Muscle Injury, Vimentin Expression, and Nonsteroidal Anti-inflammatory Drugs Predispose to Cryptic Group A Streptococcal Necrotizing Infection

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Background. Myonecrosis due to group A streptococci (GAS) often develops at sites of nonpenetrating muscle injury, and nonsteroidal anti-inflammatory drugs (NSAIDs) may increase the severity of these cryptic infections. We have previously shown that GAS bind to vimentin on injured skeletal muscles in vitro. The present study investigated whether vimentin up-regulation in injured muscles in vivo is associated with homing of circulating GAS to the injured site and whether NSAIDs facilitate this process.

Methods. M type 3 GAS were delivered intravenously 48 h after eccentric contraction (EC)–induced injury of murine hind-limb muscles. Vimentin gene expression and homing of GAS were followed by real-time reverse-transcriptase polymerase chain reaction and quantitative bacteriology of muscle homogenates, respectively. In separate experiments, ketorolac tromethamine (Toradol) was given 1 h before GAS infusion.

Results. Vimentin was up-regulated ~8-fold 48 h after EC. Significantly more GAS were found in moderately injured muscles than in noninjured controls. NSAIDs greatly augmented the number of GAS in injured muscles.

Conclusions. Vimentin may tether circulating GAS to injured muscle, and NSAIDs enhance this process. Strategies targeting the vimentin–GAS interaction may prevent or attenuate GAS myonecrosis. Use of NSAIDs should increase suspicion of cryptic GAS infection in patients with increasing pain at sites of nonpenetrating muscle injury.

The incidence of invasive infections with group A streptococci (GAS), such as necrotizing fasciitis, myonecrosis, and streptococcal toxic shock syndrome (StrepTSS), remains at 3.5 cases per 100,000 population per year [1]. Despite better clinical recognition of StrepTSS and intense research on streptococcal virulence factors, morbidity is high, and the mortality rate remains between 30% and 70% [1].

Nearly half of all patients with StrepTSS who have necrotizing fasciitis or myonecrosis develop deep-seated infection at the site of minor, nonpenetrating trauma or muscle strain [2–4]. For instance, of the 20 patients with invasive streptococcal infection described by Stevens et al. [2], 12 had a defined portal of entry. Of the remaining 8 patients, 1 had a superficial bruise to the hand, and the portal of entry was entirely unknown in the other 7 [2]. Thus, 8 (40%) of 20 patients in this series had no defined portal of entry, and the overall mortality rate was 30% [2]. Similarly, a report by Adams et al. [3] documented 21 cases of life-threatening GAS infection; 19 patients lacked an obvious portal of entry, and 18 (85.7%) died. Finally, a recent case-control study found that nonpenetrating trauma was significantly associated with GAS necrotizing fasciitis [4]. In these cryptic infections, the correct diagnosis is often delayed until after shock and organ failure are manifest [1], often causing the mortality rate to exceed 70% [3]. Survivors undergo emergent amputation or extensive surgical debridement and prolonged hospitalization [1, 2, 5].

After minor injury, nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently self-prescribed or given in emergency departments to alleviate pain. Because NSAIDs dysregulate production of the cytokines that mediate septic shock, Stevens [6] proposed that NSAIDs...
may actually predispose individuals to more-severe streptococcal infection. Some clinical epidemiologic studies have confirmed the association between NSAIDs and streptococcal infection, yet controversy still exists (reviewed in [7]).

Our initial analyses of the molecular mechanisms responsible for these cryptic necrotizing GAS infections demonstrated that injury of cultured human skeletal muscle cells increases the binding of GAS and that increased levels of surface-expressed vimentin correlates with enhanced adhesion [8]. In addition, we have shown that GAS bind to vimentin on the surface of skeletal muscle cells in vitro and that GAS are found in association with vimentin-positive necrotic muscle in vivo [8].

