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Complement Deficiency Promotes Cutaneous Wound Healing in Mice

Stavros Rafail,* 1 Ioannis Kourtzelis,* 1, x 2 Periklis G. Foukas, † Maciej M. Markiewski,* Robert A. DeAngelis,* Mara Guariento,* Daniel Ricklin,* Elizabeth A. Grice, † and John D. Lambris* *2

Wound healing is a complex homeostatic response to injury that engages numerous cellular activities, processes, and cell-to-cell interactions. The complement system, an intricate network of proteins with important roles in immune surveillance and homeostasis, has been implicated in many physiological processes; however, its role in wound healing remains largely unexplored. In this study, we employ a murine model of excisional cutaneous wound healing and show that C3−/− mice exhibit accelerated early stages of wound healing. Reconstitution of C3−/− mice with serum from C3+/+ mice or purified human C3 abrogated the accelerated wound-healing phenotype. Wound histology of C3−/− mice revealed a reduction in inflammatory infiltrate compared with C3+/+ mice. C3 deficiency also resulted in increased accumulation of mast cells and advanced angiogenesis. We further show that mice deficient in the downstream complement effector C5 exhibit a similar wound-healing phenotype, which is recapitulated in C5aR1−/− mice, but not C3aR−/− or C5aR2−/− mice. Taken together, these data suggest that C5a signaling through C5aR may in part play a pivotal role in recruitment and activation of inflammatory cells to the wound environment, which in turn could delay the early stages of cutaneous wound healing. These findings also suggest a previously underappreciated role for complement in wound healing, and may have therapeutic implications for conditions of delayed wound healing.

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Materials and Methods

Animal studies

Full-thickness 6-mm excisional wounds were created by skin biopsy punches on mice lacking specific components of the complement system and on C57BL/6J wild-type or littermate (if available) controls. The animal groups included C3−/−, C5ar1−/−, and C5ar2−/− mice on a C57BL/6 background compared with their littermates and C5−/− mice on a C57BL/6 background with littermate or C57BL/6J wild-type controls. Mice were shaved and cleaned with a depilatory cream, swabbed with povidone-iodine, followed by an alcohol swabbing, and allowed to rest for 1 d prior to the operation. For each anesthetized mouse, two 6-mm punch biopsies were performed on the dorsum using sterile, single-use skin biopsy punchers according to the manufacturer’s instructions (AcuPunch; Acuderm), and their borders were marked using indelible, nontoxic ink. After shaving, each animal was housed individually to prevent any potential wound disturbance by other animals. Postsurgical pain relief was not required because the animals did not exhibit signs of pain. The sizes of the wounds were monitored at regular time points, and the results were recorded by digital photography. Wound surface area was measured as a percentage compared with the initial wound surface area on day 0 using the software ProgRes CapturePro 2.7 (Zeiss Stemi 2000C microscope).

Colonies of complement-deficient mice and littermate controls were maintained by our laboratory (in the case of C5−/− and C5ar1−/− mice) in a barrier facility at the University of Pennsylvania on a 12-h light/dark cycle or by The Jackson Laboratory (Bar Harbor, ME) at their facility in a colony specifically for our use (in the case of C3−/−, C5ar1−/−, and C5ar2−/− mice). Sera and feces were tested for common rodent infections as part of routine maintenance. Water and standard rodent diet were provided ad libitum. All mice were used with the approval of the University of Pennsylvania Institutional Animal Care and Use Committee and according to criteria outlined in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Generation of C5−/− mice on a C57BL/6 background

C5−/− mice on a C57BL/6 background were not previously available. Thus, we generated this strain by standard backcrossing. Male C5−/− mice on a C57BL/10 background from The Jackson Laboratory (stock number 000461) were first mated with female wild-type C57BL/6J mice (stock number 000462). The resulting N1 male heterozygous offspring were then mated with female wild-type C57BL/6J. For each generation, genotype was confirmed using PCR analysis of tail DNA.

