Does calcium contribute to the CD95 signaling pathway?

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Death receptors play a crucial role in immune surveillance and cellular homeostasis, two processes circumvented by tumor cells. CD95 (also termed Fas or APO1) is a transmembrane receptor, which belongs to the tumor necrosis factor receptor superfamily, and induces a potent apoptotic signal. Initial steps of the CD95 signal take place through protein/protein interactions that bring zymogens such as caspase-8 and caspase-10 closer. Aggregation of these procaspases leads to their autoprocessing, to the release of activated caspases in the cytosol, which causes a caspase cascade, and to the transmission of the apoptotic signal. In parallel, CD95 engagement drives an increase in the intracellular calcium concentration $(Ca^{2+})_i$ whose origin and functions remain controversial. Although Ca^{2+} ions play a central role in apoptosis/necrosis induction, recent studies have highlighted a protective role of Ca^{2+} in death receptor signaling. In the light of these findings, we discuss the role of Ca^{2+} ions as modulators of CD95 signaling. ***Anti-Cancer Drugs*** **22:481–487 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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So-called death receptor CD95

CD95 (APO1/Fas) has been initially cloned as a death receptor [1]. The receptor CD95 is resolved in an SDS-polyacrylamide gel electrophoresis weighing approximately 40 and 50 kDa and this transmembrane receptor belongs to the tumor necrosis factor receptor superfamily [2]. In contrast to the ubiquitously expressed CD95, its cognate ligand, CD95L shows a restricted pattern of expression and is expressed at the surface of activated T-lymphocytes [3] and natural killer cells [4] in which it plays a pivotal role in the elimination of transformed and infected cells and in immune homeostasis. This ligand can be cleaved by metalloproteases, such as MMP3 [5], MMP7 [6], MMP9 [7], or ADAM10 [8,9] and is released in the connective tissue and blood circulation. In contrast to the membrane-bound CD95L, cleaved CD95L, which is found increased in certain patients affected by cancers, does not trigger any apoptotic signal [10,11] and its physiopathological role remains unknown. Recently, it has been shown using a knock-in mouse model that cleaved CD95L may aggravate autoimmune disorders and tumor occurrence through the activation of the nuclear factor-κB proinflammatory signal [12]. In agreement with the notion that the couple CD95L/CD95 may exert nonapoptotic functions, a recent study pinpointed that CD95 engagement contributed to liver and ovarian carcinogenesis through the activation of the c-Jun N-terminal kinase (JNK) signaling pathway in mouse xenograft models and tissue-specific CD95 knockout mice [13]. In addition, CD95 stimulation promotes liver regeneration after partial hepatectomy [14], raising the possibility that in a certain context, CD95 may exert dominant pro-proliferative activities. At present, the main challenges remain to decipher how the death receptor CD95 can be converted into a pro-oncogenic receptor and to show that this process can occur in humans.

The CD95-mediated apoptotic signal: protein/protein interactions

CD95 is a type I transmembrane protein whose intracellular region encompasses a domain termed death domain (DD). Similar to the death receptors belonging to the tumor necrosis factor receptor family, CD95 does not exhibit any enzymatic activity and the transmitted signal is ignited through the formation of a plasma membrane platform that promotes protein/protein interactions [1,15]. Indeed, on the binding of membrane-bound CD95L, CD95 is clustered to form nanometer-scaled structures called signaling protein oligomerization transduction structures [16] and/or SDS-stable microaggregates [17,18] and then, to constitute micrometer-sized platform called CD95-CAP [19]. Meanwhile, CD95 manifests modifications of its DD conformation, which allows the recruitment of the adapter protein Fas-associating protein with DD (FADD) [20], which in turn recruits zymogens named caspase-8 and caspase-10. This complex is called the death-inducing signaling complex (DISC) [21] and it includes factors such as cellular FADD-like interleukin-1 β-converting enzyme-inhibitory protein (c-FLIP) [22,23] and PED/PEA-15 [24], which hamper the ignition of the apoptotic signal. According to the efficiency of the DISC formation, cells have been

