Molecular Pathogenesis of Necrotizing Fasciitis

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Key Words

group A *Streptococcus*, severe invasive infection, flesh-eating disease

Abstract

Necrotizing fasciitis, also known as the flesh-eating disease, is a severe invasive infection associated with very high rates of human morbidity and mortality. It is most commonly caused by group A *Streptococcus* (GAS), a versatile human pathogen that causes diseases ranging in severity from uncomplicated pharyngitis (or strep throat) to life-threatening infections such as necrotizing fasciitis. Herein, we review recent discoveries bearing on the molecular pathogenesis of GAS necrotizing fasciitis. Importantly, the integration of new technologies and the development of human-relevant animal models have markedly expanded our understanding of the key pathogen-host interactions underlying GAS necrotizing fasciitis. For example, we now know that GAS organisms secrete a variety of proteases that disrupt host tissue and that these proteolytic enzymes are regulated by multiple transcriptional and post-translational processes. This pathogenesis knowledge will be crucial to supporting downstream efforts that seek to develop novel vaccines and therapeutic agents for this serious human infection.

INTRODUCTION

Group A *Streptococcus* (GAS): a gram-positive organism that causes a variety of human infections, including the life-threatening necrotizing fasciitis (flesh-eating disease)

Sepsis: syndrome consisting of a whole-body inflammatory state, often accompanied by hemodynamic compromise and multiple end-organ damage, usually in the context of a severe invasive infection

Historically, group A *Streptococcus* (GAS) has caused epidemics of scarlet fever, rheumatic fever, and puerperal sepsis (childbed fever) (1). Although GAS most commonly causes innocuous infections such as pharyngitis (strep throat) and impetigo, this organism has recently received much public attention due to its ability to also cause necrotizing fasciitis, a devastating infection colloquially known as the flesh-eating disease (2). Since the mid- to late 1980s, there has been an unexplained resurgence in several forms of severe invasive GAS disease, including necrotizing fasciitis, necrotizing pneumonia, and sepsis syndrome (3). There are approximately 10,000 cases of invasive GAS infection in the United States each year, and mortality rates exceed 50% in some reports (4). In particular, GAS necrotizing fasciitis was once considered a fairly uncommon entity, but population-based studies estimate that as many as 1500 to 3000 cases of necrotizing fasciitis occur annually (5). Coupled with this increased incidence, the very dramatic clinical presentation and the exceptionally high morbidity and mortality rate of this disease have drawn much interest to its study (6). The current lack of rapid diagnostic techniques and an effective vaccine contributes a great deal to this health care problem. Furthermore, despite decades of study, many fundamental questions bearing on GAS pathogenesis in necrotizing fasciitis and other invasive infections remain unanswered (7). This knowledge deficit has driven an intense research effort to improve our understanding of the epidemiology, molecular mechanisms, and host-pathogen interactions underlying GAS necrotizing fasciitis in human patients. These recent discoveries are reviewed herein.

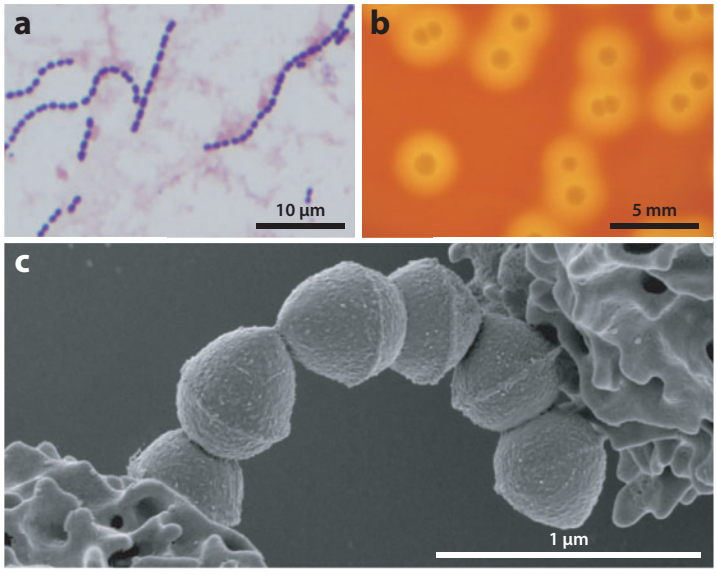


Figure 1

Morphology of group A *Streptococcus* (GAS). (*a*) Micrograph of GAS isolated from the blood of a human patient. GAS are gram-positive cocci that grow in pairs and chains. (*b*) Photograph of GAS grown on solid agar media supplemented with sheep blood. GAS forms small, gray-colored, smoothly contoured, round colonies with a distinct zone of beta hemolysis (area of complete clearing around each colony). (*c*) Scanning electron micrograph of GAS organisms interacting with human neutrophils). Pathogen-host interactions and immune evasion are important themes in necrotizing fasciitis pathogenesis. Printed with permission from Frank DeLeo.

NECROTIZING FASCIITIS

Microbiology of Necrotizing Fasciitis

Clinicians have traditionally categorized necrotizing fasciitis on the basis of the causative organism. As mentioned above, GAS is widely recognized as the most common agent of the flesh-eating disease (**Figure 1**). Other pathogenic gram-positive cocci such as *Staphylococcus aureus* are important but less frequent causes of necrotizing fasciitis (8). Although this organism is a very interesting pathogen in its own right, a complete discussion of staphylococcal infections is beyond the scope of this manuscript, and we direct the reader to a recent review (9). However, we point out that *S. aureus* possesses a unique set of virulence factors that share many overlapping mechanistic themes with GAS, such as epithelial cell adhesion, tissue destruction, and immune evasion. Thus, lessons learned from the study of GAS invasive infections may be extended to necrotizing fasciitis pathogenesis overall.

Several recent publications have reported human necrotizing fasciitis cases that are attributed to a polymicrobial infection. These cases typically involve one pathogen such as

GAS or *S. aureus* that is commonly associated with necrotizing fasciitis, plus a multitude of other less virulent gram-positive bacteria such as alpha-hemolytic *Streptococcus* spp. or *Staphylococcus epidermidis* (10). It remains to be determined whether these polymicrobial cases represent a primary monomicrobial invasive infection that has been subsequently colonized by environmental flora or whether the additional organisms truly participate in a symbiotic infectious process (11). This is a matter of ongoing investigation that may have important implications to pathogenesis research.

Finally, gram-negative bacilli such as *Escherichia coli* and *Bacteroides fragilis* and gas-forming organisms such as *Clostridium* spp. may also cause a distinct type of necrotizing fasciitis known as Fournier's gangrene. This term encompasses a broad spectrum of necrotizing infections that are limited to the perineal, perianal, and genital regions. Often associated with a recent surgical or traumatic disruption of the skin, Fournier's gangrene typically occurs as a secondary complication of other anatomically localized pathologies such as colorectal malignancy, diverticulitis, or anal fissures. Given the restricted microbial and anatomical distribution of Fournier's gangrene, this disease is generally considered to be a different entity from the stereotypical GAS necrotizing fasciitis discussed herein. For further information on Fournier's gangrene, we direct the reader to a recent review (12).

Historical Perspectives on Necrotizing Fasciitis

The earliest report of necrotizing fasciitis is attributed to the fifth century BCE writings of Hippocrates, who described a severe invasive infection that affected many patients during an outbreak of erysipelas (13). His records capture the massive tissue destruction and dramatic clinical presentation that is characteristic of GAS necrotizing fasciitis:

> . . .[T]he erysipelas would quickly spread widely in all directions. Flesh, sinews and bones fell away in large quantities. . . . Fever was sometimes present and sometimes absent. . . . There were many deaths. The course of the disease was the same to whatever part of the body it spread.

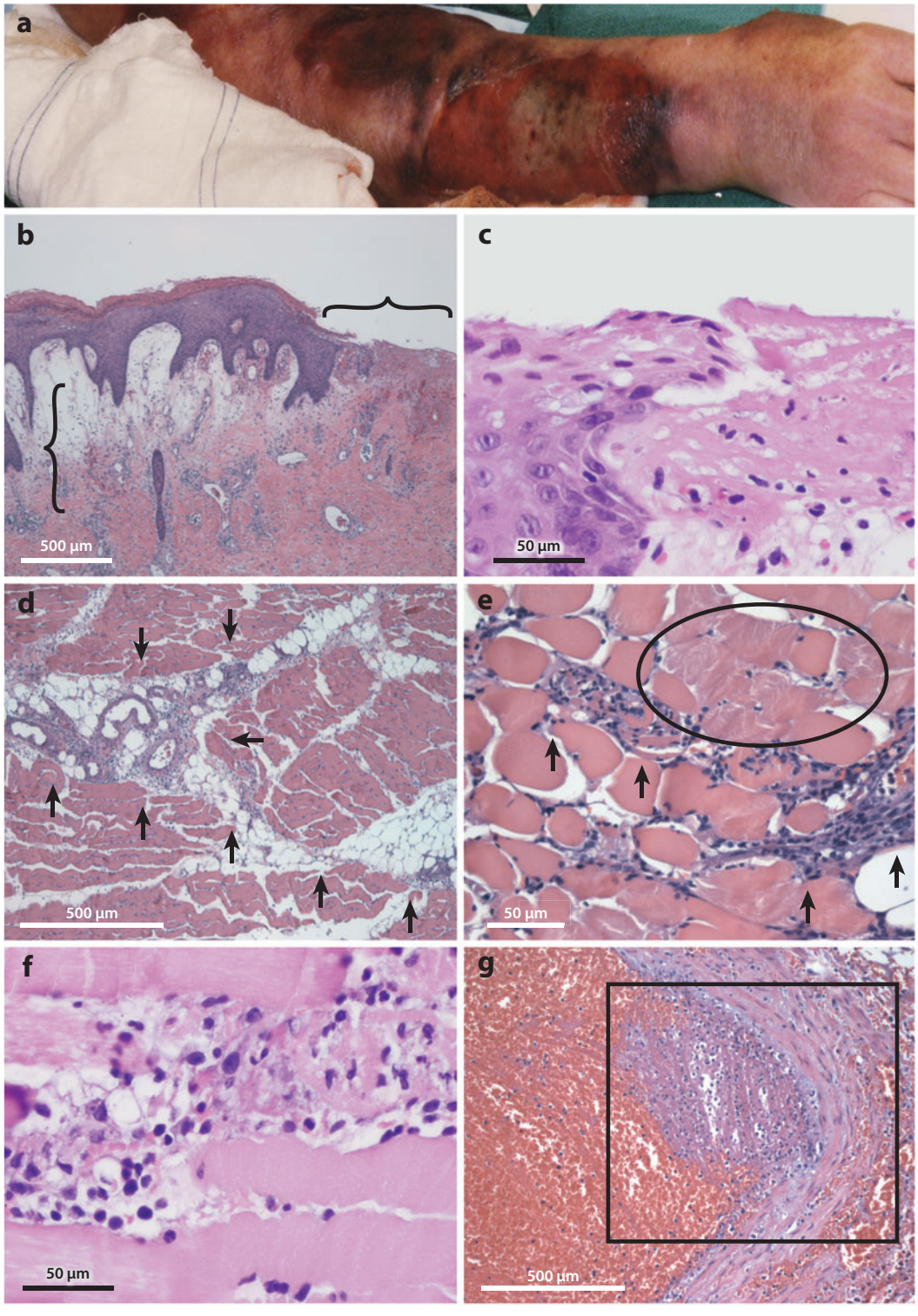
During the eighteenth and nineteenth centuries, British naval surgeons referred to necrotizing fasciitis as hospital gangrene. The first modern report giving a detailed clinical description of hospital gangrene in the United States was written by Joseph Jones, a Confederate Army surgeon practicing during the American Civil War. Over the years, many other terms have been used for necrotizing fasciitis, including flesh-eating bacteria syndrome, suppurative fasciitis, streptococcal gangrene, and necrotizing erysipelas. The moniker necrotizing fasciitis was proposed by Wilson in 1952 (14) to emphasize the predominant histopathological feature of this disease, a vigorous necrotizing infection involving the deep fascia and soft tissue (**Figure 2**).

