-

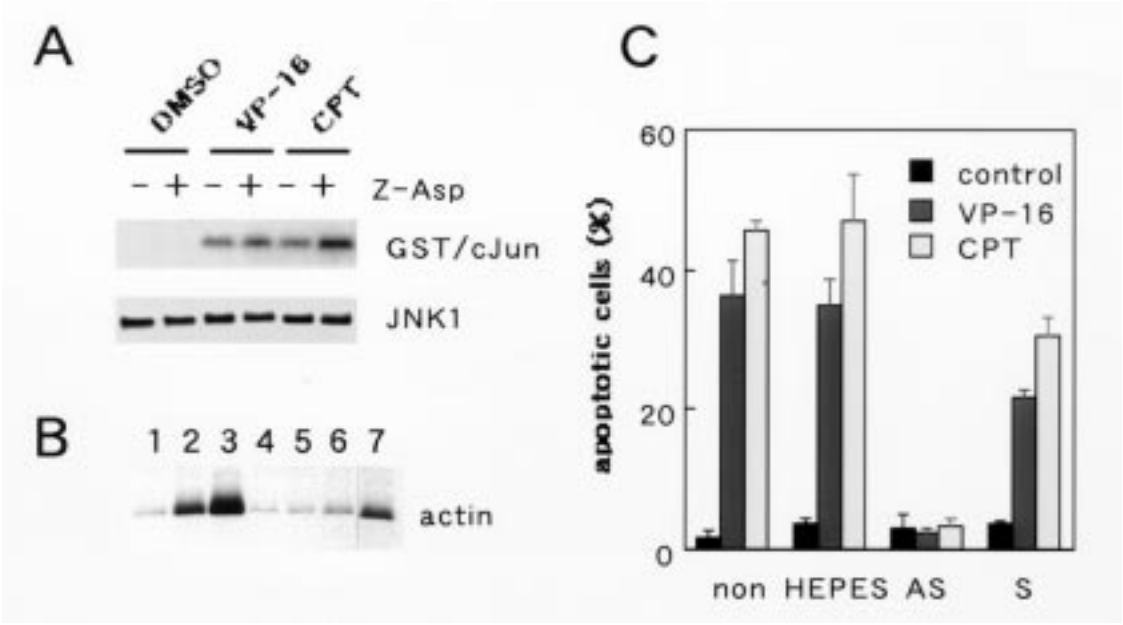

Figure 6. JNK1 activation precedes caspase activation and apoptosis. *A*, effect of Z-Asp-CH_2-DCB on JNK1 activation. Cells were treated with 10 mg mL^{-1} of either VP-16 or CPT with or without 50 mg mL^{-1} Z-Asp-CH_2-DCB for 3 h. JNK1 activity was monitored as in Figure 2. *B, C*, effect of *JNK1* antisense oligonucleotide on actin cleavage and apoptosis of U937 cells. Cells were treated with *JNK1* sense (SON) or antisense (ASON) oligonucleotides for 72 h followed by exposure to 10 mg mL^{-1} of either VP-16 or CPT for 4 h. *B*, actin-cleaving activity. Lanes 1, 2, 3, HEPES buffer; 4, 5, 6, ASON; 7, SON; 1, 4, no treatment; 2, 5, VP-16; 3, 6, 7, CPT. *C*, drug-induced apoptosis. Cells were scored for the incidence of apoptotic changes in morphology by a photomicroscopy (more than 300 cells per sample were counted). AS, ASON; S, SON.

tosis) [99]. Although the expression of p53 is not sufficient to induce apoptosis in other p53-deficient tumor cell lines, it render the cells more sensitive to chemotherapeutic drugs-induced apoptosis [100]. In clinical situation, loss of p53 appears to play an important role in the treatment of cancer. Actually, it was reported that the defect in p53 caused marked resistance to some antitumor agents [101]. These observations suggest that inactivation of p53 could cause resistance to chemotherapy in tumor cells and also indicate that p53 could be another effective target for cancer chemotherapy.

