Agent-based model of inflammation and wound healing: insights into diabetic foot ulcer pathology and the role of transforming growth factor-β1

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Manuscript received: May 25, 2006
Accepted in final form: March 12, 2007

DOI:10.1111/j.1524-475X.2007.00271.x

ABSTRACT

Inflammation and wound healing are inextricably linked and complex processes, and are deranged in the setting of chronic, nonhealing diabetic foot ulcers (DFU). An ideal therapy for DFU should both suppress excessive inflammation while enhancing healing. We reasoned that biological simulation would clarify mechanisms and help refine therapeutic approaches to DFU. We developed an agent-based model (ABM) capable of reproducing qualitatively much of the literature data on skin wound healing, including changes in relevant cell populations (macrophages, neutrophils, fibroblasts) and their key effector cytokines (tumor necrosis factor-α [TNF], interleukin [IL]-1β, IL-10, and transforming growth factor [TGF]-β1). In this simulation, a normal healing response results in tissue damage that first increases (due to wound-induced inflammation) and then decreases as the collagen levels increase. Studies by others suggest that diabetes and DFU are characterized by elevated TNF and reduced TGF-β1, although which of these changes is a cause and which one is an effect is unclear. Accordingly, we simulated the genesis of DFU in two ways, either by (1) increasing the rate of TNF production fourfold or (2) by decreasing the rate of TGF-β1 production 67% based on prior literature. Both manipulations resulted in increased inflammation (elevated neutrophils, TNF, and tissue damage) and delayed healing (reduced TGF-β1 and collagen). Our ABM reproduced the therapeutic effect of platelet-derived growth factor/platelet releasate treatment as well as DFU debridement. We next simulated the expected effect of administering (1) a neutralizing anti-TNF antibody, (2) an agent that would increase the activation of endogenous latent TGF-β1, or (3) latent TGF-β1 (which has a longer half-life than active TGF-β1), and found that these therapies would have similar effects regardless of the initial assumption of the derangement that underlies DFU (elevated TNF vs. reduced TGF-β1). *In silico* methods may elucidate mechanisms of and suggest therapies for aberrant skin healing.

Wound healing is a process that involves both inflammation and the resolution of the inflammatory response, which culminates in remodeling.[1,2] The first phase of the wound-healing response involves the degranulation of platelets and infiltration of inflammatory cells, followed by proliferation of fibroblasts and epithelial cells that deposit collagen and cause contraction of wounds. Rodent models of diabetes display impaired wound repair, with decreased wound tensile strength and collagen deposition in implanted sponges.[3] Collagen organization in healing wounds is also poor.[3] Furthermore, diabetic wounds have deficits in neovascularization[4] and decreased levels of DNA and protein.[3] Proinflammatory cytokines such as tumor necrosis factor (TNF)-α[5] and interferon (IFN)-γ[6] inhibit wound healing both in vitro and in vivo. Interleukin (IL)-6, a cytokine central to inflammation,[7] is also necessary for proper healing.[8] IL-10, a potent antiinflammatory cytokine, appears to suppress inflammation and induce the remodeling necessary for proper wound healing.[9] One cytokine that is central to the wound-healing cascade is transforming growth factor (TGF)-β1.[10] TGF-β1, like all isoforms of TGF-β, is produced in a latent form, which must be activated in order to exert its biological effects. The TGF-β1 precursor homodimerizes intracellularly, and is then cleaved extracellularly to yield the active TGF-β1 dimer as well as the remaining portion of its dimerized precursor, the latency-associated peptide (LAP). Under physiological conditions, TGF-β1 is expressed almost completely in its latent form, which

ABM	Agent-based model
TGF-β1	Transforming growth factor-β1

consists of the active TGF-β1 dimer bound noncovalently to LAP. Additional proteins such as α2-macroglobulin, latent TGF-β-binding proteins, or proteoglycans (e.g., decorin) are bound to latent TGF-β1 in what is known as the large latent complex. LAP and other proteins must be dissociated from latent TGF-β1, in a process known as activation, before TGF-β1 gains biological activity.[11] Of note, there are multiple regulatory intersections among TNF, IL-6, IL-10, IFN-γ, and TGF-β1,[12] and much of this complexity has been captured in our equation-based models of inflammation.[13,14]

Both inflammation and wound healing are deranged in chronic, nonhealing foot ulcers, constituting a major complication of diabetes. Diabetic foot ulcers (DFU) are caused by both vascular and neurologic complications of diabetes, in combination with persistent opportunistic infections[15,16] and deficient wound healing.[17] Over 10 million Americans carry a diagnosis of diabetes, and an estimated 5 million more are undiagnosed diabetics.[18] The incidence of foot ulcer in this population approaches 2% per year.[19] With reported average treatment costs ranging from $2,500 to almost $14,000 per year, DFU represent a significant financial burden on society.[17,19] Additionally, DFU are responsible for more than 50,000 major lower extremity amputations in the United States every year.[20] Notably, diabetics with foot ulcers have more than twice the mortality of diabetics with healthy feet.[21] Diabetics are known to have elevated levels of TNF,[22] and studies have suggested a relatively reduced expression of active TGF-β1 in DFU.[23]

A standard treatment for DFU is wound debridement, which is effective in approximately 25% of patients.[24] Models of diabetic wound healing have demonstrated the efficacy of single growth factors such as platelet-derived growth factor (PDGF),[25] fibroblast growth factor (FGF, acidic or basic),[3] or TGF-β1;[3] these results often have not borne fruit when carried to clinical trials of DFU.[26] Although these therapeutic failures suggest that studies should be carried out on DFU patients and tissue/cells derived from these ulcers, we believe that such studies alone are likely insufficient due to the complexity of the wound-healing process,[27] the complexity of inflammation from which this process stems,[12] the co-morbidities,[15] and genetic variability in genes such as TGF-β1[28] in the inflammation/wound-healing responses of individual patients. Nonetheless, our experience tells us that both systemic and local factors can delay the healing of both acute and chronic wounds and thereby modify their trajectories.[29]

Mathematical modeling of complex systems is emerging as an approach by which to tame the seemingly unpredictable behavior of such biological phenomena and account for the plethora of known and unknown interactions among biologic pathways, including both acute inflammation[13] and wound healing.[27,30–39] However, these intertwined processes have not been simulated as a cohesive whole in the setting of DFU. Herein, we describe the development of an agent-based model (ABM) of inflammation and wound healing in the skin. We show how this simulation is capable of reproducing qualitatively much of the phenotype of skin wound healing, including changes in relevant cell populations (macrophages, neutrophils, fibroblasts) and, importantly, proinflammatory cytokines such as TNF, and antiinflammatory and prohealing cytokines such as TGF-β1. We demonstrate how we can simulate the phenotype of DFU using this ABM, and furthermore, simulate the modulation of PDGF/platelets, debridement, TNF, and TGF-β1 in the setting of DFU with the goal of suggesting novel therapeutic approaches.

