Ionic Modulation of Bacterial Virulence and Its Role in Surgical Infection

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Abstract

Background: Bacterial virulence is a dynamic property of pathogens that is expressed in a context-dependent manner. For a bacterial pathogen, the expression of virulence is a tradeoff, as there is an energy cost that can disturb other functions. As a result, virulence is activated only when bacteria sense the need for it.

Methods: Recent work from our laboratory has identified many of the local cues in the environmental context that activate bacterial virulence during surgical injury, resulting in bacterial invasion, tissue inflammation, and, in some cases, lethal sepsis.

Results: After surgical injury, cytokines, opioids, and end-products of ischemia can activate bacterial virulence circuits, such as the quorum-sensing signaling system, directly. However, when key ions are present, such as phosphate and iron, certain pathogenic bacteria become insensitive to these incoming host cues.

Conclusion: In this review, we provide molecular insight into the process by which certain surgical infections may be prevented by ionic modulation of the local microenvironment.

Keywords: injury; phosphate; pseudomonas; sepsis; stress; trace metals; trauma

AMONG THE MANY MECHANISMS by which bacteria have evolved to become highly successful pathogens is their ability to process information from their local microenvironment [1]. Bacteria have created exquisite systems to gather, process, and transduce both physico-chemical cues and host-derived elements released during physiologic stress and injury [2]. Among the various physico-chemical cues are pH, redox state, and osmolality, as well as various transition metals and compounds such as iron, phosphate, and zinc [3]. Highly developed two- and three-component systems exist for many of the trace metals and compounds whereby products such as phosphate are ligated onto external appendages and transported into the cell cytoplasm. Once they are present internally, phosphor-sensory and phosphor-regulatory circuits process the information and connect directly to the quorum-sensing signaling system of virulence activation. For example, in the case of phosphate, the well-described PsTS system binds extracellular phosphate and is a receptor to transduce signals in response to the extracellular phosphate concentration [3,4]. When this concentration falls below a certain threshold, bacterial virulence is activated. When it remains above this threshold, bacterial virulence is suppressed. Ostensibly, this system evolved to allow bacteria to sense the degree of public goods available in their surrounding ecosystem as a mechanism to conserve energy and invade cells within a host only when necessary [1]. As

intra-cellular phosphate is abundant in most host cells, bacteria such as *Pseudomonas aeruginosa* become highly invasive when extra-cellular phosphate falls below a particular threshold. Under most conditions of health, extra-cellular phosphate is abundant in intestinal and respiratory mucus in the form of organic phospholipids and as dietary phosphate in the intestinal tract. However, for bacteria to colonize, they must adhere to either the intestinal, respiratory, or other mucosal surface mucins; otherwise, they will be swept away by peristalsis or ciliary action. In normally sterile sites, such as a surgical incision, the only available phosphate is within cells.

In this review, we limit our discussion to phosphate and iron but touch on the topics of zinc and manganese. In each case, we demonstrate that extra-cellular depletion of these life-sustaining ions is an important trigger to which bacteria respond with expression of an invasive phenotype. This finding may have important implications for post-surgical and post-trauma infection and sepsis pathogenesis and may inform novel strategies for prevention of these complications [5].

Depletion of Extra-Cellular Phosphate during Catabolic Stress

Hypophosphatemia is common after severe catabolic stress and was recognized recently as a prognostic sign of a

worse outcome, in particular as a risk factor for surgical infection, sepsis, and death after critical illness [6,7]. Yet it has long been recognized that phosphate excretion into the urine is increased after surgical injury by mechanisms that involve the circulating hormone phosphatonin [8]. In both animals and human beings, recognized initially after open versus laparoscopic cholecystectomy and then after open cardiac surgery, enhanced phosphate excretion has been observed in conjunction with an increase in the serum hormone phosphatonin, which drives phosphate mobilization and urinary excretion. Although this hormone surge is a function of the degree of the surgical stress, the enhanced phosphate excretion does not necessarily result in overt serum hypophosphatemia. Yet when stress is severe, such as after burn injury or severe acute pancreatitis, serum phosphate can become markedly depleted, which portends a poor prognosis [8,9]. Whether the hypophosphatemia is a surrogate marker for the severity of stress, or itself a factor that impairs recovery, has not been established. Particularly, why the body would rid itself of phosphate at a time when it is maximally stressed is not clear, especially given the important need for phosphate for the brain, heart, and all intracellular processes involving protein phosphorylation. However, the degree to which intracellular phosphate stores are depleted after catabolic stress and whether this is significant for the outcome is not clear.