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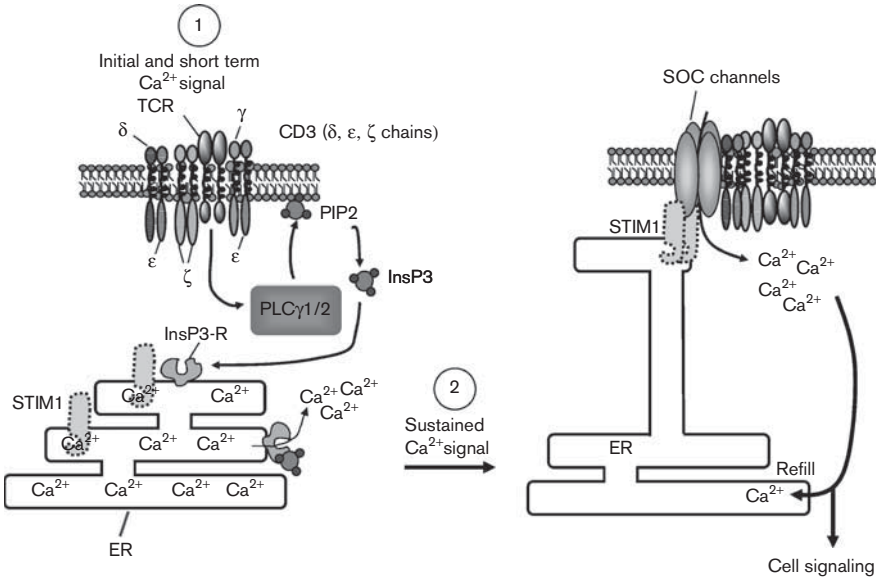
classified as type I (efficient) and type II (inefficient) [25]. The redistribution of CD95 into plasma membrane subdomains termed as lipid rafts or detergent-resistant membranes [26], the post-translational modification of CD95 (myristoylation [27,28] and glycosylation [29]), or the reprogramming cellular process designated as epithelial–mesenchymal transition [30] affects the efficiency in forming the DISC and consequently have been reported to participate in type I/type II behaviors. Nevertheless, molecular mechanisms enhancing or preventing DISC formation remain to be defined.

The CD95-mediated sustained calcium signal

Although it is well accepted that the activation of the death receptor CD95 promotes a sustained calcium signal, to date, no consensus has been found regarding the molecular mechanisms responsible for this CD95-induced calcium signal. Initial studies ascertained that CD95 engagement drives a rapid and sustained increase in the intracellular concentration of Ca^{2+} $(Ca^{2+})_i$ and this response relied on both intracellular and extracellular pools of calcium [31]. More recently, it was definitively proved that CD95 engagement elicits the production of inositol 1,4,5-triphosphate (InsP3) through the activation of phospholipase-Cγ1. In turn, $InsP_3$ binds to and activates $InsP_3$ receptors that orchestrate the subsequent release of Ca^{2+} from endoplasmic reticulum (ER) stores [32] (Fig. 1). It still remains to define the molecular process causing sustained Ca^{2+} response on addition of CD95L. On T-cell receptor (TCR) engagement, a sustained Ca^{2+} increase occurs through a biphasic signal caused by the activation of $InsP_3$ receptor ($InsP_3$-R) and the release of the ER-stored Ca^{2+}. Depletion of the intracellular Ca^{2+} stores leads to the activation of Ca^{2+} influx by the store-operated Ca^{2+} (SOC) channels (reviewed in [33,34]; Fig. 1). The Ca^{2+} release-activated Ca^{2+} (CRAC) channels belong to the SOC channel family, which also consists of some of the transient receptor potential (TRP) cation channels. The CRAC-mediated Ca^{2+} influx not only participates in replenishing the Ca^{2+} stores and perpetuating Ca^{2+} signaling in lymphocytes, but also plays pivotal roles in proliferation, cytokine generation, and differentiation (reviewed in [35]; Fig. 1). Recently, different groups have identified stromal-interacting molecule 1 as the putative ER store Ca^{2+}-sensing molecule [36–38] coupling store depletion to aggregation and activation of the plasma membrane CRAC channels, which in turn lead to Ca^{2+} influx (Fig. 1). Further investigations showed that Orai-1 is part of the SOC and constitutes the molecular basis of the store-operated calcium entry (SOCE; for review see [39]). In this regard, depletion of the ER-stored Ca^{2+} evokes the prompt translocation of stromal-interacting molecule 1 to the plasma membrane in which it forms punctuate clusters and interacts with Orai-1 [40–43] (Fig. 1). This interaction seems to be necessary to elicit calcium entry. Several studies indicate that other proteins constitute the SOC such as TRP cation channel family C