MATERIALS AND METHODS

ABM of inflammation and wound healing

The popularity of ABM lies in the fact that this type of model can simulate the behavior of complex systems in which agents interact with each other and with their environment following local rules based on known physiology. Moreover, the ABM framework accounts for the stochastic nature of biological processes, in that each rule is a probability of a given event happening; thus, each simulation leads to a unique outcome and can be considered as a separate experiment (or—virtual patient").[13,40] A typical ABM includes three types of elements: region, patch, and agent. The region consists of small patches that are uniquely characterized by spatial position, and contain local information. Agents are the objects that can move in the region. The motion of all agents is due to both chemoattraction and stochastic walk, as described in greater detail in the Supplementary Materials. We designed an ABM to simulate inflammation and wound healing in a physical domain including skin and underlying soft tissue (the tissue), using Netlogo software (Center for Connected Learning and Computer-Based Modeling, Northwestern University, Evanston, IL). First, we created two regions to simulate blood (the source of some of the inflammatory cells that infiltrate injured tissue) and the tissue itself (which contains some inflammatory cells as well as the fibroblasts that will eventually act to heal the injured tissue). The two regions (blood and tissue) do not intersect: the tissue region is circular and surrounded by the blood region. We used agents to represent tissue damage (induced by the initial injury as well as by subsequent inflammation, and also a stimulus for further inflammation), as well as resting and activated inflammatory cells (neutrophils, macrophages, and fibroblasts). We also used patch variables to represent latent TGF-β1 and the mediators produced by these cells during the inflammation and wound-healing stages. The mediators include the pro-inflammatory cytokines IL-1β and TNF (both produced by neutrophils and macrophages); the anti-inflammatory cytokines TGF-β1 and IL-10 (both produced by macrophages); and collagen (produced by fibroblasts).[41] Initially, some resting macrophages, neutrophils, fibroblasts, and latent TGF-β1 are present with a random distribution both in tissue and blood. By stimulating the tissue with damage in the middle of the region, the model creates a chemoattractant gradient (induced by platelet degranulation),[42] which acts to induce the infiltration and activation of both neutrophils and macrophages. Fibroblasts are activated at a later stage both by damage and TGF-β1, to produce collagen that acts to repair both the initial and inflammation-induced damage.[42] For a detailed description of rules, please see Supplementary Materials.

RESULTS

Simulating normal tissue healing

Our ABM was capable of reproducing the qualitative features and general time course of skin wound healing, with regard to the dynamics of neutrophils, macrophages, and fibroblasts (Figure 1A), and the inflammatory cytokines IL-1, IL-10, and TNF (Figure 1B). The values in the figures are averaged over the entire space (also in all subsequent figures). Collagen deposition and tissue damage variables served as surrogates for wound healing, and these, too, exhibited the expected qualitative behavior, with wound resolution occurring in approximately 1 month (Figure 1C). The simulation presented is one run, representative of the behavior of the ABM under these baseline conditions. In later simulations, we show the variability across simulations at defined time points in the inflammation/healing process. Notably, this simulation did not address aspects of longer-term collagen remodeling because this aspect of healing was not incorporated into the ABM.

Comparison of normal vs. DFU healing

We hypothesized that, because inflammation is the initial driver of wound healing, inflammatory derangements seen in DFU might underlie the delayed healing characteristic of these lesions. Previous studies have suggested that macrophages from diabetics exhibit elevated TNF production,[22] and other studies have shown reduced expression of active TGF-β1.[23] TNF and TGF-β1 cross-regulate their own expression and activity in diverse and complex ways, with TNF generally inducing the expression of TGF-β1 and TGF-β1 suppressing the expression of TNF.[43] Accordingly, we hypothesized that either derangement alone might be sufficient to result in altered healing. To test this hypothesis, we simulated the effects of elevated TNF or reduced TGF-β1. In Figure 2, we show the healing trajectories (shown as dynamics of tissue damage) of normal (solid line, reprised from Figure 1C), TNF-high DFU (dotted line), and TGF-β1-low DFU (dashed line). As can be seen, simulated damage in the DFU settings remains elevated as compared with normal wound healing, which we interpret as delayed healing. Thus, as suggested previously,[29] the healing trajectories of DFU in these simulations are clearly delayed as compared with normal skin healing. Importantly, this is an emergent property of the system, because the various observations indicative of DFU-like healing have not been programmed into the simulation but rather emerge as a result of changing a single variable (either TNF or TGF-β1).

Simulating clinical variability and known therapies for DFU

We wished to determine whether our ABM would result in the sort of patient-to-patient variability that is typically observed clinically with regard to DFU healing. Accordingly, we carried out multiple simulations and examined our scenarios of normal (Figure 3A), TNF-high (Figure 3B), and TGF-β1-low (Figure 3C) wound healing. These figures show that TNF-high and TGF-β1-low conditions result in higher levels of tissue damage, although our ABM exhibits the type of inter-individual variability previously shown in ABM of acute inflammation.[40]

Debridement, or physical removal of dead or dying tissue, is part of the routine care for DFU, and has been reported to improve healing in ~25% of patients.[24] Accordingly, we simulated this procedure by making the assumption that approximately 75% of damaged tissue would be removed at either day 7 or 14 into the time course of healing. Simulated tissue damage was then assessed at day 30. These simulations were carried out in the presence

