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REVIEW

Cytotoxic Signal Transmission Pathways via TNF Family Receptors

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Abstract—Studies indicating an important role of the TNF-receptor family in control of cell proliferation, differentiation, and death have drastically increased in number in recent years. The main function of many members of this family is cell death triggering, and this is apparently the only function for some of them. Studies on the molecular mechanisms of cell death activated by members of the TNF-receptor family revealed and identified proteins directly or indirectly associated with TNF receptors. Pathways of cytotoxic signal transduction by some members of the TNF-Rs family based on currently proven protein–protein interactions and the role of distinct proteins in these processes are summarized in this review.

Key words: apoptosis, TNF-receptor family, tumor necrosis factor, TNF-RI, TNF-RII, Fas-ligand, Fas-antigen, caspases, Bcl-2 protein family

Two cell death types, apoptosis and necrosis, can be identified by analysis of morphological and biochemical changes in dying cells [1]. Necrotic death is supposed to be a passive cell response to exposures damaging primarily the plasmatic membrane [1, 2]. Removal of necrotic cells in the organism is accompanied by the activation of inflammatory reactions [3]. Apoptosis is considered to be an active suicidal process directed to the prevention of DNA-dependent synthetic reactions and obligate for normal processes of embryo- and morphogenesis, homeostasis, and functioning of various differentiated cells [2, 4]. Apoptotic cell death occurs without the induction of inflammation [5, 6]. The interest in this cell death type increased in recent years in connection with the establishment of the important role of this phenomenon in many pathological processes, such as oncological, infectious, neurodegenerative, and other disorders [7-9]. The great majority of pathological situations proved to be associated with changes in expression and/or functioning of distinct TNF-receptor family members and TNF-ligands including so-called "death receptors" and their ligands and associated molecules [10-12].

Normally, receptors and ligands belonging to these families are involved in differentiation of various tissues, particularly lymphoid, osseous, and nervous tissues, and also in regulation and operation of immune and inflam-

Abbreviations: AP1) Activator Protein-1 transcription factor; Apaf**) Apoptotic protease-activating factor; ASK1) Apoptosis Signal-regulating Kinase 1 (JNK kinase kinase); aPKC) atypical Protein Kinase C; BTK) Bruton's Tyrosine Kinase; CARD) Caspase Activation and Recruitment Domain; c-FLIP) cellular FLICE-Inhibitory Protein; c-IAP**) cellular Inhibitor of Apoptosis Proteins; CRADD) Caspase and RIP Adapter with Death Domain; DAXX) Death Associated protein; DFF40) 40-kD DNA Fragmentation Factor; DFF45) 45-kD inhibitor of DNA Fragmentation Factor; ERK) Extracellular Regulated Kinase; FADD) Fas-Associated Death Domain protein; GCKR) Germinal Center Kinase-Related kinase; ICAM**) Intercellular Adhesion Molecule; I-κB) Inhibitor of NF-kappa B; IKK**) I kappa B Kinases; JNK) c-Jun N-terminal Kinase; MAPK**) Mitogen-Activated Protein Kinases; MEK**) MAPK/ERK Kinases; MEKK**) MAPK/ERK Kinase Kinases; NF-κB) Nuclear Factor kappa B; NIK) Nuclear factor kappa B-Inducing Kinase; PED/PEA15) Phosphoprotein Enriched in Diabetes/15-kD Phosphoprotein Enriched in Astrocytes; PML) Promyelocytic Leukemia Protein; RING) Really Interesting New Gene; RIP**) Receptor Interacting Proteins; SAPKs) Stress-Activated Protein Kinases; SODD) Silencer Of Death Domains; TANK) TRAF-Associated NF-kappa B activator; TNF) Tumor Necrosis Factor; TNF-Rs) Tumor Necrosis Factor Receptors; TRADD) TNF-R1-Associated Death Domain protein; TRAF**) TNF Receptor-Associated Factor; VDAC) Voltage Dependent Anion Channel; **, numeral or letters denote protein isoforms.

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mation reactions [13-16]. Cell death initiation (triggering) is one of the main functions of many members belonging to these families. The studies of the last decade have extended substantially a conceptualization of molecular apoptotic mechanisms induced by the interaction between TNF-receptors and their ligands. One of the possible reaction consequences starting from the ligand binding to the receptor on the cellular membrane and ending with internucleosomal chromatin degradation has been followed for one of the family member, Fas-receptor [17, 18]. At the same time, an inundation of experimental evidence obtained on various cell death models induced by TNF family members not only has answered earlier questions concerning identification of distinct molecules involved in apoptosis, but also brought forward new problems. The main task of the present review is to attempt to reply to questions of whether several independent (autonomous) ways exist for cytotoxic signal transduction or, alternatively, that trigger mechanisms mediated by various TNF-receptor family members activate a universal chain of events resulting in cell death, and what is the role and place of known proteins involved in apoptosis regulation (p53, Bcl-2 family, and other) in the sequence carried out with cell death.

TNF-RECEPTOR AND TNF-LIGAND FAMILY

The TNF-receptor family (TNF-Rs) includes at present TNF-RI (p55R/CD120a), TNF-RII (p75R/CD120b), TNF-RIII (TNF-Rrp/LTβ-R), Fas (Apo-1/CD95), NGF-RI (p75NGF-R/p75NTR), CD27, CD30, CD40, OX40, 4-1BB, DR3 (Apo-3/WSL-1/LARD/TRAMP), DR4 (TRAIL-R1), DR5 (KILLER/TRAIL-R2), DR6, RANK, OPG (OCIF/ FDCR-1/TR1), GITR (AITR), and TNFRSF19 [10, 12]. Besides these human proteins, the receptor CAR1 revealed in chicken cells [19], and also herpes virus-binding protein HVEM (TR2) [20] and poxvirus proteins encoded by *crmB*, *crmC*, and *crmD* genes and their homologs [21, 22] are included in this family. A special group of proteins belonging to this family consists of so-called "decoy" receptors: DcR1 (LIT/TRID/TRAIL-R3), DcR2 (TRAIL-R4), and DcR3 (TR6), which are truncated variants of TNF-Rs family molecules [12].

The members of TNF-Rs family are structurally similar membrane proteins of type I. They consist of extracellular (N-terminal), transmembrane, and intracellular (C-terminal) parts. Ligand-binding domains of the extra cellular part are characterized by the presence of 2 to 6 repeats of about 40 amino acids enriched in Cys (CRDs). The body of biochemical and crystallographic data first obtained in the study of TNF-RI shows that cysteines on neighboring receptor molecules can form intermolecular disulfide bonds stabilizing activated receptors in the form of dimers or trimers [23]. CRD2 and CRD3 are found to be involved in the activated TNF-R1, TNF-R2, and Fas trimer formation [23, 24], whereas CRD1 provides ligand-independent receptor oligomerization on the cell surface [25, 26]. A homologous sequence named the death domain was found in the cytoplasmic part of some family members. This domain was first identified by Tartaglia and coauthors, who showed that the 80-amino acid residue sequence on the C-end of the intracellular receptor part is necessary and sufficient for triggering cytotoxic signal transduction [27]. The experiments with TNF-RI and analysis of other TNF-Rs family members containing the "death domain" have established that the functional properties of this domain are independent of its position relative to the extracellular part of the receptor.

