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THE MULTILEVEL REGULATION OF CD95 SIGNALING OUTCOME

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CD95, also called Fas or APO-1, was the first death receptor (DR) identified and characterized. Studies on CD95 receptor signaling revealed the versatile principles of cell fate regulation via DR. DRs could exert both pro- and anti-apoptotic effects depending on clustering, internalization or signaling thresholds and other extracellular signals. It became clear that molecular network regulating cell death and survival is under the multilevel control. In this Review we focus on the regulation of CD95 signaling and provide brief analysis of molecular switches of its pro- and antiapoptotic functions. At least five levels of life-death cell regulation via CD95 could be tracked: extracellular, membrane, DISC, mitochondrial, and miRNA. The cellular outcome of signaling via DRs depends on other extracellular signals and availability of different intracellular components of signal transduction pathways. This article is part of a Special Issue entitled "Apoptosis: Four Decades Later".
***Key Words*: CD95, apoptosis, death receptors, signaling.**

Starting from the concept of natural cell death proposed in 1842 by Carl Vogt and the first morphological documentation of programmed cell death provided in 1885 by Walther Flemming till the introduction of the term of apoptosis in 1972 our knowledge of this process was restrained to descriptive observations [1].

The key milestones on the way to understanding the molecular network that regulate cellular life and death decisions were the discovery of Bcl-2 family proteins, revealing the role of p53 in apoptosis and characterization of CD95-mediated signaling pathways. Actually, CD95, also called Fas or APO-1, was the first death receptor (DR) identified and characterized.

There are multiple examples of novel antigen identification using monoclonal antibodies (mAbs). Hybridoma technology of mAbs generation includes an element of discovery, especially when whole cells or cellular components are used for immunization. We have many examples of novel antigen identification independently in different laboratories that used this approach. One of such examples is CD95 discovery.

Apoptosis got special attention from oncologists, since revolving tumor cell fate from proliferation to apoptosis seemed to be an ideal scenario for antitumor therapy. In fact the paper of Kerr *et al.* [2] with definition of term apoptosis was published in the British Journal of Cancer. The general idea of 1989 year publication of Peter Kramer laboratory at German Cancer Research Center in Heidelberg was to characterize cell surface molecules involved in control of growth of malignant lymphocytes [3]. Among mAbs against the human B lymphoblast cell line SKW6.4 they identified one mAb anti-APO-1 that blocks growth and induces apoptosis of target cells *in vitro*. Moreover, anti-APO-1 was able to induce *in vivo* apoptosis of Burkitt lymphoma cell line BJAB xenotransplant [3]. In parallel, Shin Yonehara *et al.* [4] published a paper on a new Fas antigen (FS-7-associated surface antigen) involved in induction of cell death. They accidentally found mAb that had a cytopathic activity against human FS-7 cells [4]. It should be noted that practically at the same time the mAb IPO-4 against lymphocyte activation antigen was developed at the Institute of Problems of Oncology in Kyiv (Ukraine) [5–7]. In this case a mouse was immunized with B-lymphoblastoid cell line RPMI-1788 [5]. During Fifth International Workshop on White Cell Differentiation Antigens it was shown that mAbs Apo-1, anti-Fas, IPO-4 and also 7C11 recognize closely related epitopes on antigen that in 1993 received an international nomenclature CD95 [8]. Expression cloning of CD95 cDNA [9] showed that this antigen belongs to TNF-receptor superfamily (TNFRSF) that according to HUGO Gene Nomenclature Committee up to date embraces 26 genes (http://www.genenames.org/genefamily/tnfrsf.php).

