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Hydrogen peroxide fuels aging, inflammation, cancer metabolism and metastasis

The seed and soil also needs "fertilizer"

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In 1889, Dr. Stephen Paget proposed the "seed and soil" hypothesis, which states that cancer cells (the seeds) need the proper microenvironment (the soil) for them to grow, spread and metastasize systemically. In this hypothesis, Dr. Paget rightfully recognized that the tumor microenvironment has an important role to play in cancer progression and metastasis. In this regard, a series of recent studies have elegantly shown that the production of hydrogen peroxide, by both cancer cells and cancer-associated fibroblasts, may provide the necessary "fertilizer," by driving accelerated aging, DNA damage, inflammation and cancer metabolism, in the tumor microenvironment. By secreting hydrogen peroxide, cancer cells and fibroblasts are mimicking the behavior of immune cells (macrophages/neutrophils), driving local and systemic inflammation, via the innate immune response (NFκB). Thus, we should consider using various therapeutic strategies (such as catalase and/or other antioxidants) to neutralize the production of cancer-associated hydrogen peroxide, thereby preventing tumor-stroma co-evolution and metastasis. The implications of these findings for overcoming chemo-resistance in cancer cells are also discussed in the context of hydrogen peroxide production and cancer metabolism.

Key words: hydrogen peroxide, tumor stroma, microenvironment, aging, DNA damage, inflammation, NFκB, cancer metabolism, HIF1, autophagy, hypoxia, oxidative stress, seed and soil hypothesis

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The "free radical theory of aging" states that progressive defects in mitochondrial function lead to the increased production of reactive oxygen species (ROS), such as hydrogen peroxide, resulting in accumulated DNA damage.[1-3] Ultimately, this DNA damage also increases our susceptibility toward the onset of cancer.[4-6]

But what if cancer cells could facilitate this process too, by producing hydrogen peroxide themselves? In fact, oncogene-induced transformation of cells results in hydrogen peroxide and ROS production[7-10] (**Fig. 1**). And treatment with antioxidants blocks oxidative stress, and in some cases is sufficient to reverse cell transformation.[11-13] Similarly, non-transformed cells, including stem cells, require ROS production for cell proliferation.[14,15]

Normally, epithelial cells only produce hydrogen peroxide during wound healing, initiating the onset of inflammation and myofibroblast conversion.[16] In this regard, epithelial cells behave like inflammatory cells, such as macrophages and neutrophils, which are responsible for the vast majority of hydrogen peroxide production during the inflammatory immune response.[8-10] Similarly, oxidative stress, via hydrogen peroxide production, is sufficient to convert normal fibroblasts to activated myofibroblasts.[17] These activated myofibroblasts then produce hydrogen peroxide themselves, propagating the inflammatory signal.[18]

In normal wound healing, the production of hydrogen peroxide is shut off. However, in cancer cells and tumor tissues,

the production of hydrogen peroxide continues, leading to further DNA damage, inflammation and changes in cellular metabolism. Hydrogen peroxide drives the onset of inflammation, via the activation of NFκB, a master regulator of the innate immune response.[19] Hydrogen peroxide also damages DNA, cellular membranes and organelles, resulting in the onset of autophagy and mitophagy, and HIF1 activation.[20-24] Mitophagy (the autophagic destruction of mitochondria) results in aerobic glycolysis and lactate production under conditions of oxidative stress.[22,25]

Similarly, during scar formation, keloid fibroblasts produce hydrogen peroxide, undergo aerobic glycolysis and secrete lactate.[26-28] Thus, myofibroblasts also have the capacity to undergo the Warburg Effect (i.e., aerobic glycolysis),[26,28] a process that was previously thought to be confined to cancer cells.[29,30]

This is all consistent with the idea that cancers are wounds that do not heal,[31] likely due to the continued production of hydrogen peroxide.

