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RhoA signaling in phorbol ester-induced apoptosis

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Summary

Exposure of cells to phorbol ester activates protein kinase C (PKC) to induce apoptosis or differentiation, depending on the cellular context. In erythroblastic cell lines, TF-1 and D2, upregulation of the RhoA signaling promotes phorbol ester-induced apoptosis through activating Rho-associated kinase (ROCK)/phosphorylation of myosin light chain (MLC), thus generating membrane contraction force. As a result, cell adhesion is inhibited and death receptor-mediated death pathway is activated in these cells with a concurrent changes in nucleocytoplasmic signaling for protein trafficking. A microtubule-regulated GEF-H1, which is a specific RhoA activator, was identified to contribute to RhoA activation in these cells. Thus, a cytoskeleton-regulated RhoA signaling cooperates with PKC activation constitutes a cellular context to determine the cell fate in response to phorbol ester stimulation.

Introduction

Tissue homeostasis in multicellular organism is achieved by coordination and balance between cell proliferation, differentiation and apoptosis. The molecular mechanisms for integrating signals from the environment to achieve this orchestral coordination are complicated and dynamic. To dissect the interaction between signal pathways from environment cue and the existing cellular context, it is necessary to first define the molecular context responsible for cell fate decision in response to one particular type of stimulus. When exposed to phorbol-12-myristate-13-acetate (PMA), an activator of novel and classical PKCs, hematopoietic cells can be induced to differentiation or apoptosis depending on the cell types [1–6]. Presumably, signal from PKC activation emerges different signal pathways in the cells, resulting in differential cellular responses [7]. We previously used erythroblastoma cells as a model system to study the molecular context responsible for PMA-induced apoptosis, which led us to find the involvement of RhoA signal pathway. In this review article, we will discuss how the molecular context regulated by RhoA signaling leads to PMA-induced apoptosis.

The growth of myeloid progenitor TF-1 cells is dependent on granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin 3 [8]. Upon treatment with PMA, approximately 15% of the cells differentiate into macrophage-like cells and attach to the culture dishes, whereas the other 85% of the population remain in suspension and become apoptotic [9]. A few TF-1 variants, which grow autonomously in medium without supplementation of GM-CSF or interleukin 3, have previously been selected and characterized [9]. D2 is one of the variant cell lines that show 50% of the cells differentiating and the other 50% apoptotic after PMA stimulation [9]. Removal of serum resulted in all D2 or TF-1 cells adherent to the culture dishes and prevented cells from PMA-induced apoptosis, while addition of

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lysophosphatidate (LPA) to serum-free medium increased PMA-induced apoptosis [10]. Prevention of cell adhesion by plating cells onto hydrogel-coated dishes caused all cells apoptosis upon PMA treatment [11], indicating that the LPA-mediated signal pathway changes cell adhesion capacity, thus promoting PMA-induced apoptosis. Expression of C3, a specific inhibitor of RhoA, was able to abrogate LPA-mediated promoted apoptosis, and expression of dominant active form of G α12/13 increased PMA-induced apoptosis in serum-free medium. These evidence clearly show that LPA/GPCR/G α12/13/RhoA pathway contributes to a cellular context that affects the cellular response to PMA induction [10]. On this basis, a series of downstream events of RhoA pathway were found to be involved in PMA-induced apoptosis in this model system.