Besides transmembrane forms, soluble receptor forms are known for most of the family members. Soluble molecules are formed due to the splitting off of the extracellular part of the membrane-bound receptors (TNF-RI, TNF-RII, CD27, CD30, CD40) or alternative splicing leading to the elimination of the transmembrane part (Fas, 4-1BB). DcR3 (TR6), one of the "decoy"-receptors, is soluble, because its corresponding gene is lacking the sequence encoding a transmembrane region [28, 29].

Ligands recognized by the TNF-Rs receptor family members form a family of TNF-ligands: TNF, LT-α, LT-β, FasL (CD95L), CD27L (CD70), CD30L (CD153), CD40L (CD154), OX40L (gp34), 4-1BBL, Apo-3L (TWEAK), Apo-2L (TRAIL), RANKL (ODF/TRANCE/OPGL), GITRL (AITRL/TL6), LIGHT (HVEM-L), APRIL, BAFF (THANK/TALL), VEGI, and TNFSF20. Nerve growth factor, NGF, interacting with NGF-RI is structurally distinct from other TNF-ligands and is not included in this family. In contrast to corresponding receptors, ligands belonging to this family are type II proteins containing an extracellular (C-terminal), a transmembrane, and a short intracellular (N-terminal) region. All the active TNF-ligands are supposed to exist in homotrimer forms, so inducing a stabilization of the corresponding receptor oligomers [23, 30, 31]. LT-α and LT-β are the only ligands known at present that are able to form heterotrimers (LT-α_3, LT-α_1/β_2, and LT-α_2/β_1) [32]. The ligands belonging to this family are synthesized as membrane-bound proteins; however, soluble forms presented by the extracellular parts of their molecules have been also shown to exist for most of them. A specific proteolysis of the transmembrane forms with the formation of secreted TNF-ligands is catalyzed generally by some metal-dependent proteases [33, 34]. The functional activity of soluble and transmembrane ligands may vary drastically [35]. Different affinity of two ligand forms to the ligand-binding domain of corresponding receptor probably due to their various structural properties is the most probable reason for this observation.

Interactions between ligands and receptors of these families may induce various cell responses depending on

Properties of TNF-receptors and TNF-ligands

No.	Receptor	Presence of "death domain"	Ligand	Cell response to receptor activation	References
1	TNF-RI (p55R/CD120a)	+	TNF, LT-α	death, proliferation, differentiation	[36-41]
2	TNF-RII (p75R/CD120b)	—	TNF, LT-α	death, proliferation, differentiation	[42-44]
3	TNF-RIII (TNF-Rrp/LT-β-R)	—	LT-α_1/β_2,LT-α_2/β_1, LIGHT	death, differentiation	[45-47]
4	Fas (Apo-1/CD95)	+	FasL (CD95L)	death, proliferation	[37, 48, 49]
5	NGF-R1 (p75NGF-R/p75NTR)	+	NGF, BDNF, NT-3, NT-4	death, differentiation, proliferation	[15, 50, 51]
6	CD27	—	CD27L (CD70)	differentiation, proliferation	[52-54]
7	CD30	—	CD30L (CD153)	death, differentiation, proliferation	[55-58]
8	CD40	—	CD40L (CD154)	death, differentiation, proliferation	[59-61]
9	OX40	—	OX40L (gp34)	differentiation, proliferation	[62, 63]
10	4-1BB (CD137)	—	4-1BBL	proliferation	[64, 65]
11	DR3 (Apo-3/WSL-1/LARD/TRAMP)	+	Apo-3L (TWEAK)	death, differentiation (?)	[66-68]
12	DR4 (TRAIL-R1)	+	Apo-2L (TRAIL)	death	[69-71]
13	DR5 (KILLER/TRAIL-R2)	+	Apo-2L (TRAIL)	death	[70-73]
14	DR6	+	?	death	[74]
15	RANK	—	RANKL (ODF/OPGL/TRANCE)	differentiation, proliferation	[75-77]
16	OPG (OCIF/FDCR-1/TR1)	+	RANKL (ODF/OPGL/TRANCE), TRAIL	differentiation	[14,78-80]
17	GITR (AITR)	—	GITRL (AITRL/TL6)	inhibition of death	[81-83]
18	CAR1	+	?	death	[19]
19	HVEM (TR2)	—	LT-α, LIGHT	death, differentiation	[20, 84-86]
20	CrmB, CrmC, CrmD	—	TNF	inhibition of death	[21, 22, 87]
21	DcR1 (LIT/TRID/TRAIL-R3)	—	Apo-2L (TRAIL)	inhibition of death	[73, 88, 89]
22	DcR2 (TRAIL-R4)	+	Apo-2L (TRAIL)	inhibition of death	[90, 91]
23	DcR3 (TR6)	—	FasL, LIGHT	inhibition of death	[28, 29]
24	TNFRSF19	—	?	?	[92]
25	?		APRIL	proliferation	[93]
26	?		BAFF/THANK	death, proliferation	[94, 95]
27	?		VEGI	death, proliferation	[96, 97]
28	?		TNFSF20	death, proliferation	[98]

the cell types, their metabolic state, and the environment. The ligand–receptor pair variants and some examples of cell reactions activated by the binding of the TNF-Rs family receptors to their ligands are given in the table. First, note that the components of these families, receptors and ligands, are very numerous, wherein their tissue specificity is quite conditional, and, second, cross-reactivity is notable between various receptors and ligands, when one receptor recognizes various ligands and vice versa. All these facts together with the wide distribution of membrane-associated and soluble forms of TNF-ligands and TNF-receptors suggest that, on one hand, direct contact and humoral interaction between cells of very different cell types is possible due to the receptors and ligands belonging to this family, and, on the other hand, the existence of mechanisms strictly regulating the expression of these proteins and respectively the intercellular interactions. Possible cytotoxic signaling routes mediated by some TNF-Rs receptor family members are considered below on the basis of protein–protein interactions determined to date.

TNF-RI MEDIATED SIGNAL TRANSDUCTION

The experiments with transgenic *tnf-RI* "knock-out" animals demonstrated that physiological role of this receptor is probably consists in regulation of dendritic cell differentiation, formation of germinal centers, and immunoglobulin G production [13, 99]. Besides, it turned out that the activation of TNF-RI controls also the synthesis of molecules that determine infiltration of monocytes and neutrophils into various organs and tissues [100]. There are data indicating that sleep duration is also determined by TNF-RI functioning [101]. Remember that this receptor may be activated not only by the interaction with TNF, but also with LT-α_3 [102].