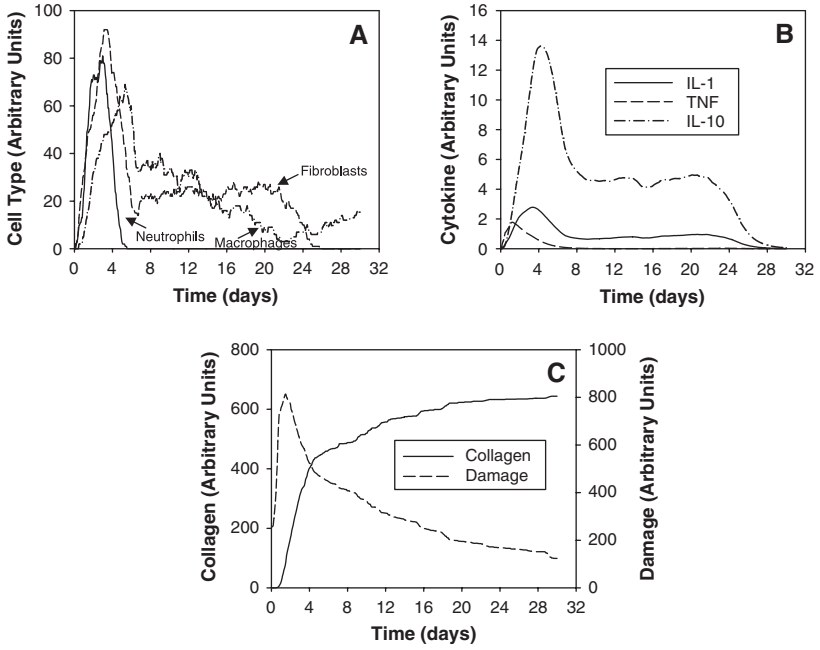

Figure 1. Simulations of baseline wound healing. Simulations using the agent-based model were carried out for 30 days, and show the dynamics of inflammatory cells (A), cytokines (B), collagen (C, left *y*-axis), and tissue damage (C, right *y*-axis).

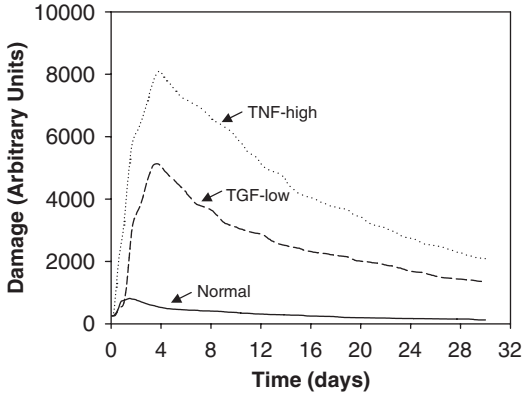

Figure 2. Simulations of healing trajectories in Normal, TNF-high, and TGF-β1-low cases. The simulated recovery of normal skin tissue damage (i.e., wound healing; solid line) is compared with one of two hypothetical derangements underlying DFU: elevated TNF production (dotted line) or reduced capacity to produce TGF-β1 (dashed line). Details of normal, TNF-high, and TGF-β1-low simulations are provided in the Supplementary Materials. TGF, transforming growth factor; TNF, tumor necrosis factor; DFU, diabetic foot ulcers.

of the inter-individual variability depicted in Figures 3A–C, in an attempt to determine whether the type of variability observed in clinical trials would be seen. Figure 4A shows the elevated damage at simulated day 30 in both TNF-high and TGF-β1-low DFU as compared with normal healing. Interestingly, our simulation suggests that collagen content would be elevated relative to normal healing if DFU were caused by elevated TNF, but the predicted collagen content of DFU derived from low TGFβ1 is predicted to be no different from that of normal controls (Figure 4B). Debridement at day 7 was predicted to result in statistically significant reductions in tissue damage in both TNF-high and TGF-β1-low DFU (Figure 4A). Interestingly, this effect of simulated debridement did not reach statistical significance when simulated debridement was performed at day 14. In contrast, debridement was not predicted to result in decreased collagen at day 7 or 14, in either TNF-high or TGF-β1-low DFU. These results are in general agreement with clinical studies of debridement.[24] We note that in subsequent simulations below, debridement was not simulated in order to be able to gain an insight into the single manipulation being studied.

We also simulated biological therapies for DFU. Although multiple randomized prospective clinical trials have been performed using growth factors in the treatment of DFU, only PDGF has been approved for use.[44] PDGF, which is released from platelet granules,[45] has been found to increase healing in diabetic neuropathic foot ulcers and is marketed as REGRANEX™.[46] There is also limited evidence to suggest clinical benefit from a platelet releasate that contains many growth factors including PDGF.[26,47–49] In order to further validate our ABM, in which platelets are one class of agent, we assessed predicted tissue damage in the setting of increased platelet-derived factors. We simulated this increase by increasing separately the chemoattractant effect of platelets on macrophages and neutrophils (both effects being parameters in our ABM; see Supplementary Materials). As can be seen in Figure 5, increasing the chemoattractant effect of platelets on macrophages by 70% and the chemoattractant effect of platelets on neutrophils by 18% resulted in reduced damage under both the increased TNF (dotted line) and reduced TGFβ1 (solid line) cases (compare with the same simulations in Figure 2).

This finding arose from a systematic modulation of the relative chemoattractant effects of platelets on macrophages and neutrophils, in a further attempt to explore

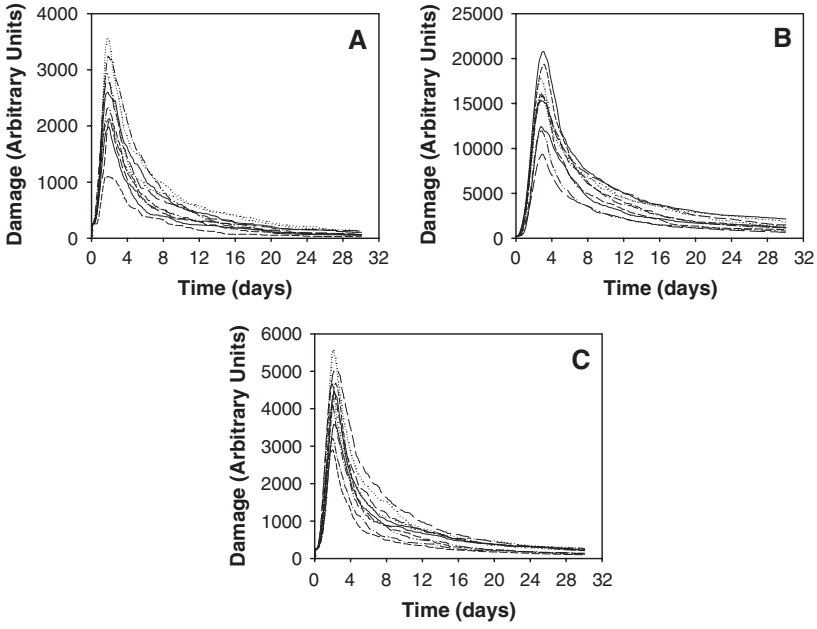

Figure 3. Simulation of the variability in healing trajectories in Normal, TNF-high, and TGF-β1-low cases. Ten simulations for each case of normal, TNF-high, and TGF-β1-low skin healing were carried out, and the time courses of predicted damage/dysfunction are shown. Details of normal, TNF-high, and TGF-β1-low simulations are provided in the Supplementary Materials. TGF, transforming growth factor; TNF, tumor necrosis factor.