RhoA-signaling and morphological change

The Rho GTPases are a family of proteins involved in multiple cellular processes, including cytoskeletal organization, gene expression and transformation [12, 13]. Among them RhoA is known to coordinate with other signal pathway to control dynamic rearrangements of the actin cytoskeleton, which are central to the morphological changes observed in apoptosis [14–16]. It has been shown that activation of RhoA is responsible for the contraction observed in the apoptotic cells induced by serum starvation, and ectopic expression of dominant active form of RhoA is sufficient to induce apoptosis [17–19]. Among the diverse downstream effectors of RhoA, two forms of serine/threonine kinases (ROCK I and ROCK II) have been shown to mediate RhoA signaling in membrane contraction force during apoptosis [20, 21]. The kinase activity of ROCK is autoinhibited via the interaction between its C-terminal inhibitory domain and N-terminal kinase region. Upon binding to the GTP-bound form of RhoA at its C-terminal inhibitory region, the kinase activity of ROCK is enhanced due to the relief of autoinhibition [20, 22]. ROCK regulates the phosphorylation of myosin light chain (MLC) by directly phosphorylating MLC at Thr18 and Ser19, and by the inactivation of myosin phosphatase [23, 24]. Phosphorylation of MLC activates myosin ATPase activity, which couples with actin-myosin filaments to the plasma membrane, thus increasing the actomyosin force generation and cell contractility. It has been shown that in apoptotic cells ROCK I is cleaved by caspase-3, which removes its C-terminal inhibitory region [25, 26], thus activating its kinase function and increasing MLC phosphorylation, resulting in membrane contraction. This mechanism contributes to formation of membrane blebbing in apoptotic cells. Distinct from these late-stage-apoptotic cells with caspase-3 activation, in PMA-induced pro-apoptotic cells ROCK-mediated MLC phosphorylation indeed occurs earlier than caspase-3 activation [11]. In fact, inhibition of ROCK by its specific inhibitor, Y27632 [27], prevented PMA-induced activation of caspase-3 in suspension cells, thus abrogating apoptosis. Therefore, ROCK activation in PMA-induced pro-apoptotic cells acts as an upstream event required for membrane contraction and the subsequent caspase-3 activation during PMA stimulation [11].

We have compared the distribution of F-actin in PMA-induced pro-apoptotic and differentiating cells. As a contrast to untreated D2 cells where cortical F-actin was detected, one pronounced F-actin aggregate was specifically observed in pro-apoptotic population of cells [unpublished data], accompanied by a significant increase of MLC phosphorylation. Perhaps, too much contraction in pro-apoptotic cells caused a severe collapse of F-actin network, disrupting the interconnection between plasma membrane and F-actin-mediated structure. This structural change may therefore affect membrane microdomain, disabling the function of membrane receptors required for adhesion and death signaling.

Activation of RhoA signaling in PMA-induced pro-apoptotic cells

Rho A cycles between a GDP-bound inactive state and a GTP-bound active state. Activation is accomplished by guanine nucleotide exchange factors that (GEF) favor the release of bound GDP and subsequent GTP loading. Currently, LARG [28, 29], PDZ-RhoGEF [30], p115RhoGEF [31], AKAP-Lbc [32], and GEF-H1 are the known RhoA GEF proteins controlling RhoA activation. By GST-RhoA pull-down analysis, we identified GEF-H1 abundantly present in the cytosolic soluble fraction of D2 cells [33]. GEF-H1 is a

microtubule-associated GEF, whose function in RhoA depends on its subcellular localization [34–36]. In HeLa cells, GEF-H1 associates with microtubules, where GEF-H1 loses its RhoA activation function. During microtubule depolymerization in mitosis or nocodazole-treated cells, GEF-H1 is released to the cytoplasm and activates RhoA [34], which plays a role in generating the contractile force during mitosis [37]. Consistent with its localization-dependent function, in PMA-induced pro-apoptotic cells, GEF-H1 stayed in cytoplasm fraction, whereas in the attached cells GEF-H1 is associated with microtubules [33]. Depletion of GEF-H1 expression in D2 cells under serum-free incubation condition caused a significant reduction of RhoA activity. As a result, these cells did not undergo PMA-induced contraction and the subsequent cell death was prevented. Accordingly, our current hypothesis is that cytosolic GEF-H1 in suspension D2 cells constitutes a cellular context for sustained activation of RhoA, which is required for ROCK-dependent contraction in response to PMA stimulation. In PMA-induced differentiating cells, GEF-H1 is sequestered by the more organized microtubules and is unable to activate RhoA/ROCK signaling, thus allowing cells to survive with adhesion and spreading. At present, the mechanism underlying the differential subcellular localization of GEF-H1 in PMA-induced pro-apoptotic and differentiating cells remains to be investigated. Since microtubule organization is very different in PMA-induced pro-apoptotic cells and adherent cells, it is likely that there is positive feedback in RhoA activation by microtubule disassembly during apoptotic induction.

