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The beneficial role of extracellular reactive oxygen species in apoptosis-induced compensatory proliferation

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ABSTRACT
Apoptosis-induced proliferation (AiP) maintains tissue homeostasis following massive stress-induced cell death. During this phenomenon, dying cells induce proliferation of the surviving cells to compensate for the tissue loss, and thus restore organ size. Along with wound healing and tissue regeneration, AiP also contributes to tumor repopulation following radiation or chemotherapy. There are several models of AiP. Using an “undead” AiP model that causes hyperplastic overgrowth of Drosophila epithelial tissue, we recently demonstrated that extracellular reactive oxygen species (eROS) are produced by undead epithelial cells, and are necessary for inducing AiP and overgrowth. Furthermore, hemocytes, the Drosophila blood cells, are seen adjacent to the undead epithelial tissue, and may secrete the TNF ortholog Eiger that signals through the TNF receptor to active Jun-N-terminal kinase (JNK) in the undead tissue and induce proliferation. We propose that undead epithelial tissue triggers an inflammatory response that resembles recruitment of macrophages to human epithelial tumors, and that these tumor-associated macrophages release signals for proliferation and tumor growth of the epithelium. This Extra View article summarizes these recent findings with a focus on the role of eROS for promoting regeneration and inflammation-induced tumorigenesis.

Synopsis of our recently published work
Compensatory proliferation is a mechanism that maintains tissue homeostasis after significant stress-induced cell death. Compensatory proliferation initiated by active caspases is termed apoptosis-induced compensatory proliferation (AiP). The role of AiP in tissue regeneration is demonstrated in multiple models including Hydra, Planaria, Drosophila, Xenopus and mice. In mice and potentially in humans, AiP can contribute to tumor repopulation following radiation or chemotherapy, where the dying tumor cells promote the proliferation of surviving tumor cells. In Drosophila, several studies have identified different factors that contribute to AiP. These studies make use of the “undead” model of AiP, in which the apoptotic cascade is initiated by expressing pro-apoptotic factors such as hid, but the execution of cell death is inhibited by co-expression of the effector caspase inhibitor p35 (ref. 7) (Fig. 1). In this experimental setup, the active initiator caspase Dronc (the Drosophila Caspase-9 ortholog), while unable to kill cells due to p35 expression, continues to induce the release of signals for AiP, thus causing hyperplastic overgrowth of the epithelial tissue (Figs. 1, 2). Using an eye-specific undead AiP model (ey-Gal4 UAS-hid UAS-p35 (ey->hid-p35)), we recently demonstrated that continued signaling by active Dronc in undead cells leads to generation of extracellular reactive oxygen species (eROS) via the activity of NADPH oxidases, in particular Dual Oxidase (Duox) (Fig. 1). These eROS drive AiP and cause overgrowth of the undead tissue as loss of Duox or mis-expression of extracellular catalases (hCatS) suppresses the overgrowth phenotype (Fig. 2). Thus, eROS are necessary for AiP; however, if they are sufficient to cause overgrowth of undead tissue needs to be determined. One function of eROS is the activation of hemocytes, Drosophila macrophages, on the undead epithelial tissue. Activated hemocytes attached to the undead cells secrete inflammatory cytokines such as the TNF ortholog Eiger, which triggers activation of JNK back in the undead cells through activation of the TNF receptor Grindelwald (Fig. 1). JNK signaling then promotes the
release of mitogens such as Wingless (Wg) which triggers AiP. In undead cells, JNK also triggers transcription of the pro-apoptotic gene hid which sets an amplification loop in motion. Factors involved in apoptosis are indicated in blue, in JNK activation in green and in production of ROS in red. Question marks indicate unknown mechanisms.

**Figure 1.** Schematic view of our current understanding of the cellular mechanisms of apoptosis-induced compensatory proliferation (AiP). Undead cells can be induced by co-expression of the pro-apoptotic gene hid and the effector caspase inhibitor p35. Because P35 specifically inhibits the effector caspase DrICE (and Dcp-1; not shown), the initiator caspase Dronc cannot induce apoptosis ("undead" state), but remains active for apoptosis-independent functions. One of these functions is the activation of the transmembrane NADPH oxidase Duox, which generates extracellular ROS (eROS) such as superoxide and hydrogen peroxide. eROS activate and change the behavior of hemocytes to become proliferation-promoting. This is accomplished through release of Eiger which activates the JNK pathway in undead and neighboring surviving cells. JNK stimulates the production of mitogens such as wingless (wg) (and dpp and spi; not shown) for AiP. In undead cells, JNK also induces expression of hid which sets an amplification loop in motion. Factors involved in apoptosis are indicated in blue, in JNK activation in green and in production of ROS in red. Question marks indicate unknown mechanisms.

**eROS – damage response or redox signaling?**

Reactive oxygen species (ROS) are partially reduced metabolites of oxygen, which include superoxide anion (O$_2^-$), hydroxyl anion (OH$^-$) and hydrogen peroxide (H$_2$O$_2$). ROS are either generated as by-products of aerobic respiration via electron transfer or generated by the cell to modulate signaling pathways. In the context of AiP, ROS are generated by the transmembrane NADPH oxidase Duox, which is activated upon apoptosis induction. These ROS play a role in the activation of the JNK pathway and the production of mitogens such as Wingless (Wg).

