Oxygen 2002: Wounds

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INTRODUCTION

The years 2001 and 2002 were productive for oxygen research. During that time, we came to better understand the role of oxidants in the mechanism of healing, a large step. The list of known roles for oxygen lengthened. We found more proof that oxygen therapy is useful for wounds and wound infections. We learned better than before when hyperbaric oxygen therapy (HBO_2) will be effective and who will benefit from it.

We ended the twentieth century with the certainty that oxygen has its place in all major components of wound healing. In addition, we now know that those oxidants derived directly from oxygen, reactive oxygen species (ROS), are vital to the signaling process that transcribes collagen genes [1]). We learned that this effect applies to many cell types, including vascular endothelial cells. Oxygen, we realized, is absolutely required for the resistance to bacterial infection [2], but we now know that the bacteria-killing ROS are also fundamental to the entire process of healing, a rare theoretical simplification that we encountered in this area of research.

Going into the new millennium, we suspected that peroxide and other oxidants might regulate all of these events. The growing interest in nitric oxide (NO) upon the discovery that arginine accelerates wound healing was our first clue [3]). Since then, our suspicion has developed into fact, though the interest in NO as far as wounds are concerned, seems to have receded somewhat. The concept of increasing tissue oxygen tensions to promote angiogenesis and defeat infections is now defended by more than just a few qualitative clinical observations. Instead, we have a coherent theory, supported by known basic mechanisms.

To understand the advances, we must first disclaim the idea that reactive-oxygen species are usually harmful. In fact, the presence of ROS is a mainstay of life. They occur in countless, essentially chemical reactions, both enzymatic and non-enzymatic [4]. Oxidants become problematic only in excessive amounts [5]. Even though wounds are hypoxic by nature, it is clear they heal in an oxidative milieu. The H_2O_2 concentration in wound fluid is normally about 5 to 15 micromolar (unpublished). It is higher in neutrophils at the healing edge of the wound [2, 6]. The concentrations of other oxidants are unknown, but given the instability of most regulatory oxidants, their concentrations are probably not descriptive of their function.

The easiest and the least painful way to explain the new conception of wound healing, including the roles of ROS, is through the actions of oxygen in wound immunity. In this sense, wounds can be regarded as essentially inflammatory lesions.

Immunity

Wounds represent a severe break in immunity to bacterial infections. The more hypoxic a wound, the more vulnerable it is. Oxygen, through the production of ROS, is essential to resist infection [2, 7, 8]. For instance, in the case of staphylococcus, the major wound pathogen, oxidants generated by leukocytes seem to be the only bactericidal mechanism. Tissue hypoxia is probably the most significant background for vulnerability to wound infection [7].

The killing of bacteria within leukocytes has many mechanisms, usually divided into oxidative and non-oxidative. Non-oxidative mechanisms provide immunity to the run-of-the-mill bacteria that infest wounds only in the immune-suppressed patient. Oxidative killing, on the other hand, is responsible for eliminating the species that commonly infest wounds. Hence, we can conclude that hypoxia is the weak spot in the defensive barriers. Nevertheless, the 'spectrum' of oxidative killing is wide. Recent observations suggest that all kinds of leukocytes produce bactericidal oxidants and employ them as lethal agents to kill bacteria and even tumor cells. All wound cell types, fibroblasts, endothelial cells, neutrophils, and macrophages generate these oxidants [4]. However, polymorphonuclear leukocytes seem to provide most of the action, at least in wounds in which these professional bactericidal cells kill almost all wound pathogens by engulfing them and exposing them to ROS. When oxygen tension is low, they become fewer and thus less effective.

The mechanism of bacterial killing is deceptively simple. Upon entry into the wounded area, leukocytes are 'primed' for greater activation by exposure to complement, antibody-opsonized bacteria, etc. When they ingest debris or bacteria and become fully activated, an enzyme complex, the NADPH or phagocytic oxidase (more precisely an 'oxygenase' and more easily called 'phox'), is assembled from its pre-existing, separated, components in the cell [9]. There, then, begins a rapid increase in the cell's consumption of molecular oxygen, as much as fifty-fold, during which almost all consumed oxygen is converted to superoxide ($^{\cdot}O_2$). Through various means, $^{\cdot}O_2$ is thence converted to other oxidants, such as hydrogen peroxide, aldehydes, NO, etc. This is called the oxidative (or respiratory) burst. The genetic absence of phox causes a severe susceptibility to infection, mainly due to the types of bacteria that are common pathogens in wounds [10, 11].

The rough equation (the sum of several) for the oxidative burst is:

$$\text{Glucose} + \text{oxygen} \longrightarrow {}^{\cdot}O_2^- + \text{Lactate} + H^+$$

The activation of phox materially lowers the already jeopardized PO_2 in the wound, and raises the lactate and ROS concentrations. Most important is that the reaction proceeds at a rate proportional to the local concentration of oxygen (PO_2). The Km for oxygen is about 50mmHg.