To further investigate the role of vimentin in GAS infection at sites of muscle injury, the present study used a murine model of eccentric contraction (EC) [9, 10] to mimic the antecedent injury in the “no-portal” group of human GAS myonecrosis cases. Here, the anterior hind-limb muscles were electrically stimulated to contract while the foot was mechanically flexed in the opposite direction, thereby creating a muscle strain. At 48 h after EC-induced injury, when vimentin was maximally expressed, M type 3 GAS were delivered intravenously. In separate experiments, animals received a nonselective NSAID before GAS infusion. The homing of GAS to injured muscle was determined by comparing the number of bacteria in exercised and contralateral, nonexercised muscle homogenates. The results demonstrated that moderate injury is sufficient to initiate GAS infection and that NSAIDs greatly augment this process.

**METHODS**

**EC exercise protocol.** Animal studies were approved by the Boise Veterans Affairs Medical Center’s Subcommittee on the Use and Care of Animals in Research. Adult Swiss Webster mice (female; weight, 22–28 g; Charles River Laboratories) were anesthetized (2% isoflurane) and positioned on a temperature-controlled platform adjacent to a muscle physiology apparatus (modification of model 360B; Aurora Scientific) that permits measurement and control of both ankle dorsiflexion torque and muscle length (position) [9]. The tibialis anterior (TA) and extensor digitorum longus (EDL) muscles were stimulated electrically at a frequency of 100–150 Hz by sterile 28-gauge needle electrodes placed subcutaneously 2–3 mm apart near the right peroneal nerve, just lateral to the midline and distal to the knee joint. The maximal mean isometric torque (MIT) generated by 2 type 3 GAS were stimulated electri-

The opposite leg of each animal was not exercised and served as a control. A separate group of animals was similarly treated, except that the foot was held stationary (isometric contraction).

After completion of the exercise protocol, maximal MIT was again measured. After this, the electrodes were removed, and the animal was immediately treated with a single dose of the analgesic buprenorphine at 0.1 mg/kg in 0.5 mL sterile saline given subcutaneously in the scruff of the neck. The animal was allowed to recover from anesthesia and returned to its cage. EC is a well-characterized model for muscle injury that demonstrates (1) injury and repair-specific alterations in cytoskeletal architecture and (2) alterations in muscle-specific gene expression, including vimentin up-regulation [10].

**EC-induced vimentin gene expression.** The TA muscles from both exercised and contralateral, nonexercised legs were harvested at 12, 24, 48, or 72 h after exercise and immediately flash-frozen in liquid nitrogen for subsequent analysis of vimentin expression by real-time reverse-transcriptase polymerase chain reaction (rtRT-PCR). A separate group of animals (n = 4) underwent the isometric exercise, and their muscles were harvested at 48 h. Frozen muscles were treated for 16–24 h with a commercial RNA stabilization reagent (RNAlater; Ambion) at −20°C and then ground using an RNase-free mortar and pestle (Kontes). The lysates were passed through a column-based tissue shredder (QIAshredders; Qiagen) to complete the cellular disruption. Total RNA was extracted and cleared of contaminating genomic DNA by means of a commercial kit (RNaseasy Fibrous Tissue Mini Kit; Qiagen). RNA quantity and quality were assessed by absorption spectrometry and gel electrophoresis, respectively.

RNA (1 μg) was reverse transcribed using the ReactionReady First Strand cDNA Synthesis Kit (SuperArray), in accordance with the manufacturer’s instructions. RNA with no reverse transcriptase served as the negative control. The resultant cDNA or a vimentin–positive control plasmid (OriGene) was added to 96-well reaction plates (MicroAmp Fast Optical plates; Applied Biosystems) containing SYBR Green/ROX PCR Master Mix (SuperArray), along with primers for vimentin (Sigma; [10]) or murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH; SuperArray). rtRT-PCR was performed on an ABI 7500 Fast PCR machine (Applied Biosystems) with the following parameters: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. A dissociation (melting) curve was run immediately after the PCR program to verify product integrity.