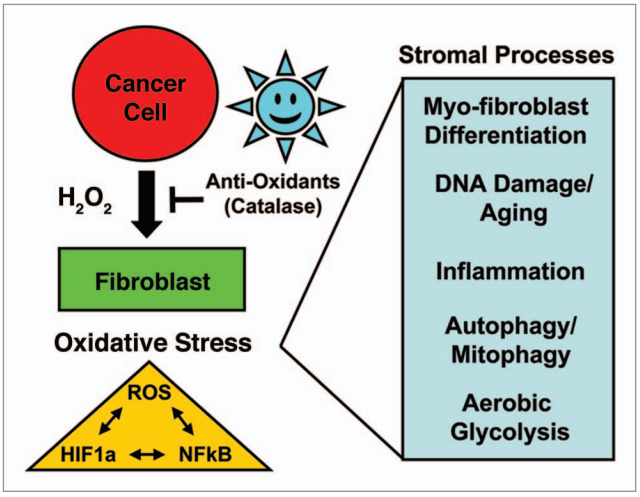

Figure 1. Cancer cells initially produce hydrogen peroxide, which "fertilizes" the tumor microenvironment. See text for details. In this model, cancer cells initially produce and secrete hydrogen peroxide that induces oxidative stress in adjacent cancer-associated fibroblasts. Then, cancer cells mount an anti-oxidant defense by expressing key proteins, such as the peroxiredoxins and TIGAR. Oxidative stress and ROS production in cancer associated fibroblasts then "fertilizes" the tumor microenvironment via myofibroblast differentiation and DNA damage, autophagy/mitophagy, aerobic glycolysis and inflammation. Oxidative stress activates two major transcription factors in cancer-associated fibroblasts, namely HIF1α (aerobic glycolysis) and NFκB (inflammation), which both contribute to the induction of autophagy and mitophagy. ROS production in the tumor microenvironment also has a mutagenic "Bystander Effect" on cancer cells, driving their evolution toward a more aggressive phenotype, aneuploidy and genomic instability. Importantly, anti-oxidants that neutralize hydrogen peroxide [such as catalase and N-acetyl-cysteine (NAC)] should prevent "fertilization" of the soil.

Does Hydrogen Peroxide Function as "Fertilizer" for Tumor Growth and Metastasis?

In the "seed and soil" hypothesis, the cancer cells (the seeds) require the proper local and systemic environment (the soil) to facilitate tumor growth and metastasis.[32-34] Several independent lines of evidence now suggest that hydrogen peroxide may function as the "fertilizer" in this process, by driving accelerated aging, DNA damage, inflammation and cancer metabolism.[35-39]

Catalase is one of the major enzymes that detoxifies hydrogen peroxide in the body, functioning as a powerful catalytic anti-oxidant. Catalase converts hydrogen peroxide to water and oxygen. It is one of the most catalytically active enzymes known; one molecule of catalase can "neutralize" 40 million molecules of hydrogen peroxide per second.

Thus, it might be predicted that a catalase-deficiency predisposes toward the development of cancer, due to increased levels of hydrogen peroxide. Interestingly, by 9 months of age, female catalase-deficient mice spontaneously develop mammary tumors, which can be prevented by treatment of these mice with dietary Vitamin E, a known anti-oxidant.[40] In contrast, wild-type control mice do not show the development of mammary cancers.[40] Thus, the inability to properly detoxify hydrogen peroxide is sufficient to promote tumor initiation.[40] A catalase-deficiency also appears to contribute toward the onset of fibrosis,[41-44] possibly further promoting tumor growth.

Conversely, in humans, the CC allele of the catalase gene is associated with increased serum catalase activity and a 20% reduction in breast cancers.[45] This reduction in breast cancer risk was increased to 40%, if women with the CC allele ate large amounts of vegetables and fruit, that contain antioxidants.[45] Similarly, treatment of breast cancer patients with antioxidants (Vitamins C/E) results in substantial reductions in both mortality and recurrence (approaching 40–50%), if patients with radiation therapy are excluded.[46]

In Japan, a plethora of pre-clinical studies have now shown that "catalase therapy" (either via i.v. or i.p. injection of catalase) in rodent animal models is sufficient to almost completely prevent both tumor recurrence and metastasis.[47-53]

Transgenic overexpression of catalase in MMTV-PyMT mice (a well-established model of breast cancer) reduces tumor aggressiveness (from high-grade to low-grade) and reduces lung metastatic tumor burden by >12-fold.[54,55]

Glutathione peroxidase family members also function to detoxify hydrogen peroxide.[56] Of the eight known family members (GPX1–8), it appears that GPX4 is the most relevant to our discussion, as it is targeted to mitochondria. Interestingly, overexpression of GPX4 in fibroblasts protects against ROS production, NFκB activation and IL-6 secretion, as well as MMP production.[56] In addition, overexpression of GPX4 in cancer cells effectively reduces cell proliferation, tumor growth and metastasis.[56]

Thus, it appears that hydrogen peroxide does function as "fertilizer" to promote tumor growth, progression and metastasis.

