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Review

Tumor cell "dead or alive": Caspase and survivin regulate cell death, cell cycle and cell survival

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Summary. Cell death and cell cycle progression are two sides of the same coin, and these two different phenomenons are regulated moderately to maintain the cellular homeostasis. Tumor is one of the disease states produced as a result of the disintegrated regulation and is characterized as cells showing an irreversible progression of cell cycle and a resistance to cell death signaling. Several investigations have been performed for the understanding of cell death or cell cycle, and cell death research has remarkably progressed in these 10 years. Caspase is a nomenclature referring to ICE/CED-3 cysteine proteinase family and plays a central role during cell death. Recently, several investigations raised some possible hypotheses that caspase is also involved in cell cycle regulation. In this issue, therefore, we review the molecular basis of cell death and cell cycle regulated by caspase in tumor, especially hepatocellular carcinoma cells.

Key words: Cell death, Cell survival, Cell cycle, Caspase family, IAP family

Fas ligand/Fas cell death-inducing system

Fas is a type-I transmembrane protein belonging to the nerve growth factor/tumor necrosis factor receptor family (Itoh et al., 1991; Nagata, 1994). Tumor necrosis factor receptor-1 (TNF-R1 and ref. Old, 1985; Tartaglia et al., 1993) and TNF-related apoptosis-inducing ligand-receptor 1/2 (TRAIL-R1/2 and ref. Pan et al., 1997; Sheridan et al., 1997) also belong to the same family and these transmembrane proteins are closely involved in cell death induction. As shown in Fig. 1A, this receptor family is characterized by a cysteine-rich extracellular NH2-terminal domain and one transmembrane domain. The four death-associated receptors, Fas, TNF-R1 and TRAIL-R1/2, contain a unique domain in the cytoplasmic region which is necessary for the cell death signaling, which is referred to as the "death domain". It is known that lpr/lpr and lpr^{cg}/lpr^{cg} mutant mice are animal models for auto-immune disease (Watanabe-Fukunaga et al., 1992), and the functional lack of Fas in these mutant mice, which was caused by ETn insertion and a point mutation in the death domain, has been documented (Watanabe-Fukunaga et al., 1992). In addition, an inhibitory domain was identified in the cytoplasmic COOH-terminal region of Fas (Itoh and Nagata, 1993). Fas was originally identified during the search for an agonistic antibody against human TNF-R1 (Yonehara et al., 1989). Fas has an essential role in cell death during clonal deletion of T cells, male and female reproductive tissue regression, estrous cycle, DES syndrome and tumor regression (Mapara et al., 1993; Nagata, 1994; Nagata and Golstein, 1995; Ogasawara et al., 1995; Suzuki et al., 1996a-c, 1997a).

Fas transduces cell death signaling upon stimulation by its cognate ligand, known as the Fas ligand (Suda et al., 1993; Nagata, 1994; Nagata and Golstein, 1995). The Fas ligand is also a transmembrane protein (type-II) belonging to the tumor necrosis factor family (Fig. 1B and ref. Suda et al., 1993; Nagata, 1994; Nagata and Golstein, 1995). It has been reported that the gld/gld mutant model mouse for autoimmune disease involves the functional loss of Fas ligand (Nagata, 1994; Takahashi et al., 1994; Nagata and Golstein, 1995). In recent years, a close involvement of the Fas ligand/Fas death induction system has been suggested in liver failure, such as fulminant and viral hepatitis (Ogasawara et al., 1993; Hiramatsu et al., 1994; Kondo et al., 1997; Rodriguez et al., 1996a,b). Thus, Fas-mediated cell death has broad implications in a variety of clinical investigations.

Caspase is the essential mediator for cell death signaling

Among the known genes associated with cell death, caspase is the most important factor, since it plays the central role in cell death signaling. Caspase is the

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nomenclature for the interleukin-1ß converting enzyme (ICE)/CED-3 gene family and belongs to the cysteine proteinase family Alnemri et al., 1996). The CED-3 death gene was identified originally from C. elegans, and ICE was detected as the human homologue demonstrating high similarity to CED-3 (Yuan et al., 1993). Since overexpression of CED-3 or ICE induced cell death in mammalian cells (Miura et al., 1993), both the ICE and CED-3 genes were characterized as the cell death-associated gene. Since then, ten genes comprise the caspase family, and these are classified into three subfamilies, the caspase 1 (ICE)-, caspase 2 (ICH-1)- and caspase 3 (CPP32)-subfamilies (Fig. 2). Caspase activation has been well documented after various stimulations, such as the Fas ligand/Fas system (Enari et al., 1995, 1996; Tewari and Dixit, 1995), TNF-α/TNF-R1 system (Miura et al., 1995; Tewari and Dixit, 1995), chemical hypoxia (Jacobson and Raff, 1995; Shimizu et al., 1995, 1996a-c), chemotherapeutic agents (Suzuki and Kato, 1996; Suzuki et al., 1997b, 1998a,b) and amyloid ß protein (Suzuki, 1997). The sequential activation of the caspase 1- and caspase 3-subfamilies is known as the "ICE cascade" (Enari et al., 1996).