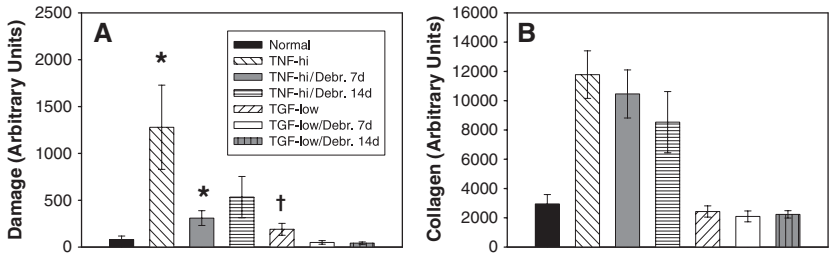

Figure 4. Simulation of debridement of DFU. Debridement (Debr.) was simulated as removal of 75% of damaged tissue at the indicated day (either 7 or 14 days post-wounding) in either of two hypothetical derangements underlying DFU: TNF-high or TGF-β1-low (see Supplementary Materials for details of normal, TNF-high, and TGF-β1-low simulations. Simulated tissue damage (A) or collagen content (B) was assessed at day 30 post-wounding. *$p < 0.05$ vs. Normal; †$p < 0.05$ vs. TNF-high. TGF, transforming growth factor; TNF, tumor necrosis factor; DFU, diabetic foot ulcers.

the potential for our ABM to reproduce interpatient variability (Tables 1 and 2). Interestingly, different ratios of the effects of platelets on macrophages and neutrophils were predicted to be either efficacious or non-efficacious at reducing tissue damage in a non-intuitive manner. In the ABM, activated macrophages are assumed to produce TGF-β1, which promotes the healing of DFU and inhibits TNF production by neutrophils (see the TGF-β1 rule in the Supplementary Materials). In turn, activated neutrophils are assumed to produce TNF, which not only causes damage but also inhibits TGF-β1 (see the TNF and damage rules in the Supplementary Materials). By changing the different potency of activation of macrophages and neutrophils by platelets, the ABM produces different amounts of activated macrophages and activated neutrophils. This change, in turn, leads to the different predicted profiles of TGF-β1, TNF, and damage. Because of these dynamics, either beneficial or detrimental effects of platelets on macrophages and neutrophils are predicted. In all cases, a benefit was predicted if the effect of platelets on macrophages was greater than the effect of platelets on neutrophils, and this held true whether we assumed that the underlying cause of DFU was elevated TNF (Table 1) or reduced TGF-β1 (Table 2). Nonintuitively, if the parameter governing the chemoattractant effect of platelets on neutrophils grew above a certain threshold, the predicted damage was higher than without therapy. We interpret this outcome as signifying that tissue damage mediated by neutrophils exceeds the beneficial effects of the prohealing elements derived from macrophages. These results may explain why some patients respond to PDGF or platelet releasate and some do not.[47,48]

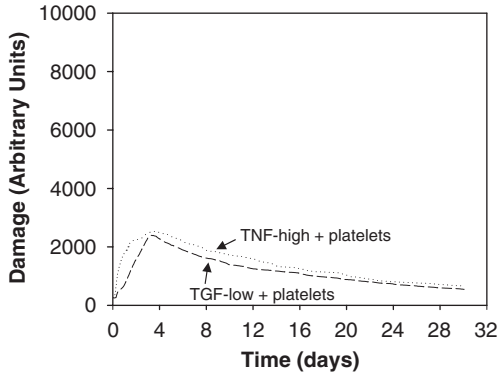

Figure 5. Simulation of PDGF/platelet release therapy for diabetic foot ulcers. The simulation of Figure 2 was repeated, this time in the presence of elevated effects of platelets (increasing the effect of platelets on macrophages by 70% and the effect of platelets on neutrophils by 18%). PDGF, platelet-derived growth factor.

Inflammatory and healing characteristics of simulated DFU

Having demonstrated that the overall qualitative simulations are valid, we next wished to examine the characteristics associated with healing in simulations of normal tissue and the two methods of simulating DFU (TNF-high and TGF-β1-low). These simulations were all examined at 2.5 days in order to examine early drivers of the inflammatory and healing responses. In all cases, we carried out ten simulations of each condition, because the ABM platform is inherently stochastic.[50] This approach allowed us to simulate several patients and also to carry out a statistical analysis (Kruskall–Wallis analysis of variance ANOVA on ranks, followed by Tukey post-hoc test) to ascertain group differences (considered significant at $p < 0.05$). As can be seen in Figure 6, the qualitative features of simulated wound healing either in the setting of elevated TNF production (gray bars) or reduced TGF-β1 production (hatched bars) as compared with normal healing (black bars) are largely similar: elevated neutrophil influx (Figure 6A), elevated TNF expression (Figure 6B), elevated IL-10 (Figure 6C), reduced collagen deposition (Figure 6E), and increased tissue damage (Figure 6F). In general, these are all hallmarks of DFU. Interestingly, decreased TGF-β1 expression as compared with normal healing was observed in the simulations in which DFU were presumed to arise from reduced TGF-β1 (as expected, Figure 6D, hatched bar), but this was not the case in simulations in which TNF was over-produced (Figure 6D, gray bar).