Clinical Course of Necrotizing Fasciitis

Necrotizing fasciitis is a rapidly progressive, highly destructive bacterial infection involving the skin, subcutaneous and deep soft tissue, and muscle. In a matter of hours to days, the infection can progress from an apparently benign-appearing skin lesion, often mistaken for a spider or insect bite, to a highly lethal disease (**Figure 2*a***). Numerous clinical studies demonstrate that rapid surgical debridement of infected tissue, within 12 to 24 h of initial clinical presentation, is essential for patient survival (15). In many cases, the tissue damage is so severe that multiple surgical procedures or limb amputation is required. The bacterial infection initially spreads along the fascial planes that separate adjacent muscle groups (**Figure 2*b***) (16). The loose organization of fibrous connective tissue and neurovascular structures within these fascial planes poses little anatomic barrier to local dissemination. Then, as GAS organisms proliferate in the normally sterile site,

Necrotizing fasciitis (flesh-eating disease): a severe invasive infection of the fascia and muscle that is characterized by widespread host-tissue destruction and very high rates of human morbidity

Invasive infection: infection of a normally sterile body site such as the blood (bacteremia), lungs (pneumonia), or fascia (necrotizing fasciitis)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there is a rapid influx of acute inflammatory cells. The combined action of many potent protease and other degradative virulence factors expressed by invading GAS organisms and the tissue-damaging enzymes released by host polymorphonuclear (PMN) leukocytes results in severe tissue damage (**Figure 2*c***). Ischemia from microvascular thromboses, a common histological feature of human GAS necrotizing fasciitis, may also significantly contribute to tissue damage (**Figure 2*d***) (17). As the infection progresses, the fascial sheaths are breached, and nutrient-rich skeletal muscle fibers are exposed. The progression of necrotizing fasciitis (**Figure 3**) to include widespread muscle cell death is termed necrotizing myositis, and it is associated with an especially poor prognosis (18). GAS organisms may also gain access to the vascular system, resulting in bacteremia, systemic dissemination, and sepsis syndrome, conditions that are also associated with a poor outcome (19). Thus, in addition to radical surgical intervention, aggressive use of intravenous antibiotics and volume resuscitation are crucial to successful therapy. Emperic agents usually include a combination of penicillin, vancomycin, and clindamycin (20). Clindamycin is thought to slow the rate of tissue destruction because of its inhibitory effect on bacterial toxin production and cell wall synthesis; however, case series have not proven a positive association between clindamycin and survival (19). Similarly, investigators have also hypothesized that intravenous polyclonal immunoglobulin (IVIG) may have a beneficial role in treating severe GAS infections by either opsonizing bacteria or modulating the immune response (21), but experimental evidence supporting its efficacy is lacking (19). Well-controlled human trials are needed to fully evaluate the therapeutic potential of both clindamycin and IVIG in GAS necrotizing fasciitis.

PMN: polymorphonuclear

Diagnosis of Group A *Streptococcus* Necrotizing Fasciitis

Early diagnosis is critical for better outcome, as delayed diagnosis is considered to be an important contributing factor to the very high morbidity and mortality rate associated with GAS necrotizing fasciitis. There is no single clinical laboratory test, imaging technique, or pathognomonic physical exam finding available (2). Patients commonly present with nonspecific symptoms such as fever, exquisitely tender skin lesions, vomiting, diarrhea, and toxemia (22). If the presenting symptoms confer a high index of suspicion, surgical exploration is needed for a definitive diagnosis. Necrotizing fasciitis is then confirmed by visual and microscopic examination of infected tissues (**Figure 2**). Microbial cultures of biopsy material can identify the causative organism and confirm its antibiotic susceptibility. Surgically resected lesions typically show an extensive field of purulent, necrotic tissue that may produce a watery, malodorous fluid. Importantly, because the infection is primarily focused in the subcutaneous and deep soft tissue, the overlying skin often has a bland appearance that underestimates the actual severity of the necrotizing infection. For this reason, patients

Figure 2

Histopathology features of human necrotizing fasciitis. (*a*) The arm of this patient infected with group A *Streptococcus* is very swollen and bruised. A large ulcer is developing on the dorsal forearm. However, the visual appearance still greatly underestimates the true severity of the underlying infection. Printed with permission from Donald Low. (*b*) Microscopic evaluation of the skin shows an erosion of the epidermis (*horizontal bracket*), edematous thickening of the dermis (*vertical bracket*), and sparse inflammatory cell infiltrate (original magnification 2×). (*c*) These histopathological features of the ulcer and dermal-epidermal junction are better seen at high magnification. (*d*) In the infected deep soft tissue, there are marked necrosis, acute inflammatory cell infiltration, and bacterial organisms that are primarily concentrated along the major fascial planes (*arrows*). (*e*) The necrosis is observed to extend beyond the fascial planes to also infiltrate along individual muscle fibers (*arrows*). Numerous nonviable muscle cells are present alongside the advancing infection (*circled region*). (*f*) At high magnification, four severely damaged muscle fibers are seen. (*g*) A characteristic feature of human necrotizing fasciitis is vascular thrombosis (*boxed region*) and hemorrhage.

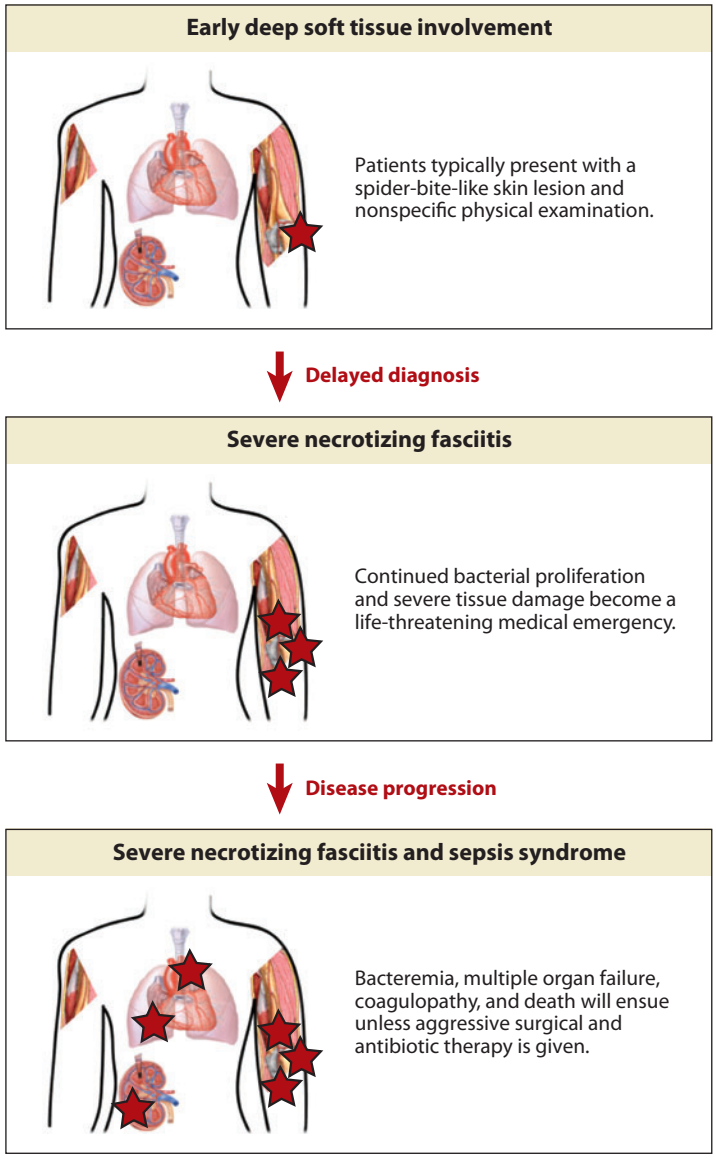

Figure 3

Clinical course of necrotizing fasciitis. Because all patients differ, the following is intended to depict an idealized model. A patient with necrotizing fasciitis will typically first present with an innocuous-appearing skin lesion. A defined site of bacterial inoculation, history of local trauma, or recent group A *Streptococcus* pharyngitis may or may not be reported. If the initial diagnosis is delayed, the deep soft-tissue infection rapidly progresses to severe necrotizing fasciitis. Life-saving treatment then requires aggressive cardiovascular support, surgical excision of infected tissue, and multiagent antibiotics. Clinical investigations also suggest that intravenous polyclonal immunoglobulin may be beneficial in some patients.

commonly complain of pain that seems deceptively out of proportion to the external examination (23). However, as the infection progresses and the neurovascular structures are destroyed, the lesion can become anesthetic. Depending on the clinical context and examination findings, the differential diagnosis of necrotizing fasciitis may include a variety of dermatological and musculoskeletal maladies such as cellulitis, erysipelas, impetigo, phycomycosis, acute neutrophilic dermatosis (Sweet syndrome), pyoderma gangrenosum, hidradenitis suppurativa, myositis, and spider or insect bite. Vascular conditions such as lymphangitis, leukocytoclastic vasculitis, and thrombophlebitis may also be considered. To date, efforts to devise a rapid diagnostic laboratory test panel have been unsuccessful (24). Serum and tissue specimens from necrotizing fasciitis patients contain increased white blood cell counts and increased acute phase response reactants including interleukin (IL)-1, tumor necrosis factor beta (TNF-β), and interferon gamma (IFN-γ); however, these are nonspecific alterations that may also present in less severe infections such as cellulitis (24). Additional studies that use nonhuman primate infection models and human case-control series are needed to identify analytes having greater diagnostic value for GAS necrotizing fasciitis.