Within the caspase family members, the caspase 3 subfamily, including caspase 3 (CPP32/Yama/Apopain and ref. Fernandes-Alnemri et al., 1994; Nicholson et al., 1995; Tewari et al., 1995) and caspase 8 (FLICE/MACH and ref. Boldin et al., 1996; Muzio et al., 1996), plays the central role for cell death induction (Hasegawa et al., 1996). Caspase 3 is the prototype factor for the caspase 3-subfamily, and its essential role in physiological cell death and disease states during neuronal development (Kuida et al., 1996) and liver failure (Rodriguez et al., 1996a,b; Suzuki, 1998) has been well documented. Upon the stimulation of cell death, active caspase 3 processes PARP (Lazebnic et al., 1994), lamin (Lazebnik et al., 1995), gelsolin (Kothakota et al., 1997), ICAD (Enari et al., 1998) and Acinus (Sahara et al., 1999) to lead cells to die.

Caspase and cell death signaling transduced by Fas

As described above, Fas transduces cell death signaling upon stimulation by Fas ligand (Nagata, 1997). The intracellular cell death signaling is mediated by caspase activation at several steps (Fig. 3), and the caspase-mediated death signaling is initially transduced by Fas-DISC (Fas-death-inducing signaling complex and ref. Kischkel et al., 1995) formation. Upon the stimulation of Fas, FADD and caspase 8 interacts with Fas-death domain, and the caspase 8 activated as a result of Fas-DISC formation proteolyses cytoplasmic serine proteinase-cleaved caspase 3 at p17 site (Suzuki et al., 1997c; Thornbery et al., 1997). In addition, it has been known that caspase 3 activation during Fas-mediated cell death is initiated by mitochondrial death pathway; caspase 9 activated by ATP, Apaf-1 and apoptogenic cytochrome c (Liu et al., 1996; Zou et al., 1997), induce caspase 3 activation (Green and Reed, 1998) and this mitochondrial death pathway is initially triggered by caspase 8-cleaved Bid (Wang et al., 1996) after Fas-DISC formation (Li et al., 1998). Thus, caspase 3 which