Simulating hypothetical therapies for DFU

We next sought to simulate therapies for DFU. Numerous studies in animal models of diabetic wound healing have demonstrated the efficacy of the topical administration of single growth factors such as PDGF,[25] acidic or basic FGF,[3] or TGF-β1.[3] However, these results have not been confirmed in clinical trials of DFU.[44] In an attempt to determine whether *in silico* methodologies could suggest

Table 1. Simulation of DFU therapy

*P*1 (% increase over *P*1 in the TNF-high simulation; Figure 2)	*P*2 (% increase over *P*2 in the TNF-high simulation; Figure 2)	Damage (arbitrary units)	Healing (relative to no treatment)
129	19	892	Yes
129	39	5,600	Yes
129	≥ 59	$> 8,000$	No
95	19	2,000	Yes
95	39	2,070	Yes
95	≥ 59	$> 8,000$	Yes
69	19	2,700	Yes
69	≥ 39	$> 8,000$	No
44	19	3,100	Yes
44	≥ 39	$> 8,000$	No
34	19	4,000	Yes
34	≥ 39	$> 8,000$	No
22	≥ 19	$> 8,000$	No

The predicted effect on tissue damage of increasing the chemoattraction of platelets on macrophages (*P*1) and neutrophils (*P*2) was simulated. Macrophages are activated by platelets if the number of platelets is greater than $(100/(1+M\times M))/(1+P1)$, and neutrophils are activated by platelets if the number of platelets is greater than $(100/(1+M\times 1.7)\ (M\times 1.7))/(1+P2)$. At baseline, $P1=P2=0$. In this case, DFU were assumed to arise from elevations in TNF production. Healing (assessed at 4–6 days) was assumed to occur if the predicted damage was lower than that predicted for the untreated DFU (8,000 arbitrary units; see Figure 2, dotted line).
DFU, diabetic foot ulcers; TNF, tumor necrosis factor.

Table 2. Simulation of DFU therapy

*P*1 (% increase over *P*1 in the TGF-β1-low simulation; Figure 2)	*P*2 (% increase over *P*2 in the TGF-β1-low simulation; Figure 2)	Damage (arbitrary units)	Healing (relative to no treatment)
170	19	1,400	Yes
150	19	1,600	Yes
129	19	2,000	Yes
129	> 39	$> 3,000$	No
95	≥ 19	$> 3,000$	No
69	19	2,700	$\sim$Equal
69	≥ 39	$> 3,000$	No
44	19	2,300	Yes
44	≥ 39	$> 3,000$	No
34	19	3,500	No
34	≥ 39	$> 3,000$	No
22	≥ 19	$> 3,000$	No

The predicted effect on tissue damage of increasing the chemoattraction of platelets on macrophages (*P*1) and neutrophils (*P*2) was simulated. Macrophages are activated by platelets if the number of platelets is greater than $(100/(1+M\times M))(1+P1)$, and neutrophils are activated by platelets if the number of platelets is $(100/(1+M\times 1.7)\ (M\times 1.7))/(1+P2)$. At baseline, $P1=P2=0$. In this case, DFU were assumed to arise from reduced TGF-β1 production. Healing (assessed at 4–6 days) was assumed to occur if the predicted damage was lower than that predicted for the untreated DFU (3,000 arbitrary units; see Figure 2, dashed line).
DFU, diabetic foot ulcers; TGF, transforming growth factor.

some insights into therapy design for DFU, we applied simulated therapeutic strategies to our simulations of delayed healing depicted in Figure 6. Because both elevated TNF and reduced TGF-β1 gave generally similar qualitative patterns of inflammatory and healing derangements, we tested the simulated therapies under settings of either elevated TNF (Figure 7) or reduced TGF-β1 (Figure 8). The therapies we chose to simulate are directly related to these derangements: neutralizing antibodies to TNF (anti-TNF), agents that increase the rate of TGF-β1 activation, and latent TGF-β1 itself. The rationale for these interventions was the following. In the case of anti-TNF, FDA-approved anti-TNF antibodies are available for the treatment of several inflammatory diseases, with further indications likely;[51] additionally, we have carried out previous work on simulating mathematically the actions of anti-TNF in the setting of sepsis.[52] TGF-β1 modulation (either provision of TGF-β1 or its inhibition) has been proposed as a possible therapy for various aspects of aberrant wound healing.[53] TGF-β1, like all other isoforms of TGF-β, is synthesized in a biologically inactive (latent) state and must be activated through various mechanisms in order to bind to its cognate receptor complex and exert its diverse biological functions.[11] Treatment with active TGF-β1 as well as TGF-β2 has been attempted in the setting of DFU, with initially promising results but ultimately with lack of statistically significant efficacy.[54,55] One reason for this lack of overall efficacy might be that active TGF-β1 has a shorter half-life than latent TGF-β1.[11] In the case of TGF-β1 activation, we have described such effects of NO[56,57] and have suggested that this, among other effects of NO on cytokines, may underlie the generally beneficial effects of NO in wound healing.[58] We hypothesize that a class of agents such as NO that could selectively augment or suppress TGF-β1 activation may have therapeutic utility in wound healing. As can be seen in Figures 7 and 8, we carried out ten simulations of each condition, and assessed statistical significance by Kruskall–Wallis ANOVA on ranks, followed by Tukey post-hoc test.

Regardless of whether DFU healing was simulated as stemming from elevated TNF production (Figure 7) or reduced TGF-β1 production (Figure 8), all three therapies (anti-TNF [hatched bars], latent TGF-β1 [open bars], or