Wound histology and immunohistochemistry

Tissue from wounded areas was excised and either snap frozen in liquid N2 or fixed in formalin and then embedded in paraffin. The excised tissue, which included the wound bed and the surrounding normal skin, was sectioned through the center of the wound, so that each section included a full diametric part of it. Wound morphology, inflammatory cell infiltrate, and vessel density were assessed in a blinded fashion by light microscopy (Nikon Eclipse E400 of H&E- and immunohistochemistry-stained sections by two pathologists (M.M.M. and P.G.F.).

Inflammatory cells were counted per high-power field (original magnification ×400) in a square below the epidermis, hypodermis (white adipose tissue), and adventitia (s.c. tissue); the last two regions are separated by the panniculus carnosus muscle (19). Neutrophils and foam histiocytes were assessed morphologically in H&E-stained sections, whereas mast cell density was assessed in sections stained with a Naphthol AS-D Chloroacetate (Specific Esterase) Kit (91C; Sigma-Aldrich). vessel cell density was evaluated per high-power field (original magnification ×400) following immunohistochemistry of formalin and paraffin tissue sections for α-smooth muscle actin (α-SMA), the expression of which is relatively restricted to vascular smooth muscle cells. Briefly, immunohistochemistry was performed on 3-μm paraffin sections using a DAKO Autostainer Plus device. Slides were immersed in a high-pH target retrieval solution (DMM82; DAKO), boiled in a microwave at 650 W for 20 min, and subsequently cooled at room temperature for 20 min. Endogenous peroxidase activity was blocked by means of a peroxidase-blocking reagent (SM801; DAKO). Primary Ab against α-SMA (mouse monoclonal, clone 1A4, 1:200 dilution; DAKO) was used for detection. The Ag–Ab complex was visualized using diaminobenzidine as a chromogen for 1 min, following incubation with EnVision FLEX+, Mouse, High pH (K8002; DAKO) for 30 min. As a negative control, the same immunohistochemical procedure was followed, replacing the primary Ab with TBS. All sections were lightly counterstained (25 s) with hematoxylin prior to mounting.

Human C3 purification

C3 was purified from human plasma, as previously described (20), with the following modifications. Briefly, the plasma was fractionated with 12% (w/v) PEG 3350 (Sigma-Aldrich), and the pellet was resuspended in 3 mM phosphate buffer containing 50 mM NaCl (pH 7.4) (buffer A) and applied to a column packed with Source 15Q resin (GE Healthcare). The elution was carried out with a two-step gradient (0–50% buffer B in 15-column volumes; 55–100% buffer B in 15-column volumes) between buffer A and buffer B (3 mM phosphate buffer containing 240 mM NaCl, pH 7.4). The C3 was further purified on a Mono Q column (GE Healthcare) with a two-step gradient (0–50% buffer C in 3-column volumes; 50–100% buffer C in 15-column volumes) between buffer A and buffer C (3 mM phosphate buffer containing 335 mM NaCl, pH 7.4). Finally, the C3 was buffer exchanged into PBS and sterile filtered.

Reconstitution studies

Mice in each group received 500 μl of either wild-type or C3−/− freshly isolated serum. A day before wounding (on day 0), the animals were treated i.p. with their respective serum, and two skin wounds per animal were created with an AcuPunch, as previously described. Similarly, for human C3 reconstitution, C3−/− animals received 0.8 mg C3 i.p. 2–3 h before wounding.

Data analysis

A p value ≤0.05, based on an unpaired, two-tailed t test with GraphPad Prism 4 (GraphPad Software, San Diego, CA), was used to indicate statistically significant differences between groups.