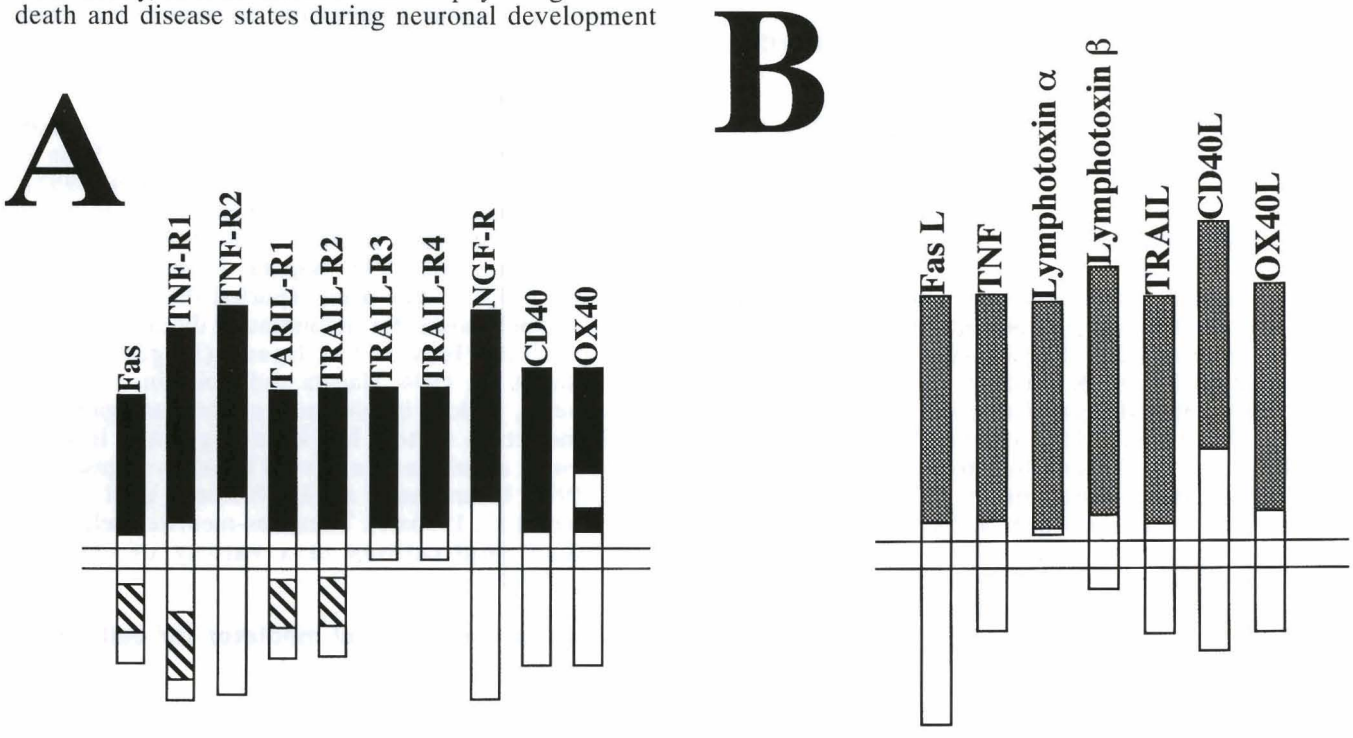

Fig. 1. Schematic model for NGF/TNF receptor family **(A)** and TNF family **(B)**. Closed box **(A)** is the extracellular cysteine-rich domain and shaded box **(A)** is the cytoplasmic "death domain". Shaded box in **B** is the high homology region within TNF family members.

was activated as a result of Fas-DISC recruitment and/or by mitochondrial death pathway, proteolyses several proteins.

IAP family: Caspase 3 inactivator

Caspase 3 activation has been well documented in Fas-mediated cell death, and its expression is observed endogenously in cells (Hasegawa et al., 1996). Caspase 3 is the most influential cell death factor and its activation by a variety of stimuli means "instantaneous death". Why do cells hold such a dangerous factor? To address our question, we sought to identify an endogenous suppressor of cell death, assuming that cells have a pathway that confers resistance against caspase activation.

Bcl-2 is a known cell death suppressor and was identified originally as a proto-oncoprotein through the study of the t(14;18) translocation present in human B cell follicular lymphomas (Tsujimoto et al., 1984). Bcl-2 localizes to the membrane of organelles and can be encountered on the nuclear membrane, endoplasmic reticulum and mitochondrial membrane (Akao et al., 1994). Bcl-2 is unique in that it inhibits apoptosis rather than promoting cell proliferation (Tsujimoto, 1989; Vaux et al., 1988). Recently, multiple members have been identified within the Bcl-2 family, some functioning, such as Bcl-xs, Bax and Bak (Oltvai et al., 1993; Chittenden et al., 1996), to drive the death mechanism and others, such as Bcl-2 and Bcl-xL (Boise et al., 1993; Tsujimoto, 1989; Vaux et al., 1988), acting against apoptotic cell death. Bcl-2 expression suppressed Fas-mediated cell death (Itoh et al., 1993). Recently, the mitochondrial channel VDAC was identified as the target molecule of Bcl-2 and Bcl-xL (Shimizu et al., 1999). By the interaction of Bcl-2 or Bcl-xL with VDAC, caspase 3 activation is prevented as a result of suppression of cytochrome c release from mitochondria (Shimizu et al., 1999). Thus, Bcl-2 and Bcl-xL indirectly suppress caspase 3 activation. In contrast, IAP family proteins are direct inactivators of caspase 3 (LaCasse et al., 1998). The ability to suppress cell death by viral gene products, such as crmA (cowpox virus and ref. Ray et al., 1992; Gagliardini et al., 1994; Miura et al., 1995; Tewari and Dixit, 1995; Tewari et al., 1995) and p35 (baculo virus and ref. Clem and Miller, 1994; Xue and Horvitz, 1995), has been reported and the proteins suppress caspase activation by direct interaction. In recent years, ILP on the X chromosome, also called XIAP, has been identified as a member of the mammalian IAP family (Duckett et al., 1996; Deveraux et al., 1997).

Human hepatoblastoma HepG2 cells do not express Bcl-2 (Suzuki et al., 1999c). However, the cells show resistance to Fas-mediated cell death in the absence of actinomycin D (Suzuki et al., 1998a). Fas-mediated cell death in HepG2 cells was also mediated by caspase 3 activation (Suzuki et al., 1998a). Interestingly, the proteolytic activity of human recombinant active caspase 3 was suppressed by the addition of cell extracts from normal untreated HepG2 cells, and we identified ILP as the direct suppressor of caspase 3 activated during Fas-mediated cell death in HepG2 cells (Suzuki et al., 1998a). However, we also demonstrated that ILP interacted only with active caspase 3, but not with procaspase 3, and co-immunoprecipitation analysis identified p21, the cell cycle suppressor (Nakanishi et al., 1995; Sherr and Roberts, 1995), as the interactive

Caspase (ICE/CED-3 cystein proteinase) family

Caspase 1 (ICE) Caspase 4 (ICErel-II/TX/ICH-2) Caspase 5 (ICErel-III)	Caspase 1 (ICE) subfamily
Caspase 3 (CPP32/Yama/Apopain) Caspase 6 (Mch2a) Caspase 7 (ICE-LAP3/Mch3a) Caspase 8 (FLICE/MACH) Caspase 10 (Mch4)	Caspase 3 (CPP32) subfamily
Caspase 2 (ICH-1) Caspase 9 (ICE-LAP6/Mch6)	Caspase 2 (ICH-1) subfamily

Fig. 2. The classification of caspase family members.