**Figure 2.** Undead tissue displays eROS-dependent hyperplastic overgrowth. Co-expression of hid and p35 in the developing eye imaginal disc using eyeless-Gal4 (ey-Gal4 UAS-hid UAS-p35 (ey>hid-p35)) promotes hyperplastic overgrowth of head cuticle with pattern duplications compared to wild-type control (A, B). Simultaneous expression of an extracellular catalase (hCatS), which neutralizes H$_2$O$_2$, suppresses overgrowth and normalizes the pattern of the adult head (C). Scale bars, 200μm.
reactions in the mitochondria, or via membrane-associated NADPH oxidases such as Nox and Duox. An electron reduction of molecular $O_2$ generates highly reactive and unstable $O_2^-$, which is rapidly converted into more stable $H_2O_2$, a weak oxidizing agent able to diffuse across cellular membranes. Due to their reactive nature, accumulation of $O_2^-$ and $OH^-$ radicals are more associated with oxidative stress in the cell causing damage to DNA, proteins and lipids which can lead to various pathologies and diseases. However, at optimal concentrations, ROS, in particular $H_2O_2$, can function as secondary messengers or signaling molecules to regulate normal cellular functions as part of controlled redox signaling.11,13

**Control of hemocyte activity by eROS**

Duox-generated eROS, especially $H_2O_2$, have a well characterized function during wound repair processes in embryos where they attract and activate hemocytes to the wound site.14 Two recent reports, including our own, demonstrated that repair processes in imaginal discs also require ROS.9,15 In the first report, ROS were generated in response to a short pulse of apoptosis in larval wing imaginal discs. Similar to embryonic wound repair, ROS are exclusively restricted to tissue repair and overgrowth was not observed. 15 A role of hemocytes was not addressed in this work. In our report, we demonstrated that eROS trigger over-proliferation of undead epithelial tissue in eye and wing imaginal discs.9 Intracellular ROS – if they are produced – have no or very little contribution to the overgrowth phenotype. In this case, eROS activate hemocytes, which induce proliferation of undead epithelial cells causing overgrowth.

Usually, hemocytes do not control proliferation and instead are involved in non-proliferative processes such as phagocytosis of apoptotic cells, host defense against invading pathogens and tissue repair processes.16 Therefore, in the context of undead tissue, hemocytes appear to adopt novel properties that enable them to promote proliferation.9 Similar observations have also been reported for a *Drosophila* tumor model.17 How eROS trigger this proliferation-inducing property of hemocytes is unknown. However, because eROS activate hemocytes for non-proliferative wound repair in embryos, it is likely that undead tissue generates additional signals that together with eROS mediate the proliferation-inducing property of hemocytes.

It is interesting to note that hemocytes on undead tissue change their morphology and location. On control eye-antennal imaginal discs, they form large cell aggregates along the morphogenetic furrow at the eye portion and at the antennal portion of the disc. However, in response to exposure to eROS generated by undead tissue, hemocytes separate from the cellular clusters, are less spherical and extend cellular protrusions which make contact with the epithelial tissue of the imaginal discs7 (Fig. 1). Because hemocytes usually do not promote proliferation, these observations suggest that they are “alternatively activated,” similar to tumor-associated macrophages in human cancer.18

Mammalian macrophages are a functionally and phenotypically diverse group of innate immune cells. They are generally classified by their activation states into M1 macrophages, which represent the “classically activated” cells, and M2 macrophages which include the “alternatively activated” macrophages.19 M1 polarized macrophages are primed for pro-inflammatory responses, and display microbicidal and tumoricidal properties. M2 macrophages on the other hand promote anti-inflammatory responses, and are involved with tissue repair and tumor promotion.18,19 Most human solid tumors have high density of macrophage infiltration, which usually correlates with poor patient prognosis. These tumor-associated macrophages (TAMs) show characteristics of alternatively-activated M2 state. TAMs are responsible for promoting tumor inflammation, DNA damage, metastasis and tumor repopulation.18 Like mammalian macrophages, *Drosophila* hemocytes show functional plasticity; however, whether the hemocytes undergo differential activation is something that is not well understood. Thus, it is tempting to assume that the tumor or the undead epithelium in *Drosophila* promotes activation of hemocytes to induce tumor progression and overgrowth.

**Molecular mechanisms of ROS action**

It is unknown how Duox-generated eROS from undead tissue activate hemocytes. Redox sensitive signaling events are often triggered close to the source of ROS production, and because hemocytes are already attached to eye-antennal imaginal discs,9 they may be directly activated by Duox-generated ROS. Indeed, the redox reporter GST-D-GFP20 is active in hemocytes
attached to undead eye imaginal discs (Fig. 3). However, the reporter is also induced in hemocytes attached to the control eye discs (Fig. 3). This finding may suggest that hemocytes do not further respond to eROS. Nevertheless, another possibility to explain this result is that hemocytes at control discs are primed for redox signaling and can respond rapidly when they actually are exposed to eROS.