In accordance with this simple notion, hydrogen peroxide production and chronic inflammation (also due to viral and bacterial infections), may be the major drivers of tumor initiation. In fact, the most common "root causes" of cancer worldwide are thought to be due to infectious agents, such as in liver (HBV),[57] cervical (HPV),[58] nasopharyngeal (EBV),[59,60] lung (TB),[61] and stomach (*H. pylori*) [62] cancers. Similarly, chronic inflammatory diseases, such as scleroderma,[63] ulcerative colitis,[64] and Crohn's disease,[65] all predispose to the onset of cancer. ROS-producing bacteria have even been implicated in the pathogenesis of colon cancer, which induce aneuploidy (genomic instability) in normal colonocytes via a "bystander effect."[66]

Likewise, *H. pylori* increases the local production of ROS species (including hydrogen peroxide) and RNS (such as nitric oxide) in the stomach.[62] This also fits well with the possibility that Wasabi consumption [a condiment that actively produces hydrogen peroxide (horseradish)] could explain the observation that Japanese men have the highest rate of gastric cancer in the world, ~7–10 times higher than in the United States.[67]

As discussed below, ROS-induced cytokine production and inflammation[65] also further drive autophagy (the production of high-energy nutrients) in the tumor microenvironment,[36] thereby producing "fuel" to feed "hungry" cancer cells.