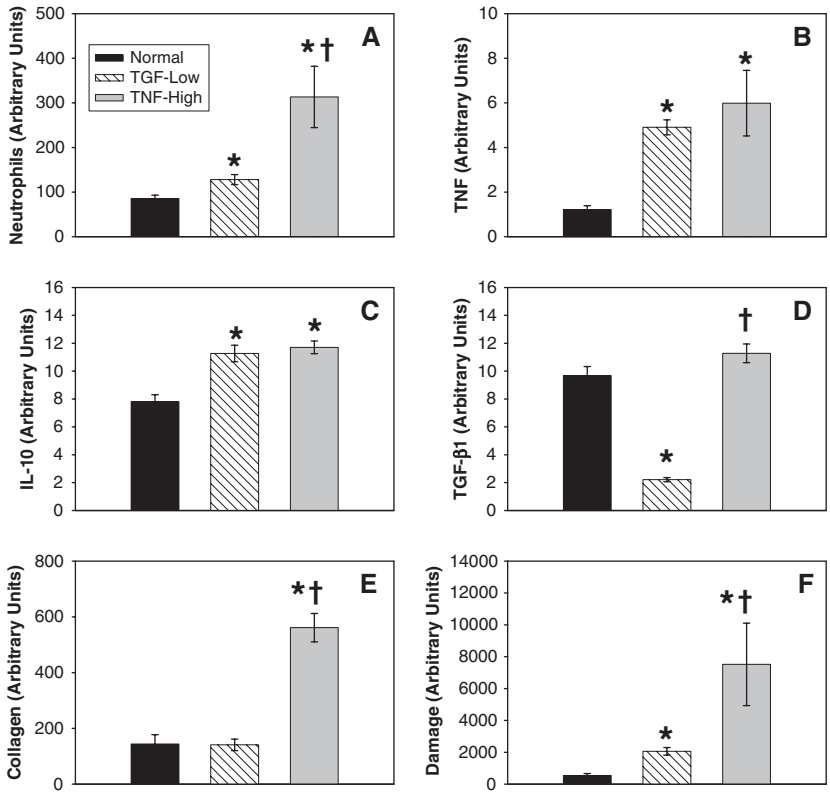

Figure 6. Simulations of inflammation and healing parameters in normal, TNF-high, and TGF-β1-low cases. The simulated levels of neutrophils (A), TNF (B), IL-10 (C), TGF-β1 (D), collagen (E), and tissue damage (F) are shown for normal skin healing (black bars) and two hypothetical derangements underlying diabetic foot ulcers: elevated TNF production (gray bars) or reduced capacity to produce TGF-β1 (hatched bars). $*p < 0.05$ vs. Normal; $^{\dagger}p < 0.05$ vs. TGF-β1-low (all by Kruskall–Wallis analysis of variance on ranks, followed by Tukey post-hoc test). IL, interleukins; TGF, transforming growth factor; TNF, tumor necrosis factor.

TGF-β1 activation [gray bars]) were predicted to suppress neutrophil influx (Figures 7A and 8A) and tissue damage (Figures 7F and 8F) to the same, statistically significant degree as compared with the DFU baseline (black bars). Interestingly, our simulations suggested that all three therapies would result in a reduction of TNF production in DFU tissue (Figures 7B and 8B), although the effect of anti-TNF would not be statistically significant if the cause of DFU was elevated TNF production. Our simulation suggested that provision of latent TGF-β1 or activation of endogenous TGF-β1 would elevate overall TGF-β1 expression regardless of the presumed cause (high TNF or low TGF-β1) of DFU pathology (Figures 7D and 8D). Interestingly, only the TGF-β1 activator was predicted to increase collagen deposition in a statistically significant fashion, and only upon assumption of reduced baseline TGF-β1 as a cause of DFU. Another non-intuitive finding was the suggestion that anti-TNF would decrease collagen levels below those of baseline DFU (Figures 7E and 8E), while at the same time reducing overall tissue damage (Figures 7F and 8F).

DISCUSSION

Chronic wounds are a serious health care problem, costing billions of dollars each year and carrying unaccounted but considerable suffering and anguish. DFU, in particular, are a major cause of amputation.[17,19] There has been great interest in treating DFU with growth factors in recent years. One might suggest that the use of growth factors to heal a DFU would result in improved healing and a lowered amputation rate.[44] Unfortunately, the number of amputations performed in the United States each year remains essentially unchanged.[17,19]

In order to attempt to break the logjam of compounds available for clinical trials in the setting of DFU, we created an ABM simulation of the inflammation/wound-healing process. Our specific objectives were (1) to use this ABM to test hypotheses regarding the genesis of DFU and (2) to test *in silico* possible therapies for DFU. In the process of validating our ABM, we were able to simulate existing therapies for DFU (debridement and platelet releasate/PDGF).

We note that other groups have also published studies on modeling wound healing, but have not focused on the interrelation between inflammation and wound healing in the setting of DFU as we have. Current mathematical modeling of wound healing has focused mainly on two areas: epidermal wound healing and dermal wound healing. For epidermal wound healing, Sherratt and Murray[32] proposed a two-dimensional diffusion-reaction type of partial differential equation model based on a set of biological experiments. Their model consists of epithelial cell density per unit area and the concentration of mitosis-regulating substances.[31] Recently, Walker et al.[38,39] used an ABM to simulate wounded epithelial cell monolayers, and they suggest that based on the simple rules, it is sufficient to