At present, three main molecular level effects are elucidated that determine the way of realization of various cell responses due to the interaction between TNF and TNF-RI—these are the activation of: a) transcription factor NF-κB; b) stress-activated protein kinases (SAPKs), so-called p38 kinase and c-Jun N-terminal kinase (JNK), and c) apoptosis [39]. A simplified scheme of signaling pathways mediated by TNF-RI can be presented as follows (Fig. 1).

Normally, the "death domain" of the intracellular part of transmembrane TNF-RI is bound to the SODD protein preventing the homo-association of the receptor "death domains" and its interaction with any adapter proteins [103]. Interestingly, SODD is identical to Bag-4, one of the members of molecular chaperon regulator family, being presumably the key link forming the interconnection between the signal transduction induced by cytokines and protein "folding" and transport in the cell [104]. Bound TNF initiates the TNF-RI trimer activation with the result that "death domains" become associated [37, 105]. This process develops very quickly, and 1 min after the TNF addition the TNF-RI "death domains" cluster binds to the TRADD adapter protein [106, 107]. TRADD contains its own "death domain" on its C-terminal part by which TRADD homo-association and association between TRADD and TNF-RI are realized [108]. Besides, TRADD can interact with TRAF2, RIP, and FADD (MORT1) proteins. TRADD–TRAF2 complex formation results in activation of NF-κB and JNK; TRADD–FADD complex is necessary for triggering apoptosis; TRADD–RIP can transmit signals activating both NF-κB and JNK and cell death [39, 109, 110].

Signal transduction mediated by the TNF-RI–TRADD–TRAF2 complex. The amino acid sequence of TRAF2 is characterized by the presence of two domains, RING and TRAF, each composed of several structures of "zinc finger" type and responsible for protein-to-protein interactions [111, 112]. The JNK activation mediated by TRADD–TRAF2 complex is supposed to develop through the signal transduction via the following kinase chain: TRADD–TRAF2 either through ASK1 kinase or directly activates GCKR, a serine-threonine

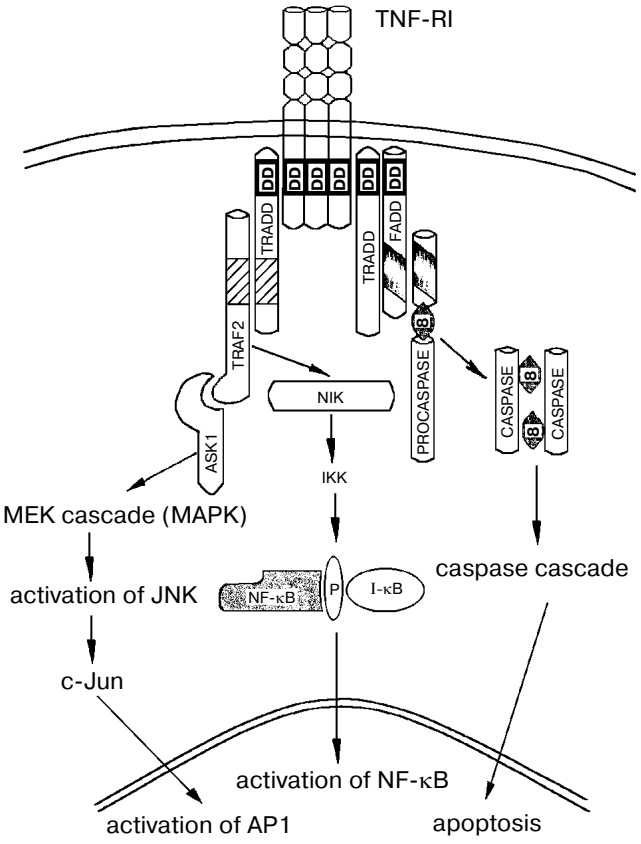

Fig. 1. Scheme of TNF-RI mediated signal transmission (see description in text).

kinase, that phosphorylates MEKK1 kinase. MEKK1 activates MEK kinase that phosphorylates JNK kinase. SAPK/JNK in turn activates the transcription factor AP1 via phosphorylation of its constituent c-Jun [113-117]. A signaling pathway from TRADD–TRAF2 to the transcription enhancement of AP1-dependent genes induce, particularly, the synthesis of various cytokines including pro-inflammatory and immunomodulatory ones, and is connected with the terminal differentiation, proliferation and, possibly, cell death under the action of TNF.

TRADD–TRAF2 complex is also involved in the activation of NF-κB transcription factor [39, 110]. In this case, the chain of events is as follows. Binding to TRADD–TRAF2, NIK kinase phosphorylates IKK kinase complex [118]. Phosphorylated IKKα and IKKβ, in turn, phosphorylate I-κB (an inhibitor of NF-κB) [119]. I-κB phosphorylation results in its degradation and NF-κB release from the inactive complex I-κB–NF-κB, translocation of the latter into the nucleus, and the activation of NF-κB-regulated gene transcription. NF-κB activation in cell induces terminal differentiation or proliferation and inhibits an apoptosis.

NF-κB was shown to be able to activate *traf2*, *c-iap1*, and *c-iap2* gene transcription [120-122]. Products of *c-iap1* and *c-iap2* genes are proteins inhibiting apoptosis, which physically associate with TRAF2. An elevated expression of c-IAP2 stimulates NF-κB activation and prevents TNF-induced cell death. Thus, TRAF2–c-IAP1 complex formation can be considered as an alternative way for TNF-RI-dependent NF-κB activation through a positive feedback mechanism. c-IAP1 and c-IAP2 can also be bound to caspases -3, -7, and -9, blocking their activity [123, 124], which indicates the possible existence of an additional route for TRAF2-mediated cytotoxic signal inhibition.

Note, that the experiments with transgenic murine cells TRAF2$^{-/-}$ have shown that the SAPK/JNK activation level drastically decreases in the absence of TRAF2, whereas NF-κB activation is reduced only slightly [125]. Besides, the defects were found in transgenic animals that are not associated apparently with TNF-induced signaling pathways (impairment of T-helper dependent immune response, CD40-mediated proliferation, and associated drastic decrease in B-cell precursors) [126]. This suggests, first, the existence of TRAF2-independent pathway of NF-κB activation, and second, the entity of additional TRAF2 functions.