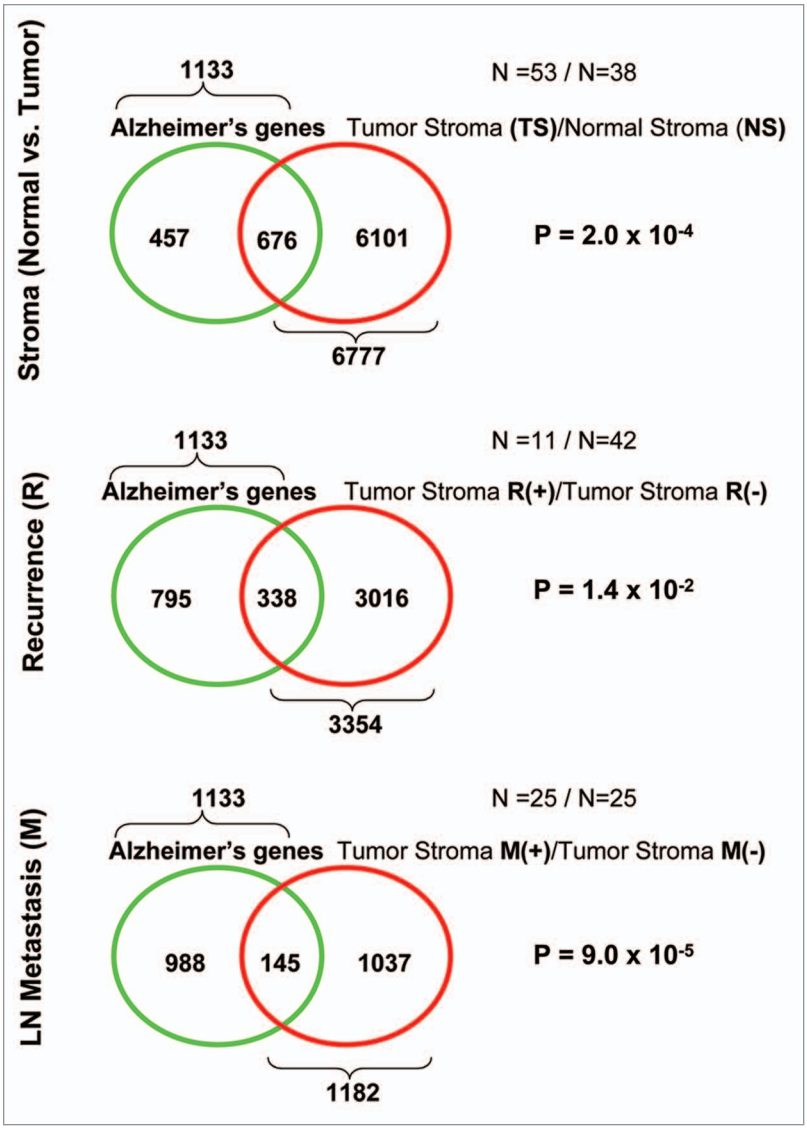

Figure 2. Transcriptional overlap between Alzheimer brain disease and the breast cancer tumor stroma: association with metastasis. Venn diagrams show that the gene signature for Alzheimer disease brain (1,133 gene transcripts; a signature for inflammation and oxidative stress) is most closely related to the primary tumor stroma of breast cancer patients that will undergo metastasis (1,182 gene transcripts), with a p value of nearly 10^{-6} (lower part). This finding is consistent with the idea that oxidative stress in the tumor stroma is associated with metastasis. Virtually identical results were also obtained with the gene signature for Cav-1-deficient stromal cells (a model for oxidative stress), which showed striking similarities toward both Alzheimer disease brain and breast cancer metastasis. Reproduced with permission from.[72,73]

Hydrogen Peroxide and the Warburg Effect in the Tumor Stroma: Metabolic Coupling

If cancer cells produce and secrete hydrogen peroxide, then this also has important metabolic consequences for the tumor microenvironment.

These stromal effects were recently observed by co-culturing MCF7 breast cancer cells with immortalized fibroblasts.[23,24,68] Interestingly, at day 2 of co-culture most of the ROS production occurred in MCF7 cells.[35] This ROS production was reduced to baseline levels by co-incubation with extracellular catalase, identifying the predominant ROS species as hydrogen peroxide.[35] In contrast, by day 5, most of the ROS production occurred in the cancer-associated fibroblasts.[23,35] Thus, it appears that cancer cells initially secrete hydrogen peroxide, which then triggers oxidative stress in neighboring fibroblasts.

Importantly, we observed using this MCF7-fibroblast co-culture system, that hydrogen peroxide secretion activates NFκB and HIF1 in cancer-associated fibroblasts, driving stromal inflammation and aerobic glycolysis, as well as autophagy.[23,24] Then, autophagy, mitophagy and aerobic glycolysis in cancer-associated fibroblasts provides high-energy nutrients and recycled building blocks (such as lactate, ketones and glutamine) to literally "feed" cancer cells.[23,24] These high-energy nutrients (such as lactate) are sufficient to promote mitochondrial biogenesis and oxidative mitochondrial metabolism in cancer cells, thereby driving tumor growth.[23,24,69,70] Under these

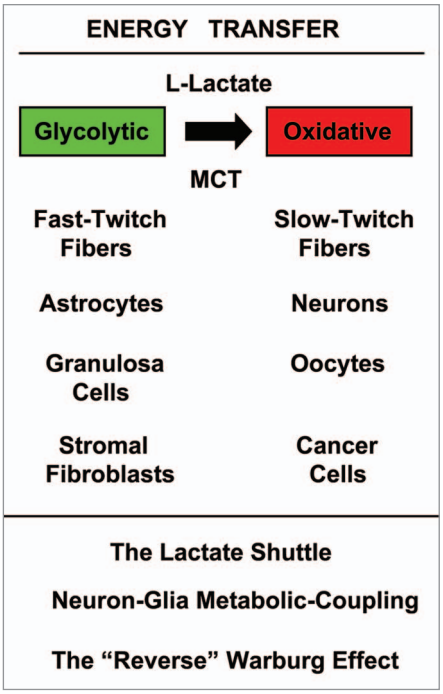

Figure 3. Energy transfer in normal metabolism and cancer: the lactate shuttle. The concept that glycolytic (green) and oxidative (red) cells can share L-lactate is shown. This is known as the "lactate shuttle," and normally occurs in skeletal muscle, the brain and the female genital tract. In skeletal muscle, fast-twitch fibers are glycolytic and slow-twitch fibers are oxidative. In the brain, astrocytes take up glucose and secrete lactate that is then transferred to neurons. This is known as "Neuron-Glia Metabolic Coupling." In the female genital tract, granulosa cells are glycolytic and produce L-lactate to "feed" the oocyte, which is oxidative and uses mitochondrial metabolism. Thus, this metabolic-coupling mechanism is widely used by the body, to maintain proper homeostasis or energy balance. Similarly, in human tumors, cancer-associated fibroblasts are glycolytic and cancer cells are oxidative. This is known as the "reverse Warburg effect." Mono-carboxylate transporters (MCTs) function to shuttle the L-lactate from glycolytic cells (MCT4) to oxidative cells (MCT1/2).