Resistance to Chemotherapy-Induced Apoptosis in Solid Tumor

Apoptosis in leukemia and solid tumors

Many chemotherapeutic drugs can trigger apoptosis in cancer cells. Does the drug-induced apoptosis correlate with chemotherapy of cancer? If so, could chemotherapy-sensitive cancers be easy to be induced to undergo apoptosis, and *vice versa* could chemotherapy-resistant tumors be difficult to be triggered for apoptosis?

To answer these questions, we have treated many kinds of human cancer cells from diffrent tissues or organs with several chemotherapeutic drugs, and tested whether the cells could undergo apoptosis. As shown in Table II, almost all drugs used caused apoptosis rapidly in leukemia U937, THP-1 and HL-60 cells. But it was difficult under the conditions to trigger rapid apoptosis in solid tumor such as colon cancer HT-29 cells and lung cancer A549 cells. Long time treatment of these cells with anti-cancer drug, however, induced 50kb to 300kb DNA fragment, and apoptotic morphology. These results indicate that drug dose and treatment time could be very impotant factors in triggering apoptosis, since it usually takes several days to induce apoptosis in most of solid tumors. While, in leukemia cells, it just takes several hours to trigger apoptosis. Clinically, it was reported that 70% of blastic cells in patiant blood were induced to undergo apoptosis after 8 hours' treatment of leukemia patiant with chemotherapeutic agents such as mitoxantrone and etoposide [102]. It suggests that drug-induced apoptosis could be clinically impotant and correlates with chemotherapy of leukemia patiants. Generally, leukemia cells are more sensitive to chemotherapy

Table 2. Apoptosis induced by chemotherapeutic agents. The cells were treated with anticancer drugs for 16 h and the induction of apoptosis was determined by DNA fragmentation assay

Drug	Dose	Cell line				
	(μg mL^{-1})	U937	THP-1	HL-60	HT29	A549
VP-16	10	+	+	+	–	–
ADM	1	+	+/–	+	–	–
DNR	1	+	+	+	–	–
CPT	1	+	+	+	–	–
Ara-C	10	+	+/–	+	–	–
Act-D	5	+	+	+	–	–
COL	1	+/–	–	+	–	–
SPM	1	+	+	+	–	–
STS	1	+	+	+	+/–	–
CHX	100	+	+	+	–	–

than solid tumors; maybe it is because anti-cancer drugs can induce apoptosis rapidly in leukemia while it takes longer time to trigger apoptosis in solid tumors.

Chemotherapy-induced apoptosis in human ovarian carcinoma cells

To clarify the molecular mechanism of anticancer drug-induced apoptosis in solid tumors, human ovarian carcinoma OVCAR-3 and OVCAR-8 cells were used as a model [103].

When human ovarian carcinoma OVCAR-3 cells were treated with cisplatin or etoposide, cell growth was significantly inhibited, and cell death was induced in a dose dependent manner. To elucidate whether this drug-induced cell death was apoptosis, we examined the nuclear changes by DAPI staining. Figure 7 represents fluorescence microscopy of DAPI-stained OVCAR-3 cells after treatment with 3 μg mL^{-1} of cisplatin for 2 days. Some of the drug-treated cells contained condensed and fragmented nuclei, which are characteristic features of apoptosis. The flow cytometric analysis showed that the apoptotic cell population in cisplatin and VP-16 treated cells were about 28 and 15%, respectively. This apoptosis process is time and dose dependent. The time-course of the cisplatin treatment at 10 μg mL^{-1} indicated that no significant change was apparent in OVCAR-3 cell morphology at 4 h after the drug treatment. Morphological change was observed after 12 h, and about 15% of cells appeared to detach from the cell layer with apoptotic characteristics as determined by flow cytometric analysis. By 24 h, these changes were more pronounced, and after 48 h of drug treatment, the apoptotic cells increased to about 55%; although some cells were still attached to the tissue culture plate.