Nucleocytoplasmic trafficking

The nucleocytoplasmic shuttling of signaling factors confers the spatial regulation of protein activity and may also acts as an integral part of the apoptotic program by controlling apoptotic regulators migrated in and out of the nucleus. Several proteins implicated in apoptotic execution including FADD [38], TRADD [39], DEDD [40], and Apaf-1 [41, 42], have been shown to change their nucleoplasmic trafficking in response to apoptotic stimulation. Relevant to this, our laboratory has previously shown that activated extracellular signal-regulated kinase (ERK) is unable to be translocated into nuclei in PMA-induced pro-apoptotic TF-1 and D2 cells, and this disabled translocation of activated ERK is responsible for lack of cyclin kinase inhibitor p21 induction in response to PMA in this population of cells [43]. Inhibition of ROCK by its specific inhibitor Y27632 could rescue these cells from PMA-induced apoptosis and restore nuclear translocation of activated ERK, indicating that ROCK-mediated signal also affects protein trafficking during apoptosis [43]. Here, it should be mentioned that the nuclear translocation of other protein with NLS-signal is not affected by PMA-induced contraction in our cell system. Although it has shown that adhesion is required for ERK nuclear translocation, we found that the relief of contraction force by inhibiting actin polymerization can restore nuclear translocation of phosphorylated ERK and prevent apoptosis in the suspension D2 and TF-1 cells, indicating that adhesion does not play a necessary role in ERK translocation. Rather, it is surely that contraction force specifically exerts an inhibitory effect on its transport machinery.

In addition to the effect on phosphorylated ERK nuclear translocation, ROCK activation in PMA-induced pro-apoptotic cells causes cytosolic translocation of nuclear protein hnRNP C1/C2 independent of caspase activation [44]. This translocation phenomenon can also be observed in TNF α-induced apoptotic NIH3T3 cells, where ROCK I activation is dependent on caspase-3-mediated cleavage. The hnRNP C1/C2 proteins are members of a large family of RNA-binding proteins that influence pre-mRNA processing, mRNA metabolism, and mRNA transport [45–50]. Because other major hnRNP proteins, hnRNP A2/B1, nuclear matrix proteins, lamin A/C, and general transcription factor Sp1 still stay in the nuclear compartment of PMA-induced pro-apoptotic cells, efflux of hnRNP C1/C2 is a rather specific event controlled by ROCK activation [44]. Unlike other hnRNP members which shuttles between nucleoplasmic and cytoplasm compartments, hnRNP C1/C2 are nuclear restricted proteins due to the presence of their nuclear retention sequence (NRS) [51]. Various C-terminus deleted forms of hnRNP C1, which contain NRS, are retained in nuclei irrespective to expression of dominant active form of ROCK(CAT), indicating

that the efflux of hnRNP C1/C2 proteins is not due to the impairment of NRS-mediated nuclear retention mechanism. Since overexpression of dominant active form of ROCK is sufficient to induce a nuclear reporter YFP fused with C-terminal 40 amino acids of hnRNP C1, this sequence is considered as a novel ROCK-responsive nuclear export sequence (NES) [44].

Since depletion of hnRNP C1/C2 does not cause cell death [52], the physiological meaning of ROCK-responsive translocation of hnRNP C1/C2 in apoptosis awaits further investigation. It will be interesting to know whether this efflux process may mediate the exclusion of other protein important for nuclear structure and function, thus making a significant alteration in nuclear morphology and function in apoptosis. In addition to the function of hnRNPC1/C2 in nuclei, the hnRNPC1/C2 proteins have also been found to bind to mRNA to mediate cap-independent translation by the mechanism involving internal ribosome entry site (IRES) [53–55]. Member of the inhibitor of apoptosis (IAP) (XIAP) is known to be translated using an IRES, allowing for the continued translation of XIAP under condition where cap-dependent translation is inhibited in apoptosis. It has been shown that the binding of hnRNP C1/C2 to IRES increases XIAP IRES function. Accordingly, it is, therefore, possible that ROCK-mediated shuttling hnRNP C1/C2 to cytoplasm may also provide a means to regulation XIAP protein expression under pro-apoptotic stress.