Association of Necrotizing Fasciitis with Antecedent Injury

Necrotizing fasciitis may affect any anatomic site. The lower and upper extremities are most commonly involved (10, 23), but numerous published reports also include cases from the head and neck, upper torso, and abdominal wall (25). The infection begins locally, often at the site of an antecedent trauma. Approximately one-half of necrotizing fasciitis patients report a penetrating or crush injury that serves as an apparent inoculating event for infection (26). In a finding with important implications to pathophysiology, case-control studies estimate that compared to patients with GAS cellulitis, necrotizing fasciitis patients are almost

six times more likely to have a recent history of trauma (27). However, the large number of patients who anecdotally report only incidental or no preceding injury indicates that other routes of inoculation must exist. For example, many reports of innocuous injuries such as muscle strains or joint sprains suggest that any disruption of the normal musculoskeletal anatomy may be a key step in GAS necrotizing fasciitis (28). Similarly, many patients also describe instances of recent trauma that are distant from the actual necrotizing lesion.

To explore the hypothesis that antecedent remote trauma contributes to GAS necrotizing fasciitis pathogenesis, Seki et al. (29) used a mouse model in which the mice were artificially bruised in a limb region distant from the primary inoculation site. Compared to unbruised controls, the bruised mice had a significantly higher mortality rate, greater bacterial burden in the injured limb, and earlier onset of bacteremia. These findings suggest that distant injuries may serve as foci for bacterial proliferation and/or dissemination. Thus, even in the absence of grossly compromised skin integrity, necrotizing fasciitis may be initiated by hematogenous seeding of soft tissues by GAS organisms. Investigators have hypothesized possible sources to include transient bacteremia from a subclinical pharyngitis or an asymptomatic nasopharyngeal colonization (30).

In further support of this concept, Bryant et al. (31) have recently shown that the intermediate filament vimentin is significantly upregulated 1–2 days following an acute soft-tissue injury. Regenerating muscle fibers and immature muscle cell precursors were shown to express vimentin on their surface in vitro, providing an adhesive tether for GAS organisms. Hamilton et al. (32) subsequently demonstrated that GAS injected intravenously in mice homed to the site of muscle injury and colocalized with vimentin expression. Importantly, administration of nonsteroidal anti-inflammatory drugs (NSAIDs) enhanced this process, providing the best experimental evidence to date that NSAID use may be a true risk factor for GAS necrotizing fasciitis. NSAIDs are often used to alleviate pain and inflammation resulting from minor injuries. Because NSAIDs are known to dysregulate the expression of cytokines, chemokines, and other inflammatory mediators, Stevens (33) proposed that NSAIDs may predispose individuals to severe GAS infections such as toxic shock syndrome. Clinical epidemiologic studies have shown an inconsistent association between NSAIDs and invasive disease (4, 34). Well-designed animal studies are needed to definitively test the hypothesis that an altered immune response, whether due to pharmaceutical agents or other immunosuppressive comorbidities, contributes to pathogenesis. These studies may explain the reported epidemiological association between necrotizing fasciitis and diabetes mellitus, hepatitis B or C virus infection, and autoimmune disease (4, 6, 18).

GROUP A *STREPTOCOCCUS*

Microbiological Features of Group A *Streptococcus*

Strains of *Streptococcus pyogenes* are serologically categorized based on the scheme originally devised by Rebecca Lancefield and colleagues during the early 1900s (35). These Lancefield types were designated through use of type-specific sera prepared for precipitin reactions on clinical isolates. The carbohydrate antigen (also known as the C substance) studied by Lancefield and coworkers is now recognized to be a major polysaccharide cell wall constituent of *S. pyogenes*. In the case of GAS, the group A C substance antigen is composed of N-acetyl-beta-D-glucosamine linked to a polymeric rhamnose chain.

GAS is a gram-positive, nonmotile, nonspore-forming coccus (**Figure 1*a***). Individual cells are round to ovoid and are 0.6–1.0 μm in diameter. These cocci divide in a single plane, so they occur in pairs or short chains. Longer chains of GAS are often recovered from pure cultures grown in enriched liquid media. As a

member of the lactobacilli, GAS is a fermentative, facultatively anaerobic organism that grows best in nutrient-rich environments. Its characteristic morphological features become most evident when grown on solid agar containing intact red blood cells (**Figure 1*b***). These features include an abundant hyaluronic acid capsule and a prominent zone of beta hemolysis. As discussed below, expression of various secreted and cell-bound virulence factors mediates the pathogen-host interactions underlying infectious disease pathogenesis (**Figure 1*c***).

Beta hemolysis: complete lysis of red blood cells by the bacterium that results in a clear zone surrounding the colony grown on blood agar

M protein: a major group A streptococcal surface protein and virulence factor that forms the basis of the commonly used GAS serotyping classification system

Clinical Diseases Caused by Group A *Streptococcus*

GAS is one of the most frequently found pathogens in humans (5). In addition to causing skin infections such as impetigo, cellulitis, and erysipelas, it is best known as a causative agent of bacterial pharyngitis. Although less common in the modern antibiotic era, postinfectious sequelae remain an important cause of human disease, particularly among medically underserved populations (5). For example, rheumatic fever is the most common preventable cause of pediatric heart disease worldwide, and numerous outbreaks of life-threatening poststreptococcal glomerulonephritis have been reported (5, 36). However, GAS can also infect a variety of normally sterile sites and can cause invasive infections such as necrotizing fasciitis, pneumonia, toxic shock syndrome, and puerperal sepsis. Importantly, the rate of invasive GAS infection has increased in recent decades to more than 3 per 100,000 people per year in developed countries (37). Large, population-based surveillance studies have also shown that GAS exhibits epidemic behavior. Studies seeking to understand the molecular cause of this apparent evolution toward increased virulence have resulted in many important contributions (7, 38). We now have an improved understanding of the temporal-spatial expression of virulence factors that enable GAS organisms to cause these varied infection types in different anatomical sites. These data have important implications for vaccine development.

Group A *Streptococcus* Serotype-Disease Phenotype Associations

As a result of the work initiated by Rebecca Lancefield and colleagues, GAS strains have been subclassified on the basis of serologic differences in the M protein (35, 38). The M protein, encoded by the *emm* gene, is a major secreted surface antigen and multifunctional virulence factor for GAS. Today, DNA sequencing of the *emm* hypervariable region has largely replaced serological typing methods in most laboratories. Although well over 100 different M types have been described, epidemiologic studies have repeatedly identified nonrandom associations between particular GAS M protein serotypes and specific disease phenotypes. For example, serotype M18 GAS strains are strongly associated with acute rheumatic fever, whereas serotype M59, M60, and M61 strains are more commonly isolated from skin infections (38). In comparison, serotype M1, M3, and M28 GAS strains cause both pharyngitis and invasive infections (39). Importantly, the serotype M3 strains are associated with a disproportionately large number of severe invasive infections and deaths (40). This association between GAS strain serotype and patient disease phenotype has led investigators to hypothesize that differences in gene content, and consequently differences in virulence factor expression, between different GAS strains are responsible for these specific disease manifestations. The recent availability of multiple complete GAS genome sequences, in vitro and in vivo transcriptome data sets, and secreted proteome analyses has provided much insight into the determinates of GAS virulence, infection character, and epidemic behavior.

Group A *Streptococcus* Genome Analysis

As the first step in investigating the molecular genetic basis of GAS virulence in humans, whole-genome sequencing projects have been performed (41). Thirteen complete GAS genomes have now been published (42–48).

These strains include serotypes M1, M2, M3, M4, M5, M6, M12, M18, M28, and M49. The availability of these genomic data from different strains causing distinct human diseases such as pharyngitis, acute rheumatic fever, acute poststreptococcal glomerulonephritis, puerperal sepsis, and necrotizing fasciitis has yielded a tremendous amount of new information.

All GAS strains have a genome size of 1.9 Mb, similar G+C content, highly conserved ribosomal RNA operons, and a core set of virulence genes. Approximately 90% of the total gene content is shared among all strains, forming the core GAS genome. The other ~10% of variable genetic material is attributed to the presence or absence of multiple prophages or prophage-like elements that encode specific virulence factors. Each published genome contains between three and eight different prophages (42–48). The majority of these mobile genetic elements encode one or more proven or putative extracellular virulence factors, including secreted toxins, adhesins, degradative enzymes, superantigens, and drug-resistance genes. Importantly, GAS genome–sequencing projects are responsible for identifying many previously unrecognized prophage-associated virulence factors. Whole-genome studies have also identified previously unknown virulence factors such as two-component regulatory genes. Because mobile genetic elements can be readily exchanged between different GAS organisms, prophages represent an important mechanism for strain diversification. All GAS strains also differ in small insertion sequences, small deletion sequences, and single nucleotide polymorphisms (SNPs). The core genome of GAS strains from different M protein serotypes differs by approximately 14,000 SNPs. In comparison, many GAS strains of the same M protein serotype differ by only several hundred SNPs (30).

Variable gene content, either encoded by prophages or due to SNPs, is now hypothesized to be the key contributor to the above-mentioned differences in disease manifestation and pathogenesis observed between different GAS strains. It may also be responsible for epidemic GAS behavior. In support of this hypothesis, Smoot et al. (47) performed DNA microarray analysis on 36 serotype M18 strains collected from varied geographic locations over 50 years. The transcriptome analysis showed that prophages and prophage-like elements were the primary source of variation in gene expression. Thus, when one particular GAS organism acquires a new virulence factor gene via prophage integration into its genome, the resulting bacterium may emerge with new pathogenesis capability. Alternatively, one particular GAS organism may acquire an SNP in an important regulatory gene, thereby significantly altering the expression of several other virulence factors. In either scenario, the new subclone may gain an enhanced ability to infect humans, be transmitted between patients, or cause severe invasive disease. As discussed in the next section, SNP analysis has yielded novel insight into the molecular pathogenesis of GAS necrotizing fasciitis by identifying the *mtsR-prsA*-SpeB axis (49).