We became interested in the phosphate economy after catabolic stress as it occurs at the intestinal mucosal level, given that phosphate depletion is a trigger for highly pathogenic bacteria such as *P. aeruginosa* to express virulence. When mice were subjected to a standard 30% hepatectomy, an otherwise completely survivable surgical injury, we observed that their mucosal phosphate stores in mucus within the ileum and cecum became depleted to less than 90% of normal [10]. This reaction decreased the mucus phosphate concentration to below the threshold at which virulence expression in *P. aeruginosa* is suppressed, thus resulting in its expression of a more invasive and toxigenic phenotype. When mice were inoculated intestinally with a strain of *P. aeruginosa* and underwent a hepatectomy, virulence was triggered, leading to *P. aeruginosa* gut-derived sepsis [10]. When phosphate was supplied directly at the site of the intestinal inoculum, however, no sepsis was observed; and the mice recovered normally. In strains obtained from phosphate-depleted versus phosphate-sufficient mice after hepatectomy, *Pseudomonas* virulence activation was predictive of a lethal outcome as a result of sepsis. This finding could not be reproduced when mice were given oral or intravenous phosphate, indicating that locally delivered phosphate is necessary to prevent lethal gut-derived sepsis. The ostensible reason for this result is that oral inorganic phosphate is absorbed in the proximal small bowel and is excreted rapidly during catabolic stress (i.e., a 30% hepatectomy) and therefore never replenishes the local phosphate stores at the site of the infectious inoculum, which in this case was the cecum. Similarly, intravenous phosphate administration will not reverse local phosphate depletion at the site of the intestinal inoculum, given that circulating phosphatonins will force its excretion in the urine (Fig. 1).

As a result of these findings, a local phosphate delivery system was developed that could provide a functionally durable degree of phosphate sufficiency directly at the site of the host-pathogen interaction, in this case where *P. aeruginosa* adhered to the cecal mucosa [10]. Taken together, these studies

demonstrate the importance of understanding both the regional and the spatial distribution of key ions during catabolic stress and its effect on the host-pathogen interface. As one might imagine, this involves serial sampling at specific regiono-spatial sites to uncover this important dynamic. Of course, this procedure is challenging in both animals and humans and therefore will require further technical consideration.

To overcome this obstacle, our laboratory developed a muco-adhesive high-molecular-weight polyethylene glycol carrier molecule to which phosphate was bonded covalently [11,12]. In this manner, we were able to provide the solution orally, and it distributed distally and remained functionally durable, eluting phosphate over days. This molecule was able to prevent both gut-derived sepsis caused by *P. aeruginosa* and anastomotic leak secondary to *Enterococcus faecalis* and *Serratia marcescens*, two organisms that express the tissue-destroying enzyme collagenase in a phosphate-dependent manner. A common theme emerging from these observations is that by identifying the local cues that trigger pathogens to express virulence and modifying the ecology of their environment by enriching it with compounds such as phosphate, one can prevent virulence activation and its downstream damage.

Phosphate prevention therapy also has been described for burn wound sepsis. *Pseudomonas aeruginosa* is the most common cause of burn wound sepsis; and despite antibiotics and topical antimicrobials, it persists as a major infection threat to burn patients. Mohammadi-Samani et al. exploited the phosphate-dependent virulence activation of *P. aeruginosa* in a rabbit model of burn injury by creating wound dressings rich in phosphate [13]. Remarkably, compared with antibiotic dressings, burn wound sepsis caused by *P. aeruginosa* was prevented with phosphate-rich wound dressings. Again, this finding reinforces the idea that the suppression of virulence may be more efficacious than microbiocidal or microbiostatic antibiotics and certainly is an ecologically neutral approach to preventing infection during catabolic stress.

Depletion of Iron during Acute Catabolic Stress

That serum-free iron becomes depleted during both acute and chronic illness has been known for decades. When acute-phase proteins such as transferrin and lactoferrin increase in response to catabolic stress, they bind iron and deprive erythrocytes of a key substrate needed for erythropoiesis. As a result, iron-deficiency anemia is a common observation in acute and chronic diseases and has been postulated to be protective against subsequent infection by depriving pathogenic organisms of a key nutrient needed for growth and reproduction [14]. Indeed, iron is among the most potent elements added to bacterial media to promote growth. Most bacteria have iron-scavenging systems, much like PsTs for *Pseudomonas*, that allow them to gather, process, and transduce iron from the extra-cellular environment and, when needed, from the intra-cellular environment. Siderophores such as *Escherichia coli* and *Staphylococcus aureus* have well-described systems for iron acquisition that connect to their virulence-activation systems. Thus, similar to phosphate, when extracellular iron is in short supply, pathogens can activate an invasive phenotype, enter cells, and obtain iron from the host. In many cases, the unwanted activation of inflammation that occurs as these bacteria obtain the

iron they need to support their growth can become a major problem and reduce the probability of recovery.

