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The Early Wound Signals

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Abstract

Wounding of tissue barriers, such as epithelia, disrupts homeostasis and allows infection. Within minutes, animals detect injury and respond to it by recruitment of phagocytes and barrier breach closure. The signals that activate these first responses are scarcely known. Commonly considered are cytoplasmic factors released into the extracellular space by lysing cells (Damage Associated Molecular Patterns, DAMPs). DAMPs activate transcription through pattern recognition receptors. But the promptness of wound responses is difficult to explain by transcriptional mechanisms alone. This review highlights the emerging role of nonlytic stress signals in the rapid detection of wounds.

Introduction

With every minute that an open wound exposes the organism's interior to the outside environment, the likelihood of infection and secondary host damage rises. Owing to the frontline role of epithelia in pathogen protection, evolution of efficient wound detection mechanisms is driven by high selective pressure. This review briefly summarizes conserved damage and stress signals involved in early wound detection (Figure 1).

New approaches for studying early wound signaling in vivo

Systematic research into early wound signaling has been greatly facilitated by imaging approaches that allow measuring cell biological responses to tissue injury in real-time within the intact organism. To this end, animal models such as *C. elegans*, *drosophila* and zebrafish larvae have become popular owing to their microscopic and genetic tractability. For example, the caudal tail fin fold of zebrafish larvae is a small, flat, non-vascularized, transparent tissue that is excellently suited for live imaging of inflammatory and healing responses to wounding by a laser beam or a microneedle. The innate immune system of zebrafish closely reflects that of higher vertebrates. All major cell types, including neutrophils and macrophages, are present. The model provides a simple, yet powerful way to study how leukocytes, and other cell types, rapidly perceive information on tissue damage

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Conflict of interest

The author declares no conflict of interest.

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over distances within an intact tissue. Fluorescent biosensors for wound signals, such as Ca^{2+} or hydrogen peroxide (H_2O_2) can be expressed in wound-relevant cell types (e.g., immune/epithelial/endothelial cells, etc.) using tissue-specific promoters. Imaging biochemical together with morphological responses reveals when and where a cell senses a wound signal, and whether or how it directly reacts to it. In combination with molecular pathway perturbation and in vitro reconstitution, this general approach has been applied to tease apart some of the early wound signaling circuits discussed below.

Cell lysis signaling by DAMPs

Even before biosensor imaging enabled direct peeks into the biochemical events following injury, it was clear that cell lysis presents a crucial initial tissue damage cue in many situations. Cell lysis at the wound site causes DAMP leakage into the extracellular space. Some DAMPs, such as formylated peptides and ATP, can act as chemoattractants by binding to G-protein coupled receptors on leukocytes [2–6]. Formylated peptides are released by damaged host cells or bacteria. Host derived formylated peptides attract interstitial neutrophils to sites of liver damage [3], but do not mediate early neutrophil recruitment to dermal injury in mouse [7]. Also the chemotactic activity of ATP is context dependent [8–10]. Aside from sporadically described chemotactic activity, most DAMPs are best known for activating proinflammatory gene transcription through Toll-like (TLR), NOD-like (NLR), RAGE, Mincle, or interleukin 1 (IL1) receptors in tissue resident sentinel cells (for example, Kupffer cells in the liver). After DAMP exposure, these cells synthesize various inflammatory mediators, including IL1. Pro-IL1 is processed by caspase-1 into the mature cytokine. Caspase-1 is activated by the inflammasome, a macromolecular complex of NLR subunits that senses molecular signs of host damage or infection. IL1 coordinates local and systemic inflammatory response, such as vascular adhesion of leukocytes or fever. The above mechanisms are extensively reviewed elsewhere [1,11,12].

In the zebrafish tail fin wounding model, first motile responses of leukocytes and epithelial cells are observed within 5 minutes after injury [10,13,14], too fast to be regulated by de novo gene expression [15]. Aside from sporadic cases of DAMP-induced chemotaxis (see above), other immediate wound signaling functions of DAMPs are little described, but conceivable. Morpholino depletion of Myd88, an essential adaptor protein for DAMP signaling through TLR and IL1-receptors, suppresses immediate leukocyte recruitment to zebrafish tail fin wounds [16] consistent with an involvement in early, chemotactic wound signaling. Likewise, caspase-1 can trigger rapid eicosanoid synthesis [17], pointing out a potential link between inflammasome-mediated sensing of DAMPs, and nontranscriptional, inflammatory signaling. With internal tissue damage, it is not difficult to picture how DAMPs released by cell lysis are retained as extracellular gradients near the injury site for a while, considering the interstitial confinement of the tissue. The same is more difficult to imagine for epithelial wounds exposed to a large volume of environmental or luminal fluid, such as fish epidermis or stomach mucosa. Here, DAMPs are likely to be instantaneously rinsed out and diluted. Are there mechanisms that could provide more reliable damage detection under such circumstances? Recent concepts of epithelial wound detection highlight the role of prompt enzymatic synthesis of paracrine regulators by sublethally stressed cells at the injury site. Two important classes of such signals are reactive oxygen