The members of TNFRSF are type-I transmembrane proteins with N-terminal extracellular ligand-binding part, single transmembrane region and C-terminal cytoplasmic tail [9, 10]. The hallmark of TNFRSF proteins is up to six cysteine-rich domains (CRDs) that are typically defined by six highly conserved cysteines, which formed three intrachain disulfide bonds [10]. CD95 possess three CRDs, with ligand contacts occur mainly in the second and third CRDs [10]. The characteristic feature of CD95 and five more members of TNFRSF (TNFR1/CD120a, DR3, DR4/TRAILR1, DR5/TRAILR2 and DR6) is the presence of moderately well conserved region of about 80 residues called the "death domain" (DD) in cytoplasmic part of the receptor [11]. In addition to the full length mRNA, at least

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Abbreviations used: c-FLIP – cellular FLICE/caspase-8-like inhibitory protein; CRD – cysteine-rich domains; DD – death domain; DED – death effector domain; DISC – death-inducing signaling complex; DR – death receptor; FADD – Fas-associated death domain; IAP – inhibitor of apoptosis; MAPK – mitogen activated protein kinases; NF-kB – nuclear factor kB; PLAD – preligand assembly domain; RIP1 – receptor-interacting protein 1; SPOTS – signaling protein oligomerization transduction structures; TNF – tumor necrosis factor; TNFRSF – TNF-receptor superfamily.