Signal transduction mediated by the TNF-RI–TRADD–FADD complex. MORT1/FADD (23-kD protein) does not possess any enzymatic activity and contains two functional domains—a "death domain" in its C-terminal part and N-terminal "death effector domain" similar to the first one in its sequence and structure [109, 127-129]. TRADD and FADD are supposed to form TRADD–FADD by binding via their own "death domains" [129]. Normally, the FADD "death effector domain" is enclosed by PED/PEA15 protein carrying its own "death effector domain" [130]. FADD–PED/PEA15 complex dissociates after the addition of TNF, and released "effector domain" of FADD interacts with the analogous tandem repeated domains of pro-caspase-8 [131]. Caspases form a family of evolutionarily conservative cysteine proteinases existing in the cell as inactive precursors [132]. FADD induces caspase-8 oligomerization activating, in turn, self-splitting of the enzyme with formation of the active proteinase [133]. Caspase-8 then triggers the cascade activation reaction of effector proteinases, the consequence of which may vary apparently depending on the cell type and state. The following chain of events seems to be the most probable: caspase-8 cleaves caspase-9; caspase-8 and/or caspase-9 activates caspases-3 and -7, whereupon caspase-3 induces caspase-6 [134]. The protein DFF45 (DNA fragmenting factor) found among various substrates of caspases is cleft by caspase-3 and inhibits the nuclease CPAN/DFF40 providing internucleosomal chromatin fragmentation in dying cells [18, 135, 136].

Experiments with dominant negative mutant FADD have demonstrated that the TRADD–FADD complex is obligatory for TNF-RI – mediated cytotoxicity, however it does not participate in the SAPK/JNK and NF-κB activation [137]. An additional role of FADD in cell metabolism was established in experiments on transgenic animals with mutant or deleted *fadd* gene. Drastic decrease in numbers of peripheral T-cells and thymocytes and cardiac failure signs were found in such animals [138, 139]. The authors suppose that not only apoptosis-specific receptors, but also others can use FADD for cell proliferation and activation signaling.

Signal transduction mediated by the TNF-RI–TRADD–RIP complex. RIP (74-kD protein) is a prototype in the RIP family of serine-threonine kinases distinguished for the presence of a kinase domain on the N-terminal parts of their molecules and "death domain" or "death effector domain" on their C-termini [140-143]. The ability of RIP to trigger the reactions determining cell survival as well as cell death in response to TNF is due to the presence of functionally different domains.

RIP-dependent and also TRAF2-dependent SAPK/JNK activation is realized via a cascade phosphorylation reaction of signaling kinases. In this case, signal transduction may fit the following scheme [115]:

$$\text{TRADD–RIP} \rightarrow \text{MEKK1} \rightarrow \text{MEK} \rightarrow \text{JNK} \rightarrow \text{AP1}.$$

Similar signaling ways of AP1 activation are characteristic of other RIP-family members as well [144].

The role of RIP and particularly its kinase domain in NF-κB activation was revealed already in the initial experiments devoted to this adapter protein [141, 145]. At least two ways of NF-κB activation involving RIP are presently known. The first is essentially indistinguishable

from the signaling pathway mediated by TRAF2, with the exception that RIP cannot form fast associations with NIK and phosphorylates it due to its own kinase activity [11, 118]. The second NF-κB activation route is specific for RIP, not for TRAF2. TRADD–RIP complex interacts with p62 protein, the C-terminal part of which is responsible for RIP binding, whereas the N-terminal part – for the atypical protein kinase C (aPKC) binding. Being in a complex with TRADD–RIP–p62, aPKC activates IKKβ that, in turn, phosphorylates I-κB, causing its degradation [146]. Functional consequences of NF-κB activation via various RIP-dependent mechanisms remain unclear.

Although apoptosis- and NF-κB-inducing properties of RIP were found simultaneously, the concrete molecular mechanism(s) of RIP-dependent death remain(s) unknown. One possible variant of cytotoxic signaling is based on a) the ability of RIP to interact with adapter proteins TRADD and FADD [147], b) the inhibition of RIP-mediated apoptosis by the mutant FADD and an inhibitor of caspase activity [148] and c) on the absence of data on specific binding of RIP with any procaspase causing its activation. The model suggests the TRADD–RIP–FADD complex formation and consequent triggering the cascade protease reaction by analogy with the reaction induced by TRADD–FADD complex. The scheme does not contradict the experimental data; however, the most important question on the role of RIP in this chain remains unclear. Taking into account the possibility of phosphorylation of TRADD and FADD by the kinase activity immanent for RIP, one can admit that phosphorylated TRADD and FADD acquire other properties rather than those in the TRADD–FADD complex and become accessible for another interactions, which differ from known ones.

Recently, two research groups have proposed independently an original explanation for RIP-dependent cytotoxicity [149, 150]. The authors discovered a specific cleavage of RIP by caspase-8 under the treatment of the cells with TNF. It is accompanied by the blockade of TNF-induced NF-κB activation and enhancement of the TRADD–FADD association. Since NF-κB stimulates particularly the transcription of anti-apoptosis genes [151] and TRADD–FADD complex triggers apoptosis, RIP cleavage can be regarded as a strong pro-apoptotic signal.

Finally, the third variant is the involvement of additional adapter protein CRADD in RIP-mediated apoptosis [152]. Like FADD, CRADD possesses a two-domain structure: C-terminal "death domain" and N-terminal sequence that is able to interact with caspase-2. TNF can induce in this case TRADD–RIP–CRADD complex formation activating caspase-2. In this case, the problem is the fact that caspase-2 is presently believed to be able to activate only pro-caspase-2, but not other caspases [153]. It cannot be excluded however, that the cleavage of other, different from caspases, intracellular targets by caspase-2 is sufficient for all the apoptosis signs. Thus, elucidation of the molecular mechanisms of RIP-activated apoptosis requires further investigations.

Although the above-given schemes of TNF-RI-mediated signal transduction have reliable experimental basis and enable satisfactory explanations for most known TNF effects, they are apparently far from complete and single. Other reactions are described apart from the TNF-RI–TRADD interaction and corresponding signal pathways. TNF-RI associates physically with the MADD/DENN protein "death domain" causing activation of MEKK1 and JNK activation and phospholipase A2 phosphorylation [154, 155]. Sentrin (sentrin/SUMO-1/DAP-1/PIC1), another protein interacting with TNF-RI "death domain", is homologous to ubiquitin and participates in the control of TNF-induced cell death [156, 157]. Data are known on complex formation between TNF-RI and phosphoglycerate mutase suggesting TNF-RI involvement in regulation of glycolysis [158]. A number of works demonstrate an intriguing connection between TNF-RI and tyrosine kinase receptors via Grb2 adapter protein. This process involves FAN, another adapter protein with yet unknown but obligate function in the process, which interacts with a specific cytoplasmic TNF-RI site differing from "death domain" and is responsible for neutral sphingomyelinase activation [158, 159].

Thus, the key role in cell response directing induced by TNF binding to TNF-RI receptor belongs to factors determining the expression and modification of proteins physically associated with TNF-RI.

SIGNAL TRANSDUCTION MEDIATED BY TNF-RII

Transgenic mice with destroyed *tnf-RII* gene are characterized by elevated resistance to the cytotoxic effect of TNF, but T-cell development and activity in these animals are undistinguished from the control [160]. TNF-RII was shown to be involved in the regulation of hematopoietic cell proliferation and ICAM-1 expression in microvascular endothelial cells of the brain [161, 162].