species (ROS) and polyunsaturated fatty acids (PUFAs), namely arachidonic acid (AA) in animals, and linolenic acid (LA) in plants. Each class is triggered by elevation of cytoplasmic calcium concentration ($[Ca^{2+}]$).

Cell stress signaling by ROS

ROS production is a conserved wound response of plants and animals [18]. ROS are a group of small, oxygen containing molecules generated as constitutive by-products of aerobic metabolism, or after cell stimulation. Primary sources of cellular ROS are mitochondria and NADPH oxidases. ROS ions or radicals (O^{2-} , $HO\bullet$) are highly reactive and oxidize any biomolecule they encounter. They cannot diffuse far from their source, and are little suited as paracrine signals. In contrast, H_2O_2 is less reactive and can travel over microns, or tens of microns in the cytoplasm or interstitial space, respectively. This principally allows H_2O_2 to convey long-range signals. H_2O_2 reversibly modifies cysteine residues thereby altering protein function [19]. Regulatory ‘thiol switches’ occur in various signaling proteins, including tyrosine kinases, phosphatases, and small GTPases, and are involved in rapid wound signaling. In injured zebrafish larvae, the Ca^{2+} -dependent NADPH oxidase Duox generates an H_2O_2 gradient that extends ~ 150 μm from the wound site into the unwounded tissue, and mediates rapid leukocyte recruitment as shown by live imaging [13]. A similar mechanism was described for drosophila larvae [20]. Also plants use a Ca^{2+} activated NADPH oxidase (RBOHD) to generate ROS in response to wounding [21]. To stimulate immune cell chemotaxis and possibly DAMP recognition, extracellular H_2O_2 enters the cytoplasm to modify a thiol switch in the tyrosine kinase LYN [22,23]. In addition, ROS influence later wound responses by promoting chemokine transcription [24,25] and regenerative events in zebrafish and *Xenopus* tadpoles [26–29]. In *C. elegans* a thiol switch in the small GTPase RHO-1 controls epidermal wound closure by regulating actin polymerization at the wound margin [30]. Inactivation of RHO-1 through cysteine oxidation promotes actin polymerization, and accelerates epidermal closure. Wound-induced ROS in *C. elegans* derive from mitochondria that undergo a Ca^{2+} dependent permeability transition [30].

It is still unclear how redox sensitive signaling proteins can compete with highly reactive and abundant cellular antioxidants, such as peroxiredoxins, for diffusible oxidants, such as H_2O_2 . Peroxiredoxins react multiple orders of magnitude faster with H_2O_2 than known redox-sensitive cysteines in transcription factors, kinases or phosphatases. This raises the question under which specific conditions signaling proteins may encounter levels of oxidants sufficient to directly turn their thiol switches. Possibly, thiol switches are not the only way by which ROS and NADPH oxidases exert regulation during wound detection [31]. In zebrafish larvae, the oxidized AA derivate 5-oxo-ETE, or a closely related chemical, mediates early attraction of leukocytes to tail fin wounds [10]. 5-oxo-ETE is generated by activity of a NADP⁺ dependent enzyme 5-hydroxyeicosanoid dehydrogenase from the 5-lipoxygenase product 5-HETE. Oxidative stress and NADPH oxidase activity consumes NADPH and generates NADP⁺. This dramatically increases 5-oxo-ETE production [32]. Thus, NADPH consumption by antioxidant circuits or NADPH oxidases may enhance the production of paracrine wound signals, but to date this idea has not been directly tested.