When assembled, phox is located in the phagosomal membrane. It adds an electron and injects the resulting $^{\cdot}O_2$ into the phagosome, where it, and other oxidants that derive from it, kill bacteria [12]. This is the first function of oxidants. The degree to which it is performed is dependent on the oxygen and glucose that diffuse through the cell membrane. By supporting local PO_2 by providing oxygen and local warmth, wound infections that follow contaminated surgical procedures can be reduced by over sixty percent.

Lactate and the relatively long-lived, freely diffusible H_2O_2 escape into the cytoplasm and the extracellular space, where their next function is to act as extra- and intra- cellular 'messengers' in a process called redox signaling [13].

Redox Signaling

Among other functions, these longer-lived and diffusable oxidants are signals that one cell sends to itself or to another, directing the activities of the recipient cell. The paracrine mechanisms, most commonly employing H_2O_2, are best known.

By 2002 we already felt that hyperoxia is helpful to angiogenesis, despite conclusive data proving that vascular endothelial growth factor (VEGF) is a product of hypoxia. We have resolved this apparent paradox and can ascribe the solution to redox signaling, as well. Even in hypoxic circumstances, a low level of H_2O_2 instigates the production of angiogenic growth factors [14], while oxygen itself regulates vessel growth [15].

Oxygen, then, has several major roles in wounds. There is the production of energy, of course, but little respiration occurs in wounds, where energy is produced largely by glycolysis. Oxygen is important to structural protein synthesis, since hydroxylation of proline in procollagen gives collagen its tensile properties. The next, and perhaps last, function to be explored will be oxidants for signaling.

Lactate

Seen from a linear, rather than dynamic, point of view, the healing mechanism now briefly splits into two arms. Lactate enters, as well as leaves, cells. Intracellular and extracellular lactate is in equilibrium. When it exists in significant concentrations, it chelates iron (particularly intracellularly), thereby modifying Fenton chemistry and leading to more oxidant production, now intracellularly, and with an emphasis on hydroxyl radical. That is, the normal reaction between Fe^{++} and O_2 that releases H_2O_2 is changed somewhat to produce relatively more hydroxyl radicals (*OH) [16, 17]. This highly energized radical has a both a short life and a short path through which it can produce its effects. In the extracellular space, hydroxyl radical is scavenged by antioxidants. In the cell, it is released into or close to the nucleus and can exert many actions. For instance, oxidants are known to enhance the transcriptions of several pertinent genes, including those of collagen and vascular endothelial growth factor (VEGF) [18]. Loosely bound Fe^{++} is present intracellularly.

In a parallel step, lactate accumulation forces reduction of NAD+ to NADH via lactate dehydrogenase. This decreases the amount of NAD+ that is available for ADP-ribosylation [19]. This is known to enhance collagen gene transcription and control the activity of VEGF [19-22]. The details are not pertinent to oxygen, except that its existence allows NAD+ to be decreased, despite adequate oxygenation [23, 24]. Thus, the signaling for healing that resides in this reaction can persist, despite a relatively high PO_2.

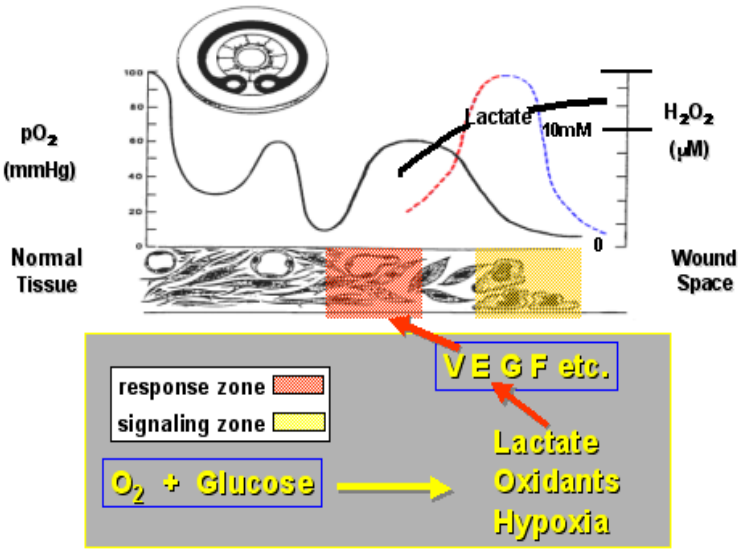
Adding lactate to closed wounds increases VEGF, TGF-β, and IL-1 several-fold (for a shorter time). It also raises collagen and angiogenesis at least forty percent. In the deposited tissue, collagen rises, while total DNA and total protein do not, suggesting that the lactate effect of ADP-ribosylation is particularly important to collagen [25].

From this discussion, it should be clear that the accumulation of lactate in wounds is not due to hypoxia [24]. Lactate is produced largely in the oxidative burst (above) and in the "Warburg phenomenon" [26], in which rapidly dividing cells rely heavily on aerobic glycolysis

—the metabolism of glucose to lactate in the presence of oxygen — as their prime energy source.

