Neutrophil spontaneous death is mediated by down-regulation of autocrine signaling through GPCR, PI3K γ , ROS, and actin

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Neutrophil spontaneous apoptosis plays a crucial role in neutrophil homeostasis and the resolution of inflammation. We previously established Akt deactivation as a key mediator of this tightly regulated cellular death program. Nevertheless, the molecular mechanisms governing the diminished Akt activation were not characterized. Here, we report that Akt deactivation during the course of neutrophil spontaneous death was a result of reduced PtdIns(3,4,5)P3 level. The phosphatidylinositol lipid kinase activity of PI3K γ , but not class IA PI3Ks, was significantly reduced during neutrophil death. The production of PtdIns(3,4,5)P3 in apoptotic neutrophils was mainly maintained by autocrinely released chemokines that elicited PI3K γ activation via G protein-coupled receptors. Unlike in other cell types, serum-derived growth factors did not provide any survival advantage in neutrophils. PI3K γ , but not class IA PI3Ks, was negatively regulated by gradually accumulated ROS in apoptotic neutrophils, which suppressed PI3K γ activity by inhibiting an actin-mediated positive feedback loop. Taken together, these results provide insight into the mechanism of neutrophil spontaneous death and reveal a cellular pathway that regulates PtdIns(3,4,5)P3/Akt in neutrophils.

Akt | apoptosis | reactive oxygen species

Neutrophils are the most abundant cell type among circulating white cells and are the major players in the innate immune system. Neutrophils are terminally differentiated and normally have a very short lifespan (7–20 hr) in circulation and in tissue (1–4 days) (1). The daily turnover of human neutrophils is $0.8\text{--}1.6 \times 10^9$ cells per kg of body weight. The same number of neutrophils need to die to keep cellular homeostasis under physiologic condition.

Neutrophils die even in the absence of any extracellular stimuli; thus, this type of death is also called spontaneous death. It shares many features of classical apoptosis, such as cell body shrinkage, cellular crenation, exteriorization of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane, vacuolated cytoplasm, mitochondria depolarization, nuclear condensation, and internucleosomal DNA fragmentation (2, 3). Neutrophil death can be modulated by various extracellular stimuli such as proinflammatory cytokines, cell adhesion, phagocytosis, red blood cells, and platelets. Under most conditions, neutrophils will be exposed to both pro- and antiapoptotic factors. The net effect on neutrophil death and survival reflects a balance between the activities of such factors. Constitutive neutrophil death is associated with up-regulation of death signaling and down-regulation of survival signaling. We recently reported that the activity of protein kinase B (PKB)/Akt, a well known prosurvival and antiapoptotic factor, decreases dramatically during the course of neutrophil death. Both PI3 kinase and Akt inhibitors enhance neutrophil death. Conditions delaying neutrophil death, such as treatment with GM-CSF, G-CSF, or IFN- γ , restore Akt activity. Neutrophils depleted of PTEN, a phosphatidylinositol 3'-phosphatase that negatively

regulates Akt activity, live much longer than wild-type neutrophils (4, 5). However, the molecular mechanisms by which PtdIns(3,4,5)P3/Akt activity is down-regulated during neutrophil spontaneous death remain ill defined.

In the present study, we identified an autocrine signal pathway that is involved in the down-regulation of PtdIns(3,4,5)P3/Akt activity during neutrophil spontaneous death. Our data demonstrate that the activity of Akt in apoptotic neutrophils is mainly maintained by autocrinely released chemokines that elicit PI3K γ activation via G protein-coupled receptors. Reactive oxygen species accumulated in apoptotic neutrophils, by blocking an actin-mediated positive feedback loop, serve as a physiological negative regulator of PI3K γ and the subsequent PtdIns(3,4,5)P3 production and Akt activation.

Results

Akt Deactivation During the Course of Neutrophil Spontaneous Death Is a Result of Reduced PtdIns(3,4,5)P3 Level. We have demonstrated that Akt deactivation is a causal mediator of neutrophil spontaneous death, but the molecular mechanisms by which Akt activity is down-regulated have not been fully investigated (4). Akt activation relies on its membrane translocation mediated by its specific association with PtdIns(3,4,5)P3 on the plasma membrane. Only the Akt molecules on the plasma membrane can be phosphorylated and activated. The level of active Akt (phospho-Akt) drastically declines during neutrophil death, whereas total Akt does not change (Fig. 1A–C), suggesting that the decrease of Akt activity is not a result of protein degradation. Akt membrane translocation and subsequent activation was previously thought to depend solely on concentrations of PtdIns(3,4,5)P3 in the membrane (6, 7). Recently, we demonstrated that two inositol phosphates, InsP7 and Ins(1,3,4,5)P4, compete for Akt-PH domain binding with PtdIns(3,4,5)P3 both in vitro and in vivo, providing another level of regulation for Akt membrane translocation and activation (8, 9). However, the levels of InsP7 and Ins(1,3,4,5)P4 are extremely low in unstimulated neutrophils, suggesting that the decreased Akt activation is likely caused by the decrease of PtdIns(3,4,5)P3 production (8) (Fig. S1). To confirm this, we measured the level of PtdIns(3,4,5)P3. Our results show that during the course of neutrophil death, levels of PtdIns(3,4,5)P3 decrease dramatically, whereas levels of PtdIns(4,5)P2, the substrate of PtdIns