The involvement of death pathway

In PMA-induced apoptotic D2 cells, activation of caspase-3 is seen after 6 h of PMA treatment. Co-incubation with general caspase inhibitor does not affect PMA-induced contraction, but abrogates cell death, indicating that contraction acts upstream of caspase activation [11]. It is well established that both intrinsic and extrinsic pathways can activate caspase-3 in response to various apoptotic stimuli [56]. A pro-apoptotic signal generated from death receptor (extrinsic) and mitochondria (intrinsic) can activate an initiator or upstream caspase, which usually possesses a long NH_2-terminal prodomain such as caspases-8, −10, and −9, respectively [57–59]. In turn, these initiators can activate the effector caspases, such as caspases-3, −6, which result in apoptotic execution. One of the best-defined apoptotic pathways is mediated by the death receptors such as CD95 or tumor necrosis factor receptors (TNFRs). Upon ligand binding, the intracellular death domain of death receptor recruits Fas-associated death domain protein (FADD) through protein–protein interaction. FADD links the receptor to the apoptotic caspase, pro-caspase-8 or −10, through homotypic interactions of death effector domains (DED), to form a death-inducing signaling complex (DISC); this in turn leads to oligomerization and activation of these two zymogens by self cleavage and the subsequent apoptotic cascade [60–63].

Unlike the finding showing that ROCK is activated by caspase-3-mediated cleavage in apoptotic cells, in PMA-induced apoptotic D2 cells, ROCK activation works upstream of caspase-3 activation. In these apoptotic cells, we found that both caspase-8 and −10 are the apical caspases and their activation is a result of membrane contraction dependent on ROCK, by which effector caspase-3 is activated to trigger this apoptotic pathway. Moreover, complex containing endogenous FADD with procaspase-10 is preferentially formed in PMA-induced pro-apoptotic cells, but not the survived cells [11]. Since inhibition of ROCK prevents caspase activation, we conclude that activation of RhoA/ROCK/MLC phosphorylation pathway in cooperation with PMA signaling in cells provides a cellular context that generates an initial membrane contraction, leading to activation of caspase-8 and −10 through a mechanism involving membrane receptor-mediated signaling. Relevant to this observation, it has been reported that MLCK-mediated MLC phosphorylation increases translocation of tumor necrosis factor receptor (TNFR) to the plasma membrane independent of TNF signal, which in turn activates caspase-8 to initiate the apoptotic pathway [64]. Other study has also shown that treatment of cells with cytoskeleton disturbing reagent cytochalasin B increases clustering of the CD95 receptor to activate caspase-8 and enhances UV-induced apoptosis [65]. Therefore, it seems to be general that actomyosin-mediated contraction force can act as a factor promoting activation of death receptor pathway.

Distinct to our findings, other study has shown that phorbol ester induces apoptosis in U937 cells,

in part, through a pathway, which requires endogenous production of TNF α depending on activation of MEK/ERK during stimulation [66]. In the case of PMA-induced apoptosis of androgen-dependent prostate cancer cells, it has been shown to PKC δ participates in activation of the extrinsic death receptor pathway via TNF α and TRAIL receptors by stimulating the releases of their ligands [67]. However, PMA-induced apoptosis in TF-1 cells did not require newly synthesized protein, neither did pre-incubated cells with recombinant TNF receptor R1 protein to sequester TNF ligand prevents PMA-induced apoptosis, thus excluding the possibility that TNF ligand binding is involved in this apoptotic process [11]. It should be mentioned that expression of dominant negative form of FADD decreased but not completely abolished PMA-induced apoptosis in D2 and TF-1 cells, implying that the death receptor-mediated pathway probably only plays a partial role in PMA-induced apoptosis. It has been demonstrated that unligated integrins recruit caspase-8 to the membrane and form the DISC without FADD, suggesting the presence of a death receptor-independent caspase-8 activation mechanism [68]. Therefore, it will be interesting to further look at the involvement of other receptor pathway in this apoptotic process.