ROS can modify proteins by oxidizing key residues in proteins causing alterations in protein structure and/or function. Oxidative modification of cysteine residues is the most common protein modification mediated by H₂O₂. This modification can cause formation of intra- and/or inter-disulfide bridges and can lead to changes in the protein activity levels, or may facilitate formation of protein complexes. H₂O₂ can also regulate several cell adhesion molecules like P-selectins, E-selectins, ICAM-1 and VCAM-1, either by direct oxidation or transcriptionally via redox-sensitive transcription factors, thereby regulating adhesion and migration of inflammatory blood cells.

Along with cell adhesion molecules, H₂O₂ also affects junction proteins, thereby causing changes in cell-cell adhesion. H₂O₂ also catalyzes the dityrosine-dependent crosslinking of extra-cellular matrix (ECM). For example, a homolog of Duox in C. elegans has been shown to induce dityrosine crosslinks of collagen to stabilize the ECM. In Drosophila, H₂O₂ generated by Duox is involved in maturation of the wing during the last day of pupal development, likely by physically crosslinking the dorsal and ventral surface cuticles.

It will be an interesting focus for further study to examine whether the eROS from undead cells cause any oxidative modifications on cell adhesion molecules or the ECM of undead cells, or if they directly modify the activity of hemocytes.

Stress activated signaling cascades that activate JNK or p38 kinases are also responsive to ROS. For example, ROS can activate Apoptosis signal-regulating kinase 1 (ASK1), a MAPKKK, by causing its dissociation from its inhibitor Thioredoxin, and recruitment of TRAF2 and TRAF6 to form a multimeric complex.

Figure 3. The redox reporter GST-D-GFP is expressed in hemocytes attached to both control and undead eye-antennal imaginal discs. Shown are eye-antennal imaginal discs of (A) ey-Gal4 UAS-p35 (ey > p35; control) and (B) ey-Gal4 UAS-hid UAS-p35 (ey > hid-p35; undead) genotype. Hemocytes are labeled using the NimC antibody (red in A, B; gray in A, "B"). GST-D-GFP is labeled in green in (A, B) and gray in (A', B'). NimC and GFP labeling overlap. Shown also in (A, B) is labeling with ELAV which marks photoreceptor neurons in the posterior part of the eye disc. ey-Gal4 is only expressed in the anterior portion of the eye disc, but when expressing hid and p35, ey > Gal4 drives overgrowth of anterior tissue into the posterior part at the expense of photoreceptors. Scale bars, 100μm.
with ASK1, leading to activation of downstream signaling pathways, including JNK and p38. ASK1 thus acts as an important molecular switch, responding to oxidative changes and activating stress response signaling pathways. It is involved in mediating apoptosis via TNFα-induced JNK activation, or ATP-induced activation of p38. (ref. 27-28) ASK1 also causes production of inflammatory cytokines in response to ROS in macrophages and other innate immune cells, and is required for recruitment and activation of macrophages to skin wounds in mice, which then mediate hair regeneration. While we have not found a requirement of ASK1 in the undead epithelial cells for activation of JNK, we have observed active JNK in hemocytes (unpublished data), and an interesting follow-up question would be to examine if eROS from the undead tissue enters the cytosol of hemocytes stimulating activation of ASK1 and downstream JNK signaling activity. This could be one of the mechanisms by which eROS activate hemocytes on the undead tissue, ultimately leading to production of TNF Eiger from hemocytes.

**Differential requirement of ROS for wound repair, regeneration and over-proliferation/cancer**

Many recent studies explored the requirement of ROS to induce regenerative responses, not only in flies but also other organisms, indicating an universal function of redox signaling for regeneration and tissue repair. These studies indicate that ROS act in the initial stage of wound detection, causing attraction of inflammatory cells to wounds and initiating an inflammatory response, culminating in regeneration and repair of the wound. Along with regeneration, ROS have also been shown to be important for driving tumorigenesis. Cancer cells show high levels of ROS, which can cause an increase in activation of mitogenic signaling pathways in these cells promoting tumor formation. As the production of ROS is observed in both the regenerative response as well as cancer cells, it will be important to understand what distinguishes both responses in terms of ROS action. Whether it is the duration of ROS production – short pulse in regeneration vs. sustained production in cancer cells – or the threshold levels of ROS will be an interesting area for further studies. Very relevant in this respect is an old theory that classifies tumors as “wounds that do not heal.” Potentially, the level, type and duration of ROS shift the response from wound healing to cancer progression.

A recent study demonstrated that damaged cells can induce organ-level quorum sensing wherein stress beyond a particular threshold leads to regeneration via recruitment of macrophages to the wounded skin in mice, which then secrete TNFα and other inflammatory cytokines. It is tempting to speculate that ROS may act as a quorum sensing molecule determining the degree of stress, and signaling to macrophages to induce either a regenerative response or promoting overgrowth as is seen in the undead model.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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