conditions, most of the DNA damage occurs in the cancer-associated fibroblasts, as the MCF7 cancer cells effectively mount an anti-oxidant defense by upregulating key proteins, such as peroxiredoxin-1 and TIGAR.[23,24] Thus, hydrogen peroxide helps produce "glycolytic fibroblasts" to feed hungry "oxidative cancer cells."

In order to pheno-copy the effects of hydrogen peroxide on cancer-associated fibroblasts, we overexpressed activated HIF1α or NFκB in normal stromal fibroblasts.[22] Notably, both HIF1α and NFκB-expressing fibroblasts undergo mitophagy, produce more lactate and stimulate the growth of human breast cancer xenografts, by up to ~3-fold.[22] Similarly, a loss of caveolin-1 (Cav-1) occurs in fibroblasts undergoing oxidative stress, as it is destroyed by lysosomal degradation/autophagy.[23,24,68] In this sense, loss of stromal Cav-1 is a marker for the effects of hydrogen peroxide and oxidative stress.[37,71,72] Cav-1-deficient fibroblasts show increases in mitochondrial oxidative stress, with a shift toward aerobic glycolysis.[71-74] In this regard, they behave like "glycolytic" myofibroblasts, which also show a loss of Cav-1 expression.[26,75,76] Most importantly, Cav-1-deficient fibroblasts promote tumor growth up to ~4-fold, when co-injected with MDA-MB-231 triple negative human breast cancer cells.[77,78] Cav-1-deficent fibroblasts and hydrogen peroxide treated fibroblasts also share the same proteomic profile, with the upregulation of myofibroblast markers, glycolytic enzymes and anti-oxidant proteins.[22,74,78-80]

Consistent with these findings, a loss of stromal Cav-1 is a powerful biomarker for a lethal tumor microenvironment, in breast and prostate cancers.[37,81-89] More specifically, in breast cancer patients, a loss of stromal Cav-1 is associated with early tumor recurrence, metastasis, tamoxifen-resistance and poor clinical outcome.[84] For example, in triple negative breast cancer patients, a loss of stromal Cav-1 predicts a 5-year survival of less than 10%. In the same patient cohort, high stromal Cav-1 was associated with a good prognosis, with >75% survival at 12 years post-diagnosis.[83]

Laser-capture micro-dissection of the tumor stroma from Cav-1-negative human breast cancer patients also reveals the presence of transcriptional gene signature(s) that are consistent with the local damaging effects of chronic hydrogen peroxide production.[37] These gene signature(s) include aging, Alzheimer disease, DNA damage, oxidative stress, inflammation, HIF1 and NFκB-activation, as well as autophagy and aerobic glycolysis.[37] Thus, localized hydrogen peroxide production may underlie the prognostic value of a loss of stromal Cav-1 as a biomarker. In this context, the Alzheimer disease brain gene signature (a marker of oxidative stress) was specifically associated with breast cancer metastasis (**Fig. 2**).[72,73]

Hydrogen Peroxide and the Lactate Shuttle

As discussed above, cancer cells use hydrogen peroxide as a weapon to extract nutrients from adjacent fibroblasts, via the stromal induction of autophagy and mitophagy.[23,24,35] This results in the production of L-lactate in cancer-associated fibroblasts, via aerobic glycolysis. Then, these fibroblasts secrete L-lactate as a waste product and cancer cells use L-lactate as "fuel" to burn in their mitochondrial TCA cycle, via oxidative phosphorylation.[23,24,35] As such, there is metabolic-coupling between "glycolytic" fibroblasts and "oxidative" cancer cells.[90] We have previously termed this phenomenon the "reverse Warburg effect," as aerobic glycolysis takes place in fibroblasts, rather than cancer cells.[74]