However, unlike the phosphate story, which was developed long after investigators began speculating about the value of iron during critical illness, the provision of iron during illness, be it chronic or acute, has been much more controversial. One of the earliest reports on the protective effect of iron deficiency on infectious disease was from a group studying subjects at risk for malaria, brucellosis, and tuberculosis in an area endemic for these infectious diseases in Somalia. Subjects were determined to be iron deficient, ostensibly as a mechanism to deprive the iron-loving mycobacteria and the other agents of a major growth factor. Subjects were then randomized to receive iron supplementation or placebo; the results demonstrated a significant (>50%) incidence of infections in the iron-treated group compared to 10% in the placebo group [15]. In this case, iron supplementation was determined to be contraindicated in a situation in which homeostasis has shifted to a new state of equilibrium in which less host iron availability is tolerated as a tradeoff to deprive a lethal pathogen of overgrowth and invasion. This also seems to be the case in areas where malaria is endemic, where iron deficiency can be seen as a host adaptation to an environmental ecology in which exposure to iron-seeking pathogens and parasites is ever present [16]. Yet when iron is provided to infants in formula, the opposite seems to be true [17]. Iron-supplemented infant formula seems to reduce the overall incidence of respiratory infections while preventing anemia [18].

At one time, critical illness and acute surgical injury were deemed to be relative contraindications to iron supplementation, again with the reasoning that such patients were at high risk for secondary infections by healthcare-acquired pathogens that would use the supplemental iron to grow and invade. Yet the scientific and evidence-based principles on which this practice was founded seemed to be misguided, and recent trials have shown a benefit to providing iron to the critically ill and injured. In fact, a recent randomized placebo-blinded trial of iron supplementation in the critically ill patient demonstrated no adverse effect on infection incidence or severity with a benefit in improving anemia [19].

So how do we reconcile the findings that when iron is provided to patients, their serum is more supportive of bacterial growth [20] with the fact that iron supplementation during critical illness does not increase the incidence of infection [19]? These observations seem to be incongruent with the idea that bacterial growth is enhanced in the presence of iron supplementation virtually across all human pathogens of interest.

Much of the confusion and misinformation about the role of ions such as phosphate, iron, zinc, and magnesium on bacterial pathogenesis has at its root a misconception, a lack of understanding of the difference between bacterial growth and bacterial virulence. For example, in virtually every medium for gram-negative bacteria, growth is supported by the addition of iron. For example, blood agar is a highly supportive growth medium for many bacteria. Clinicians tend to view virulence as a property of growth only and sometimes of resistance to antibiotics. Yet there is much more at play in the pathobiology of an invasive toxigenic and lethal pathogen. Although some pathogens are labeled “opportunistic,” in fact, all pathogens are opportunists that obtain nutrients when needed to support their growth and replication. Yet, as mentioned above, when extracellular phosphate stores are plentiful, bacterial growth is supported, and virulence is suppressed. Bacteria tend to invade tissues only when extracellular nutrients are scarce, having learned through evolutionary time that the intracellular milieu of a host is a rich source of food. In our studies of sepsis and anastomotic leak, local provision of phosphate stimulates and enhances pathogen growth while suppressing pathogen virulence, the net result of which is prevention of bacterial invasion, toxin production, sepsis, and anastomotic leak. We have demonstrated this same phenomenon for iron in the case of *Acinetobacter baumannii* infection.

We created a novel model of wound infection caused by this pathogen [21]. This pathogen was recently identified as highly problematic in complicating wounds during Iraqi Freedom. Improvised explosive devices (IED) were especially problematic in causing extremity blast injuries. Once a soldier’s wound became infected with *A. baumannii* after an IED injury, the case fatality rate exceeded 50%. Something about a highly traumatized wound renders *A. baumannii* particularly virulent. To study this infection in mice, we created a novel model of *A. baumannii* wound infection in which the abdominal muscle was both locally injured, by causing muscle trauma with a dissecting instrument (fine tooth pickup), and causing ischemia by ligating the epigastric vessels to the abdominal wall. We discovered that the ability of *A. baumannii* to cause a gross infection in a traumatized wound relies on its ability to scavenge iron [21]. Like many other pathogenic bacteria that are siderophores, *A. baumannii* scavenges iron by releasing acinetobactin into the local environment, and it can bind iron and return it to the organism. As in experiments employing *S. aureus*, *A. baumannii* growth can be enhanced by adding iron-binding agents to the growth