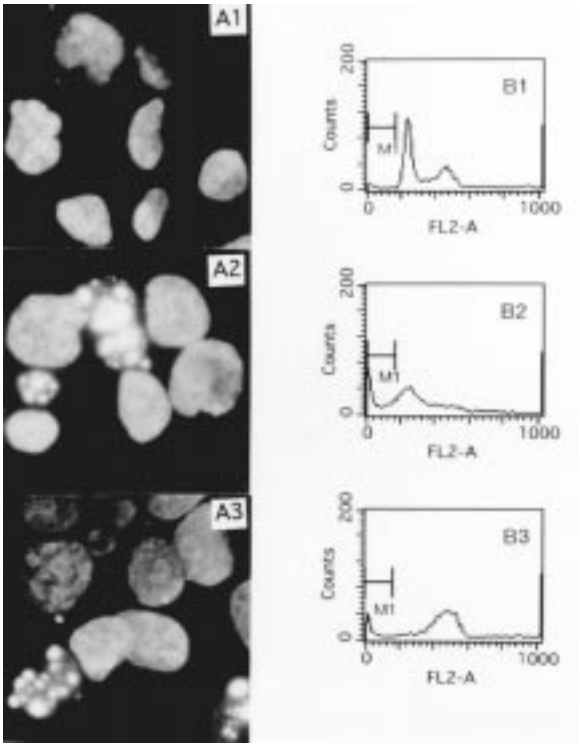

Figure 7. Apoptosis in human ovarian carcinoma cell lines treated with cDDP or VP-16. OVCAR-3 cells were incubated with vehicle DMSO (1), 3 μg mL^{-1} cDDP (2), or 10 μg mL^{-1} VP-16 (3) for 48 h. (A) the cell morphologic analyses after staining with DAPI, (B) the flow cytometric analysis.

In OVCAR-8, another human ovarian carcinoma cell line which is relatively resistant to chemotherapy, no apoptosis was observed after 2 days of treatment with 3 μg mL^{-1} cisplatin. Instead, the growth inhibition accompanied G2 arrest.

As mentioned above, activation of intracellular proteases is a crucial event in apoptosis. A series of caspase genes have been isolated, and apoptosis was induced by the overexpression of these proteases in a number of cells. We have shown that there is an actin cleavage activity (ACA) that occurs in human myeloid leukemia U937 cells during apoptosis induced by antitumor agents, and the activity could be attributed to caspase-3 [34–36]. We tested whether the cell death of ovarian carcinoma cells could accompany the activation of the proteases [103]. As shown in Figure 8, the actin-cleavable caspase-3 activity was detected in the apoptotic OVCAR-3 cell lysate treated with chemotherapeutic agents. When OVCAR-3 cells were treated with 3 μg mL^{-1} cisplatin for 48 h, actin was cleaved to 15 kDa fragment as observed in drug-treated U937 cells. Western blotting analysis of cisplatin-treated OVCAR-3 cell lysate by using the antibody raised against GQVIT peptide, the amino terminal sequence of the 15 kDa actin fragment, indicated that actin was cleaved into 15-kDa fragment in cisplatin-treated OVCAR-3 cells as in the drug-treated U937 cells. Consistant with the observations, after cisplatin treatment for 24–48 h, the caspase-3 activity in the OVCAR-3 cell lysate increased according to the increased population of apoptotic cells. A similar result was obtained when OVCAR-3 cells were treated with etoposide. Figure 8 represents the difference between the relatively chemotherapy-sensitive OVCAR-3 cells and chemotherapy-resistant OVCAR-8 cells. When OVCAR-3 cells were treated with 3 μg mL^{-1} cisplatin for 2 days, about 28% underwent morphological apoptosis, and a relevant caspase-3 activity was detected in the OVCAR-3 cell lysate. On the contrary, when chemotherapy-resistant OVCAR-8 cells were treated in the same way and at the same time, the cells showed no morphological changes characteristic of apoptosis, and no caspase-3 activity was detected in the cell lysates. The results indicate that OVCAR-8 cells are resistant to chemotherapy-induced apoptosis compared with OVCAR-3 cells, and the activation of actin-cleavable caspase-3 by the drugs were blocked in resistant OVCAR-8 cells. To characterize the apoptosis-associated caspase-3 activity, we tested the effect of caspase inhibitors, such as Z-EVD-CH_2DCB and Z-VAD-CH_2DCB, on the development of apoptosis. The inhibitors completely prevented cisplatin-induced morphological changes of apoptosis and caspase-3 activity in the cytosolic fraction. These observations showed that caspase-3 also plays an important role in chemotherapy-induced apoptosis in human ovarian carcinoma cells, while the blockade of protease activation signaling could cause resistance to chemotherapy in some solid tumors such as OVCAR-8 cells.