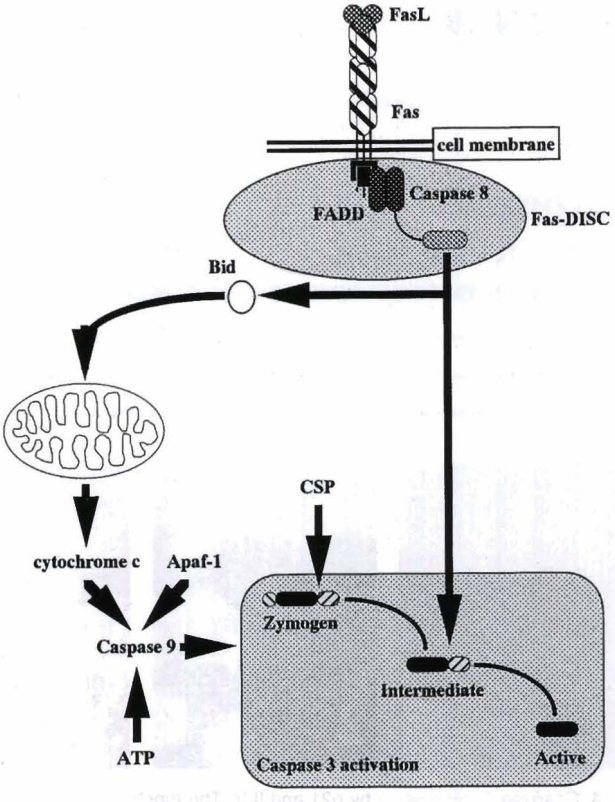

Fig. 3. The schematic model for Fas-transduced cell death signaling.

protein with procaspase 3.

Caspase 3 inactivation by p21 and ILP

As described above, we identified several caspase 3 inactivators, including p21 (for procaspase 3) and ILP (for active caspase 3). As shown in Fig. 4, the binding domain for p21 or ILP was detected in the NH2-terminus (p21) or in the active region (ILP) of caspase 3 (Suzuki et al., 1999a). Interestingly, the p21-binding domain of caspase 3 overlapped the p3 cleavage site for a serine proteinase (Suzuki et al., 1997c, 1999a). When p21 suppressed caspase 3 activation during Fas-mediated cell death, we demonstrated proteolytic activity of a p3 cleavage serine proteinase, suggesting that p21 suppresses caspase 3 activation by blocking the p3 cleavage site as a result of the interaction. On the other hand, the ILP-binding domain was found in the active region of caspase 3. Since IAP family members, including crmA, p35 and ILP, can directly suppress caspase activation (Clem and Miller, 1994; Gagliardini et al., 1994; Miura et al., 1995; Suzuki et al., 1998a, 1999a; Tewari and Dixit, 1995; Tewari et al., 1995; Xue and Horvitz, 1995), it appears that the detection of an ILP-binding domain in the active region is reasonable. Many proteinase inhibitors, including proteins and chemical compounds, have a direct interaction with the active region of their target proteinase. ILP may be a better inhibitor of caspase 3. As a first step for caspase 3 inactivation, p21 interacts with procaspase 3 to shield the p3 site cleavage, and ILP suppresses caspase 3 activation in the case of emergency. Thus, cells are protected from "sudden death" or "accidental death" by this two-step caspase 3 inactivation (Suzuki et al., 1998a, 1999a).

Protein phosphorylation and procaspase 3/p21 complex formation

p21 was originally reported as a cell cycle suppressor (Harper et al., 1993), and p21 suppresses the kinase activity of cyclin-dependent kinase and regulates p53 to arrest the cell cycle at G1 (Li et al., 1994; Sherr and Roberts, 1995). The role of p21 in cell death induction has been reported, and we suggest that this p21-associated cell death induction is due to p53 whose expression is induced by p21, since p53 induces apoptotic cell death via the Fas ligand/Fas system (Hueber et al., 1997; Bennett et al., 1998). In contrast, cell death suppression by p21 was recently reported from our (Suzuki et al., 1998a, 1999a,b, 2000c) and other