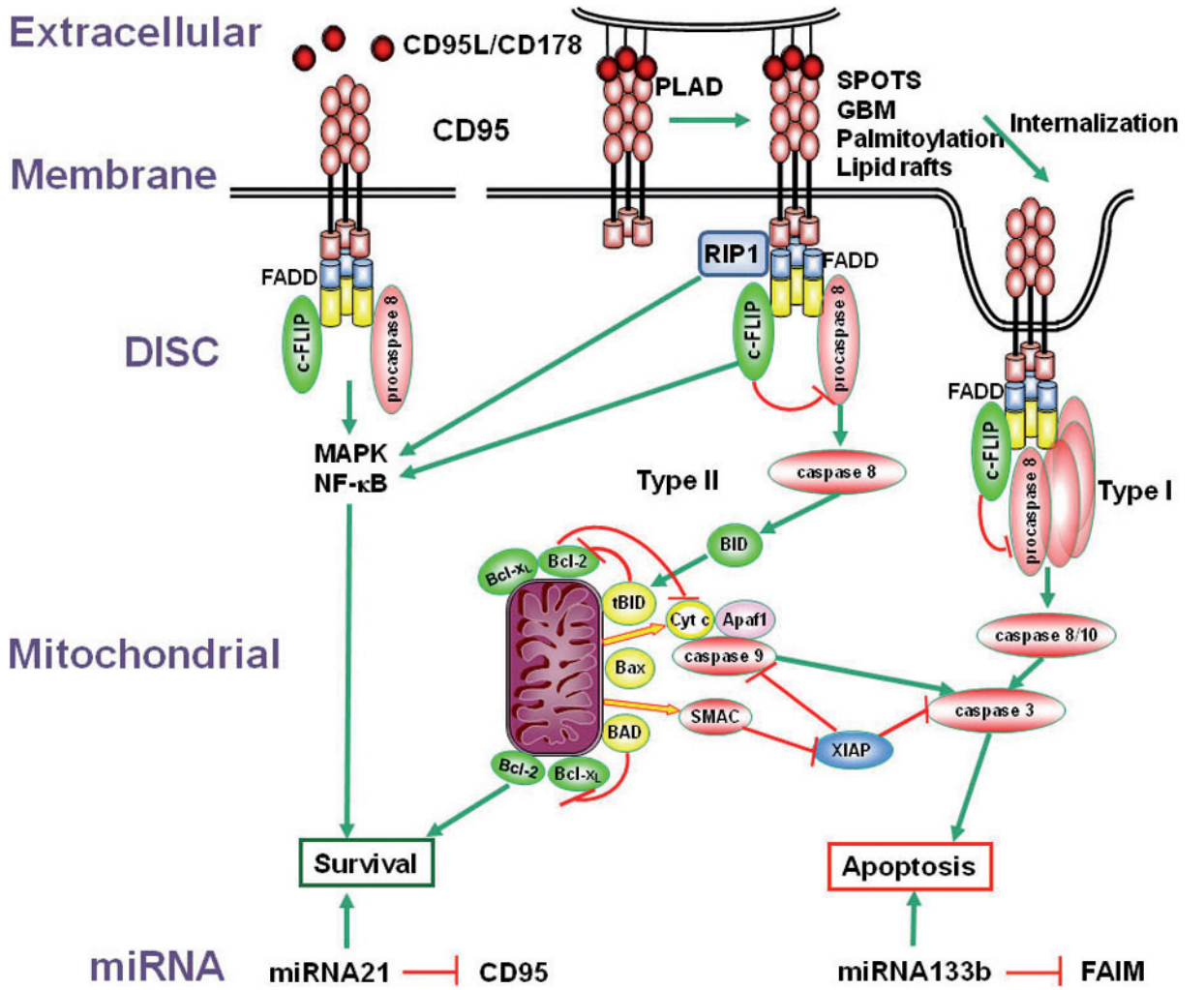

Figure. Schematic representation of multilevel regulation of CD95 signaling outcome. CD95-mediated signaling could result in cell survival or apoptosis depending on fine balance of molecular switches in cells, and activation or inhibition of a range of signaling pathways upon receptor ligation. The initial event for CD95-mediated signaling is trimerization by specific ligand CD95L/CD178. The membrane-bound CD95L is essential for the cytotoxic activity, while soluble CD95L promote non-apoptotic pathways. The initial concentration of CD95L also contributes to the cellular decisions for CD95-induced life and death. After ligation CD95 receptors are internalized through clathrin-mediated endocytosis and delivered to the early endosomal compartment, where most of the Death-Inducing-Signaling-Complex (DISC) formation occurs. Procaspase-8, c-FLIP proteins and their cleavage products play a prominent role in the regulation of CD95-mediated pro-apoptotic as well as survival signaling. The ratio of c-FLIP to procaspase-8 at the DISC plays the central role in the regulation of MAPK induction by defining the amount of active caspase-8/10 generated at the DISC. Active caspases 8 and 10 initiate a signaling cascade that results in activation of the effector caspases (caspases-3, -6, and -7) either by directly processing the effector caspases themselves (type I cells) or by engaging the mitochondrial death pathway mediated by the cleavage of Bid protein (type II cells). XIAP (X-linked inhibitor of apoptosis) could directly bind to caspase-3, -7 and -9 and by this block their function. The release of proapoptotic proteins from the mitochondria such as cytochrome *c* (Cyt *c*) and SMAC (XIAP inhibitor) promotes effector caspase activation and apoptosis. CD95 signaling outcome was shown to be regulated by miRNAs. miRNA-21 indirectly targets the CD95 expression in cancer cells, and antiapoptotic cytosolic protein Fas Apoptosis Inhibitory Molecule (FAIM) is an immediate miRNA-133b target

five alternatively spliced isoforms encoding soluble CD95 lacking transmembrane region have been identified [12, 13].

CD95 was first identified on cells of hematopoietic system [3, 5]. While expressed on minority of resting T, B, NK cells, and monocytes, this antigen is strongly upregulated after cell activation. Moreover, it is widely expressed on cell lines of T, B, NK and myeloid origin, and also on some primary leukemia and lymphoma cells [7, 14]. Since it was shown that CD95 is also expressed in liver, pancreas, heart, kidneys and brain, the initial idea to use anti-CD95 antibodies as possible anticancer agent was not followed up. Nevertheless, studies of CD95-mediated signal transduction pathways and their modulation revealed the common multilevel regulation of "death receptors" functions that determine cell fate.