The threshold cycle (Ct) for each transcript and the change in threshold cycle (ΔCt) for the vimentin/GAPDH pair of transcripts were monitored for each amplification reaction using Applied Biosystems software (version 1.3.1), and the data were analyzed using the ΔΔCt values for the vimentin expression to calculate the fold change between the exercised and nonexercised muscles.

**Streptococcus pyogenes strain.** GAS strain 88-003 is an M type 3 GAS isolated from a fatal human case of StrepTSS with myonecrosis [2]. This strain has been characterized elsewhere [2] and has been submitted to the American Type Culture Col-
lection (ATCC 51550). It has also been shown to bind to immobi-

 EC with GAS bacteremia. At 48 h after EC, animals were
given $8.2 \times 10^3$–$1.3 \times 10^7$ cfu/50 µL washed, log-phase GAS
delay strain 88-003 intravenously via the lateral tail vein. At 6 – 8 h after
GAS infusion, heparinized blood samples were obtained by ret-
roorbital puncture, and spleens as well as TA and EDL muscles
from both exercised and nonexercised legs were harvested and
weighed. Preliminary experiments demonstrated this to be an
optimal time for recovery of GAS from muscles. Muscles and
spleens were homogenized using a mortar and pestle (Kontes) in
300 µL of ice-cold sterile normal saline, and samples (50 µL)
were plated in duplicate on sheep blood agar plates. Colonies
were counted the following day. Data are expressed as the num-
ber of colony-forming units per milligram (wet weight) of tissue,
with a minimum level of detection of 3 cfu/muscle.

 EC, NSAIDs, and GAS bacteremia. At 47 h after EC, ani-
mals ($n = 16$) were treated with either ketorolac tromethamine
(Toradol; Hospira; 7.5 mg/kg in 0.5 mL intraperitoneally) or an
equal volume of sterile saline. One hour later, animals were given
GAS ($3.6 \times 10^6$ cfu/50 µL) via the tail vein, and tissues were
harvested 6 h after bacterial infusion.

RESULTS

Vimentin gene expression in mouse skeletal muscle after
EC. Up-regulation of vimentin is a marker of skeletal muscle
injury because healthy mature skeletal muscles do not express
vimentin [11–14]. Vimentin gene expression was followed over
time by rtRT-PCR in hind-limb muscles from EC- and isomet-

tically exercised animals and compared with that in the nonex-
exercised, contralateral control muscles. In the EC-exercised TA
muscles, vimentin gene expression was increased 2–4-fold at
12 h, was maximal at 48 h (mean, 7.8-fold; range, 2.8–19.2-fold;
$P < .05$ for the comparison with the control, by Duncan’s test),
and began to decline by 72 h (figure 1). The EC-induced increase
in vimentin expression at 48 h was significantly greater than that
at 12 or 24 h (figure 1) or than that measured 48 h after isometric
exercise (mean, 3.1-fold; range, 1.4–5.0-fold).

Sufficient total RNA for rtRT-PCR was not obtained from
individual EDL muscles. Thus, total RNA was prepared from
pooled EDL homogenates from 4 animals whose injured TA
muscles demonstrated a mean 6.1-fold increase in vimentin gene
expression compared with the nonexercised control TA muscles
at 24 h after EC. There was no difference in vimentin gene ex-
pression between exercised and nonexercised EDL muscles (data
not shown).

The change in the MIT from before to after exercise was used
as a surrogate for vimentin expression to assess muscle injury in
real time. EC exercise reduced the MIT by 11%–83% (mean
± SD, 43.9% ± 19.8%). In contrast, isometric exercise reduced
this value by 0%–36% (mean ± SD, 22.8% ± 11.2%). The EC-
induced reduction in MIT and the temporal dynamics of vimen-
tin gene expression in the present study are comparable to those
obtained elsewhere in a rat model of EC-induced muscle injury
[10]. The decrease in MIT after isometric exercise was attributed
to fatigue, given the short duration of rest (30 s) between the
periods of exercise [10]. This conclusion is supported by the low
level of vimentin gene expression after isometric exercise, a find-
ing that is also consistent with other reports [15]. Thus, EC exercise caused definitive muscle injury as indicated by vimentin up-regulation. Because vimentin expression was maximal 48 h after EC, this time point was chosen for the administration of GAS in subsequent experiments.