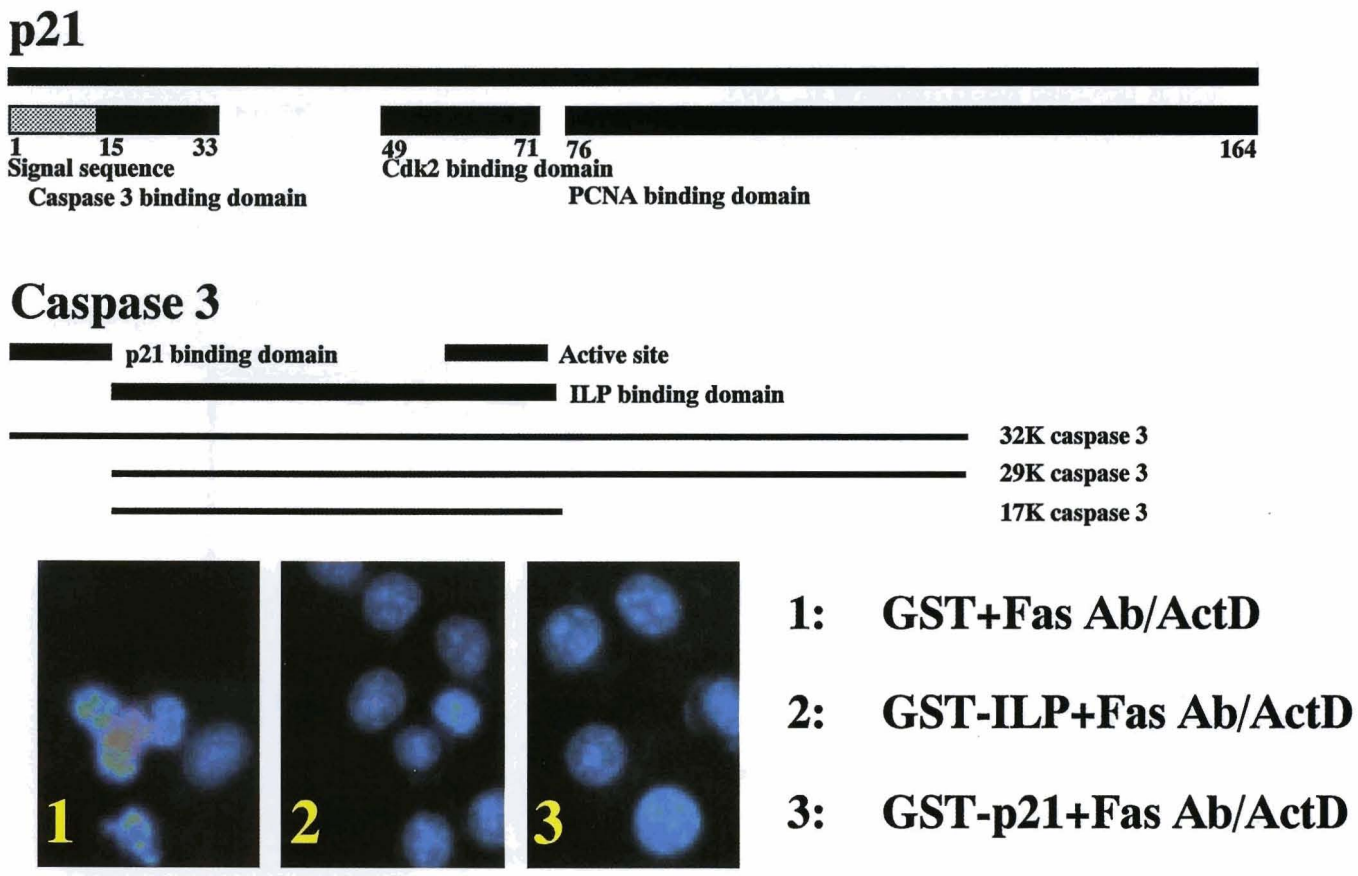

Fig. 4. Caspase 3 inactivation by p21 and ILP. The functional domains in p21 and caspase 3 are drawn schematically. The typical suppression of Fas-mediated cell death by GST-p21 and GST-ILP was observed with Hoechst 33342/PI staining.

(Hobeika et al., 1999; Xu and El-Deiry, 2000) laboratories. Furthermore, a recent study demonstrates an interesting role for p21 in cell cycle progression, in that cell cycle arrest by p53 is regulated by Arf which is the alternative reading frame product of tumor suppressor INK4a (Hirai et al., 1995; Quelle et al., 1995), but not by p21 (Weber et al., 1999). Moreover, p21 is involved in cell cycle initiation by acting as a stabilizer of cyclin and cyclin-dependent kinase (Cheng et al., 1999; Sherr and Roberts, 1999). Thus, the role of p21 in cell death and cell cycle remains unclear.