Crosslinking of CD95 with its natural ligand CD95L/CD178 or with agonistic antibodies may have a different cellular outcome. CD95 ligation exerts the cytotoxic effect by inducing apoptosis and even necrosis [9, 15, 16]. Conversely, CD95 was shown to promote cell proliferation, migration, differentiation, liver and peripheral nerve regeneration, etc. [17–19]. CD95 has been initially cloned as a DR and is classified as a tumor suppressor gene. However, it also promotes early carcinogenesis [20–22]. CD95 expression was found on neoplastic cells in tumors of different histogenesis [23], but many of these cells lack sensitivity to CD95-

mediated cytotoxic signal [24]. Despite the reports on loss-of-function mutations in *CD95* gene, they cannot account for all cases of resistance to CD95-mediated apoptosis in tumors [22, 25]. Detailed dissection of CD95-mediated signal transduction pathways revealed the multilevel regulation of this dual-function receptor signaling in normal and malignant cells. Different levels of CD95 signaling regulation and outcome are presented in schematic form (Figure).

EXTRACELLULAR LEVEL

The initial event for CD95-mediated signaling is trimerization by specific ligand [26]. In the meanwhile, the ligand-independent preassembled receptor complex formation through highly specific preligand assembly domain (PLAD) in the membrane-distal cystein domain was also reported [27, 28]. CD95L/CD178 is a preassociated homotrimer of type II membrane protein that can be released in soluble form after cleavage between Ser-126 and Leu-127 by metalloproteinases [29, 30]. Ligand contacts with CD95 occur mainly in the second and third CRDs [10]. It has been demonstrated that membrane-bound CD95L is essential for the cytotoxic activity, while soluble CD95L promote non-apoptotic pathways [31]. Moreover, the initial concentration of CD95L is one of the most important factors contributing to the cellular decisions for CD95-induced life and death [32]. Many tumor cells acquired protective mechanisms to avoid elimination by CD95–CD95L system, one of which is downregulation of *CD95* expression or *CD95* mutation [33]. These could be regarded as an extracellular level of CD95 signaling regulation.

MEMBRANE LEVEL

After ligation of CD95 by CD95L or agonistic antibodies, receptors are internalized through clathrin-mediated endocytosis and delivered to the early endosomal compartment, where most of the Death-Inducing-Signaling-Complex (DISC) formation occurs [11]. Both internalization of the CD95 receptor and localization to the endosomal compartment are required steps for efficient DISC assembly, amplification of the signal, and execution of apoptosis [34]. The early detectable events after CD95 ligation are formation of Signaling Protein Oligomerization Transduction Structures (SPOTS) [35] and SDS-stable aggregates of receptors [36]. These processes involve palmitoylation of the membrane proximal Cys-199 in the CD95 cytoplasmic region [36, 37]. The post-translational modification of CD95 by palmitoylation is the targeting signal for receptor localization to lipid rafts. Palmitoylation is required for the CD95 redistribution to actin and ezrin-mediated cytoskeleton associations, which are necessary for the efficient DISC assembly and receptor internalization [37].

The conserved extracellular glycosphingolipid-binding motif (GBM) of CD95 was identified as one of the regulatory elements in the selection of its internalization route. This motif is required for clathrin-mediated CD95 internalization, which allows the transduction of apoptotic signal. The loss of function of GBM switches the activated CD95 to an alternative internalization ezrin-dependent route and promotes its non-death functions [38].

Cholesterol- and sphingolipid-rich membrane domains (lipid rafts) were shown to be very important for effective induction of DR-mediated apoptosis. Recently, the membrane level of DR-signaling regulation appeared in the focus of anti-cancer drug development. An increasing number of antitumor drugs have been found to induce apoptosis through recruitment of CD95 into membrane rafts, and some of these compounds accumulated in raft membrane domains. Edelfosine, which is their alkyl-lysophospholipid analogue, was the first antitumor drug reported to induce apoptosis in cancer cells through co-clustering of lipid rafts and CD95 DR [39]. It was shown that pharmacologically modulated membrane microdomains can act as scaffolds for CD95 death signaling [39]. Therefore, the development of drugs, which modulate CD95 accumulation in lipid rafts, have become a promising task for cancer chemotherapy.