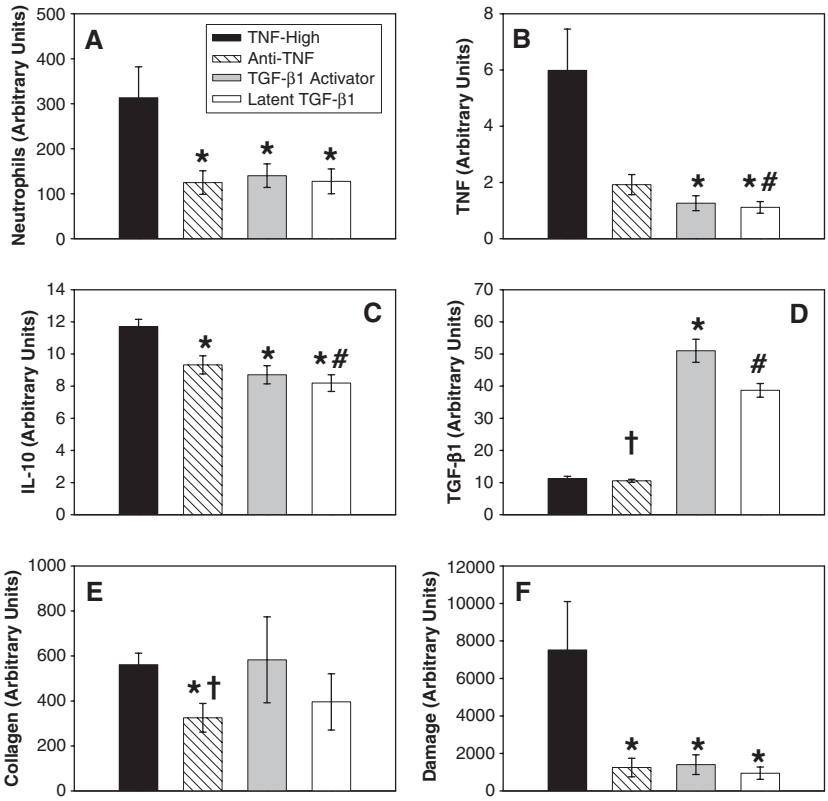

Figure 7. Simulations of therapies in TNF-high DFU. The simulated levels of neutrophils (A), TNF (B), IL-10 (C), TGF-β1 (D), collagen (E), and tissue damage (F) are shown for skin healing in DFU assumed to arise due to elevated TNF production (black bars). Also simulated are three hypothetical therapies: anti-TNF neutralizing antibodies (hatched bars), an agent that activates endogenous latent TGF-β1 (gray bars), and treatment with latent TGF-β1 (open bars). $^{*}p < 0.05$ vs. TNF-high baseline; $^{\dagger}p < 0.05$ vs. TGF-β1 activator; $^{\#}p < 0.05$ vs. anti-TNF (all by Kruskall–Wallis analysis of variance on ranks, followed by Tukey post-hoc test). IL, interleukins; TGF, transforming growth factor; TNF, tumor necrosis factor; DFU, diabetic foot ulcers.

qualitatively predict the calcium-dependent pattern of wound closure observed in vitro.

For dermal wound healing, the first mathematical models were derived from the Murray-Oster mechanical theory by Murray and Tranquillo,[30,33,34] and these models include the mechanisms involved in dermal wound contraction. As then, several more complex models have been developed. These models include multiple cell types and multiple types or phases of the viscoelastic extracellular matrix (ECM).[31,35,36] Other models include additional equations/behavior.[31,36,37]

The literature regarding the inflammatory genesis of DFU is sparse. However, two possible mechanisms stand out: elevated TNF[22] and reduced TGF-β1.[23] Because both of these cytokines are highly interrelated in their biology, we incorporated several of these interactions into our model. Our simulations suggest that for many indices of inflammation and healing, the effects of elevated TNF and reduced TGF-β1 are very similar due to the interrelationships between TNF and TGF-β1, both elevated TNF and reduced TGF-β1 are predicted to be associated with increased inflammatory infiltrates, elevated TNF and IL-10, reduced collagen, and elevated tissue damage. However, only the simulation in which TGF-β1 is reduced, and not the case in which TNF is elevated, is predicted to be associated with the reported decreased expression of TGF-β1 in DFU.[23] Thus, our simulations support the hypothesis that a central derangement in skin healing that leads to DFU is the reduced expression of TGF-β1. Nonetheless, it is possible or perhaps even likely that more than one cause of DFU exists. Given the overall qualitative similarity between the features of healing in the setting of reduced TGF-β1 and elevated TNF, both mechanisms (and others as well) may be operant in DFU. Further clinical studies are needed in order to address this issue. In any case, these different assumptions can be used in the *in silico* design and testing of DFU therapeutics, because this variability could be used to create simulated clinical trials; we[13,52] and others[40] have demonstrated the utility of this approach in the setting of sepsis.

Whether elevated TNF or reduced TGF-β1 underlies the pathology of DFU, our ABM is capable of reproducing the effect of known therapies for DFU. A major type of intervention is debridement, in which necrotic and/or infected areas of a DFU are removed surgically. Studies have shown that debridement improves healing in $\sim$25% of patients.[24] When we simulated the removal of 75% of damaged tissue at either 7 or 14 d from the onset of a wound, our ABM suggested that this would result in a statistically significant reduction in tissue damage at 30 d without a change in collagen levels, interpreted by us to mean improved healing. It might be argued that our finding of reduced tissue damage upon simulated removal of damaged tissue would seem obvious, but the finding that collagen levels remain the same suggests that indeed our ABM is depicting healing.

Although multiple randomized prospective clinical trials have been performed using growth factors in the treatment of DFU, only one growth factor, PDGF, has been approved for use.[44] PDGF has been found to

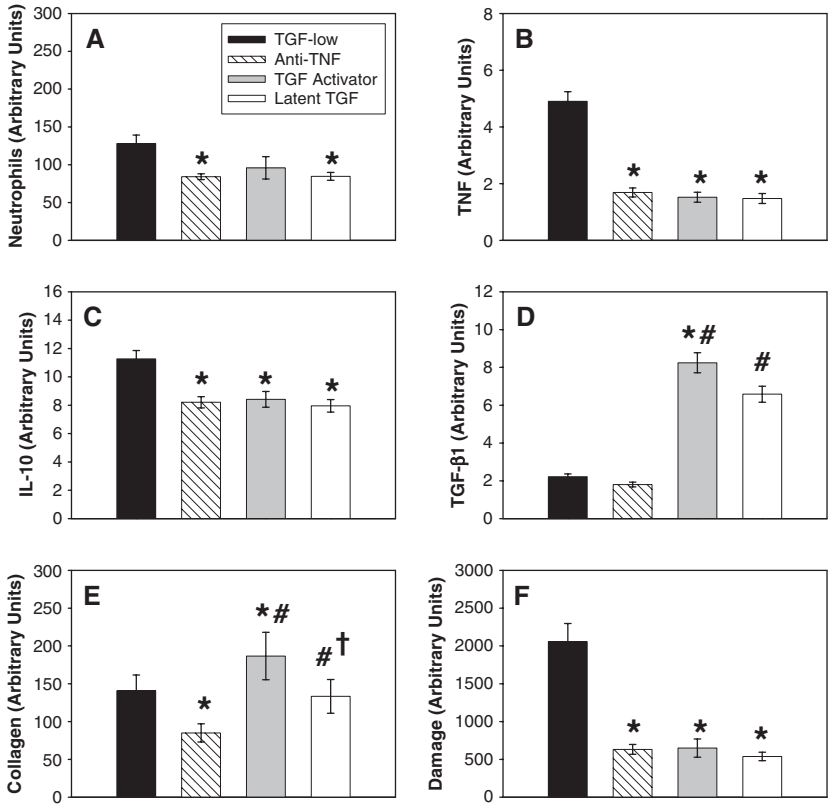

Figure 8. Simulations of therapies in TGF-β1-low DFU. The simulated levels of neutrophils (A), TNF (B), IL-10 (C), TGF-β1 (D), collagen (E), and tissue damage (F) are shown for skin healing in DFU assumed to arise due to reduced TGF-β1 production (black bars). Also simulated are three hypothetical therapies: anti-TNF neutralizing antibodies (hatched bars), an agent that activates endogenous latent TGF-β1 (gray bars), and treatment with latent TGF-β1 (open bars). $^{*}p < 0.05$ vs. TGF-β1-low baseline; $^{\dagger}p < 0.05$ vs. TGF-β1 activator; $^{\#}p < 0.05$ vs. anti-TNF (all by Kruskall–Wallis analysis of variance on ranks, followed by Tukey post-hoc test). IL, interleukins; TGF, transforming growth factor; TNF, tumor necrosis factor; DFU, diabetic foot ulcers.