SNP: single nucleotide polymorphism

SpeB: streptococcal cysteine protease B

However, many important questions bearing on GAS genomics remain unanswered. For example, the actual genetic diversity existing among strains of the same and/or different M protein serotype isolates collected from different geographic locations, at different times and associated with different infectious disease outbreaks, has not yet been fully studied. To expand the power of these initial studies, many additional GAS genome sequences are needed for analysis. Genomic data generated from additional M protein serotype strains will improve our rudimentary understanding of the associations between strain serotype and disease phenotype. Likewise, genomic data derived from additional strains within each M protein serotype will guide the formation of new hypotheses bearing on molecular pathogenesis and subclone emergence. Now that a core genomic template has been defined for several GAS M protein serotypes, next-generation sequencing technologies can be leveraged to rapidly assemble hundreds of new genome sequences from strain collections. These investigations are crucial for

supporting downstream investigations, including vaccine development.

INTEGRATED MOLECULAR APPROACH TO STUDYING INVASIVE GROUP A *STREPTOCOCCUS* PATHOGENESIS

Overview

An integrated systems biology strategy to investigate the molecular pathogenesis of invasive GAS infections has been relatively effective in enhancing our understanding of pathogen-host interactions (41). Whole-genome sequencing, comparative genomic resequencing, genome-wide expression microarray analysis, proteomic analysis, and bioinformatic analysis have been performed in studies using cell culture, mouse and nonhuman primate infection models, and human patient specimens (30, 42, 47, 50–53). Importantly, there is a growing body of evidence that suggests genetic changes such as acquisition of a bacteriophage-encoded gene or selection of an SNP may confer substantial effects on GAS strain virulence and human infection character.

Role of the *mtsR-prsA*-SpeB Virulence Axis in Necrotizing Fasciitis

The molecular genetic landscape of invasive GAS infections caused by serotype M3 strains has been extensively studied. Using a panel of isolates collected during an 11-year comprehensive population-based molecular epidemiology study conducted in Ontario, researchers have explored the relationship between human invasive infection phenotypes and bacterial strain genotypes at the nucleotide level (30, 42, 52). As mentioned above, serotype M3 GAS strains are excellent model organisms because of their disproportionate association with severe invasive infections (54). Based on a detailed genetic analysis of these 255 isolates, Beres et al. (30, 52) defined six distinct subclones within the strain collection. Importantly, strains of genetically related subclone 5 were epidemiologically associated with a significantly decreased number of human necrotizing fasciitis cases, but they caused normal levels of other invasive infections such as pneumonia. One particularly interesting genetic feature unique to the subclone 5 strains was a 1-bp insertion in the *mtsR* (*metal transporter of* Streptococcus *regulator*) gene. Olsen et al. (49) subsequently hypothesized that *mtsR* inactivation via the single nucleotide insertion caused the observed decrease in necrotizing fasciitis. To test this hypothesis, the virulence of the wild-type, isogenic *mtsR*-deletion mutant and the naturally occurring *mtsR* mutant GAS strains was investigated. Compared with the wild-type strains that have an intact genomic copy of *mtsR*, the *mtsR*-deficient strains have a significantly decreased capacity to cause necrotizing fasciitis in mice and nonhuman primates (49). The *mtsR*-mutant GAS strains caused fewer deaths, smaller lesions with less tissue destruction, and less bacteremia with systemic dissemination. Expression microarray analysis was then used to compare the transcriptome of the wild-type and *mtsR* mutant strains in vitro. Results showed that inactivation of *mtsR*, a transcriptional repressor, upregulated the expression of *prsA*, a peptidyl-prolyl isomerase required for post-translational maturation of the broad-spectrum cysteine protease virulence factor SpeB (55). This finding was notable due to a long-standing interest in SpeB as a virulence factor in invasive GAS infections (56). Subsequent mouse and nonhuman primate experiments demonstrated that isogenic strains overexpressing *prsA* or lacking *speB* reproduced the *mtsR*-negative necrotizing fasciitis phenotype. Thus, through use of an exhaustive systems biology approach, dysregulation of the *mtsR-prsA*-SpeB virulence axis by a single nucleotide mutation was shown to significantly reduce the human necrotizing fasciitis capacity of subclone 5 strains. Furthermore, the study provides a model strategy for understanding the molecular mechanisms underlying unique patient infection phenotype–GAS strain genotype relationships observed in human infectious disease research.

ANIMAL MODELS FOR STUDYING GROUP A *STREPTOCOCCUS* NECROTIZING FASCIITIS PATHOGENESIS

The characteristic clinical disease manifestation and tissue-destruction phenotype of GAS necrotizing fasciitis result from a complex interaction between the virulence factors expressed by invading bacterial organisms and their host-tissue target molecules. Because these invasive infections occur in a heterogeneous environment containing multiple host-tissue types, sophisticated animal models are needed to recapitulate this dynamic in an in vivo pathogen-host relationship (**Figure 4**). Thus, the in vitro and ex vivo assays commonly used by GAS pathogenesis researchers can only test hypotheses under carefully defined conditions, which are not fully representative of in situ infection. Furthermore, because GAS is a human-specific pathogen—that is, it only naturally infects humans and has no known animal reservoir—many of its virulence pathways have been specifically adapted to target human molecules (57). As such, all animal models of GAS-host interaction are limited. Subtle differences between the human target molecules and their homologs in other species can significantly reduce GAS virulence activity. Although necrotizing fasciitis and other invasive infections can be readily modeled in mice by inoculating bacteria directly into sterile sites such as limb muscle, the results must be interpreted cautiously. As the first step in developing improved animal models, investigators have used transgenic mice and other humanized mouse models that express human homologs of the target host proteins (58). These experimental mouse models are likely to better recapitulate the human disease process and to provide a valuable tool for conducting molecular pathogenesis research. However, nonhuman primate infection models are an even more important experimental system for studying GAS necrotizing fasciitis (49, 59). In any typical molecular pathogenesis experiment, a defined number of colony-forming units (CFUs) of one or more GAS strains (i.e., a wild-type strain, an isogenic mutant

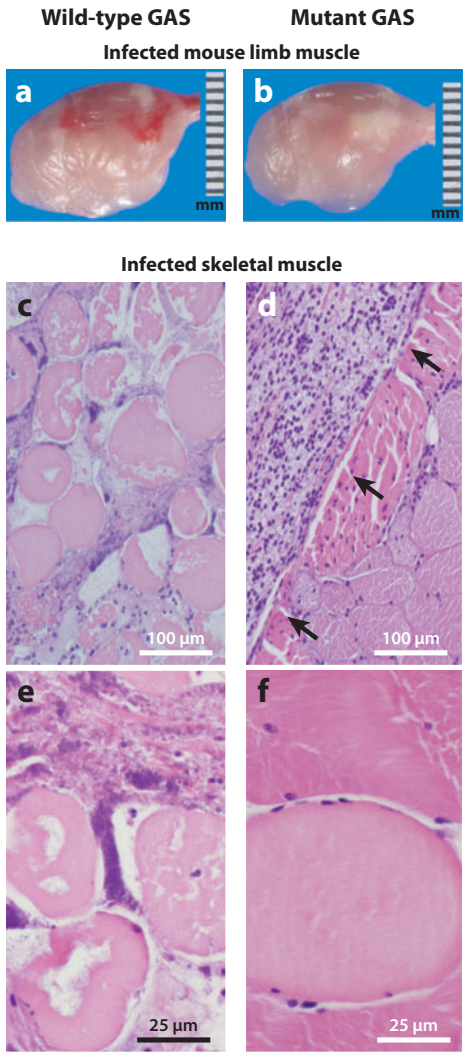

Figure 4

Animal models of group A *Streptococcus* (GAS) necrotizing fasciitis. To investigate a particular gene of interest, animals are infected intramuscularly with either the wild-type or the isogenic mutant strain, and parameters of disease severity are compared. (*a*, *b*) Photographs of infected mouse limbs demonstrate that the wild-type GAS strain (panel *a*) causes larger, more hemorrhagic lesions compared to the mutant strain (panel *b*). (*c*, *d*) Micrographs of infected nonhuman primate limb muscle excised from the inoculation site show that the wild-type GAS strain causes much more severe, widespread fascial and myocyte necrosis (panel *c*) compared to the confined lesion (*arrows*) caused by the mutant strain (panel *d*). (*e*, *f*) These features are better seen at high magnification (panel *e* compared to panel *f*).

strain, and a complemented mutant strain) are inoculated into a group of animals (i.e., via intramuscular injection of 10^6 CFU GAS into the hind limb), and various disease parameters are measured (**Figure 4**). Commonly collected data include overall survival, histopathological progression, and bacterial burden. Comparison of these results between animals infected with the different strains enables a quantitative or semiquantitative measure of the virulence effect attributed to the gene being studied.

GROUP A *STREPTOCOCCUS* PHARYNGITIS PATHOGENESIS IN THE CONTEXT OF NECROTIZING FASCIITIS

Overview

As discussed above, only a fraction of patients with necrotizing fasciitis report an antecedent penetrating injury that likely serves as the inoculation event leading to their invasive infection (60). This recurrent clinical scenario has led investigators to hypothesize that necrotizing fasciitis may sometimes result from the hematogenous seeding of deep soft tissue from a GAS reservoir in the oropharynx or other anatomic site. Asymptomatic carriage and transient colonization may also play an underappreciated role in GAS transmission, particularly among close contacts such as school-aged children and military personnel (61). Moreover, the presence of GAS in the oropharynx is generally considered to be a prerequisite for developing rheumatic fever (5). Thus, GAS pharyngitis pathogenesis, in the context of GAS necrotizing fasciitis pathogenesis, is briefly discussed below.

Epithelial Cell Adherence

GAS adherence to pharyngeal epithelial cells has been extensively investigated (62). Many different GAS molecules have been shown to contribute to cellular adherence, including fibronectin (Fn)-binding proteins (FnBPs) such as protein F1 (PrtF1/SfbI), protein F2 (PrtF2), and Fba (**Figure 5*a***) (63). Fn-binding or adherence activity of protein F3 (PrtF3), a homolog of PrtF2 that is not universally present in all GAS serotypes but is present in several of the highly virulent serotype M3 strains, has yet to be experimentally confirmed (42). Serum opacity factor (SOF) also participates in Fn-mediated host cell adherence (**Figure 5*a***) (64). Although immunization with SOF was previously shown to elicit a robust immune response and to protect mice against intraperitoneal challenge (65), a more recent publication (66) reported that intranasal immunization with SOF was not protective against mucosal challenge. This apparent contradiction may be due to differences in the animal models. Also, the possible synergistic effect of other bacterial organisms present in the oral flora on GAS adherence remains largely untested (11). Furthermore, investigations must be performed to determine whether Fn-mediated binding mechanisms also contribute to GAS virulence in deep soft tissue. It is possible that Fn molecules expressed on host fibrovascular connective tissues and muscle cells facilitate early pathogenesis events in necrotizing fasciitis. For example, the GAS receptor for vimentin binding is unknown (31).