Cell stress signaling by PUFAs

Similar to ROS production, release of free fatty acids (FFAs) by phospholipases is a general tissue damage response conserved from plants and animals [33–38]. Free AA and its oxidized derivatives, collectively termed ‘eicosanoids’, are early regulators of wound detection. AA is a nonessential carboxylic acid with a 20-carbon chain and four cis-double bonds. It is synthesized by animals and fungi, and required for the production of bioactive eicosanoids. AA and other FFAs are released from the sn-2 position of membrane phospholipids by A2-type phospholipases (PLA₂s). Ca²⁺ dependent cytosolic phospholipase A₂ (cPLA₂) is a ubiquitously expressed fatty acid hydrolase with high specificity for AA, which catalyzes the rate-limiting enzyme of eicosanoid synthesis. Lipoxygenases and cyclooxygenases metabolize AA into leukotrienes, oxo-eicosanoids, thromboxanes, prostaglandins, and other bioactive lipids. Eicosanoids control early and late wound responses, such as vascular permeabilization, leukocyte chemotaxis, inflammatory resolution, and healing, through G-protein coupled receptor signaling, and peroxisome proliferator-activated nuclear receptors (PPARs) [39,40].

Free PUFAs have an ancient history in stress and wound signaling that ranges back to the plant kingdom [41]. Although most plants produce insignificant amounts of AA, it is enriched in plant pathogens, such as phytophthora and related oomycetes. AA is a strong elicitor of host defense responses acting akin to an animal DAMP or PAMP [42]. Like animals, plants also use endogenous FFAs to regulate wound responses. LA, a PUFA abundant in plants, is the primary fatty acid substrate for jasmonic acid (JA) production through the octadecanoid pathway, the plant counterpart to the eicosanoid cascade of animals. Analogous to oxo-eicosanoids and leukotrienes synthesis in animals, JA production in plants requires PUFA lipoxygenation. In plants, JA is a central regulator of systemic wound responses that accumulates within second-to-minutes after mechanical injury [43,44]. On a similar timescale, oxo-eicosanoids and leukotrienes mediate leukocyte migration to sites of skin injury in animals [7,10].

Ca²⁺ initiates eicosanoid synthesis by promoting binding of cPLA₂ to the nuclear envelope, where its preferred phosphatidylcholine substrates with esterified AA reside. cPLA₂ activation integrates several biochemical and physical cues commonly associated with tissue injury. Beside Ca²⁺ and phosphorylation [45], also cell swelling activates cPLA₂ [10,46]. After tissue damage, cells swell when they run out of ATP required to keep their osmotic balance. This happens, for example, during necrosis [47]. Swelling renders host cells permissive for repair and defense signaling. In addition to cPLA₂, cell swelling triggers the inflammasome pathway [48], secretion of ATP [14], and diverse ion fluxes including Ca²⁺ [49]. Zebrafish are freshwater animals. In wounded zebrafish larvae, cell swelling primarily results from hypotonic fresh water entering the organism. When larvae are wounded while being immersed in isotonic liquid that mimics their interstitial osmotic pressure, early wound closure and leukocyte recruitment are severely impaired, although wound-induced cell lysis is unaltered [10,14]. By combining intravital imaging in zebrafish larvae with in vitro reconstitution in isolated nuclei and artificial lipid vesicles, it was very recently demonstrated that cell swelling activates cPLA₂ through increases in cytoplasmic [Ca²⁺] and nuclear swelling/membrane tension, with both cues being directly sensed by the

enzyme [50]. Surprisingly, this study exposed the cell nucleus as mechanotransducer of tissue damage-induced inflammation, a function previously not associated with this organelle. Swell-sensing by cPLA₂ provides an attractive way for zebrafish to monitor their epithelial integrity and respond to its disruption by triggering eicosanoid-mediated chemotaxis within seconds-to-minutes [51]. Whether osmotic surveillance also contributes to wound detection in mammalian epithelia covered by hypotonic liquid, for example, the saliva-exposed linings of mouth and esophagus, stands to be seen. Other types of tissue damage, for example, ischemic organ damage or blunt trauma, do not expose the organism to a hypotonic environment, yet cause prominent cell swelling classified as ‘cytotoxic edema’ by pathologists [47]. Cytotoxic edema is accompanied by AA release and recruitment of antimicrobial leukocytes (“sterile inflammation”). Unlike epithelial injury, internal cytotoxic edema does not imply infection. Sterile leukocyte recruitment to ischemic sites worsens tissue damage, and may reflect the maladaptation of the beneficial, epithelial wound response to types of tissue damage that exert little selective pressure, such as ischemia.