Results

Complement C3 deficiency accelerates the initial rate of excisional wound healing

We hypothesized that complement influences wound healing because the inflammatory response, which is in part regulated by complement components, plays a key role in various stages of wound healing (8). To test this hypothesis, we created 6-mm full-thickness excisional wounds on the dorsum of C3-deficient (C3−/−) and littermate wild-type (C3+/+) mice. Wound size was determined by surface area measurement and recorded on days 0, 1, 2, 3, 4, 7, and 14. We found that wounds healed faster in C3−/− mice when compared with C3+/+ mice and that the acceleration of healing was clearly seen until the third day after wounding (Fig. 1). C3−/− wounds demonstrated, on average, 38% greater closure on day 1, compared with C3+/+ wounds (p = 0.009). This trend continued through days 2 and 3, with average wound closure significantly differing by 29.0% (p = 0.005) and 29.3% (p = 0.001), respectively. The scab disappeared in both C3−/− and C3+/+ mice after ≈3 wk. Because C3 deficiency eliminates the majority of complement effector functions, these findings suggested a key role of complement in initial phases of the cutaneous wound-healing process.

To confirm the specificity of this data, we examined wound healing in C3−/− mice reconstituted with sera from C3+/+ mice or purified human C3. In both situations, C3 reconstitution in C3−/− mice significantly decreased the rate of healing at days 1 and 2, respectively.
Complement blockade promotes angiogenesis

Because angiogenesis plays a key role in wound healing and complement has been suggested to be involved in angiogenesis (9, 21), we stained wounded tissue sections with an Ab against α-SMA to assess vascularization. We detected significantly increased numbers of α-SMA+ vessels in the hypodermis of C3−/− mice compared with C3+/+ mice (p = 0.001) (Fig. 3G–I). This finding suggests that accelerated wound healing in C3−/− mice may, in part, arise from increased tissue angiogenesis.

Complement is involved in the early stages of wound healing through C5a

The comparison of wound histology from C3−/− mice and littermate controls suggested that accelerated wound healing in complement-deficient mice could be related to decreased inflammation and injury combined with increased angiogenesis. Because C5a is a potent chemoattractant, which recruits leukocytes to sites of inflammation and activates these cells, we examined the role of this anaphylatoxin in the healing process. Because eliminating C5 also eliminates C5a, we examined wound healing in mice deficient in C5. Because the only commercially available C5−/− animals were on a different background (C57BL/10) than the other complement-deficient animals used in this study (C57BL/6J), we backcrossed them for 10 generations before we included them in our studies (see detailed methodology in Materials and Methods). These C5−/− mice also displayed significantly accelerated healing compared with wild-type controls (Fig. 4A; day 1, p = 0.017; day 2, p = 0.01; day 3, p = 0.013). Therefore, the wound-healing and inflammatory phenotypes we observe in C3+/+ mice compared with C3−/− mice may, in part, be mediated by C5a signaling.

C5a mediates its functions by binding to two known receptors, that is, C5aR1 (CD88) and C5aR2 (C5L2). Although most of the studied functions of C5a are executed through C5aR1, we examined the contribution of both receptors to wound healing. Only mice deficient in C5aR1 presented with significantly accelerated healing (Fig. 4B; day 1, p = 0.015; day 2, p = 0.004). In contrast, the rate of wound healing in mice lacking C5aR2 did not significantly differ from wild-type controls (data not shown). Because anaphylatoxin C3a also has chemotactic properties, we examined wound healing in mice treated with a C3aR antagonist. However,
the wound-healing rate in these mice was similar to that observed in wild-type controls (data not shown). Thus, we concluded that inflammation mediated by C5a signaling through C5aR1 most likely delays wound healing by exacerbating tissue injury via inflammation, thereby contributing to a refined model about the involvement of complement in the early stages of wound healing (Fig. 5).

Discussion

In this study, we showed that blocking complement activation accelerates the early healing rate in a mouse model of cutaneous wound healing. We also found that the components of the complement system responsible for this effect include C3, C5, and signaling through C5aR1, but not C5aR2 or C3aR. Furthermore, reconstitution of C3-deficient animals with purified human C3 or serum from C3+/+ mice abrogated the effect, confirming the involvement of complement in the process. The absence of these molecules resulted in a reduction in the intensity of inflammation involved in the initial events of healing. We postulate that the reduced inflammation allowed the process to advance faster to the subsequent events of healing (proliferation, maturation), thus accelerating the whole process. Moreover, we observed an increase of vascularization accompanied by a significantly higher presence of mast cells in complement-deficient mice.