Action of protein kinase C and RhoA signaling in apoptosis

PKC is a family of phospholipids-dependent serine/threonine kinases comprising 10 isoenzymes [69]. These PKC isoenzymes are subdivided into three classes (i) the 'conventional' PKCs PKC α PKC β PKC γ which can be activated by phosphatidylserine, diacyl glycerol (DAG), or phorbol esters through binding to the C-1 domain and Ca^{2+} through binding to C-2 region; (ii) the 'novel' PKCs PKC δ, PKC ε, PKC θ, PKC η lack the C-2 region and thus are calcium-independent but still DAG-, phosphatidylserine-, and phorbol ester-responsive; and (iii) the 'atypical' PKCs PKC λ/ι and PKC ξ also lack the C-2 region and a functional DAG-binding site; hence they are only responsive to phosphatidylserine but not to DAG or phorbol ester. Since PKC inhibition blocked PMA-induced apoptosis in D2 cells [unpublished data], one important question is which PKC isoform works together with RhoA signaling to trigger apoptosis. Several studies showed the linkage between PKC and RhoA signaling [70–73]. PKC-induced activation of Src kinase activity has been shown to phosphorylate p190RhoGAP, which subsequently down-regulates RhoA activity [74]. On the other hand, it has been shown that RhoA is activated by PKC-mediated phosphorylation. In our system, we found expression of dominant active form of ROCK is insufficient to induce apoptosis. Therefore, it is likely that other signal pathway via PKC activation is also required for PMA-induced apoptosis.

PKCs reside in the cytosol in an inactive conformation and translocate to the membrane upon activation where they modify various cellular functions through phosphorylation of target substrates [75]. In both PMA-induced differentiating and pro-apoptotic fractions of D2 cells, there was no difference in membrane translocation of various PKC isoforms, $\alpha, \beta, \gamma, \delta, \varepsilon$ and θ [10]. although activation of PKC δ has been shown to induce apoptosis in leukemic cells, we did not find that expression of dominant negative form of PKC δ has an effect on reducing PMA-induced contraction. Nevertheless, it is still possible that the differences in cytoskeletal reorganization of suspension and adhesion cells make the difference in downstream events of PKC activation, which emerges RhoA/ROCK signaling in membrane contraction for death receptor-mediated caspase activation. From this point, it should be mentioned again, activated ERK via PKC pathway stayed in the cytosol of suspension pro-apoptotic cells. Inhibition of ERK activation by MEK inhibitor U0126 prevented PMA-induced apoptosis, indicating PKC–ERK pathway also participates in death induction. So, how does activated ERK in cytosol get involved in contraction for apoptotic induction? It appears that death-associated protein kinase (DAPK) in cytosol interacts with ERK [76]. DAPK is a DD-containing calmodulin (CaM)-regulated serine/threonine kinase, which functions as a positive mediator of apoptosis induced by a variety of stimuli, such as interferon-γ, Fas, TNF-α, TGF-β, ceramide and oncogenes c-myc and E2F [77–83]. Phosphorylation of DAPK by cytosolic sequestered ERK increased its *in vitro* and *in vivo* kinase activity. Knock down of DAPK by RNA interference increased cell survival after PMA stimulation [76],

suggesting DAPK plays a key role in the downstream effector of PKC/ERK pathway in PMA-induced apoptosis. Although MLC is a substrate of DAPK *in vitro*, we did not find extent of MLC phosphorylation to be affected by inhibition of ERK activation, excluding the possibility that the ERK–DAPK pathway in PMA-induced pro-apoptotic cells can confer the increment of MLC phosphorylation. At present, it remains a challenge to dissect the signaling events regulated by individual PKCs and DAPK in collaboration with ROCK activation for generating the apoptotic contractility in PMA-induced pro-apoptotic cells.

Conclusion remark

In summary, studies on PMA-induced pro-apoptotic in TF-1 and D2 cells have introduced a novel paradigm that cytoskeletal organization regulated by RhoA signaling plays a pivotal role in affecting the flow of PKC signaling pathways, thus eliciting effect on both nucleoplasmic trafficking and death receptor pathways. Understanding the interaction between cytoskeletal regulation and PKC activation pathway may have significant therapeutic relevance, since PKC activators (including phorbol esters) are in clinical trials for various types of cancers [84–86].

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