In our studies, when cells overexpressed p21, Fas-mediated cell death was completely suppressed and co-immunoprecipitation analysis revealed an endogenous interaction of procaspase 3 and p21 (Suzuki et al., 1998a). We identified the binding domain for the procaspase 3/p21 complex formation in the NH2-terminal amino acid sequences (Suzuki et al., 1999a). According to the procaspase 3/p21 complex formation, p21 masked the p3 cleavage site of procaspase 3 and protected procaspase 3 from serine proteinase-induced activation (Suzuki et al., 1999a). On the basis of these results, we questioned what factor(s) initiated the procaspase 3/p21 complex formation. Immuno-fluorescence analysis revealed p21 subcellular localization in both the nucleus and the cytoplasm (Suzuki et al., 1999b), consistent with p21 involvement in the cell cycle, cell cycle arrest via p53 regulation (Li et al., 1994), or cell cycle initiation by the stabilization of cyclin and cyclin-dependent kinase (Cheng et al., 1999). Furthermore, immunoblotting analysis of fractionated proteins revealed p21 in the mitochondrial fraction (Suzuki et al., 1999b). As described above, the mitochondrion is a recent focus in the cell death research field. Therefore, we prepared cells lacking mitochondrion and showed that mitochondria are necessary for the procaspase 3/p21 complex-initiated resistance to Fas-mediated cell death (Suzuki et al., 1999b). Human hepatoblastoma HepG2 cells show Fas-mediated cell death only in the presence of actinomycin D, whereas HepG2Δm (mitochondrion-lacking HepG2) cells showed it even in the absence of actinomycin D (Suzuki et al., 1999b). In addition, while HepG2Δm cells showed dissociation of the procaspase 3/p21 complex, they did not show any changes in the expression levels of caspase 3 or p21 (Suzuki et al., 1999b). In other words, free procaspase 3 activated smoothly without a p21 influence upon stimulation of Fas in these cells. Moreover, we showed a deprivation of intracellular ATP in HepG2Δm cells. During the Fas-mediated cell death, a drastic reduction of intracellular ATP was observed (Suzuki et al., 2000c). In recent years, protein phosphorylation as a cell survival system has been investigated, and Akt and protein kinase A (PKA) are well documented as the cell survival-associated protein phosphatases. Akt is activated by PI-3 kinase as a result of phosphorylation at Thr308 and Ser473 (Ahmed et al., 1997; Kennedy et al., 1997; Datta et al., 1999) and active Akt phosphorylates the Bcl-2 family cell death agonist Bad (Datta et al., 1997; Peso et al., 1997), Forkhead transcriptional factor (Brunet et al., 1999) and caspase 9 (Cardone et al., 1998). In addition, it has been reported that active Akt suppresses Fas-DISC (death-inducing signaling complex and ref. Kischkel et al., 1995) formation (Rohn et al., 1998). PKA was also recently documented as a cell survival factor due to induction of Bad phosphorylation (Harada et al., 1999).

We showed a reduction in intracellular ATP during Fas-mediated cell death and Fas-mediated cell death in the absence of actinomycin D by intracellular ATP deprivation (Suzuki et al., 2000c). Using specific inhibitors for protein phosphatases, we demonstrated that PKA, but not Akt, was involved with the resistance to Fas-mediated cell death and procaspase 3/p21 complex formation was initiated as a result of p21 phosphorylation by PKA (Suzuki et al., 2000c). Upon the phosphorylation of p21 by PKA, the phosphorylated p21 translocates and interacts with procaspase 3 on the outer mitochondrial membrane. Thus, procaspase 3 is inactivated by an interaction with p21 under the influence of PKA and intracellular ATP, and the resultant procaspase 3/p21 complex initiates cell survival in cells (Fig. 5).

Procaspase 3/p21 complex formation as a result of cell cycle progression by survivin

Notable characteristics of tumor cells include high growth rates and cell survival stemming from resistance

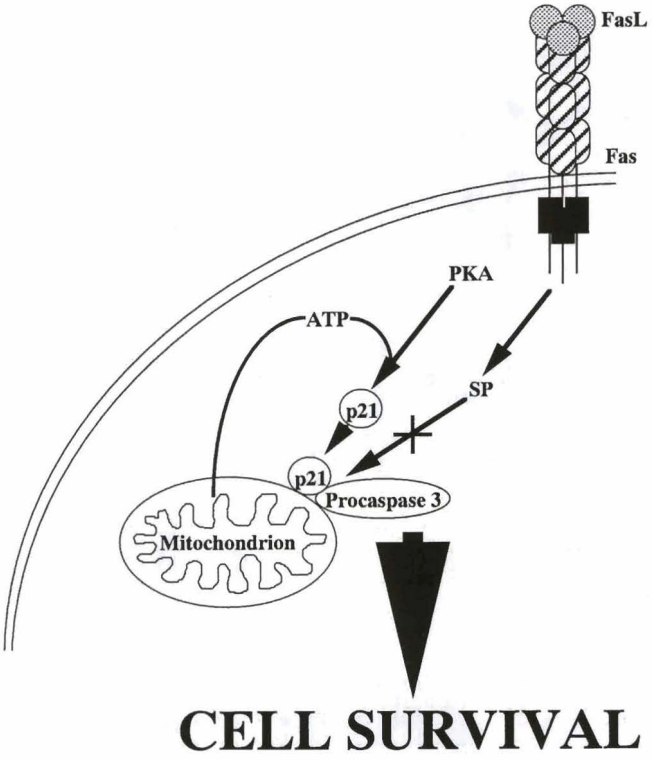

Fig. 5. The schematic model for procaspase 3/p21 complex formation.

to cell death signaling. Survivin is a recently identified IAP family protein (Ambrosini et al., 1997), and its expression is detected specifically in tumor cells (Adida et al., 1998a; Kawasaki et al., 1998; Lu et al., 1998; Swana et al., 1999) and in cells during embryonic development (Adida et al., 1998b). Compared with other IAP family members, such as crmA, p35, iap and ILP (Clem and Miller, 1994; Gagliardini et al., 1994; Miura et al., 1995; Xue and Horvitz, 1995; Duckett et al., 1996; Deveraux et al., 1997), survivin contains a single baculovirus IAP repeat, lacks a carboxyl-terminal RING finger and is unique in that its expression is observed only in proliferating cells, including tumor cells. Thus, possible involvement of survivin in cellular proliferation is suggested (Ambrosini et al., 1998). However, the molecular details are not understood.