Another recent advance in studying GAS-host cell adherence has been the discovery of pili-like cell-surface structures (67). All known GAS pili are encoded by genes located within the fibronectin, collagen-binding, T antigen (FCT) gene region. Each pilus is composed of a main subunit, which corresponds to the T antigen of the Lancefield T serotype, and two ancillary proteins that are assembled by a sortase and a signal peptidase (68). Importantly, immunization of mice with a pilus protein cocktail was shown to be protective against mucosal challenge (67). On the basis of comprehensive *tee* gene sequencing studies, Falugi et al. (69) estimated that a vaccine cocktail composed of 12 backbone T variants would protect against approximately 90% of currently circulating strains. Information acquired from pilus studies performed in other bacteria, including group B *Streptococcus* and *S. agalactiae*,

may provide additional insight for pathogenesis research (70).

Of particular interest to invasive GAS infection research is that a novel extracellular secreted phospholipase A_2 (SlaA) has also recently been shown to contribute to epithelial cell adherence (**Figure 5*a***) (71). SlaA was first identified by sequencing the genome of a serotype M3 strain isolated from a patient with streptococcal toxic shock syndrome (42). Though not widely distributed among all GAS serotypes, the bacteriophage encoding SlaA is present in almost all contemporary serotype M3 isolates. As discussed above, serotype M3 GAS strains are disproportionately associated with severe invasive infections, so SlaA may be an important contributor to the fitness of these highly virulent organisms. To test this hypothesis, Sitkiewicz et al. (71) used a nonhuman primate model of pharyngitis. The *slaA*-deletion mutant strain was significantly less virulent than its corresponding isogenic wild-type strain. Important to necrotizing fasciitis pathogenesis, the *slaA*-deletion mutant strain caused significantly less mortality, tissue destruction, and bacteremia in mice inoculated by intraperitoneal or subcutaneous injection. Also, immunization with SlaA protected mice from invasive disease (71). Thus, there is strong evidence that vaccine efforts to target GAS adherence molecules such as SOF or SlaA may have great efficacy in pharyngitis and necrotizing fasciitis. Importantly, humans with GAS infections often seroconvert to SlaA (42).

The identification and characterization of the entire GAS surface proteome have

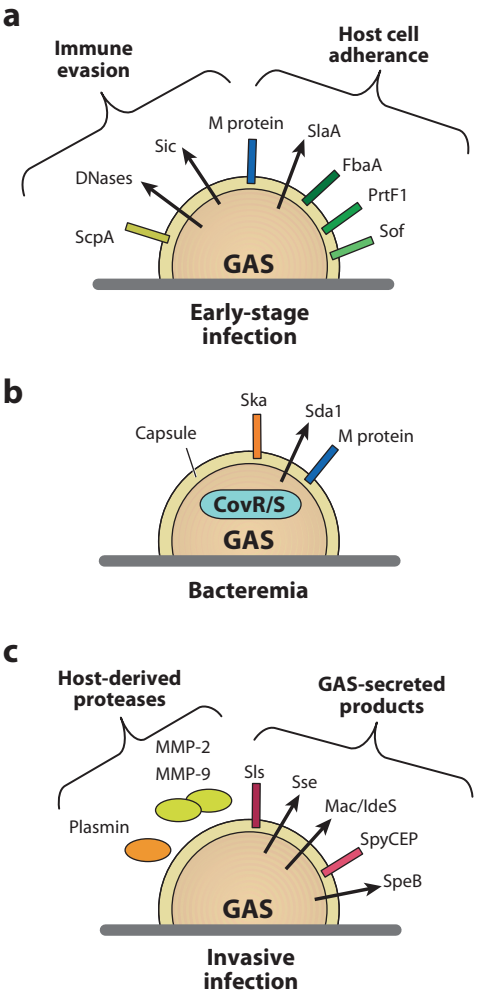

Figure 5

Group A *Streptococcus* (GAS) virulence factors are expressed in a tightly regulated temporal-spatial pattern. (*a*) During early-stage infections, virulence factors mediating host cell adherence and immune evasion are highly expressed. These virulence activities are crucial for establishing an infection. (*b*) During bacteremia, the antiphagocytic capsule and the M protein are highly expressed. Expression of the Ska and Sda1 virulence factors are also advantageous to survival in blood. Sda1 may serve as a selective force for the CovR/CovS mutation that mediates an invasive global transcriptome profile. (*c*) During invasive infections, GAS expresses numerous secreted and cell-bound virulence factors that directly damage host tissue. SpeB may be particularly important to necrotizing fasciitis. GAS also activates host-derived proteases that further contribute to tissue destruction. Abbreviations: Sic, streptococcal inhibitor of complement; SlaA, streptococcal phospholipase A_2; Sof, serum opacity factor; ScpA, streptococcal C5a peptidase; FbaA, fibronectin-binding protein A; PrtF1, protein F1; Ska, streptokinase; Sda1, streptococcal DNase; MMP-2, matrix metalloproteinase 2 (9); Sls, streptolysin S; Sse, secreted streptococcal carboxylic esterase; IdeS, immunoglobulin G degrading enzyme of *Streptococcus*; SpyCEP, *Streptococcus pyogenes* cell envelope protease; SpeB, secreted streptococcal cysteine protease B.

stimulated intense interest in screening for new vaccine candidates (72). The *Streptococcus suis* proteome has also been explored (73). However, unexpected results from molecular pathogenesis studies performed on PrtF1 require that we advance cautiously. Nyberg et al. (74) recently reported that introduction of *prtF1* into a GAS strain lacking a chromosomal copy of the gene actually decreased its virulence phenotype. It was suggested that enhanced anchoring of GAS organisms to host epithelial cells at the initial infection site reduced the likelihood of their subsequent invasion into tissue and dissemination into the vascular system. Importantly, the virulence of the PrtF1-expressing strain was partially restored in transgenic mice lacking plasma fibronectin, suggesting that GAS-fibronectin interactions occur both in tissue and in blood. These results have significant implications for vaccine research. Although vaccines against PrtF1 were once considered a promising target for intervention, investigators now believe they might have unintended consequences. If selective pressure is placed on GAS organisms in the oropharynx to downregulate PrtF1, a hypervirulent strain with enhanced invasion capability could emerge.

Nutrient Acquisition

Once introduced into the oropharynx, GAS organisms must obtain nutrients from the local environment to proliferate. Virtaneva et al. (75) used a nonhuman primate model of GAS pharyngitis to study the relationship among organism density, gene expression, and disease progression. Importantly, GAS proliferation in the oropharynx preceded signs and symptoms of clinical disease, suggesting that the pathogen obtained nutrients from saliva rather than from lysed pharyngeal epithelial cells. Because the very low concentration of glucose in human saliva is insufficient to support sustained GAS proliferation, it is possible that additional carbon sources were needed (76). The in vivo GAS expression microarray studies performed in the nonhuman primate pharyngitis model showed that genes encoding proteins that mediate maltodextrin binding, galactose metabolism, and mannose and lactose transport were upregulated (75). The cell-surface maltodextrin-binding lipoprotein MalE may be a particularly important virulence factor for pharyngitis (77). Shelburne et al. (78) have also shown that GAS organisms rely on human salivary α-amylase to initiate the polysaccharide degradation pathways necessary for downstream energy production. Thus, pharmaceutical manipulation of salivary carbohydrate concentrations or blockade of host and pathogen expressed metabolic enzymes may represent a novel strategy for treating GAS infections. Importantly, the crucial nutrient sources that initially support GAS proliferation in necrotizing fasciitis, prior to widespread tissue destruction and myonecrosis, are currently unknown. Nutrients could potentially be extracted from the rich lymphovascular networks that bathe the fascial sheaths, lysed connective tissue components, or damaged muscle cells.

Immune Evasion

GAS infection in the oropharynx, or any other anatomic site, elicits a host immune response. Immune evasion by invading GAS organisms is critical to human disease pathogenesis. Many of the GAS gene products proven to aid circumvention of the host immune system in the oropharynx probably also contribute to necrotizing fasciitis pathogenesis.

The antiphagocytic M protein and hyaluronic acid capsule are well-described virulence factors that have long been known to promote GAS survival in vivo (**Figure 4*a***) (38). Hollingshead et al. (79) found that M protein was required for GAS persistence in a rat model, and Ashbaugh et al. (80) showed that M protein–deficient GAS strains were considerably less virulent in a mouse invasive infection model. Importantly, immunological protection against GAS correlates with the presence of opsonizing antibodies against type-specific M protein (81). Although most GAS-infected humans seroconvert to conserved regions of the M protein, only antibodies targeting the

variable portion of the amino terminus that defines different M serotypes are opsonic and bind complement (82). Also, Beres et al. (30) recently reported that one of four genetically analyzed GAS strains isolated from asymptomatic carriers contained a substantial deletion in the amino terminal coding region of *emm*. As such, there is considerable interest in vaccine strategies that specifically target antigenic regions within the M protein (83). To be inclusive of all epidemiologically significant GAS serotype strains that commonly cause human disease, investigators have suggested the use of polyvalent cocktails containing as many as 26 different M protein serotypes. The clinical relevance of vaccine strategies that seek to generate a mucosal immunoglobulin A (IgA) response versus a serum IgG response remains uncertain.

Another important GAS surface molecule is the streptococcal C5a peptidase (ScpA), a serine protease that specifically cleaves and inactivates complement protein C5a (**Figure 5*a***) (84). As a result, ScpA decreases PMN leukocyte binding to GAS organisms and recruitment to the infection site. Importantly, Park et al. (85) recently demonstrated that *scpA* mutation or ScpA immunization significantly decreased GAS nasopharyngeal colonization in mice. However, many aspects of ScpA in GAS virulence have yet to be investigated. The recently published crystal structure of ScpB, the ScpA homolog in group B *Streptococcus*, may provide important new insights (86).