A major role of complement effectors is to attract, activate, and control cells of both innate and adaptive immunity. The anaphylatoxins C3a and C5a are powerful chemoattractants that guide neutrophils, monocytes, and macrophages toward the sites of complement activation (5). Neutrophils are one of the first cell populations to arrive in the wounded site, playing a bactericidal role while also cleansing the wound of debris and damaged tissue. Monocytes are another population that arrives at the wound site in response to factors released by platelets and other cells. After migration from the periphery to the wound, monocytes mature into macrophages, where they phagocytize bacteria and remove damaged tissue. Activated macrophages themselves produce C3 and participate in complement-initiated phagocytosis of intruding entities, whereas they are also involved in the clearance of apoptotic and necrotic cells (22). C5a reacts with both C5aR1 and C5aR2 in a manner that induces the “cytokine storm” in sepsis (23). Additionally, recent studies have shown that C1q can regulate the development of dendritic cells from monocytes while affecting T cell stimulation (24), although others have shown that complement promotes Th17 differentiation with the participation of TLRs through C5aR1 signaling (25). The role and importance of C1q in wound healing were also demonstrated in a recent report (9). Additionally, resident γδ T cells of the dermis help establish homeostasis after injury, because they are actively involved in the attraction and activity of macrophages and the production of growth factors such as insulin-like growth factor 1 and keratinocyte growth factor, among others (26). Their role is so vital that their absence severely impairs wound healing (27). Finally, keratinocytes, the major cell population in the skin, express proteins and receptors for several complement components and regulators (7). Our results show that, in mice lacking key components of the complement system, the inflammatory cells accumulating on the wounded site are decreased. We have also shown that this decrease involves the absence of the chemoattractant anaphylatoxin C5a.
Traditionally, cells of the immune system have been regarded as absolutely indispensable for proper wound healing. However, although immune cells are clearly essential for tissue clearance and preventing/fighting infection, the value of certain immune cells in other aspects of repair is now being challenged (3, 28). One reason for this change in view is the demonstration of the superior wound-healing capacity of fetal skin (29). In this tissue, the standard series of phases is not followed, and immune cells are practically nonexistent during the healing process. Despite the lack of immune cell involvement, fetal wounds heal very rapidly and without scar formation, essentially regenerating normal skin (29). This view has been further strengthened as more questions have been raised by studies using adult animal models devoid of specific immune cell subtypes. More specifically, animals deficient or depleted of neutrophils or macrophages exhibit accelerated healing (30–32). Finally, depletion of neutrophils in a mouse model of chronic diabetic wounds also causes faster and improved healing (33). These data come in concordance with our findings that the controlled constraint of inflammatory cells resulting from the absence of complement components can actually accelerate the rate of the healing area. Further supporting our finding, we have recently shown that complement modulates the bacterial microbiome in the skin of mice through C5aR1 signaling (34). In this study, the pharmacological blockade of C5aR1 resulted in the presence of reduced numbers of immune cells in the skin of C5aR1 antagonist-treated animals that also correlated with alterations in the bacterial content and load of the skin.