DISC LEVEL

DISC signalosome serves as third level of CD95-mediated pathways regulation with multiple molecular switches. Indeed DISC formation is necessary not only for induction of apoptosis, but also for initiation of survival pathways. Interactions between CD95 molecules stabilize the open conformation and facilitate the recruitment of the adaptor molecule Fas-Associated-Death-Domain (FADD/MORT-1) [40]. FADD binds to the intracellular DD of CD95 by homotypic interactions via its own DD [40]. FADD contains another protein–protein interaction module of about 80 residues in the N-terminal domain — death effector domain (DED) [41]. Interaction of FADD protein with the CD95 or other DD-containing receptors unmasks the DED, which allows it to recruit procaspases-8a/b and -10 [42], and also the cellular FLICE/caspase-8-like inhibitory protein (c-FLIP) [41, 43, 44]. It became clear that at the DISC two DED-containing proteins (procaspase-8 and c-FLIP) play the role of main molecular switches for the apoptotic versus non-apoptotic signaling. Pro-apoptotic as well as survival functions were reported for both procaspase-8 and c-FLIP. Procaspase-8, c-FLIP proteins and their cleavage products were demonstrated to play a prominent role in the regulation of CD95-mediated pro-apoptotic as well as survival signaling (NF-κB, MAPK, etc.) [44–46].

There are evidences for the assembling of secondary cytosolic DISC — complex II after CD95 ligation. This complex does not contain the CD95 receptor and does not depend on receptor internalization [47].

DISC serves as a platform for the oligomerization of procaspase-8 and allows two procaspase-8 homodimers to be in the close proximity leading to the initial activation of procaspase-8 followed by its subsequent cleavage. During this process procaspase-8 undergoes a substrate specificity switch [48]. Procaspase-8 at the

DISC has two substrates: itself and c-FLIP. At the same time fully activated caspase-8 can cleave the apoptotic substrates, such as effector caspases and Bid [48]. It has been recently reported using transgenic mice that the perturbation of the caspase-8 cleavage site abrogates its pro-apoptotic function without influencing its non-apoptotic function [49].

Posttranslational modifications play an important role in regulation of procaspase-8 activity. Phosphorylation of procaspase-8 at the catalytic subunits has been shown to inhibit its pro-apoptotic activity, likely by interfering with processing of procaspase-8 [50, 51]. However, polyubiquitination of procaspase-8 at the DISC stabilize the active caspase-8 heterotetramer and plays the pro-apoptotic role [52].

Regulation of caspase-8 pro-apoptotic function may also be exerted on the level of gene transcription. For example, the expression of caspase-8 is downregulated in tumor cells of neuroblastomas due to methylation of its promoter region. In this case the transcription of the gene is downregulated and cells express reduced levels of the caspase-8 protein. As a result the activation of apoptotic pathways that require caspase-8 is blocked [33].

By cleavage of c-FLIP procaspase-8 creates another molecular switch at the DISC. Procaspase-8 activity is required for generation of c-FLIP N-terminal cleavage products p43-FLIP and p22-FLIP. Three c-FLIP isoforms, long (c-$FLIP_L$), short (c-$FLIP_S$), and Raji (c-$FLIP_R$), and two procaspase-8 generated cleavage products (p43-FLIP and p22-FLIP) have been characterized so far [44]. All c-FLIP proteins are able to block procaspase-8 activation at DISC, thereby exert the survival function, especially via activation of NF-κB pathway [44, 46]. The exception is the c-$FLIP_L$ isoform, which may also induce procaspase-8 activation [44, 46]. Both c-$FLIP_L$ downregulation and overexpression were demonstrated to block procaspase-8 processing. The ratio of c-FLIP to procaspase-8 at the DISC plays the central role in the regulation of MAPK induction by defining the amount of active caspase-8 generated at the DISC [46].

Procaspase-8 activity has been suggested to be indispensible for MAPK activation, and also for the initiation of other non-apoptotic pathways [48]. In tumor cells resistant to CD95-induced apoptosis (e.g., MCF7-Fas-Bcl-xL) the triggering of CD95 was reported to result in activation of survival pathways involving NF-κB, MAPK (ERK1/2, p38, c-Jun N-terminal kinase), and Akt [33]. p43-FLIP, which is generated by procaspase-8 at the DISC, interacts with components of the TNFR-mediated NF-κB activation pathway, TNFR-associated factor 1 (TRAF1), TRAF2 and RIP, which together promote NF-κB activation. In addition, p43-FLIP generated at the DISC might directly activate the IKK complex leading to NF-κB induction [44].