increase healing in diabetic neuropathic foot ulcers and is marketed as REGRANEX™.[46] There is only limited evidence to suggest clinical benefit from other growth factors, including TGF-β1, FGF, IGF-1, GM-CSF, EGF, or a platelet releasate that contains many growth factors.[44,47,48,59–66] In our simulation, we account for the effects of platelet releasate and PDGF through the inclusion of platelets that function to chemoattract macrophages and neutrophils (see Tables 1 and 2 as well as the Supplementary Materials). Although we do not simulate PDGF explicitly, we demonstrate that modulating the actions of platelets on macrophages and neutrophils can, under certain circumstances, result in reduced tissue damage as compared with that predicted to be found in untreated DFU. Moreover, our exploration of the relative effects of platelets on macrophages and neutrophils suggested that there would be great variability in the efficacy of platelet-related therapies (either PDGF or platelet releasate). Thus, these simulations may be of benefit when attempting to personalize this type of therapy to an individual patient, if ex vivo studies could be carried out to assess the chemoattractant effect of the therapeutic agent on a patient-by-patient basis.

This interaction between inflammation and wound healing in the setting of DFU therapy may also be seen in other ways. In the clinical trials of PDGF, the importance of debridement became apparent. The first clinical trial to demonstrate benefit from PDGF found that extensive debridement, that is, wound excision down to normal tissue beyond the wound space, was associated with the highest healing rate.[24] Wound excision was favored, as this procedure removed the tissue with the highest bacterial load and the highest concentration of proteases. It may also be that the tissues removed were those trapped in the inflammatory phase of healing, with elevated TNF and reduced TGF-β1, a clinical observation that supports the findings of our simulations.

We utilized an *in silico* approach to study several hypothetical therapeutic approaches. The first agent we examined was a neutralizing anti-TNF antibody, given that elevated TNF production is a feature of diabetes[22] and given our ability to simulate many of the characteristics of DFU by assuming elevated TNF production. Several FDA-approved anti-TNF antibodies are available for the treatment of various inflammatory diseases.[51] Because we have carried out previous work on simulating mathematically the actions of anti-TNF in the setting of sepsis,[52] we sought to examine whether such a therapy might be of benefit in DFU. Our findings suggest that anti-TNF therapy for DFU should be explored.

Another therapy that we simulated was one in which exogenous, latent TGF-β1 would be provided or one that would lead to the activation of endogenous TGF-β1. TGF-β1 modulation (either provision of TGF-β1 or its inhibition) has been proposed as a possible therapy for various aspects of aberrant wound healing.[53] However, treatment with TGF-β1 was not efficacious as a DFU therapeutic.[54] We reasoned that because latent TGF-β1 has a longer

half-life than active TGF-β1,[11] it might serve as a better therapeutic agent. Our findings support this hypothesis.

We suggest at least one agent that can activate endogenous latent TGF-β1 and that may be useful as a topical drug: NO, which, we have shown in several contexts, leads to the activation of latent TGF-β1.[56,57] Nitric oxide is central to the wound-healing process[58] and is reduced in diabetic wounds.[67] We have suggested that the diverse actions of NO in wound healing may be secondary to the modulation of various cytokines including TGF-β1.[58] In the setting of DFU, chemical NO donors may be applied topically[68] or possibly delivered via gene therapy with either the inducible or constitutive NO synthase.[69] Interestingly, previous mathematical modeling approaches have examined this issue and suggested that NO production underlies keloids and hypertrophic scarring.[70] Both phenomena have also been ascribed to TGF-β1.[71]

The main disadvantage of our model is that it is based on the key mechanisms of inflammation and wound healing, but like any simulation does not incorporate all possible biological mechanisms that might be operant in the process of inflammation and wound healing. Importantly, our ABM does not account for collagen contraction as part of the wound-healing process, although it is our aim to incorporate this mechanism into later iterations of the model. It should be noted that in the ABM framework, it is often difficult to define the direct or indirect role of a given variable in the final outcome, and so the more mechanistic rules an ABM contains the less likely we are to gain this type of insight. Moreover, the more complex the ABM, the greater the computing power necessary to run any single simulation. In this manuscript, we strove to balance model realism with tractability, and believe that the overall findings justify this compromise. Also, the ABM structure contains certain assumptions regarding the stochastic nature of some of the processes being modeled, and these assumptions may not represent the exact way in which these processes occur in vivo. Another limitation relates to the way in which one models the production and clearance of a given agent, as well as the exact effects that an agent has on another agent: we have tried to base our assumptions on the literature data whenever possible, but the literature is incomplete with regard to certain specific interactions. Finally, this ABM is calibrated with regard to the literature data on skin wound healing, but has not been specifically calibrated or validated with prospective data from DFU patients, a deficiency we are currently in the process of addressing.

The clinical model presented in this manuscript could change the process of drug development for DFU. Taking a drug through basic science testing, toxicology, and clinical trials may cost hundreds of millions of dollars. If the drug could be tested in a mathematical model and found to be of benefit, a pharmaceutical company may be more willing to proceed with a clinical trial as the outcome would likely be successful.[72] Many trials fail because of noise in the system, i.e., the clinical efficacy may be masked because of differences in patient characteristics between the control and the study group. If the proper patient group was chosen for study, a clinical trial may show benefit. Mathematical modeling would enable development to be focused on agents that are likely to be of benefit.

ACKNOWLEDGMENTS

This work was partially funded by grants from the National Institutes of Health (P50-GM-5378909) to QM, BR, GC, and YV.

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