Homing of GAS to the site of muscle injury. We hypothesized that circulating GAS from transient bacteremia would localize to the site of muscle injury. To test this, organisms were delivered intravenously to animals 48 h after EC-induced muscle injury, and, 6 h later, blood, spleen, and muscles were harvested for quantitative bacteriology. Variation between animals was observed in the absolute numbers of GAS in the blood and spleen. These differences were attributed to differences in animal size, individual variation in the ability to clear the organism, and differences in the sizes of the inocula used on different days. Furthermore, the design of these experiments (i.e., the killing of animals at a predetermined time) did not allow us to follow the distribution of GAS longitudinally in a single animal or to compare courses between individual animals.

Because of the variability in the intensity of muscle injury (as measured by the reduction in MIT), animals were divided into 2 groups on the basis of a median split: “minimal injury” versus “moderate injury” (reduction in MIT of ≤44% and >44%, respectively). Irrespective of the injury category, streptococci were rarely detected in the EDL, probably because the EC exercise protocol used does not create injury in the highly elastic EDL [10]. This conclusion is supported by our observation that no increase in vimentin gene expression was found in exercised EDL muscles in this model. In contrast, GAS were consistently found in the TA muscles. However, because of the nonnormality of the measures of GAS in the these muscles, a Wilcoxon signed rank test was chosen to evaluate statistical differences between the number of GAS in EC-injured and noninjured TA muscles in these 2 injury groups. For the minimally injured group (n = 8), there was not a significant difference (S = −3.5; P = .73) in the number of GAS between injured and noninjured muscles (figure 2). In those sustaining moderate injury (n = 8), injured muscles contained significantly more GAS than did the contralateral uninjured muscles (S = 17.5; P = .039) (figure 2). Examination of the means showed a 2-fold increase in organisms in the injured muscles compared with noninjured muscles. When the median was used as a measure of central tendency appropriate for nonnormal sample distributions, the same 2-fold increase was observed. This difference did not correlate with the number of circulating GAS at the time of muscle harvest, nor was it attributable to differences in the weights of the TA muscles. In total, these results support the notion that vimentin up-regulation in injured muscles is associated with localization of GAS to the injured site.

Effects of NSAIDs on GAS infection of injured muscle. To determine the effects of NSAIDs on GAS infection of injured muscles, animals were given either ketorolac tromethamine or saline 47 h after EC and 1 h before GAS infusion. TA muscles were harvested 6 h later for bacterial quantitation. One animal in the NSAID-treated group had obvious anatomical abnormalities on dissection and was excluded from analysis. Two animals in the NSAID-treated group fell into the minimally injured category (reduction in MIT of <44%). This number did not allow
minor muscle strain, sprain, or bruise is the rule [2, 3]. In fact, several authors have concluded that nonpenetrating muscle injury may be a prerequisite for GAS necrotizing fasciitis or myonecrosis [3, 4]. This implies that there is a subtle but specific interaction between GAS and skeletal muscle that initiates invasive GAS infection at the site of minor muscle injury.

Clinical findings and laboratory evidence support this notion. First, the temporal dynamics of vimentin expression after EC injury parallels the time course for development of infection in the no-portal human GAS myonecrosis cases—most patients present to emergency departments 24–72 h after muscle injury because of increasing focal pain at the site of injury [1]. Second, large numbers of GAS adhere specifically to the surfaces of myofibrillar bundles in experimental animals with GAS myonecrosis [16]. Similar findings have been reported in fatal human cases of GAS myonecrosis [3, 8]. Third, using cultured human skeletal muscle cells, we have shown that adherence of GAS to injured cells was increased 2–5-fold over that in normal, healthy cells [8]. Fourth, we have identified vimentin as the major skeletal muscle surface protein that binds both M type 1 and 3 GAS [8]. Results from the present study confirm that skeletal muscles do not express vimentin except during regeneration after injury [11–14] and demonstrate that injured muscle tissues harbor more GAS than noninjured control muscles. Taken together, these data suggest that injury-induced up-regulation of vimentin facilitates the development of GAS myonecrosis at sites of muscle injury.