Interestingly, the concept of a "lactate shuttle" and that glycolytic and oxidative cells share L-lactate is not new (**Fig. 3**). Lactate shuttles are now known to occur in skeletal muscle, the brain and even the female genital tract, and this is a normal physiological process. In skeletal muscle,

fast-twitch fibers are glycolytic and slow-twitch fibers are oxidative.[91] In the brain, astrocytes take up glucose and secrete lactate that is then transferred to neurons.[92-95] This is known as "neuron-glia metabolic coupling."[92-95] Finally, in the female genital tract, granulosa cells are glycolytic and produce L-lactate to "feed" the oocyte, which is oxidative and uses mitochondrial metabolism.[96-104] Thus, this metabolic-coupling mechanism appears to be widely used by the body, to maintain proper homeostasis or energy balance.

In this context, it is believed that cancer-associated fibroblasts produce ROS (including hydrogen peroxide), and that this helps to maintain their glycolytic state.[23,24,35] Similarly, fast-twitch skeletal muscle fibers are glycolytic and are the largest producers of hydrogen peroxide, as compared with slow-twitch fibers.[105] Consistent with this hypothesis, MCT4, the main transporter for the extrusion of L-lactate from glycolytic cells, is induced by oxidative stress in fibroblasts.[38]

Oxidative Stress, Metabolic-Coupling and Drug Resistance

What if acquired resistance to chemotherapeutic agents was a metabolic and stromal phenomenon? Then, we might be able to reverse drug-resistance with drug combinations that target both the tumor stroma and the epithelial cancer cells, resulting in metabolic uncoupling.

We recently tested this idea using the MCF7-fibroblast co-culture system.[35,68] MCF7 cells are a well-established ER(+) breast cancer cell line (**Fig. 4**). As predicted, MCF7 cancer cells cultured alone were extremely sensitive to tamoxifen-induced apoptosis, which targets ER-signaling.[106] Remarkably, under co-culture conditions with fibroblasts, MCF7 cells became nearly completely resistant to the pro-apoptotic effects of tamoxifen.[106]

Metabolic analysis of this phenomenon indicated that tamoxifen-resistance in co-cultured MCF7 cancer cells was due to a shift from a "glycolytic" to an "oxidative state," with increased mitochondrial activity and decreased glucose uptake.[35,106] Thus, we searched for a drug combination that could overcome this stromal-based tamoxifen-resistance. In this context, we observed that the addition of tamoxifen plus dasastinib was indeed sufficient to prevent stromal-based tamoxifen-resistance and restore sensitivity toward tamoxifen-induced apoptosis in cancer cells.[35,106] Under these conditions, we observed that MCF7 cancer cells were shifted back toward the "glycolytic" state and hydrogen-peroxide production was reduced to baseline levels.[35,106] Thus, tamoxifen plus dasatinib maintained both fibroblasts and cancer cells in a "glycolytic state," with minimal ROS production and high-sensitivity toward apoptosis, likely due to an absence of metabolic-coupling.[35,106] This is consistent with idea that tamoxifen plus dasatinib has a generalized anti-oxidant effect.

Interestingly, under these same co-culture conditions, treatment with catalase to neurtralize hydrogen peroxide, prevented ROS production and dramatically induced apoptosis in co-cultured MCF7 cancer cells.[35] Thus, in co-culture, catalase has the same anti-oxidant and pro-apoptotic effects on MCF7 cancer cells, as tamoxifen plus dasatinib.[35,106] Mechanistically, this may explain why "catalase therapy" in pre-clinical models prevents both tumor recurrence and metastasis, as it "cuts off the metabolic fuel supply."