The TNF–TNF-RII binding, like in the case with TNF-RI, is supposed to be able to cause the activation of transcription factor NF-κB, stress-activated protein kinases SAPKs/JNK, and apoptosis [11]. However, molecular mechanisms of these responses mediated by TNF-RII (Fig. 2), apparently differ from those activated by the TNF-RI. In our opinion, we give here the most reliable from numerous known data supporting this supposition. These are, first, the existence itself of two different TNF-receptor types; second, the absence of a "death domain" in the cytoplasmic TNF-RII part, and, third, the affinity of TNF-RII to TNF is at least one order of magnitude less than that of TNF-RI to TNF [163]. This

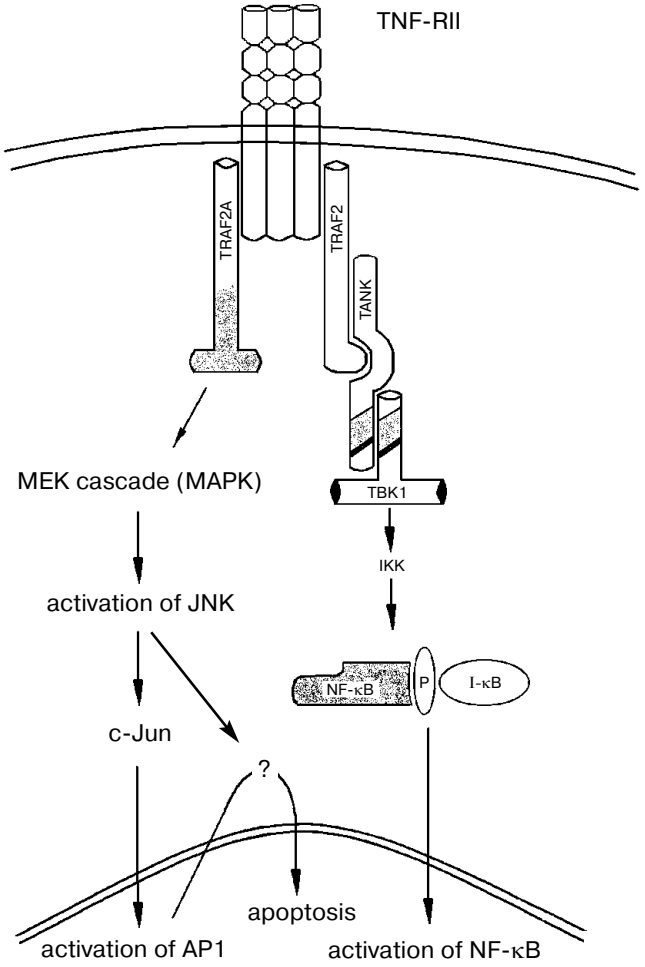

Fig. 2. Scheme of TNF-RII mediated signal transmission (see description in text).

suggests lesser stability of TNF-RII complexes with TNF and respectively with adapter proteins as compared to the complexes formed by TNF-RI.

TNF-RII and activation of NF-κB and JNK. Most of the data describing TNF-RII-dependent signaling mechanisms were obtained from *in vitro* experiments, so the question upon the situation *in vivo* remains open. TRAF2 adapter protein is believed to play a key role in TNF-RII-mediated signaling. Association of TNF-RII with TRAF2 occurs both in the presence and absence of TNF [111, 164]. TRAF2 can form either homo- or heterocomplexes with various proteins [111, 112, 165]. TRAF2 is apparently involved in formation of various complexes depending on the presence of ligand. TNF trimer binding with TNF-RII stabilizes oligomerization of the latter, although it is unknown yet, is this di- or trimerization [166, 167]. Then, according to the general scheme, cytoplasmic sites of oligomerized TNF-RII receptors interact with TRAF2 trimer [11, 168]. Then, the signaling pathways of the NF-κB or SAPKs/JNK activation are probably the same as ones mentioned in the section on the role of TNF-RI–TRADD–TRAF2 complex in cell response to TNF. Meanwhile among the proteins found in TNF-RII signaling complexes the only one (apart from TRAF2 itself) can activate NF-κB, whereas at least five proteins block it.

TANK protein that binds to TRAF2 stimulates NF-κB activation under certain conditions [169, 170]. The interaction between TNF and TNF-RII is supposed to initiate the formation of a complex composed of TNF-RII, TRAF2, TANK, and TANK-binding kinase (TBK1). In this complex, TBK1 undergoes activation and phosphorylates the kinase complex, IKK [171]. This pathway is assumed as an alternative one for TRAF2-dependent NF-κB activation.

The proteins TRAF2A, TRAF1, TRIP, A20, and filamin inhibit TRAF2-dependent NF-κB activation. TRAF2A is a product of alternative TRAF2 splicing and can bind to the cytoplasmic domain of TNF-RII and other TRAF-family proteins as well. Due to its altered RING-domain, TRAF2A is apparently not able not only to activate NF-κB, but blocks TRAF2-dependent stimulation of this transcription factor [172]. TRAF1, an another NF-κB-inhibiting protein, is found in TRAF2–TRAF1 heterocomplex. TRAF1 is characterized by the absence of a RING-domain and respectively by the ability to interact directly with TNF-RII [111]. TNF was shown to induce TRAF1 synthesis, and elevated TRAF1 expression completely blocks TNF-dependent NF-κB activation [173]. TRIP and A20 in association with TRAF2–TRAF1 complex also inhibit TRAF2-mediated NF-κB activation [174, 175]. The elevated expression of filamin, an actin binding protein that can associate physically with TRAF2, leads to the same effect [176].

The phosphorylation of TRAF-binding domain of TNF-RII by the casein kinase I-like p80TRAK kinase is another route of negative control of NF-κB activity. Phosphorylation in this site blocks NF-κB activation, possibly due to the influence on TRAF2 binding [177, 178].

The data described above suggest that TNF-RII-mediated NF-κB activation is limited in time and strictly regulated by the receptor phosphorylation and the ratio between TRAF2 and TRAF2-associated proteins in TNF-RII signaling complex.

Like the NF-κB stimulation, TRAF2 is believed to play the main role in TNF-RII-dependent JNK activation [179]. Filamin elevated expression is shown to inhibit JNK activation [176], whereas TRAF2A is a specific JNK activator [180]. Regulation of JNK activity is evidently determined by the type of proteins that are the constituents of the TNF-RII-signaling complex.

TNF-RII and activation of apoptosis. Much experimental evidence for the involvement of TNF-RII in TNF

cytotoxic signals is beyond question, but still no clear picture exists describing the molecular mechanisms of TNF-RII-dependent death. The majority of authors believes that TNF-RII-dependent apoptosis is due to the mediated activation of TNF-RI-specific cytotoxic pathways. Several routes of apoptotic signal transduction from TNF-RII onto TNF-RI have been proposed.