Caspases 3 and 7 have been identified as the target molecule binding proteins in a cell-free system (Tamm et al., 1998). When cells expressing high levels of exogenous survivin were stimulated with Fas, the ability to suppress cell death with survivin was weaker than with ILP (Suzuki et al., 2000a). We also observed survivin-interactions with caspase 3 in a cell-free system. However, co-immnuprecipitation analysis revealed that endogenous survivin interacted with cyclin-dependent kinase (Cdk) 4, but not with caspase (Suzuki et al., 2000a). In addition, we demonstrated nuclear translocation of survivin following up-regulation of cell growth (Ito et al., 2000; Suzuki et al., 2000a,b). In cells overexpressing survivin, retinoblastoma (Rb) protein phosphorylation was observed as well as upregulation of Cdk2/cyclin E complex kinase activity (S phase progression marker). Moreover, we also demonstrated that p21 was released from the Cdk4/cyclin D1 complex and translocated to the mitochondrial outer membrane, and that procaspase 3/p21 complex formation on mitochondria was accelerated (Ito et al., 2000; Suzuki et al., 2000a,b). Thus, our results suggested that survivin-initiated cell death suppression was due to accelerated procaspase 3/p21 complex formation, rather than a direct interaction with caspase.

There is some evidence that survivin is involved in cell cycle progression. First, survivin translocates into the nucleus following upregulation of cell growth activity. Second, survivin overexpression and nuclear translocation induces Rb phosphorylation, up-regulation of Cdk2/cyclin E complex kinase activity and the S phase population. Finally, survivin interacts directly with Cdk4. When survivin/Cdk4 complex formation was suppressed by a functional loss of survivin, Rb phosphorylation, and up-regulation of Cdk2/cyclin E complex kinase activity and the S phase population was prevented (Suzuki et al., 2000b), suggesting that survivin/Cdk4 complex formation is an important element for cell cycle progression. It has been reported that INK4a directly interacts with Cdk4 and leads to G1