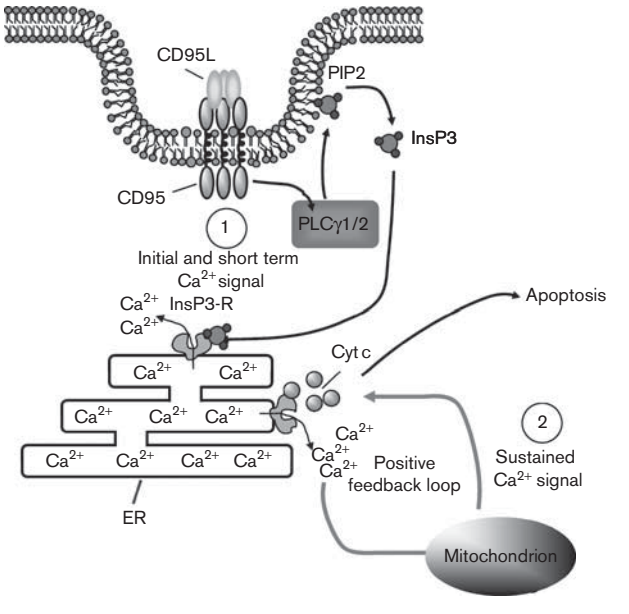
Fig. 1



The biphasic Ca^{2+} response elicited on T-cell receptor (TCR) engagement. The Ca^{2+} signal induced on TCR engagement (encountering the antigen/major histocompatibility complex) is shown. TCR engagement leads to a rapid and transient release of Ca^{2+} from endoplasmic reticulum (ER) followed by a long-lasting stimulation of the Ca^{2+} entry through Ca^{2+} release-activated Ca^{2+} channels. The TCR-associated CD3 consists of chains γ, δ, ε, and γ as indicated in the figure. InsP3, inositol 1,4,5 trisphosphate; InsP3-R, InsP3 receptor; PLC-γ1, phospholipase-Cγ1; STIM1, stromal interaction molecule 1; SOC, store-operated calcium.

(a member of the TRP superfamily of channels), the sarco-ER calcium ATPase, and the microtubule end-binding protein (for review see [44]). According to these findings, we may envisage that similar to the TCR engagement, CD95 engagement may lead to the activation of the plasma membrane CRAC channels. However, a recent study showed a different mechanism for CD95 to orchestrate the sustained rise of cytosolic Ca^{2+} [32]. Indeed, Boehning's group pointed out that the CD95 signal triggers the release of cytochrome c by the mitochondria, which interacts with $InsP_3$-R and by this way, is supposed to fuel Ca^{2+} response, which in turn promotes the apoptotic signal [32] (Fig. 2). The notion that CD95 may induce a CRAC-dependent sustained increase in cytosolic Ca^{2+} is also challenged by observations that CD95 activates sphingomyelinases [45,46], which produce ceramide, whose sphingosine backbone hampers the activation of CRAC channels [47]. As CD95 rapidly triggers acid sphingomyelinase (increase in ceramide dosed 30 s after addition of the agonist anti-CD95 mAb) [19], one could surmise that CRAC channels are unusable in this context and thus, the mitochondrial release of cytochrome c may circumvent the blockade of CRAC channels to allow a sustained Ca^{2+} signal. However, these assumptions have to be proved, as a minimum of 1 h preincubation with CD95L is required to switch off CRAC channels in activated T-lymphocytes and thus, to prevent sustained Ca^{2+} signal on TCR activation [47].