Upon DISC formation, two CD95 apoptotic signaling pathways were revealed that correspond to two types of cells [53]. Type I cells are characterized by high levels of CD95 DISC formation and increased amounts of active caspase-8. Activated caspase-8 directly leads to the activation of downstream effector caspases-3 and -7. Lower levels of DISC formation and as a result lower levels of active caspase-8 were found in type II cells. For these cells an additional amplification loop is needed for effective apoptotic signaling that involves the cleavage by caspase-8 of the Bcl-2 family protein Bid to generate truncated (t) Bid and subsequent tBid-mediated release of cytochrome *c* from mitochondria. Upon release of cytochrome *c* from mitochondria, it binds to the cytosolic adaptor protein Apaf1 (apoptotic protease activating factor 1) to facilitate the formation of apoptosome followed by recruitment and activation of procaspase-9, which in turn cleaves downstream effector caspases [54]. It should be noticed, that type I cells are characterized by rapid CD95 internalization while it was delayed in type II cells [55]. The definition of type I and II cells and also assembly of complex II could be applied to other DRs.

Another molecular switch of CD95-mediated signaling on the level of DISC may be provided by RIP1, DD-containing receptor-interacting protein 1 [56, 57]. This serine-threonine kinase binds to all DRs as well as to DD-containing adaptors. RIP1 posttranslational modifications like phosphorylation and ubiquitination regulate this kinase involvement in TNFR1 signaling cascades that determine cell fate. RIP1 phosphorylation is necessary for ERK activation, and polyubiquitination is critical for NF-κB activation, while deubiquitination of RIP1 results in enhanced formation of RIP1/FADD/procaspase 8 complexes, followed by caspase 8 activation and apoptosis [56]. The RIP1 kinase domain is important for survival signals such as those mediated by ERK, while RIP1 DD is required for TNF-induced cell death [56–58]. The RIP1 involvement in CD95-mediated signaling is less clarified, however it was shown that RIP1 DD is critical for RIP1 and CD95 association to mediate anoikis and RIP1 shuttles between CD95-mediated cell death and integrin/focal adhesion kinase-mediated survival pathways [58]. Also RIP1 contributes to CD95-mediated NF-κB activation [56]. Moreover, RIP1 plays a central role in CD95-mediated necrosis in the absence of caspase-8 [15, 56].

Mutations in the *CD95* gene are frequently found in human cancers and the vast majority of the mutations were reported for DD coding region (exon 8 or the part of exon 9) [33]. Although a large number of tumors have congenital or somatic mutations in the CD95 DD, the loss of heterozygocity was found quite rarely. Thus, maintaining one wild-type receptor may confer an oncogenic advantage [33]. This advantage is likely due to the fact that CD95-induced apoptosis requires two functional CD95 alleles in order to ensure efficient DISC formation [59]. In contrast, the signaling threshold to activate NF-κB is significantly lower and can be achieved even in the presence of only one functional CD95 allele [59].

MITOCHONDRIAL LEVEL

Regulation of CD95-induced apoptosis on mitochondrial level would apply for Type II cells, which require activation loop for signal amplification. Apoptosis could be blocked at the level of mitochondria by the expression of the anti-apoptotic members of the Bcl-2 family proteins (Bcl-2, Bcl-x_L). Briefly, these proteins as well as pro-apoptotic Bcl-2 family proteins (Bax, Bak, Bcl-x_S, etc.) have an opposite effect on mitochondrial outer membrane permeabilization by regulating the inner mitochondrial permeability transition pore [60]. Anti-apoptotic Bcl-2 family proteins prevent the formation of the mitochondrial apoptosis-induced channel required for cytochrome *c* release from mitochondria and apoptosome formation [61].