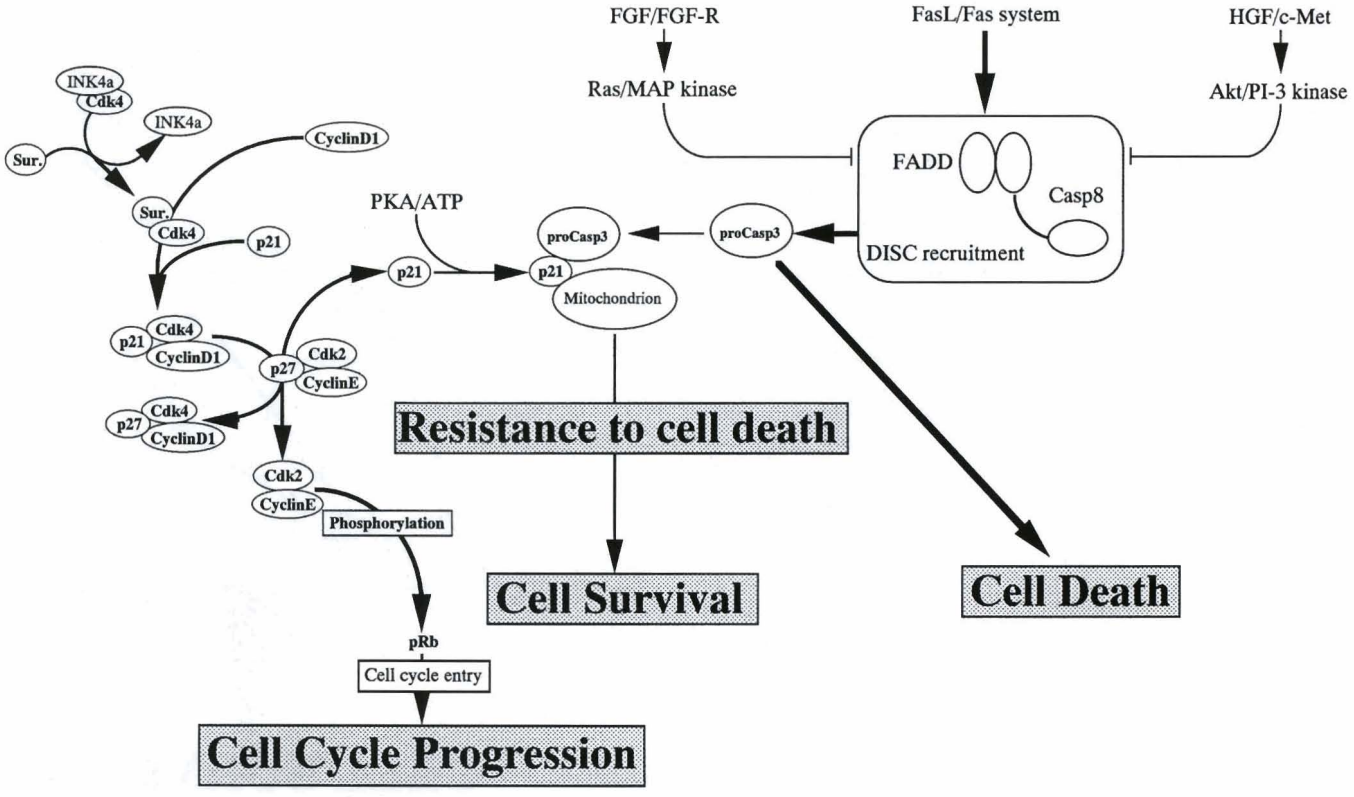

Fig. 6. The correlation of cell death, cell cycle and cell survival.

arrest (Hirai et al., 1995; Byeon et al., 1998; Parry et al., 1999; Stein et al., 1999). Interestingly, we also demonstrated that INK4a overexpression leads to G1 arrest, but survivin prevented this as a result of a competitive interaction of survivin with the Cdk4/INK4a complex (Suzuki et al., 2000b). Survivin appears to displace INK4a from its complex with Cdk4.

Resistance to Fas-mediated cell death by HGF: Akt activation and Fas-DISC recruitment

The resistance to Fas-mediated cell death has been well documented when cells were treated with some growth factors, such as basis FGF (fibroblast growth factor and ref. Kazama and Yonehara, 2000). In recent years, cell survival signaling transduced by hepatocyte growth factor (HGF) has also been well investigated. HGF initiates cell survival signaling in several cells, such as ovarian cell, renal cell and neuronal cell (Liu, 1999; Ueoka et al., 2000; Zhang et al., 2000). This cell survival signaling is mediated by Akt/PI-3 kinase pathway after c-Met interaction and we have demonstrated that human HCC (hepatocellular carcinoma cell) lines showed the cell survival mediated by HGF-activated Akt (Suzuki et al., 2000d).

It is known that HGF activates Akt/PI-3 kinase and/or Ras/MAP kinase pathway (Jones et al., 1999; Ueoka et al., 2000; Zhang et al.,, 2000). HGF suppressed HCC cell death transduced by Fas activation and the cell death signaling was reacquired by Akt inhibition, but not by MAP kinase inhibition (Suzuki et al., 2000d).

Furthermore, we demonstrated that activated Akt by HGF suppressed Fas-DISC recruitment (Suzuki et al., 2000d). As described above, it has been documented that active Akt inactivated Bad and caspase 9, and suppressed Fas-DISC recruitment (Datta et al., 1997; Peso et al., 1997; Cardone et al., 1998; Rohn et al., 1998). HCC lines do not express Bad (Suzuki et al., 1999c) and the death signaling by Fas is not mediated by caspase 9-involved mitochondrial death pathway (Suzuki et al., 1999b, 2000a). Ras/MAP kinase pathway also suppresses Fas-mediated cell death (Kazama and Yonehara, 2000) and HGF activates Ras/MAP kinase pathway (Jones et al., 1999). However, our results showed no involvement of Ras/MAP kinase pathway in the resistance to Fas-mediated cell death by HGF in HCC lines, at least.

Cell death, cell survival and cell cycle in HCC

Until recently, most studies of cell death, cell cycle and cell survival have progressed independently. There is now greater interaction between the research fields and, importantly, a greater appreciation that research of cell death, cell cycle and/or cell survival are two sides of the same coin.

Through the results of our work and those of others, molecular machinery and essential regulators for cell death, cell cycle and cell survival have been elucidated (Fig. 6). Both cell cycle progression and cell survival are essential for tumor cells, including HCC. Caspase 3, whose activation is initiated as a result of Fas-DISC recruitment, is the most essential killer factor and plays the central role during Fas-mediated cell death. Caspase 3 activation is suppressed by p21: Procaspase 3/p21 complex formation on mitochondria is under the influence of PKA and intracellular ATP and this complex formation is initiated as a result of p21 release from its complex form with Cdk4 by survivin. The survivin/Cdk4 complex up-regulates Rb phosphorylation through Cdk2/cyclin E complex activation and leads cells to S phase entry. Thus, p21 released during the survivin-induced cell cycle progression suppresses cell death and the cells acquire resistance to cell death and cell cycle progression.

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