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Fig. 2. Deactivation of PtdIns(3,4,5)P3/Akt signaling during neutrophil spontaneous death is a result of reduced PI3K γ activity. (A) The level of PTEN in normal and apoptotic neutrophils. Shown is the result of a representative experiment that was repeated three times. (B) The level of SHIP in normal and apoptotic neutrophils. Total and phosphorylated SHIP were detected by Western blot using anti-SHIP and anti-phospho-SHIP antibodies, respectively (Cell Signaling). Shown is the result of a representative experiment that was repeated three times. (C–E) The enzymatic activity of PI3K class IA is not altered during neutrophil spontaneous death. (C) The three isoforms of PI3K class IA were pulled down with a PI3K p85 antibody (Upstate Biotechnology). Neutrophil whole-cell lysates and immunoprecipitated samples were blotted with indicated PI3K antibodies. Shown is the result of a representative experiment that was repeated three times. (D) PI3 kinase activity of the immunoprecipitated enzymes. Shown is the result of a representative TLC plate. At each indicated time point, the kinase reaction was stopped and the lipids were extracted and analyzed by TLC. The positions of individual phosphatidylinositol were assigned from their migration distance matching those of corresponding authentic 32 P-labeled standards. The amount of 32 P-labeled PtdIns(3)P was quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics). The PI3 kinase activity was expressed as the percentage of activity at time 3 hr (healthy neutrophils). (E) The data of the densitometric analyses are expressed as the percentage of the normal neutrophil control (3 hr). All values represent mean $\pm$ SD of three separate experiments. (F and G) The enzymatic activity of PI3K class IB (PI3K γ) is down-regulated during neutrophil spontaneous death. (F) PI3K γ was pulled down with a p110 γ antibody (Upstate Biotechnology). Neutrophil whole cell lysates and immunoprecipitated samples were blotted with a rabbit polyclonal anti-p110 antibody. Shown is the result of a representative experiment that was repeated three times. (G) PI3 kinase activity of the immunoprecipitated enzymes. Shown is the result of a representative TLC plate. (H) The data of the densitometric analyses are expressed as the percentage of the normal neutrophil control. All values represent mean $\pm$ SD of three separate experiments.

control cells cultured in the presence of serum (Fig. 3A). This result is consistent with the fact that class IA PI3Ks are not involved in regulating PtdIns(3,4,5)P3 signal during neutrophil death (Fig. 2).

In neutrophils, PtdIns(3,4,5)P3 signal can also be elicited by heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins)-coupled receptors. Chemokines bind receptors on cell membrane and induce the dissociation of a specific G protein into α and $\beta\gamma$ subunits. Released $\beta\gamma$ subunits are able to directly initiate activation class IB PI3K (PI3K γ) (1, 6). We have shown that deactivation of PI3K γ , but not class IA PI3Ks, was responsible for Akt deactivation during neutrophil death, suggesting the involvement of GPCR-mediated pathways in regulating neutrophil death. We explored the role of GPCR using a bacterial-derived toxin, pertussis toxin (PTX), which catalyzes ADP ribosylation of G proteins and thus suppresses their activation. We found a significant accelerated death in PTX-treated neutrophils (Fig. 3B). At 9 hr in culture, PTX-treated neutrophils showed nearly one fold higher death rate than untreated neutrophils. Similar results were obtained at 15 and 24 hr. The difference became nonsignificant at 48 hr, because most untreated neutrophils also became apoptotic.

Because serum deprivation did not affect the half-life of neutrophils, the factors leading to GPCR activation might be produced by the cultured neutrophils in an autocrine manner. To test this, we cultured neutrophils in serum-free medium and

examined the secreted “GPCR activating activity” in the supernatants (Fig. 3C). Uniform treatment of freshly isolated neutrophils with chemokines or formyl-peptide (e.g., fMLP) elicits instant GPCR activation and elevation of PtdIns(3,4,5)P3 in the plasma membrane (17). We evaluated GPCR-elicited PtdIns(3,4,5)P3 signaling by measuring the level of endogenous Akt phosphorylation. Before chemoattractant stimulation, Akt phosphorylation was virtually undetectable in neutrophils (5). Upon stimulation, neutrophils showed maximum Akt phosphorylation at 2 min, which then declined marginally by 5 min. We used the level of Akt phosphorylation at 3 min after stimulation to assess GPCR activation. Our results showed that a large amount of “GPCR activating activity” was secreted and accumulated in the culture medium (Fig. 3C). Its ability to induce Akt phosphorylation was completely inhibited by PTX, further demonstrating that the activity of these secreted factors was indeed mediated by GPCR. Supporting this autocrine chemokine release mechanism, a significant amount of CXC chemokine IL8 was detected in the neutrophil culturing medium (Fig. 3D). IL8 can bind and activate G protein-coupled CXCR1 and CXCR2 receptors leading to activation of PI3K γ and Akt (18). Thus, IL8 should be one of the neutrophil-released chemokines that support neutrophil survival. However, numerous chemokines can be produced by neutrophils; it is unlikely that IL8 will be the only one playing a role in neutrophil spontaneous death.