Mitochondria-dependent level of apoptosis regulation also involves the family of inhibitors of apoptosis (IAP) [62, 63]. These are functionally and structurally related proteins, which could bind effector caspases and serve as endogenous IAP. A common feature of all IAPs is the presence of a Baculovirus IAP Repeat (BIR) 70 amino acid domain in one to three copies. The human IAP family consists of 8 members, and IAP homologs were identified in numerous organisms [63, 64]. The most described family member is XIAP (X-linked inhibitor of apoptosis). XIAP directly bind to caspase-3, caspase-7 and caspase-9 and by this block their function. Activity of XIAP is blocked by binding to SMAC (second mitochondria-derived activator of caspase) and HTRA2 (high temperature requirement protein A2) proteins released from mitochondria upon induction of pro-apoptic stimuli. Other IAP proteins are not potent inhibitors of caspases. Instead, they bind to SMAC with high affinities and prevent it from blocking XIAP-mediated inhibition [63, 64]. Several IAP proteins are crucial regulators of NF-κB, particularly c-IAP1 and c-IAP2 [64].

Members of the IAP protein family are frequently deregulated in cancer and contribute to chemoresistance and treatment failure [65]. Genetic evidence indicates that *cIAP1* and *cIAP2* are proto-oncogenes that are affected by chromosomal aberrations in cancers including esophageal carcinoma, hepatocellular carcinoma, cervical cancer, liver cancer, medulloblastoma, glioblastoma, non-small-cell lung cancer, small cell lung cancer and pancreatic cancer [64]. High level of XIAP expression significantly correlated with poor clinical outcome for patients with diffuse large B cell lymphoma, clear-cell renal cell carcinoma, bladder cancer, hepatocellular carcinoma, breast carcinoma, cervical squamous cell carcinoma [64]. Taking into account the important role of IAPs for tumor cell survival, these proteins became an attractive targets for therapeutic intervention in human malignancies [64, 65].

MicroRNA LEVEL

MicroRNAs (miRNAs), a class of endogenous noncoding highly conserved RNAs, are involved in regulation of gene activity at the post-transcriptional level. They negatively regulate translation or directly cleave the targeted mRNA. MiRNAs are highly expressed ubiquitous regulators, which were shown to be engaged (implicated) in regulation of cell differentiation, proliferation and programmed death. It was shown that several miRNAs are able to control pro- or antiapoptotic processes and, importantly, tumorigenesis [66, 67]. A miRNA gene array analysis revealed the differential miRNAs expression between Type I and Type II cells: the members of let-7 family were preferentially expressed in Type II cells [66]. Also, miRNA-21 indirectly targets the CD95 expression in cancer cells [67]. And recently the miRNA direct target of CD95-mediated pathway was found. It was shown that antiapoptotic cytosolic protein Fas apoptosis inhibitory molecule (FAIM) is an immediate miRNA-133b target [68]. The molecular mechanisms of FAIM action are not understood in detail [69]. However, it is clear that it is working upstream from PARP, caspase-8 and caspase-3 [70]. It was suggested that CD95 ligation induces the release of splice variant FAIM-L from CD95, allows the binding of FADD and caspase-8, and leads to apoptosis activation [71]. Post-transcriptional level of apoptotic cell-signaling has not been extensively explored nevertheless increasing evidences are indicating that miRNAs may serve as a controller of receptor mediated apoptosis in normal and tumor cells.

We learned the important lessons from studies on CD95 receptor signaling that revealed the versatile principles of cell fate regulation via DRs. First, the DRs are the dual-function receptors. Second, molecular network that regulates cell death and survival is under the multilevel control. Third, at least five levels of life-death cell regulation can be tracked: extracellular, membrane, DISC, mitochondrial, and miRNA. Fourth, the signal transduction pathways, which regulate cell life and death decisions, have illusory boundaries. Fifth, the cellular outcome of signaling via DRs depends on other extracellular signals and availability of different intracellular components of signal-transduction pathways.

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