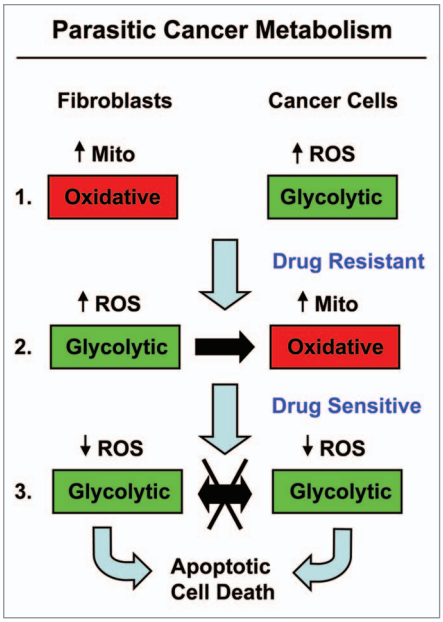

Figure 4. Understanding the stromal and metabolic basis of resistance to chemotherapy: parasitic cancer metabolism. Part 1: Fibroblasts cultured alone are oxidative, and show increased mitochondrial activity. In contrast, MCF7 cells cultured alone are glycolytic and are sensitive to tamoxifen-induced apoptosis. Part 2: However, during co-culture, cancer cells and fibroblasts undergo a complete reversal of their metabolic states. In this role reversal, MCF7 cells become oxidative and fibroblasts become glycolytic. This change is initially driven by hydrogen peroxide production in MCF7 cells, which shifts adjacent fibroblasts toward the glycolytic state, via oxidative stress. Then, under these conditions, there is a net energy transfer (in the form of nutrients, such as L-lactate; see the black arrow) from glycolytic fibroblasts to oxidative cancer cells, which confers resistance to tamoxifen-induced apoptosis. Part 3: Incubation of co-cultures with tamoxifen plus dasatinib shifts both cell types toward a glycolytic state, with decreased ROS production. As such, there is no net energy transfer. These conditions sensitize MCF7 toward apoptosis, reversing their tamoxifen-resistant phenotype. Mito, mitochondria.

Clinical Utility of Hydrogen Peroxide for Cancer Diagnosis

Since cancer cells and tumors both produce large amounts of hydrogen peroxide,[7-10] the detection of hydrogen peroxide production may be an important new approach toward cancer diagnosis and for the development of novel imaging techniques.[51,107-109]

For example, cancer patients (such as those with breast and lung tumors)