Fig. 2



Sustained Ca^{2+} signal induced on CD95 engagement. CD95-mediated Ca^{2+} response as proposed by Boehning's group [32]. ER, endoplasmic reticulum; InsP3, inositol 1,4,5 trisphosphate; PLC-γ1, phospholipase-Cγ1; PIP2, phosphatidylinositol 4,5-bisphosphate.

Role of extracellular calcium in the CD95-mediated apoptotic signal

Intracellular concentration of free Ca^{2+} is tightly controlled and consists of approximately 100 nmol/l in the cytosol of resting cells. In contrast, the extracellular medium commonly exhibits a 10 000 fold higher Ca^{2+} concentration (approximately 1 mmol/l) and various stimuli affect the $(Ca^{2+})_i$ by promoting Ca^{2+} influx by the activation of plasma membrane Ca^{2+} channels.

With regard to the CD95 signal, initial studies ascertained that engagement of the death receptor drives a rapid and sustained increase in the $(Ca^{2+})_i$ and this response relied on both intracellular and extracellular pools of calcium [31]. It is noteworthy that in this study, researchers observed that only the intracellular pools of Ca^{2+} were implicated in the CD95-mediated DNA fragmentation as the presence or absence of extracellular Ca^{2+} did not modify the intensity of genomic DNA processing [31,48]. Strikingly, the CD95-mediated apoptotic signal was significantly enhanced in γδ T lymphocytes incubated in a Ca^{2+}-free medium compared with a normal medium [48]. Margatoxin is an inhibitor of the voltage-gated K^+ channels causing a depolarization of the membrane potential, which in turn reduces the calcium influx. The association of an agonistic anti-CD95 mAb with margatoxin resulted in a significant increase in the CD95-mediated apoptotic signal [49]. Finally, two groups observed that a $(Ca^{2+})_i$ increase protects osteoclasts and astrocytes from the initial steps of the CD95 signal [50,51]. Altogether these observations suggest that extracellular calcium may protect cells from the CD95-mediated apoptotic signal.

Molecular targets of intracellular Ca^{2+}

Several proteins whose activity is controlled by $(Ca^{2+})_i$ have been implied in proapoptotic and antiapoptotic mechanisms. Among them, some were shown to be associated with CD95 signaling. An obvious link between Ca^{2+} and proteolysis of cellular targets occurs through the activation of the Ca^{2+}-dependent cysteine proteases called calpains. These proteases, which are activated in the presence of Ca^{2+} [52], have direct impact on the execution of apoptosis by processing key elements in the apoptotic machinery such as members of the Bcl-2 family [53] or Bid [54], caspase-12 [53], and the X-linked inhibitor of apoptosis [55]. Calpains are implicated in CD95 death signaling, particularly in hematopoietic cells [56,57].

In the signaling routes of apoptosis, Ca^{2+}-dependent phosphatases also play an important role. In particular, various apoptotic routes share the activation of the Ca^{2+}-dependent serine–threonine phosphatase, calcineurin [58]. Calcineurin dephosphorylates and activates the BH3-only protein BAD, thus enhancing BAD heterodimerization with BCL-X_L and promoting apoptosis [59].