FIG. 1. Proposed mechanism of phosphate economy following catabolic stress. Following injury, increased urinary phosphate excretion develops in response to elevated serum phosphatonin, the main driver of phosphate redistribution. Phosphate depletion at key site of host pathogen interactions, such as in the gut develop and trigger virulence expression and alter the microbiome.

medium. Yet in this set of experiments, we reasoned that if organisms such as *A. baumannii* seek iron when it is locally deficient, and this requires activation of their virulence repertoire, provision of iron should suppress virulence despite promoting growth, similar to what we observed with phosphate [21]. However, this view runs counter to the standard line of reasoning, because in this model, not only did we demonstrate that *A. baumannii* needs iron to support its growth, but mutants of *A. baumannii* that cannot scavenge iron (i.e., deletion of the acinetobactin gene) do not cause infection in our wound model. This finding suggests that iron deprivation, as many have demonstrated with iron-chelating and -binding agents, would be the most efficacious approach. However, in this model, we demonstrated that provision of iron directly into the wound prevented infection by suppressing *A. baumannii* virulence activation [21].

We concluded that perhaps a general principle in the prevention of clinical infection that may have been overlooked is to provide local ion sufficiency in an at-risk wound with the idea that potentially invading microbes will be disincentivized to seek intracellular sources of iron, phosphate, and other trace metals that are vital to their growth. This approach is in line with the overall economic theory that if an ecosystem is rich in public goods, more cooperative behavior, with less predatory and cheating behavior, is observed. This effect might be at play in complex microbial environments such as a wound at risk of infection.

Zinc and Manganese: Trace Metals that Affect Bacterial Behavior in a Manner Similar to Iron

Both zinc and manganese display behavior like that of iron. Similar to the behavior of iron, hosts routinely defend themselves against bacterial pathogens by sequestering zinc and manganese as a method to limit bacterial acquisition of these key growth elements. As a counter-response, many bacteria have highly evolved systems to acquire both zinc and manganese. The mechanisms of these responses are beyond the scope of this review, but their importance in host–pathogen interactions has been reviewed in detail recently [3]. Their role in surgical site infections remains to be clarified.

Embedding Functionally Durable Ions in the Host–Pathogen Interaction Without the Need to Flood the Entire Systemic Compartment

Perhaps much of the confusion about the ability of ionic modulation of the host to prevent infection lies in the challenge of delivering key transition metals and compounds such as iron, phosphate, zinc, and magnesium, to name a few, directly to the host–pathogen interface. As clinicians are accustomed to flooding the systemic compartment with the ion of interest with the hope that it will become bioavailable at the site of a pathogenic threat, this approach has led to much confusion. In vitro studies examining bacterial growth alone are used to inform systemic delivery strategies whose direct effect on host–pathogen interactions are not assessed save for tallying the incidence of clinical infection. As can be seen from the studies on iron and phosphate provision into the wound site, supporting growth while suppressing bacterial virulence, although counterintuitive, may have merit as a method to reduce infection and avoid further need for systemic antibiotics. Surgeons are well aware that there are certain tissue-level in-

fections that will not respond to either systemic or topical antibiotics. In many of these cases, such as burn wounds and soft tissue infections, only radical surgical debridement will suffice. Clinicians will argue that there is insufficient tissue penetration that prevents the efficacy of these antibiotics or that the local milieu (pH, redox, etc.) inactivates the bacteria. However, antibiotics themselves can induce bacterial virulence expression and obviously can promote the emergence of resistant strains. Perhaps it is time to consider other approaches, aimed primarily at prevention, while seemingly counterintuitively, to exploit a more molecular and evolutionarily stable strategy to allow bacteria to grow while limiting their ability to cause harm. Such an approach may be highly useful in the critically ill patient, where multiple mucosal surfaces, such as the lung and gut, are at risk for invasive colonization of healthcare-associated pathogens. Once these pathogens colonize and nutrients cannot be obtained in the usual manner, given the use of antibiotics, chemically defined diets, or parenteral nutrition, such pathogens will invade tissues and cause significant harm. Consideration of a non-microbiocidal approach such as ion supplementation at the site of the host–pathogen interaction may have merit in this context.

Author Disclosure Statement

Dr. Alverdy is the founder of Covira Surgical that is developing phosphorylated PEG polymer to prevent surgical infections. No competing financial interests exist.

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