A role for NSAIDs in the development of severe streptococcal infection has long been proposed, yet controversy remains. For example, in one study, 92% of patients with StrepTSS reported antecedent NSAID use [17]. Similarly, in a case-control study of children with varicella, 42% of patients who developed life-threatening GAS necrotizing fasciitis had received NSAIDs, compared with only 15% of patients who developed other, non-necrotizing soft-tissue infections [18]. Another prospective study in children with primary varicella demonstrated only a slightly increased risk of GAS infection in those receiving both acetaminophen and ibuprofen compared with controls [19], causing the authors to doubt the association between NSAIDs and GAS infection. A recent prospective epidemiologic study in the United Kingdom found that use of NSAIDs was independently associated with increased risk for StrepTSS [20].

Despite this protracted controversy, until now no experimental studies have been undertaken to test the possible link between NSAIDs and GAS infection in general, and certainly none have specifically examined this relationship after muscle injury. The data presented here demonstrate for the first time that GAS travel from the bloodstream to sites of moderate muscle injury, likely via a vimentin-mediated process. Furthermore, NSAID administration at the time of peak vimentin expression markedly enhances GAS infection of injured muscles. Thus, 3 ingredients contribute to the development of severe, cryptic GAS

**DISCUSSION**

A critical role for antecedent skeletal muscle injury has been well established for some bacterial infections, such as clostridial myonecrosis, in which a deep, penetrating injury directly introduces organisms (or spores) into devitalized tissues. Although the rate at which GAS myonecrosis progresses is comparable to that of clostridial gas gangrene (inches per hour), the antecedent injuries predisposing to GAS infection are distinctly different. In the no-portal group of patients with streptococcal myonecrosis, a

![Figure 3. Augmentation of infection of injured skeletal muscles with group A streptococci (GAS) by nonsteroidal anti-inflammatory drugs (NSAIDs). Animals with moderate eccentric contraction (EC)-induced muscle injury (reduction in mean isometric torque, 63.8% ± 11%) were treated with either ketorolac tromethamine (n = 5) or saline (n = 8) 1 h before intravenous administration of GAS. Six hours after bacterial challenge, muscles were harvested for quantitative bacteriology. Data are means ± SEs. There was significant interaction (P < .001), as determined by 2-by-2 analysis of variance (ANOVA), with drug type (NSAID vs. no NSAID) as the between factor and muscle treatment (injury vs. no injury) as the within factor. *P < .001, post-hoc ANOVA.](image-url)
In summary, we propose the following paradigm for the initiation of these enigmatic necrotizing GAS infections. By 24–48 h after acute injury, regenerating myoblasts maximally express vimentin on their surface. In the absence of penetrating trauma, GAS traffic to the injured site, likely via a transient bacteremia from the oropharynx, and attach specifically to vimentin expressed on injured muscles in sufficient numbers to initiate infection. NSAID-induced delays in muscle regeneration and cellular immunosuppression further enhance GAS binding and facilitate unrestrained bacterial proliferation. Once established, proliferating organisms release potent cytolysins, causing further muscle cell injury and enabling intracellular vimentin to amplify the interaction between GAS and muscle.

Translational studies are currently ongoing to identify and block the GAS adhesin that binds to vimentin and to prevent GAS binding in vivo by directly silencing vimentin gene expression in injured muscle. Future studies will explore the roles played by the different cyclooxygenase isoforms in the interaction between GAS and injured muscle. Together, these studies may uncover novel therapeutic targets to prevent or attenuate this devastating infection. Until more is revealed about the role played by NSAIDs in this process, physicians should be alerted to possible deep-seated GAS infection in persons taking NSAIDs and presenting with increasing pain at the site of nonpenetrating muscle injury.

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References