Among the various kinases directly or indirectly activated by Ca^{2+} signals, the protein kinase C (PKC) family has been proposed to play an important role in the Ca^{2+}-mediated signaling of apoptosis. The 'classical' PKCs (e.g. α, β isoenzymes) are activated by the rise of intracellular Ca^{2+} and diacylglycerol, which can be generated by the hydrolysis of phosphatidylinositol 4,5-biphosphate by phospholipase-Cγ. PKC activation enables the inhibition of TNF-related apoptosis-inducing ligand receptor-mediated apoptosis [60,61]. However, the molecular mechanism underlying this protective effect remains undefined. Gomez-Angelats and Cidlowski [62] have proposed that 'classical' PKC modulates CD95 signaling by a rapid and FLIP-independent suppression of FADD recruitment to CD95, which prevents DISC formation.

Although numerous proteins fail to bind Ca^{2+}, they undergo an alteration of their enzymatic activity on the rise of cytosolic Ca^{2+}. Indeed, the Ca^{2+}-binding protein calmodulin couples the increase in $(Ca^{2+})_i$ to the enzymatic activity of numerous proteins [63]. Three different genes (*CALM1*, *CALM2*, and *CALM3*) encode for a unique human calmodulin. On Ca^{2+} binding, the ubiquitously expressed calmodulin is activated, and mediates a plethora of fundamental processes, by its association with diverse effectors. In this regard, calmodulin is able to bind essential factors constituting the DISC such as CD95 itself [64], FADD [65], and c-FLIP [66]. Nevertheless, implication of calmodulin in the CD95-mediated apoptotic signal and even its recruitment into the DISC remain highly debated. For instance, although Ruberti *et al.* showed that the couple Ca^{2+}/calmodulin interacts with two different regions inside the DD of FADD, these interactions do not alter the CD95-mediated apoptotic signal but rather plays a crucial role in cell cycle progression [65]. In contrast, binding of calmodulin to c-FLIP or CD95 significantly hampers the CD95-mediated apoptotic signal in cholangiocarcinoma malignant cells and osteoclasts [50,64]. In addition, the same group showed that calmodulin was unable to bind FADD [66]. In summary, even if calmodulin may contribute to DISC formation and/or its regulation, it remains to decipher the molecular target(s) with which it will interact and its function in the initial steps of the CD95 signaling pathway.

Calcium/calmodulin-dependent protein kinase II (CamKII) is activated on binding to Ca^{2+}/calmodulin and it has been reported that this serine/threonine kinase prevents the initial steps of the CD95 cue [51,67,68]. For instance, CamKII activity promotes the expression of c-FLIP and PED-PEA-15 [51] that both impinge on DISC formation [22,24]. In addition, CamKII can phosphorylate c-FLIP a critical step for the recruitment of the antiapoptotic factor to the DISC [69]. Similarly, PED-PEA-15 undergoes a CamKII-driven phosphorylation on serine 116, which leads to its binding to FADD, which in turn hinders FADD/CD95 interaction [67]. Overall, these observations endow CamKII with a potent antiapoptotic function. As things cannot be so simple with Ca^{2+} signaling, a prolonged ER stress drives the increase in $(Ca^{2+})_i$ that is responsible for the activation of CamKII inducing the JNK signaling pathway, which in turn promotes the CD95 signal [70]. Consequently, the kinase activity of CamKII is able to promote the CD95-mediated apoptotic signal through the activation of JNK by the induction of a still poorly defined molecular process [71]. Cellular context and/or the $(Ca^{2+})_i$ pattern controlling the proapoptotic or the antiapoptotic role of the CamKII remain to be characterized.

Calcium

Cytosolic Ca^{2+} is the most versatile signaling molecule in biology [72]. It is a focal point of many signal transduction pathways and regulates a wide range of cellular processes including, but not limited to, fertilization, exocytosis, gene transcription, learning, and memory [73]. In addition, it is involved in exerting control over a number of opposing physiological processes, including smooth muscle contraction and relaxation [74], mitochondrial metabolism and mitochondrial-dependent apoptotic signal [75], proliferation, cell adhesion and motility [76], and apoptotic [77] and necrotic cell death [78].