One of these is "ligand passing" from TNF-RII to TNF-RI [181]. In this case, TNF-RII, the lower affinity receptor for TNF, plays a role of regulator in the reaction of binding between TNF and TNF-RI, probably, via increase in local TNF concentration on the cell membrane. The possibility of the ligand passing was confirmed in experiments on receptor immunoprecipitation in the absence and presence of the ligand [182]. The authors demonstrated that both receptors were present in precipitates obtained with anti-TNF-RI as well as anti-TNF-RII antibodies from cell lysates treated with TNF. The ligand-induced receptor complex formation was transient and had no dependence on intracellular receptor domains. Nevertheless, the authors do not exclude the possibility that the receptor heterocomplex formation not only conditions the TNF transmission from one receptor onto another, but also induces the alteration in the receptor intracellular domain conformations and respectively their ability to interact with adapter proteins.

Virtually the same conclusion upon the important role of intracellular associations binds the works explaining cytotoxic signal transduction from TNF-RII to TNF-RI via either competitive or cross-interaction of receptors with adapter proteins. The authors do not generally take into account ligand passing or they regard this event as possible but not important [183, 184]. TRAF2 apparently fulfills the key function in the interaction between TNF-RII and TNF-RI [120]. Although a particular mechanism of signal transduction remains unclear, it is suggested that the TNF-RII activation blocks antiapoptotic TRAF2-dependent signaling via the decrease in TRAF2 expression and/or induces RIP-mediated cytotoxicity via TRAF2 binding with RIP [44, 112, 164, 183, 184].

Another route of TNF-RII-mediated apoptosis through the activation of TNF-RI-dependent signaling pathways is based on the results obtained from the investigation of TNF-induced transcription. It turned out that the interaction between TNF and TNF-RII leads to drastic *tnf* gene transcription enhancement in the treated cells, probably due to SAPK/JNK activation [185, 186]. Endogenously synthesized TNF is translocated into the cell membrane and triggers cell death through binding with TNF-RI. The mechanism described allows speculation by analogy with alternative schemes of TNF-RII-mediated cytotoxicity based on the up-regulation of other apoptotic ligands under the influence of TNF on the cells. The existence of signaling pathway can be admitted particularly involving SAPK/JNK stimulation caused by the interaction between TNF and TNF-RII (see above) and following JNK-dependent transcription activation of the Fas-ligand [187, 188] which, in turn, initiates the Fas-mediated apoptosis.

To summarize the results of investigation on TNF-RII, one can arrive at a rather unexpected conclusion that unlike TNF-RI, which activates signaling cascades apparently via TNF binding only, TNF-RII functions may consist of constitutive signaling cascade activation in the absence of any ligand. One can admit that in the norm TNF-RII stimulates a transcription of protective anti-apoptotic genes and support cell metabolism through the constitutive activation of NF-κB. The interaction with TNF leads to the inhibition of NF-κB activation and respectively to the elevation in sensitivity to TNF cytotoxic effect. In parallel, TNF-RII in one or another way transmits the signal to TNF-RI initiating TNF-RI-dependent cell death. Further investigations are needed to test this supposition.

SIGNAL TRANSDUCTION MEDIATED BY Fas-ANTIGEN

The transmembrane and soluble forms of Fas-ligand as well as specific anti-Fas antibodies activate Fas-antigen via cross-linking of the receptor molecules that as a rule results in cell death by apoptosis. Besides, Fas stimulation is known to induce proliferation of cells, in particular, activated T-cells and human diploid fibroblasts [49, 189, 190].

The Fas "knock-out" results in hyperplasia of the liver and lymphoid peripheral organs in transgenic animals [191, 192]. Such mice suffer lymphocytosis that is accompanied by the infiltration of lymphocytes into lungs and liver, and also increased immunoglobulin production and peripheral T-lymphocyte clone deletion [192].

Signal transduction mediated by the Fas–FADD complex. Fas-mediated apoptotic pathway, from interaction of ligand with Fas-receptor up to chromatin degradation can be presented as follows (Fig. 3) and seems to be identical to those induced by TNF. Binding of FasL homotrimer to extracellular sites of three Fas molecules induces clustering of intracellular receptor regions due to the physical association of "death domains" [37]. In cells, the Fas "death domains" association forms a complex with analogous "death domain" of MORT1/FADD adapter protein, the main function of which possibly consists in the transmission of arising conformational changes [109, 128]. FADD activation and its following interaction with caspase-8 can be controlled by PED/PEA-15 protein expression and/or phosphorylation [130, 193]. In addition, the "death effector domains" of FADD and caspase-8 may be "blocked" by c-FLIP protein (Casper/CASH/CLARP/FLAME-1/I-FLICE/MRIT/usurpin), containing two "death effector

domains" and another domain that is homologous to the caspase-8 and -10 sequences with the exception that the amino acids necessary for caspase activity manifestation are deleted [194-197]. Note that the elevated c-FLIP expression in transformed cells leads generally to the inhibition of the death mediated by the receptors containing "death domain", whereas in normal cells its activation occurs [194-197]. Cell death inhibition or induction by c-FLIP depends on the expression of proteins, which are able to interact with c-FLIP (FADD, caspases-8 and -10, TRAF1 and TRAF2, $BclX_L$, etc.), and also various isoforms of c-FLIP itself.

Depending on cell nature and its functional status the activated caspase-8 can transduce the cytotoxic signal by two possible pathways [198, 199]. The first mechanism including immediate activation of the effector caspases (-3, -4, -5, -6, -7, etc.) by caspase-8 was described above briefly. Another one is based on apoptosis-stimulating signals of mitochondrial proteins. In this case, a pro-apoptotic member of the Bcl-2 protein family, Bid protein, a characteristic feature of which is the presence of the BH3-domain exclusively, serves as an early target of caspase-8 [200-202]. Three other domains (BH1, BH2, and BH4), which are inherent in Bcl-2 protein family and determine homo- and heterooligomer formation in these proteins, are absent from Bid. Caspase-8 cleaves Bid that is in cytoplasmic fraction of dying cells into two fragments with molecular mass of 6.5 kD (N-terminal polypeptide) and 15 kD (C-terminal polypeptide containing BH3-domain). The latter is translocated into mitochondria inducing mitochondrial cytochrome *c* release into the cytoplasm [203, 204]. The data on mechanisms of Bid-dependent cytochrome *c* release do not yet allow a clear description of this process. However, several routes may exist for activation of apoptogenic signals via Bid. In one of these routes Bid incorporates into the outer mitochondrial membrane forming a pore, thus facilitating cytochrome *c* release from the mitochondrial intermembrane space [205]. According to the other version, Bid stimulates a pore opening in the inner mitochondrial membrane due to the interaction with adenine nucleotide transporter [206]. As a result, mitochondria swell with consequent disruption of the outer mitochondrial membrane and cytochrome *c* release.