Although it was first described more than ten years ago, the in vivo relevance of streptococcal inhibitor of complement (Sic) to host immune evasion has only recently been elucidated (**Figure 5*a***) (87–89). Sic inhibits multiple host-derived molecules, including the complement membrane attack complex, lysozyme, alpha and beta defensins, secretory leukocyte proteinase inhibitor, IFN-γ/CXCL9, and LL-37 (88). Sic also interacts with human cytoskeletal proteins (89). These combined effects result in increased GAS resistance to phagocytosis and bactericidal activity (89). Importantly, Virtaneva et al. (75) demonstrated that *sic* is strongly upregulated early during pharyngeal infection of nonhuman primates. Furthermore, *sic* inactivation significantly impaired GAS proliferation in human saliva ex vivo and murine nasopharyngeal colonization in vivo (51).

Similarly, the critically important contribution of GAS DNases to pharyngeal pathogenesis was also unproven until very recently (**Figure 5*a***). Using an isogenic deletion mutant strain in which all three GAS-encoded extracellular DNases were simultaneously inactivated, Sumby et al. (53) demonstrated that DNase activity is required to cause GAS pharyngitis in nonhuman primates. Furthermore, of great importance to necrotizing fasciitis pathogenesis is that the triple DNase mutant strain was also less virulent in mouse models of skin infection and bacteremia (53). Compared to the wild-type strain, DNase inactivation resulted in increased susceptibility to phagocytosis and bactericide by host PMNs and deficient degradation of DNA. Subsequent studies have confirmed that GAS DNases function in pathogenesis by degrading host-protective neutrophil extracellular traps (90).

Intracellular Invasion

GAS is predominantly an extracellular pathogen, but there is now strong evidence that it also invades host pharyngeal epithelial cells. Moreover, intracellular persistence has been associated with penicillin treatment failure and recurrent infection (91). However, a causal relationship between cellular invasion and pharyngeal pathogenesis has yet to be definitively established. Not unexpectedly, many of the GAS adhesion proteins discussed above have also been implicated in cellular invasion. In vitro experiments have demonstrated that GAS binding to host epithelial cell integrins via FnBPs, M protein, or streptococcal collagen-like protein 1 results in actin cytoskeletal rearrangement and internalization (92). Wang et al. (93) showed that these events occur through a signal transduction pathway involving transforming growth factor beta 1. Fn-dependent internalization of adherent GAS

SpyCEP: *Streptococcus pyogenes* cell envelope protease

organisms may also be mediated by proteolytic cleavage of the cellular anchors (94). CD46 binding by GAS M protein may be important to host cell adhesion, immune evasion, and internalization. Lovkvist et al. (95) recently demonstrated that GAS organisms bind soluble CD46 molecules shed by apoptotic host cells, possibly as a strategy to avoid the immune system. Transgenic mice expressing human CD46 developed significantly higher levels of bacteremia and mortality. Although untested in experimental models, similar GAS cellular adhesion/invasion mechanisms may also contribute to connective tissue and muscle cell damage in necrotizing fasciitis. Additional studies are needed to test this hypothesis.

GROUP A *STREPTOCOCCUS* INVASIVE INFECTION PATHOGENESIS

Overview

Many recent discoveries have provided new insight into the pathogen-host interactions that underlie invasive GAS infections such as necrotizing fasciitis (**Figure 6**). This deeply seated, widely destructive, soft-tissue infection is characterized by severe tissue damage, vascular dissemination, and systemic manifestations that result in very high rates of human morbidity and mortality. As described above, fascial invasion by GAS organisms may occur by (*a*) progression of an antecedent superficial infection, (*b*) direct inoculation with a penetrating injury, or (*c*) hematogenous seeding of a previous injury site. Under any of these scenarios, key pathogenesis steps for GAS to cause necrotizing fasciitis include coordinated virulence factor expression resulting in tissue destruction and vascular dissemination, host defense subversion, and transcriptome modification. Several newly described GAS gene regulatory systems are crucial components to the rapid environmental adaptation needed for necrotizing fasciitis pathogenesis. Importantly, human genetic and acquired factors may also play an underappreciated role in infection susceptibility and severity.

Tissue Damage and Immune Evasion

Rampant tissue destruction and bacterial dissemination are hallmarks of GAS necrotizing fasciitis. For decades, investigators have hypothesized that the extracellular proteases and other secreted GAS molecules are central contributors to invasive disease, especially necrotizing fasciitis. However, until the advent of modern molecular biology techniques and improved animal models, many fundamental virulence questions regarding GAS proteases had gone incompletely answered or unaddressed (41). For example, due to the relative paucity of PMNs in human necrotizing fasciitis lesions, GAS organisms had long been thought to express one or more virulence factors that prevent PMN leukocyte migration and bacterial phagocytosis in vivo, but the identity of these secreted molecules was unknown (96). Recently, Hidalgo-Grass et al. (97) demonstrated that GAS organisms secrete the *S. pyogenes* cell envelope protease (SpyCEP), a proteolytic enzyme that has specific activity against human IL-8 (CXCL8), a host PMN leukocyte–recruiting chemokine (**Figures 5*c*** and **6**). SpyCEP also inhibits PMN leukocyte activity through cleavage of granulocyte chemotactic protein 2 (GCP-2, CXCL6) and growth-related oncogene alpha (GROα, CXCL1) (57). To test the hypothesis that SpyCEP contributes to host-pathogen interactions during invasive infections, Sumby et al. (57) compared the virulence of a wild-type strain and an isogenic mutant strain in a mouse skin infection model. Importantly, the SpyCEP-deficient strain caused greater neutrophil influx, leading to more PMN leukocyte degranulation and degradative enzyme release in situ, resulting in significantly larger skin lesions. Taken together, the implications of these SpyCEP pathogenesis studies are significant. First, the cumulative data confirm that GAS has developed specific virulence mechanisms that protect it against the human innate immune system. Second, they provide additional evidence that host-derived factors such as neutrophil collagenase and elastase contribute to the severe tissue damage characteristic of GAS

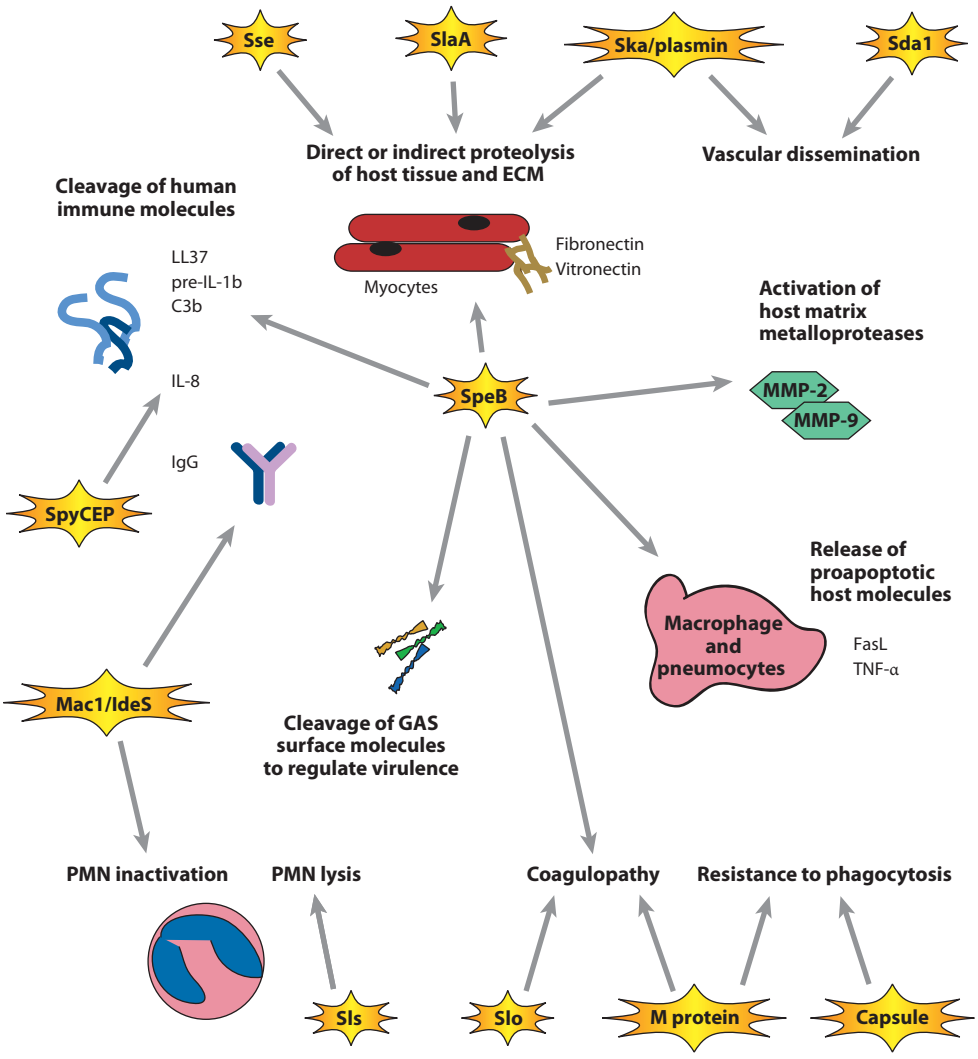

Figure 6

Group A *Streptococcus* (GAS) secretes numerous virulence factors that contribute to the molecular pathogenesis of human necrotizing fasciitis. Importantly, many complex and overlapping pathways exist, and some GAS proteins have multiple functions. A subset of virulence factors such as SpeB and Ska/Plasmin directly damage host tissues, degrade extracellular matrix proteins, and facilitate vascular dissemination via their enzymatic activity. Other virulence factors such as SpyCEP and Mac1/IdeS indirectly damage host tissue by cleaving immune molecules, inactivating or lysing polymorphonuclear (PMN) leukocytes, and stimulating release of proapoptotic molecules. Host matrix metalloproteinases (MMPs) and coagulopathy have also been implicated in GAS necrotizing fasciitis. This schematic depicts the best-studied pathogen-host interactions, but additional virulence factors and pathogenesis pathways exist. Abbreviations: ECM, extracellular matrix; Mac1/IdeS, immunoglobulin G degrading enzyme of *Streptococcus*; Sse, secreted streptococcal carboxylic esterase; SlaA, streptococcal phospholipase A_2; Ska, streptokinase; Sda1, streptococcal DNase I; IgG, immunoglobulin G; IL, interleukin; SpeB, secreted streptococcal cysteine protease B; SpyCEP, *Streptococcus pyogenes* cell envelope protease; TNF-α, tumor necrosis factor alpha; Sls, streptolysin S; Slo, streptolysin O.