As mentioned above, cytosolic Ca^{2+} originates from both internal (calcium stores) and external (channels) sources to generate signals that transduce exogenous stimulation into physiological output. It is well known that prolonged elevations of Ca^{2+} lead to cell damage or death; therefore, cells generally limit the temporal and spatial extent of their $(Ca^{2+})_i$ rises by the mean of various pumps and exchangers, which participate in returning the elevated levels of Ca^{2+} back to the resting state. Intracellular compartments also play a critical role in that they help the recovery process by taking Ca^{2+} up from the cytoplasm. Alterations in the ebb and flow of Ca^{2+} through the ER or mitochondria can lead to cell death.

Recently, evidence indicates that Ca^{2+} versatility relies on the tightly regulated spatiotemporal Ca^{2+} signals restricted to precise microdomains that contain Ca^{2+}-permeable channels, their modulators, and their downstream targets. Thus, Ca^{2+} signals with a particular spatial and temporal 'signature' could potentially underlie the generation of a specific, singular cellular process. For instance, Ca^{2+} signal triggered upon various stimuli (i.e. TCR, migration) exhibits nonhomogeneous Ca^{2+} distribution and generates the so-called Ca^{2+} microdomains or hot spots, which may account for a highly focalized function in the cell such as initial TCR activation [79] or cell steering [76]. As calcium ions normally diffuse quite fast and freely inside the cells, and thus their distribution is equalized between the different parts, cells have developed mechanisms to maintain localized calcium regions. One possibility is to gather

calcium channels in specific regions of the cell. Another possibility is to partition molecules that extrude calcium ions from the cell very close to the channels through which calcium ions flow in. In addition, membranes of intracellular organelles may form barriers limiting Ca^{2+} diffusion. Finally, high concentrations of Ca^{2+}-binding proteins to the inflowing Ca^{2+} ions can constrain the region of increased calcium concentration. Such microdomains facilitate dynamic cellular signaling by providing a perfect niche for clustering receptors and channels and bringing together signaling components that were scattered earlier.

Although most fundamental processes require Ca^{2+} release from intracellular ER stores, the influx of external Ca^{2+} participates in sustaining the Ca^{2+} response in many physiological processes. In this regard, the SOCE, which is consisted in the Ca^{2+} entry mechanism activated on Ca^{2+} depletion in the internal Ca^{2+} store, not only participates in refilling the internal ER stores, but also critically contributes to regulating cell signaling. Although the canonical members of the TRP cation channel family C and Orai-1 have been shown as putative SOC channels [33,80,81], mechanisms by which SOCE is regulated are not yet established. Lipid rafts have been shown to cluster to gather signaling complexes [82,83] and many of the regulators and ion channels orchestrating Ca^{2+} influx have been found compartmentalized into lipid rafts [84]. On the grounds that partition of CD95 into lipid rafts alters its apoptotic signal [19,26,85–91], one could argue that lipid rafts serve as platforms to bring Ca^{2+} and the CD95 machinery closer and thus, to promote or impair the initial steps of the CD95-mediated apoptotic signal. Overall, the role of calcium in the apoptotic signal induced by CD95 remains controversial and no consensus seems to be reached at this point.

Conclusion

As the $(Ca^{2+})i$ must be finely controlled temporally and spatially, disruption of the Ca^{2+}-regulating mechanisms, unless compensated by another mechanism, will result in an alteration in cell function (channelopathies). Perturbed neuronal calcium homeostasis is implicated in age-related cognitive impairment and Alzheimer's disease [92]. Ca^{2+} has also been linked to signaling pathways that are important in cancer such as migration, invasion, proliferation, and apoptosis. The role of calcium as a regulator of these processes combined with the altered expression of specific isoforms of calcium pumps and channels in some cancers has created significant interest in these proteins as novel targets for cancer drug discovery. With regard to the CD95 signaling pathway, the role of Ca^{2+} remains poorly defined but we can surmise that the recent identification of the proteins constituting the CRAC channels and their mechanisms of activation will revisit the role of Ca^{2+} influx in death receptor signaling.

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