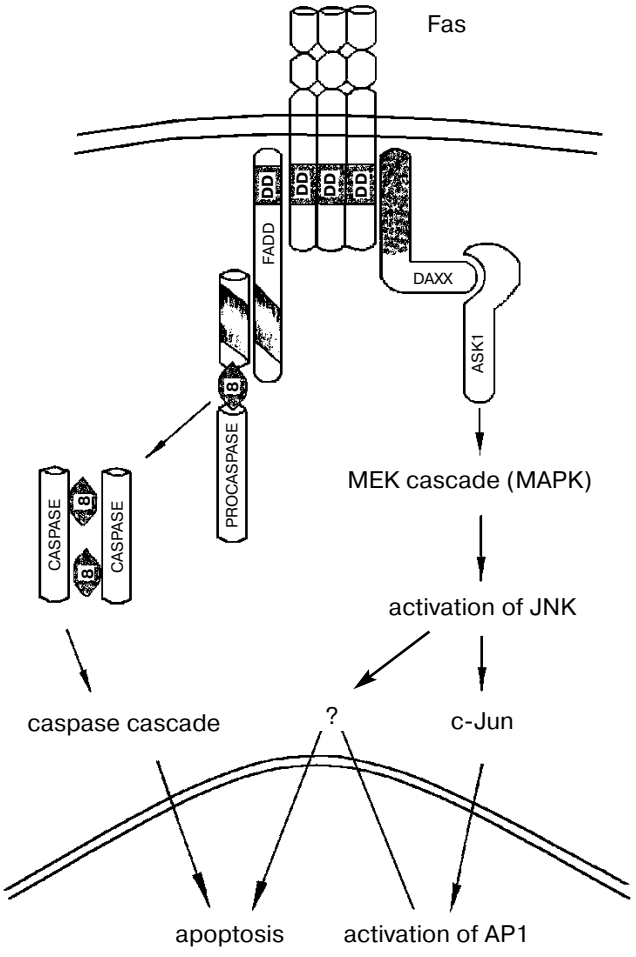

Fig. 3. Scheme of Fas-antigen-mediated signal transmission (see description in the text).

It is Martinou's opinion [207] that the role of Bid consists of oligomerization of Bax, another pro-apoptosis protein. After oligomerization, Bax either gains the capacity to fit into the outer mitochondrial membranes with pore formation [208] or induces VDAC closing and brakes ADP transport through the outer membrane. This leads to inhibition of ATP synthesis, elevation of membrane potential, mitochondrial matrix swelling and, as a consequence, to the impairment of the outer mitochondrial membrane integrity [209].

In cytoplasm, cytochrome *c* interacts with Apaf-1 protein; three functional domains are identified in its composition: CARD, the analog of "death effector domain", NOD, a domain responsible for oligomerization and nucleotide binding, and WD-40-repeats participating in protein–protein interactions [210-212]. Cytochrome *c* induces Apaf-1 oligomerization in the presence of ATP/dATP. In thus forming cytochrome *c*–Apaf-1 multimeric complex, Apaf-1 binds and activates pro-caspase-9 through the CARD-domain [213-216]. Activated caspase-9 is released from the complex, wherein WD-40-repeats of Apaf-1 play the key role in the process, and then activates caspases-3, -6 and -7, thus triggering a proteolytic cascade reaction [215, 217, 218]. Further events develop apparently by analogy much as what occurs in proteolytic cascade activation induced by caspase-8. Note that the release of cytochrome *c* into the cytoplasm may lead not only to apoptotic but also to necrotic cell death because the leakage of cytochrome *c* from mitochondria may cause an interruption of electron

transport respiratory chain and, as a consequence, the ATP level decrease and the oxygen radical production overcoming the antioxidative capacity of the cells [219, 220].

Thus, at least two mechanisms of Fas-dependent apoptosis are known to be mediated by the physical association of Fas-antigen with FADD. What cell death pathway will be activated is possibly dependent on the nature of the cells and/or their functioning state, but the specific molecular and biochemical causes determining the choice of the cytotoxic mechanism by the cell remain unstudied.

Signal transduction mediated by the Fas–DAXX complex. As shown recently, Fas-antigen stimulation can induce the JNK-activating signal transduction. As it takes place, Fas oligomerization leads to the physical association of the "death domain" of Fas-antigen with the DAXX protein [221, 222]. A sequence of DAXX has no homology with the "death domain", so the DAXX binding to Fas occurs independently of FADD. A DAXX-dependent JNK-activation pathway seems to be similar to the reaction sequence triggered by the TNF-RI–TRADD–TRAF2 complex (see above). DAXX interacts with the ASK1 kinase that launches a kinase cascade, which, in turn, modifies activities of nuclear transcription factors [223, 224]. However, the role of DAXX-mediated JNK activation remains unclear. It has been proposed in some papers that JNK activation may enhance the Fas-mediated apoptosis with the activation of caspase-8 as a necessary preceding step for the kinase cascade induction [225, 226]. Experiments with mutant Fas and DAXX have thus demonstrated that Fas-dependent cell death and JNK activation may be induced and respectively inhibited independently from each other [222, 227]. Moreover, analysis of transgenic mice with mutant DAXX has enabled to the authors to conclude that DAXX may not only not enhance Fas-mediated apoptosis, but also in some way inhibit embryonic cell death [228].

Unexpected results concerning DAXX functioning were obtained independently in several laboratories in studies of so-called "nuclear dots" (ND10/"PML nuclear bodies") [227, 229-231]. "Nuclear dots" are protein complexes which are composed by PML, Sp100, and a number of other proteins and detected as discrete dots in interphase nuclei. Functions of "nuclear dots" are not yet determined, however, they are known to participate in cell responses to various stress exposures, virus infections, and carcinogenic transformation through their influences on cell cycle [230, 232]. It turned out that DAXX is also localized in the "nuclear dots". A number of stimuli, for example, mitogenic activation of mature splenocytes, induces PML sentrinization defining in turn the ability of modified PML to bind physically with DAXX. DAXX beyond the binding capacity of PML, in the absence of PML, or when PML is inactivated is found in condensed chromatin and exhibits transcription repressor properties. DAXX was shown to inhibit gene transcription via physical association with transcription factors, for instance ETS1 and Pax3 [233, 234]. In this state DAXX has no pro-apoptotic properties. PML inhibits DAXX repressive properties by binding it in "nuclear dots", that is accompanied by cell sensitivity enhancement to apoptosis and particularly to Fas-mediated death. Direct proofs of near-membrane DAXX localization are absent, but it seems that DAXX is distributed in some way between nucleus and cytoplasm forming heterocomplexes with Fas-antigen and nuclear proteins. Fas activation leads probably to the creation of additional associations between Fas–DAXX complex and some proteins, for instance, ASK1, and to translocation of nuclear DAXX from chromatin into "nuclear dots". Taking into account on one hand the data on DAXX effect on Fas-mediated apoptosis, and on the other, on the transcription factor activity, a logical conclusion may be drawn that DAXX can be considered as a protein the main functional role of which consists in prevention rather than enhancement of apoptosis. In the absence of cytotoxic signal, nuclear DAXX apparently inhibits the transcription activity of factors regulating expression of genes which are necessary for death realization, whereas DAXX being bound to Fas, hinders the "death domain" association of Fas-antigen. Fas oligomerization induced by specific antibodies or ligand leads to redistribution of DAXX in nucleus and respectively to release and activation of transcription factors, realizing apoptotic gene transcription. The thus observed DAXX-dependent JNK activation induces stimulation of other transcription factors, for example, AP1 and SRF, but their role in apoptosis enhancement or inhibition has yet to be established.