Mast cells are derived from hematopoietic progenitors that are known to migrate to and reside within connective and mucosal tissues, where they differentiate and respond to various stimuli by releasing proinflammatory mediators, including histamine, growth factors, and proteases. Human mast cells are known to release vascular endothelial growth factor and fibroblast growth factor-2 among other angiogenic growth factors. Serine proteases such as chymase degrade the extracellular matrix, and thus prepare the surrounding area for angiogenesis (35). Mast cell metalloproteinases can stimulate the release of angiogenic factors found in the extracellular matrix with subsequent release of fragments of hyaluronic acid, which are proangiogenic (36). Our histological findings reveal that the absence of complement components results in an accumulation of mast cells in the site of early cutaneous wounds. This is supplemented by increased angiogenesis, as shown by α-SMA staining. This comes in concordance with previous findings in which mice deficient in C3 displayed increased neovascularization in a model of retinopathy of prematurity and in an in vivo Matrigel plug assay (21). The diverse role of complement components and the complexity of the wound-healing process are highlighted by recent work showing that, in later stages (14 d postwounding), the presence of C1q is essential for physiological angiogenesis in a murine cutaneous model (9). Moreover, Fukuoka et al. (37) showed that β-tryptase, a major protease of human mast cells, can directly generate bioactive C3a and C5a, whereas, in turn, these anaphylatoxins are known activators of mast cell degranulation via complement receptor

**FIGURE 4.** C5−/− and C5aR1−/− mice have accelerated wound-healing phenotypes similar to C3−/− mice. To examine the involvement of downstream complement molecules in wound healing, we wounded (A) C5−/− mice and (B) C5aR1−/− mice, and compared the rate of surface area healing with that of complement-sufficient animals over time. The data are expressed as percentage areas compared with the initial wounded area (100%) on the y-axis. We observed that both C5−/− mice and C5aR1−/− mice exhibited accelerated healing over the first days of the process, suggesting that C5a is involved in the inflammatory stage of healing.
signaling (38, 39). We, therefore, postulate that a compensatory mechanism exists in complement-deficient mice, which results in the increased number of mast cells that was observed in our study.

Apart from the role of complement on immune cells, recent work has also shown the involvement of complement in other key processes playing roles in wound healing, such as fibroblast migration and activation (40), angiogenesis (9, 21), and coagulation (41). In addition to regulating inflammation and angiogenesis, there are likely most other complement-dependent mechanisms involved in wound healing. For instance, the coagulation system is the first process that is activated after wounding. Because C5aR1-mediated production of tissue factor (41) initiates the coagulation cascade, it is possible that complement also influences wound healing through coagulation. Moreover, thrombin, which converts fibrinogen to fibrin (42, 43), is also known to activate complement through the extrinsic pathway by cleaving C5 (6). Indeed, C3 fibrinogen to fibrin (42, 43), is also known to activate complement mediated production of tissue factor (41) initiates the coagulation process that is activated after wounding. Because C5aR1-involved in wound healing. For instance, the coagulation system is involved in wound healing. For instance, the coagulation system is involved in wound healing. For instance, the coagulation system is involved in wound healing. As a result, this study.

The number and complexity of the events (such as coagulation, inflammation, angiogenesis, and epithelialization) that are involved in the process of wound healing render it a very interesting, as well as challenging, research area. Defects in any of these stages can lead to impaired healing or chronic wounds and have been implicated in several diseases (e.g., diabetes). The involvement of complement in each of these events makes it a promising target for therapeutic intervention. Our findings already show that the absence of specific components of complement can lead to accelerated healing. Our proposed mechanism of action (Fig. 5) emphasizes the importance of complement inhibition at the level of C3 or C5a, as it may result in a shortened inflammatory stage together with increased angiogenesis and high levels of mast cells that, at least in murine models of cutaneous wound healing, contribute to a more efficient healing process. Although wound healing in a mouse is fundamentally different from that in humans as it primarily occurs via contraction (44), our results show that complement’s beneficiary inhibition targets mechanisms shared between the two species. The availability of human complement inhibitors, some of which are currently being tested in clinical trials (45, 46), makes the inhibition of complement activation a promising strategy in promoting faster, safer, and more effective healing.

Disclosures

J.D.L. and S.R. are inventors of patent applications that describe the use of complement inhibitors in wound healing. J.D.L. and D.R. are inventors of patents and/or patent applications that describe complement inhibitors. J.D.L. is also the founder of Amyndas Pharmaceuticals, which is developing complement inhibitors for clinical applications.

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