IdeS: immunoglobulin G degrading enzyme of *Streptococcus*

Sse: secreted streptococcal carboxylic esterase

necrotizing fasciitis. In support of this theory, Soehnlein et al. (98) recently demonstrated that host PMN leukocyte degranulation also augments lung damage in GAS pneumonia. Importantly, SpyCEP was recently identified as a potential vaccine candidate during a GAS cell-surface proteome analysis (72).

Following a similar pathogenesis theme of secreted proteases inhibiting the host innate immune system, GAS has evolved the Mac1/IdeS virulence factor (**Figures 5*c*** and **6**). Lei et al. (99) first demonstrated that Mac1, a GAS homolog of the human leukocyte beta 2 integrin, inhibits host PMNs by binding to their surface CD16 molecules, low-affinity IgG receptors. IdeS, the same molecule as Mac1, was independently described as a secreted cysteine protease with activity specific for cleaving human IgG (100). Thus, by mimicking a host cell receptor and cleaving human IgG, Mac1/IdeS potentially enhances GAS survival in tissue. Importantly, patients with invasive GAS disease frequently seroconvert to Mac1/IdeS, confirming that it is expressed in vivo (101). However, the virulence role of Mac1/IdeS remains untested in animal models. Also, the true enzymatic activity and pathogenesis effect of allelic variants present in different serotype GAS strains are unknown (102). Of particular interest to rheumatologists and endocrinologists is that Nandakumar et al. (103) recently hypothesized that Mac1/IdeS endopeptidase activity could be exploited to target self-reactive antibodies in patients with autoimmune disease. The Mac1/IdeS crystal structures may assist in these studies (104).

The secreted streptococcal carboxylic esterase (Sse) is another recently identified virulence factor with particular importance to necrotizing fasciitis (**Figures 5*c*** and **6**). It was previously known that GAS organisms secrete a carboxylic esterase in vitro and that pharyngitis patients often seroconvert with esterase-specific antibodies (105). Because these enzymes are capable of cleaving the carboxylic acid ester bonds present in triglycerides and phospholipids, investigators had hypothesized that they may play an important role in tissue invasion and nutrient utilization in vivo. Liu et al. (106) characterized the enzymatic properties of Sse, demonstrating that active or passive immunization of mice with Sse was protective against a lethal subcutaneous GAS challenge. Similarly, the potent pore-forming cytotoxin streptolysin S (Sls) has also been shown to contribute to GAS pathogenesis in mouse invasive infection models (107).

The cellular localization of GAS proteases may have important implications that bear on their role in necrotizing fasciitis pathogenesis. For example, secreted virulence factors such as SpeB and Sse are released from the organism into the surrounding host tissue. As such, they are likely to diffusely damage fascia and muscle cells distant to the organism, preparing the anatomic region for impending GAS invasion. In comparison, the protease activity of a surface-retained molecule such as SpyCEP is concentrated near the invading organism, where it can be directed to specific targets such as basement membranes and vascular walls. Although the mouse infection model studies comparing wild-type and isogenic mutant strains demonstrated each of these GAS protease virulence factors to be significant alone, it is likely that their combined effects are required for optimal in vivo necrotizing fasciitis pathogenesis. Importantly, immunization with several newly described degradative enzymes protects mice against lethal challenge, suggesting a role for these proteases in GAS vaccine research (71, 72, 106). Additional studies in nonhuman primates and human patients are needed to fully address their potential clinical relevance.

Subversion of Host Molecules

In addition to expressing protease virulence factors, GAS organisms also usurp host-derived molecules to facilitate disease progression. This is an expanding concept in GAS pathogenesis research. One of the best-known examples of this phenomenon involves the broad-spectrum cysteine protease SpeB (**Figures 5*c***

and **6**). SpeB is well known for its ability to cleave many host molecules, including extracellular proteins such as fibronectin and vitronectin, and immunologic mediators such as pre-IL-1β and the antimicrobial peptide LL-37 (31, 108). This direct proteolytic activity has obvious implications for understanding the tissue destruction that occurs in necrotizing fasciitis lesions. However, SpeB also activates host matrix metalloproteinases (MMPs), including MMP-2 and MMP-9 (109). GAS, through an unknown mechanism, also activates MMP-13 in chondrocytes in vitro (110). Analogous to the molecular processes that underlie cancer cell metastasis, dysregulated MMP activity may contribute to necrotizing fasciitis by further degrading host-tissue components that serve as barriers to GAS dissemination. Because MMPs are also responsible for the normal remodeling processes that maintain proper structure and function of the extracellular matrix, loss of this reparative activity may further intensify tissue damage. In support of this hypothesis, SpeB-activated MMP-2 and MMP-9 have been shown to stimulate the release of proapoptotic molecules such as TNF-α and FasL from infected human macrophages in vitro and mouse pneumocytes in vivo (111). Though extensively studied in cardiac, rheumatological, and neoplastic disease, the potential therapeutic application of MMP inhibitors in invasive infections such as GAS necrotizing fasciitis has not yet been tested.

Following a similar theme of host molecule subversion to generate new virulence mechanisms, invading GAS bacteria can also commandeer the human plasmin system (**Figures 5*c*** and **6**). During an invasive infection, secreted GAS proteases such as SpeB and SpyCEP damage vascular structures. When vessel integrity is compromised, host serum proteins such as plasminogen leak into the surrounding tissue and are captured by nearby GAS organisms (112). Several GAS molecules capable of binding human plasminogen, including M protein, M protein–related protein, glyceraldehyde-3-phosphate, and streptococcal enolase (113), have been identified. GAS streptokinase (Ska) complexes with the surface-bound plasminogen proenzyme to form an activator complex that converts unbound plasminogen molecules into plasmin. Plasmin, a broad-spectrum serine protease, then becomes yet another of the degradative enzymes present within the expanding necrotizing fasciitis lesion. In support of this hypothesis, coadministration of purified human plasminogen significantly reduces the number of GAS CFUs needed to generate equivalently sized skin lesions in mice (114). Also, transgenic mice expressing human plasminogen develop significantly more severe invasive disease when infected with Ska-expressing GAS strains (58). Importantly, Ikebe et al. (115) recently showed that *ska* transcript levels are increased in serotype M49 GAS strains isolated from necrotizing fasciitis cases compared to isolates from noninvasive infections. This provides a possible genetic explanation for previous reports of invasive infection isolates having increased plasminogen-binding activity (116). Notably, the in vivo significance of a newly discovered *ska* allelic variant with decreased in vitro plasminogen activation capacity has yet to be determined (117).

Knowledge gained from these studies emphasizes a crucial concept in GAS pathogenesis research. This pathogen has evolved an extraordinary level of human-specific host adaptation. Thus, hypotheses bearing on GAS necrotizing fasciitis pathogenesis must be tested in animal models that best recapitulate the human disease condition. For example, MIP-2, the murine homolog of human IL-8, is more resistant to cleavage by SpyCEP due to amino acid differences in the cleavage site (118). Similarly, GAS Ska is specific for human plasminogen, so transgenic mice expressing the human proenzyme are needed to fully understand this system (58). For the same reason, results from our ongoing investigation of the *mtsR-prsA*-SpeB virulence axis have been confirmed in a nonhuman primate model of necrotizing fasciitis (49).

The Transition from Local to Invasive Infection

An important goal of contemporary necrotizing fasciitis pathogenesis research is to better understand how the GAS transcriptome changes at each stage of infection. A growing body of data has demonstrated that GAS organisms undergo a very complex molecular transition during the progression from a localized to an invasive infection (2). Thus, not only is the virulence potential of any GAS strain determined by its repertoire of virulence factor genes, but it is also significantly influenced by transcriptional regulators. In support of this hypothesis, Sumby et al. (119) demonstrated that many GAS isolates from human pharyngeal and invasive infections have distinct global expression microarray profiles. The pharyngeal-type profile differed from the invasive-type profile by approximately 10% of the entire transcriptome. Importantly, when pharyngeal-type profile GAS isolates were inoculated subcutaneously in mice, they acquired an invasive-type expression profile and enhanced virulence phenotype. Subsequent studies have shown that various function-altering mutations in either the sensor kinase (CovS) component or the transcriptional repressor (CovR) component of the CovR/CovS two-component regulatory system underlie these massive transcriptome changes (119). Importantly, Engleberg et al. (120) also reported the emergence of CovR/CovS mutants with enhanced virulence in vivo. Gene-expression studies performed on GAS strains with inactivating mutations in CovR/CovS revealed a marked dysregulation of many proven and putative virulence factors, including *speB*, *spyCEP*, *ska*, *sic*, and *sdaD1*. CovR/CovS also directly or indirectly influences other regulatory genes such as *two-component regulatory system X* (*trxSR*) and *multiple gene activator* (*mga*) (121). Thus, the CovR/CovS two-component regulatory system plays a central role in directing infection phenotype. These findings provide many new avenues for GAS necrotizing fasciitis pathogenesis research.

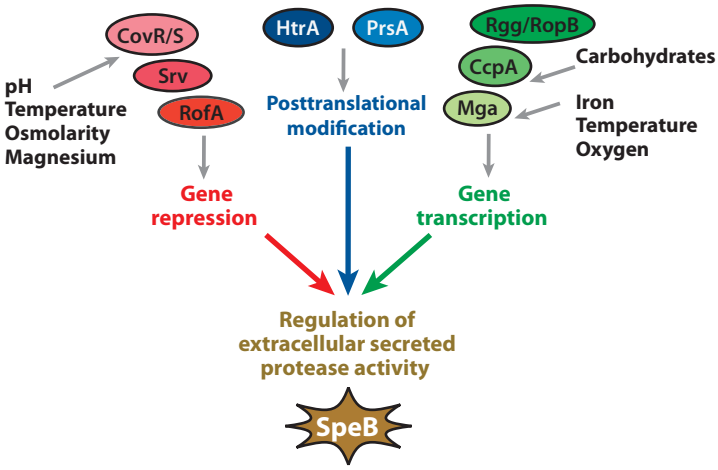

Figure 7

SpeB, a broad-spectrum cysteine protease virulence factor, is regulated by multiple intersecting and collateral pathways that respond to different environmental stimuli. SpeB is transcriptionally repressed by CovR/CovS, Srv, and RofA (*red*), whereas it is transcriptionally activated by Rgg/RopB, CcpA, and Mga (*green*). SpeB activity is also regulated by posttranslational modifications mediated by HtrA and PrsA (*blue*). The combined effects of these multiple regulatory pathways result in the in vivo temporal-spatial expression pattern of SpeB. Abbreviations: SpeB, secreted streptococcal cysteine protease B; CovR/S, control of virulence genes regulator/sensor; Srv, streptococcal regulator of virulence; RofA, regulator of protein F; RopB, regulator of proteases B; CcpA, catabolite control protein A; Mga, multiple gene activator; HtrA, serine protease with high temperature requirement A; PrsA, peptidyl-prolyl isomerase A.