Thus, although the functional significance of DAXX in cell remains unclear, its ability to form complexes with various proteins in the nucleus and Fas-antigen implies that DAXX and Fas participate not only in cell death processes but also in organization of normal cell life support.

Interactions of Fas with proteins other than FADD and DAXX. Besides FADD and DAXX, other proteins are known to be able to interact physically with Fas. Ubiquitin-conjugating enzyme, UBC-FAP, was one of the first known proteins binding with Fas "death domain" [235]. Its main function is apparently to attach sentrin to various intracellular proteins. It is assumed that sentrinization of proteins endows those with the ability to interact with other proteins, particularly nuclear membrane proteins and some intranuclear structures, and besides it is a signal for degradation of proteins which are stable against ubiquitinylation-mediated proteolysis [236, 237]. Protein modification with sentrin was shown to be necessary for the normal cell cycle, especially for G_2 stage and mitosis [236, 238, 239]. Data are available on this type of posttranslational modification of RAD 51, RAD 52, p53 proteins participating in the reparation of double-

stranded DNA breaks [240]. Note that normal p53 sentrinization leads not to degradation of this transcription factor, but to enhancement of its transcriptional activity [241, 242]. Since the substitution of mutant *p53* gene inherent in various tumor cells (wherein the product cannot conjugate to sentrin) for wild-type gene results typically in death of the transformed cells, it is believed that sentrin plays an important role not only in the cell cycle, but also in cell death regulation. Mediated regulation of cell sensitivity to cytotoxic agents may be realized via sentrinization of I-κB, an NF-κB inhibitor. In this case, sentrin hinders the I-κB ubiquitinylation and respectively its degradation. Thus, the I-κB–NF-κB complex remains unchanged and NF-κB stays inactive [243]. Finally, as noted above, sentrin can bind to TNF-R1 and Fas "death domains" inhibiting TNF-dependent and Fas-mediated cell death [156].

Taking into account the important role of protein sentrinization in cell life maintenance, it can be suggested that Fas-antigen interacting with UBC-FAP controls the ability of the latter to catalyze sentrin attachment to various intracellular targets, including itself. Fas–UBC-FAP complex formation may be considered from this point of view as yet another way of Fas-mediated signaling, which is necessary for cell division, reparation, and death. At the same time, it must be ruled out that Fas–UBC-FAP association is an intermediate step of Fas sentrinization reaction determining its subsequent degradation by sentrin-specific proteases [237]. In that event, Fas–UBC-FAP complex formation must precede the decrease in cell sensitivity to Fas-mediated apoptosis due to receptor proteolysis.

Protein tyrosine kinases p59fyn and BTK are revealed among other Fas-binding proteins [244, 245]. T-Lymphocytes isolated from transgenic mice with knocked-out *fyn* gene displayed less sensitivity to the cytotoxic effect of Fas-ligand compared to control cells. p59fyn tyrosine kinase apparently modifies the proteins participating in early steps of Fas-mediated apoptotic signal transduction, then it is cleft by caspase-3. Unlike p59fyn, BTK prevents Fas-dependent cell death. B-Lymphocytes with a disrupted *btk* gene died more effectively than control cells when Fas was activated. BTK is supposed to physically hinder Fas binding with FADD, thus blocking cytotoxic signal transduction, wherein the role of its enzymatic activity remains unclear.

Note that Fas stimulation results in alteration of various enzymatic activities and expression of some proteins. Activation of phosphatidylinositol-3′-kinase in T-lymphocyte Th2 population [246], PITSLRE protein kinase [247], double-stranded RNA activated protein kinase [248], p21-activated kinase [249], phospholipase D [250], acidic sphingomyelinase [251], and a number of other enzymes was shown. Biochemical pathways from Fas oligomerization to their activation are not yet traceable in the vast majority of cases.

Thus, Fas-antigen can induce various cell responses, such as death, proliferation, and, possibly, differentiation through the interaction with some intracellular proteins. Although some signaling pathways induced by Fas activation, particularly cytotoxic pathway involving FADD, are studied in more detail compared to the processes triggered via other receptors of TNF-Rs family, the general scheme of Fas-mediated signaling including apoptosis is far from being completed.

The existing schemes of signal transduction mediated by the activation of other TNF-Rs receptor family members are all the more fragmentary compared to TNF-RI-, TNF-RII-, and Fas-dependent pathways. More or less harmonic pictures are proposed for CD40 and DR3 [12, 252]. This is because the similarity of some distinct signaling steps known for CD40 and TNF-RII [11] and DR3 is assumed to trigger reactions that are actually identical to those activated by TNF-RI [12]. Conceptual and methodical approaches elaborated during TNF-RI-, TNF-RII-, and Fas-mediated signaling investigation will apparently enable establishment in the coming years of the exact molecular pathways induced by other receptors of TNF-Rs family. At the same time, already the signal transduction pathways from various receptors may be considered as interrelated, and the cell response to the activation of some receptor depends not only on the expression level of "own" signaling proteins, but also on proteins formally belonging to the signaling pathways triggered by other receptors.

Several levels of interactions between various signaling pathways mediated by the activation of TNF-Rs family members may be distinguished on the basis of known data. The first is interaction of common ligands with various receptors and *vice versa* (table). The expression and availability of transmembrane and soluble receptors and ligands which are capable of interaction is determined apparently by the cell type and nature of the signals received from the outside. The next level is immediate intermolecular interaction of various TNF-Rs family receptors, the possibility of which has been demonstrated so far by experiments *in vitro* exclusively [26, 37, 253]. The possibility of inter-receptor interaction depends on the expression level of receptors and proteins involved in signaling complex associated with the receptors. The adapter proteins which can physically associate not only with various receptors but also *inter se* and with enzymatic active molecules play the main role on the third level of interrelation between the signaling pathways [109, 112, 129, 148, 221]. Numerous experimental data devoted to the investigation of these molecules force us to consider them as a key link providing intersection of various signaling pathways.

The possibility of signal interchange complicates substantially the general picture of cytotoxic reactions in multicellular organism and along with this enables new

directions in investigation of mechanisms regulating expression of molecules involved in cell death and its modification.

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