Cell stress signaling by Ca²⁺

Backtracking of various wound signaling pathways leads to Ca²⁺, a universal mediator of cell and tissue stress and injury responses [52,53]. It is one of the earliest chemical wound signals known, and stimulates injury-induced ROS and PUFA release in plants and animals by activating Ca²⁺ dependent NADPH oxidases and various phospholipases [10,20,21,35]. Besides, it regulates other wound-relevant mechanisms, such as vesicle secretion, and actin-myosin contraction. The exquisite responsiveness of cytoplasmic [Ca²⁺] to wounding derives from its steep, concentration gradients maintained by cation pumps and exchangers over plasma and organelle membranes. When mechanical or chemical stresses increase membrane ion permeability through ligand-activated or mechanosensitive channels, or physical bilayer damage, Ca²⁺ rushes along its concentration gradient into the cytoplasm. In plants and animals, wounding and other stresses trigger rapid Ca²⁺ waves that often propagate hundreds of micrometers into the unwounded tissue [20,26,54,55]. In cultured corneal epithelial cells, RNAi knockdown of the P2Y₂ receptor strongly suppressed wound-induced Ca²⁺ transients [56]. Based on indirect, pharmacological evidence, a similar mechanism has been proposed for wound-induced Ca²⁺ transients in zebrafish larvae [24]. By contrast, genetic experiments suggest the involvement of transient receptor potential (TRP) channels in wound-induced Ca²⁺ waves in *C. elegans* [57] and *drosophila* [20,58].

Specificity of cell stress signaling

Ca²⁺ mediates diverse biological processes not directly related to tissue injury. How can organisms distinguish between wound-induced and physiological Ca²⁺ transients as they occur, for example, during normal heart, muscle, or nerve activity? One possibility is that the amplitude, frequency, or duration of a Ca²⁺ signal may differ either depending on its upstream trigger, or the biochemical state of the signal-receiving cell. For instance, in response to the same stimulus, an anoxic, energy-depleted cell may exhibit more sustained Ca²⁺ transients than a well-oxygenated one, because its ATP-dependent Ca²⁺ sink mechanisms are compromised. Alternatively, cells may interpret the spatiotemporal

coincidence of several, generic stress signal as ‘wound’. In zebrafish larvae, wound-induced Ca^{2+} only activates cPLA₂ in the presence of osmotic cell swelling [10, 50]. In engineering terms, cPLA₂ functions as molecular ‘AND-gate’ that delivers an inflammatory output in response to a particular input-pattern of chemical and mechanical stimuli. Such AND-gates may exist on various levels of wound signaling.

Conclusion

Our understanding of early wound signaling mechanisms has been significantly advanced by the development of biosensors and organismal model systems that enable real-time imaging of injury responses within the intact tissue. The classic DAMP paradigm defines a ‘wound’ as a focal cluster of lytic cells. More broadly, ‘wounds’ are severe, but not necessarily lytic perturbations of tissue homeostasis. Wounds trigger a characteristic, spatiotemporal code of Ca^{2+} , ROS, membrane tension, and perhaps other generic stress signals. Biological network architectures that can process many physiological inputs with a limited repertoire of host signals are termed ‘bow tie’. Such networks may warrant appropriateness and timeliness of host responses to many homeostatic insults, including epithelial wounding [59,60]. AND-gates, such as cPLA₂, rapidly translate stress signal patterns into host responses that protect the exposed tissue. In plants and animals, this integration involves redox signaling through thiol switches, and synthesis of oxidized, PUFA-derived lipid mediators. Wound detection through DAMPs or stress signals is not mutually exclusive. The type of injury probably decides which mechanism is dominant.

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Figure 1.

Idealized cartoon scheme of early wound signaling sequence. Epithelial injury triggers a momentary efflux of damage associated molecular patterns (DAMPs) from lysing cells at the onset of wounding. Seconds-to-minutes lasting cytoplasmic Ca^{2+} transients stimulate NADPH oxidases, mitochondria, and phospholipases of stressed but intact cells to release reactive oxygen species (ROS), and polyunsaturated free fatty acids (PUFAs). ROS regulate proteins involved in the wound response by oxidation of 'thiol switches', and possibly NADPH depletion (for explanation see body text). PUFAs, such as arachidonic acid and linolenic acid, are metabolized into eicosanoids and jasmonic acid, which are powerful, early wound mediators in animals and plants, respectively. All the above signals also modulate wound-induced gene transcription to influence later stages of healing.