Importantly, CovR/CovS responds to a variety of environmental cues that may signal the transposition of a GAS organism from a mucosal to an invasive site. Stimuli such as elevated temperature, acidified pH, and high osmolarity are all present in necrotic tissue (122). Other proven stressors include elevated magnesium, low iron, and the antimicrobial peptide LL-37 (**Figure 7**) (123). Thus, strategies that either alter these host environmental signals or directly block the transcriptome switch could have significant therapeutic value in GAS necrotizing fasciitis. Manipulation of small inhibitory RNA molecules may be a promising option for developing novel molecular therapeutics against GAS (124).

Coagulopathy in Necrotizing Fasciitis

Coagulopathy is a well-recognized clinical feature of GAS necrotizing fasciitis. In a

multivariate analysis of morbidity data collected during a prospective population-based surveillance study of invasive GAS infections, Mehta et al. (19) demonstrated that coagulopathy is an independent marker of mortality. Thus, dysregulation of the delicate balance between host procoagulant and thrombolytic activity may represent an additional GAS virulence mechanism (**Figure 6**). Regional hypoxia due to vascular thrombosis is believed to significantly promote tissue damage in necrotizing fasciitis, but thrombus formation may alternatively limit the hematogenous spread of bacteria from necrotic lesions (125). In support of this hypothesis, Sun et al. (126) demonstrated that genetically engineered mice with defects in clotting factor V function, thrombin generation, or fibrinogen expression were significantly more susceptible to lethal GAS challenge by subcutaneous injection. SpeB and streptolysin O (Slo) have also been implicated in vascular compromise due to their proteolytic effect on endothelial cells, platelets, and other vasoactive compounds (17). Similarly, the GAS M protein exerts procoagulant effects by inducing tissue factor expression and activating platelets (127). Thus, pharmaceutical manipulation of GAS-host coagulation pathways, which could take place either by inhibiting Ska activity or through Ska production, may have therapeutic value in necrotizing fasciitis. Published cases reporting a good outcome in necrotizing fasciitis and sepsis patients following use of the recombinant human activated protein C support this hypothesis (128). Insight gained from the crystal structures of M1 protein, Ska, and the Ska-plasminogen activator complex may help guide the rational design of these new drugs (129, 130).

Regulatory Mechanisms Controlling Virulence Factor Expression

Further adding to the complexity of our emerging molecular pathogenesis model of GAS necrotizing fasciitis is that investigators have demonstrated that temporal-spatial factors significantly contribute to virulence factor expression and infectious disease progression (**Figure 7**). For example, SpeB is expressed at very low levels during the early growth phase. Transcripts are undetectable until the mid- to late-log phase of growth in nutrient-rich liquid media (131). An initial relative lack of SpeB allows other GAS surface molecules such as M protein and SlaA to adhere to host cells and drive proliferation. Later in growth, *speB* expression is significantly upregulated in many strains (132). At this point, SpeB proteolytically inactivates GAS surface molecules, a process that may release the adherent organisms from their host cell tethers (133). Concurrently, SpeB also degrades host-derived molecules, facilitating tissue dissemination (134). Upon transitioning to a bacteremic stage, *speB* is again downregulated, rejuvenating the activity of other GAS virulence factors such as Sda1 and Ska that are advantageous to survival in blood (**Figure 5*b***) (135). Walker et al. (135) have suggested that Sda1 actually serves as a selective force for the CovR/CovS mutation during bacteremia. Importantly, however, SpeB may be upregulated when GAS organisms disseminate into tissue. In support of this last hypothesis, immunohistochemical analysis of human tissue from necrotizing fasciitis cases are strongly positive for SpeB (136). In situ, SpeB colocalizes with host defense molecules such as the antimicrobial peptide LL-37, a known cleavage target of SpeB (136). Furthermore, as discussed above, Olsen et al. (49) recently demonstrated that GAS strains with decreased SpeB secreted protease activity have a significantly impaired capacity to cause necrotizing fasciitis in mice and nonhuman primates. Researchers have also shown that intact SpeB is needed for full virulence in invasive infection models in mice (56, 137). Thus, altered SpeB protease activity in a subpopulation of invading organisms, whether due to changes in gene expression or posttranslational processing, may contribute to the overall tissue pathology caused by the total GAS population during an invasive infection (138).

During invasive infection, GAS must rapidly adapt to changing environmental stressors.

SpeB expression is coordinated through multiple collateral and intersecting gene regulatory pathways that are responsible for this temporal-spatial expression pattern (**Figure 7**) (119, 139). Rgg, also termed RopB, is the best-characterized regulator of SpeB (140). *covR/covS*, *mga*, *srv*, and *dpp* have also been suggested to directly or indirectly influence *speB* expression. As such, *speB* responds to many different environmental stimuli, including pH changes, salt concentration, and nutritional stress (**Figure 7**) (132). Other crucial GAS virulence factors are probably also regulated through similarly complex transcriptional mechanisms (135). The catabolite control protein A may also play an important role in necrotizing fasciitis by directly linking host metabolism to virulence factor expression (78).

Posttranslational events also regulate GAS virulence factor expression (**Figure 7**). For example, the peptidyl-prolyl isomerase PrsA is needed for full SpeB maturation (55). The GAS ExPortal, an organelle dedicated to extracellular trafficking of secreted proteins, has also been indirectly linked to SpeB maturation (141). Posttranslational processing may be particularly important to regulating virulence factor activity at the infection site. Additional studies are needed to test this hypothesis.

HOST FACTORS

Clinicians and researchers have long known that a single GAS strain can cause very different disease manifestations in different patients. However, the importance of host factors and genetics in susceptibility and severity has only recently become evident. Results from case-control and molecular epidemiology studies have made many of these new discoveries possible. For example, human patients with low acute-phase antibody levels to various GAS molecules are predisposed to developing severe invasive infections (142). However, once initiated, antibody levels apparently have no effect on infection severity or outcome (143). Maripuu et al. (144) have hypothesized that host antibody titer is less critical than antigen neutralization and leukocyte mitogenicity. Following a similar theme of differential host susceptibility, different HLA class II haplotypes and TNF-α microsatellites may either confer protection against or increase risk for severe disease (145). This may be related to the affinity of particular HLA haplotypes for binding GAS superantigens. Norrby-Tegland et al. (146) recently demonstrated that the superantigen SpeF is expressed in situ in high-clinical-grade lesions. Compared to patients with lower-grade lesions, these patients also had high expression levels of several potent cytokines, including IL-1, TNF-β, and IFN-γ. Taken together, these findings suggest that new vaccine and treatment strategies may need to be customized for different host genotypes. Following this line of reason, Ulrich (147) recently showed that a fusion protein containing highly immunogenic but functionally inactivated portions of the superantigen SpeA and the cysteine protease SpeB protected mice against lethal invasive infection. In addition, aside from IVIG preparations that still have an uncertain role in necrotizing fasciitis treatment, immunomodulatory agents have not been adequately studied in necrotizing fasciitis patients.

Preexisting medical conditions may also significantly contribute to necrotizing fasciitis pathogenesis. Among adult patients, diabetes mellitus, malignancy, intravenous drug abuse, alcoholism, and recent NSAID use are epidemiologically linked to severe invasive GAS infections (19, 148). This enhanced susceptibility may be due to immunosuppression from the disease process and its treatment, anatomical defects such as vascular damage and hemodynamic compromise, or other physiological mechanisms, and it may underlie the very high mortality rate of hospital-based outbreaks of GAS necrotizing fasciitis (149). A preceding varicella zoster infection or early age of first GAS exposure is strongly associated with severe invasive disease among children (150). Interestingly, GAS necrotizing fasciitis is relatively uncommon among pediatric patients, suggesting that age-dependent pathogen-host interactions may exist (148). In support of this hypothesis, an

exuberant cytokine response is associated with increased disease severity in children.

FUTURE RESEARCH

Although significant advances in our fundamental understanding of GAS necrotizing fasciitis pathogenesis have been achieved, many important questions bearing on pathogen-host interactions remain unanswered. For example, investigators have recently begun applying genome-wide approaches to learn how subtle changes in GAS gene content affect virulence and infection phenotype. However, the host factors that also contribute to tissue destruction and mortality in necrotizing fasciitis are not known. In vitro and ex vivo transcriptome studies have also contributed to our knowledge of GAS virulence factor expression and regulation, but with the exception of pharyngitis, no in vivo analyses that use animal models and human patients have been performed. It is likely that there are specific spatial and temporal transcriptome profiles associated with necrotizing fasciitis and other invasive infection types. Humanized mouse and nonhuman primate models will also be needed to advance this line of research. These molecular data will support downstream efforts to rationally develop new vaccines, diagnostics, and therapies. Finally, although much effort is being devoted to vaccine testing in animal models, it is not known whether these strategies will be effective in preventing and/or treating human infections.

SUMMARY POINTS

1. Necrotizing fasciitis, also known as flesh-eating disease, is a serious invasive infection associated with very high rates of human morbidity and mortality.
2. GAS is the most common causative agent of necrotizing fasciitis.
3. GAS has acquired an exceptionally high level of host specificity.
4. Genome-wide approaches and improved animal models have answered many key questions and identified new lines of investigation bearing on the molecular pathogenesis of GAS necrotizing fasciitis.
5. GAS expresses a variety of different virulence factors that contribute to the severe tissue destruction that is characteristic of necrotizing fasciitis.
6. Virulence mechanisms include host cell adhesion, immune evasion, and tissue destruction by GAS-derived molecules. Host-derived molecules can also be commandeered by GAS organisms to further contribute to tissue damage.
7. Multiple collateral and intersecting transcriptional and posttranslational pathways regulate GAS virulence factor expression.

FUTURE ISSUES

1. Although in vitro microarray studies have greatly contributed to our understanding of GAS gene expression under defined laboratory conditions, similar studies must be performed in vivo using animal infection models and human patient specimens.
2. Population-based studies have identified epidemiologic associations between specific GAS genotypes and human infectious disease phenotypes, but hypothesis-driven investigations are needed to confirm the underlying molecular pathways.

3. The host factors contributing to necrotizing fasciitis susceptibility, severity, and outcome remain largely unstudied. These could have important implications to vaccine development.
4. Despite decades of study, there is no commercially available GAS vaccine. Many candidate antigens have been identified, but their efficacy has not yet been tested in nonhuman primates or in humans.

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