Resistance to apoptosis signaling in solid tumor

Solid tumors are often refractory and difficult to cure with chemotherapy. One of the important reasons may be that solid tumors are more resistant to apoptosis than leukemic cells. It should be noted that the activation of caspase proteases and the development of apoptosis in OVCAR-3 cells take longer (1 to 2 days) than those in leukemic cells, which undergo apoptosis within several hours after drug treatment [103]. Solid tumor cells could have the survival advantage because, during the slow progression of apoptosis, cells could develop protective responses and repair damages caused by the anticancer drugs. Elucidation of the molecular mechanism of chemotherapy-induced apoptosis in solid tumors could enhance the development of cancer chemotherapy.

Conclusion

Tumor cells undergo apoptotic cell death when treated with several anticancer drugs. Since the agents with various cellular targets induce a similar pattern of cell death (apoptosis), it was suggested that a common pathway of apoptosis could exist in chemotherapeutic drugs-induced apoptosis, and the defect in the process could cause drug resistant phonotype of tumor cells. In this review, we have described the molecules which are critical in and possibly modify the antitumor agent-induced apoptosis. The factors involved in apoptosis signaling pathways could modulate the sensitivity of cancer cells to chemotherapeutic agents, since the defect of apoptosis signaling pathways (such as that in the mutant UK711 cells) actually protected the tumor cells from apoptosis, which resulted in a marked increase in their survival [20]. In human leukemia U937 cells, anticancer drug-induced activation of JNK/SAPK causes apoptosis by activating caspase proteases [34–36,54]. If this occurs in clinical situations, defects in such apoptosis signaling might result in therapeutic difficulties in that

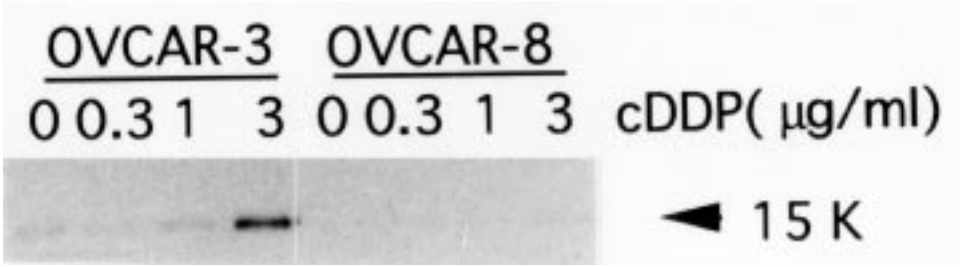

Figure 8. Difference of actin cleavage activity between OVCAR-3 and OVCAR-8 cells. OVCAR-3 and OVCAR-8 cells were incubated for 48 h with 0, 0.3, 1 or 3 μg mL^{-1} of cisplatin (cDDP) and then the actin cleavage activity by caspase-3 was examined.

cancers often exhibit broader patterns of resistances to chemotherapy and radiation than those defined by the classical MDR phenotype. Several other molecules have been identified as inducers or suppressors of apoptosis. The overexression of apoptosis inhibitors such as Bcl-2/Bcl-XL was shown to cause resistance to chemotherapy both *in vitro* and *in vivo* [93–96]. Meanwhile, the defect in the positive mediators of apoptosis such as p53 also causes resistance to various antitumor agents [100,101]. More recently, some oncogenes were shown to suppress apoptosis as well as to stimulate growth signals [104,105]. These findings strongly indicate that the modulators of apoptosis could be one of the major determinants of resistance to chemotherapy in tumor cells, and also indicate that the factors or the drugs directly targeting these molecules could be another candidates of novel chemosensitizer.

In the present clinical situations, solid tumors are difficult to cure with chemotherapy. The resistance to antitumor agents in solid tumors could be (at least in part) caused by some defects in the signaling pathways of apoptosis, although the defects are not clear at present. Further studies are needed to clarify the molecular mechanisms of apoptosis especially in solid tumor and to develop effective therapeutic approaches targeting the molecules of apoptosis.

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