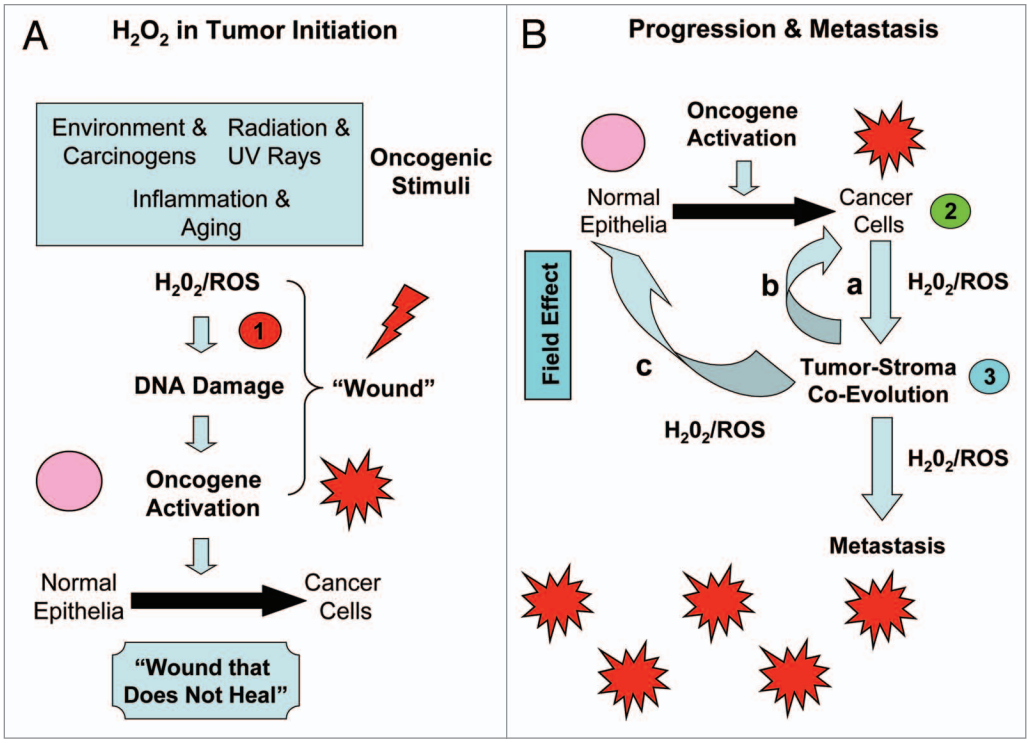

Figure 5. Three-step carcinogenesis: hydrogen peroxide in tumor initiation, progression and metastasis. (A) Tumor Initiation (step 1). Oncogenic stimuli (such as carcinogens, UV rays, inflammation and aging) initiate hydrogen peroxide production, leading to DNA damage in normal epithelial cells. Such DNA damage mimics the "wounding process," thus driving oncogene activation, tumor suppressor inactivation and cell transformation. This leads to the formation of cancer cells, via mutagenesis and ensuing genomic instability. (B) Tumor Progression and Metastasis (steps 2 and 3). Once cancer cells are formed via oncogene activation in normal epithelial cells, then these cancer cells also begin to produce and secrete hydrogen peroxide (Step 2) to promote tumor-stroma co-evolution and metastasis (Step 3). In part a, cancer cells secrete hydrogen peroxide, which induces oxidative stress in neighboring stromal cells, such as fibroblasts. In part b, oxidative stress in fibroblasts leads to ROS production in the tumor stroma, which then further mutagenizes the cancer cells, allowing them to evolve to a more aggressive state, driving stromal lactate production and metastasis. Finally, in part c, hydrogen peroxide and ROS production could also mutagenize adjacent normal epithelial cells, further driving the formation of new cancer cells. This step, part c, may also account for the "field effect," in which an entire area of tissue appears normal, but has been "cancerized" by hydrogen peroxide, oxidative stress and DNA damage.

can be distinguished from normal controls, based on the detection of hydrogen peroxide in their exhaled breath.[110-112] Consistent with the idea that hydrogen peroxide may also originate from the tumor stroma, patients with interstitial pulmonary fibrosis show increased levels of hydrogen peroxide in their exhaled breath.[113] Also, patients with pulmonary fibrosis show a loss of stromal Cav-1 expression, providing another mechanistic link.[114-116]

Finally, new imaging probes have also been invented that can detect hydrogen peroxide production in pre-clinical models, such as human tumor xenografts.[117] In this regard, PET imaging of human tumors, with Fluoro-2-deoxy-D-glucose (F-2-DG), may be already detecting cancer-associated fibroblasts,[35] that are undergoing aerobic glycolysis, due to oxidative stress induced by hydrogen peroxide production and inflammation.

In further support of this notion, PET imaging with F-2-DG can be effectively used to detect many distinct inflammatory diseases, both of infectious and non-infectious origins. These conditions include fever of unknown origin (FUO) and bacteremic foci, as well as graft rejection in liver and renal transplants.[118-124]

Summary: Hydrogen Peroxide in Tumor Initiation, Progression and Metastasis

In summary, hydrogen peroxide is a known carcinogen, and is associated with mutatgenic potential, resulting in a positive Ames test in bacteria.[125,126] Thus, hydrogen peroxide may also induce DNA damage in normal epithelial cells.[126,127] In fact, various known oncogenic stimuli are sufficient to drive hydrogen peroxide production, such as environmental stressors, carcinogens, radiation (including UV exposure), as well as inflammation and normal aging.[126-137] As such, hydrogen peroxide may be directly involved in the earliest tumor initiating events.[126,127,137,138] In accordance with this notion, addition of catalase to the extracellular tissue culture media is indeed sufficient to prevent or dramatically delay the onset of cell transformation and genomic instability, when normal cells are presented with an oncogenic stimulus.[138]

Thus, we propose that carcinogenesis may be a 3-step process, related to hydrogen peroxide production, as outlined in **Figure 5A and B**. In this cascade or chain of hydrogen peroxide production, ROS and oxidative stress is passed from the

oncogenic stimulus and normal epithelial cells (**step 1**), to the cancer cells (**step 2**), and then to the tumor stroma (**step 3**). Step 1 is hydrogen peroxide induced tumor initiation, while steps 2 and 3 are hydrogen peroxide induced tumor progression and metastasis. Hydrogen peroxide induced DNA damage in the tumor microenvironment may also account for the "field effect," such that an entire area of tissue appears normal, but has been "cancerized" by oxidative stress and DNA damage, leading to premature aging, inflammation, cancer metabolism and metastasis.[23,35,139] Indeed, lactate production in the tumor microenvironment is sufficient to promote an ~10-fold increase in lung metastasis.[70]

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