Growth Factors

Many of the growth factors/cytokines appear to be produced as a result of oxidant activity. Among these are VEGF, IL-l, TGF-β and IGF-1. A VEGF gene is known to respond to hydrogen peroxide alone [27]. The important growth factor, hypoxia inducible factor-1α (HIF-1α), which responds to hypoxia (a result of oxidant production) actually *requires* some sort of oxidant, in addition to hypoxia, for its transcription [28-30]. HIF-1α also goes on to stimulate VEGF. Also, lactate and peroxide lead to the expression of an early growth-response gene, one of the early responses to cell 'stress.' From this data, it appears as though the definition of wound could be, "a volume of tissue in which the microcirculation is injured, and at the same time, inflamed, oxygen-poor, oxidant- and lactate-rich" (Figure 1).



Fig, 1. Wound Model

Thus, *another function* of oxidants is to act as (intercellular and/or intracellular*)* messengers for the elicitation of growth factors/cytokines. This is not to say, however, that oxidants are the only path. IGF-1 also enters from blood and platelet derived growth factor (PDGF) from platelets. Oxidants also assist in the downstream effects. For instance, VEGF causes endothelial cells to migrate, multiply, and secrete proteases that clear out spaces in the pre-existing capillary membrane. The cells can then migrate toward the source of the VEGF. The function of at least one protease, MMP-9, is also redox-regulated. It is useful to visualize oxidant chemistry in a compartmentalized context.

Fibroblasts and Collagen

Procollagen gene transcription occurs upon exposure of fibroblasts to peroxide [1]. At this point, it is easiest to switch back to the subject of molecular oxygen. As noted, its concentration, (i.e. PO_2) is low within approximately a day of injury. This hypoxia becomes highly significant. In a twist that was almost unimaginable a year ago, our prior knowledge that oxygen is required at rather high PO_2 for fibroblasts and endothelial cells to produce collagen has been expanded. Adding oxygen at this point supports collagen deposition for mechanical support of tissue integrity as well as for the endothelial cells to align themselves into tubes and support

blood pressure [31], i.e., to support angiogenesis. In short, wound healing (and signaling) seems to require lactate, as well as sufficient oxygen, to support collagen deposition and oxidant production. These two processes compete for oxygen as clinical behavior predicts. Both excessive and insufficient inflammation retard healing. Therefore, the trick is to get it just right. This is where hyperbaric oxygen enters the scene.

Hyperbaric Oxygen

One might ask if the administration of more oxygen would lower lactate levels. In short, this does not happen [32, 33]. Lactate has other sources in wounds. First, rapidly dividing cells release large amounts [of lactate] (Warburg Effect) by aerobic glycolysis. Literally, all cancer cells do this. Second, as noted above, leukocytes provide the energy for oxidant production from aerobic glycolysis. That is, the production of lactate actually amplified as oxygen availability increases. This means that lactate production may increase with *rising oxygen* and release more lactate as a consequence. This occurs between PO_2 zero and about 800 mm Hg. What lactate comes from hypoxia itself, if any, is not known.

The success of periodic high-oxygen concentrations in the breathing mixture depends on its transmission to the wound. If it is not transmitted, there is no hope of increasing healing. When excess oxygen arrives, oxidant production increases. In particular, H_2O_2 is increased, leading to more VEGF, additional collagen, and increased bacterial killing. In some wounds, the H_2O_2 may briefly reach toxic levels, but prompt cessation of added oxygen allows the antioxidant defense mechanisms to operate. Then, there may be hypoxia with a second stimulation of VEGF that is translated into blood vessel growth in the next cycle. In all of this, the lactate levels remain high [33]. Thus, the very nature of HBO_2 therapy, cycles of added oxygen, may account for some of its success.

CONCLUSION

In order to accept the new data, we must modify our attitudes about oxidants. Even if one is addicted to anti-oxidants, like vitamin E, ascorbate (ascorbate, a necessary cofactor for deposition of collagen, is another pertinent and tangential subject to the oxygen story), etc., one must also admit that oxidants are essential to life. Most reactions that use molecular oxygen, of which there are many, involve the formation of a transient oxidant radical. This is exaggerated in the normal inflammation of wound healing. Thus, the architecture of wounds, the oxygen gradients that cross the inflamed zone, is the setting for their sensitivities to hypoxia and responses to oxygen [34].

The interjection of ROS has so far provided such a cohesive and continuous set of mechanisms that they might well be thought of as the core of repair. Many parts of healing, lysis of matrix, integrins, and mitogens, we suspect, will be hung on this core when the full explanation is finally disclosed.

Life seems to be perched on a narrow path between too little oxygen, or asphyxia, and oxidation (rust). However, this does not mean that we cannot supply more oxygen and more oxidants to our useful advantage for short periods of need. Important defenses, such as inflammation and wound healing, depend on them. The basic rule is that health requires short and/or gentle exposures, while long and extreme exposures are generally toxic.

Where these realizations will take us is anyone's guess, but I predict that the advances of the last two years will lead to practical application. Perhaps one might not have to think any

further than of adjusting the redox potential of wounds in order to adjust healing — whether we want less or more. It would be nice to have some agent that can act as a potent means of preventing scarring. That may be the next challenge. As of 2002, with the discovery of oxidant signaling, it seems feasible.

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