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Wound Healing Characteristics of ICAM-1 Null Mice Devoid of All Isoforms of ICAM-1

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Abstract

Background—Intercellular Adhesion Molecule-1 (ICAM-1) permits leukocyte-endothelial adhesion and transmigration during inflammation. Membrane-bound ICAM-1 knockout mice have been used to understand this molecule's role in wound-healing, but expressed spliced isoforms of ICAM-1 may have impacted results. We aimed to characterize wound-healing in an ICAM-1 null model devoid of all ICAM-1 isoforms.

Methods—Full-thickness 8-mm wounds were created on C57/BL6 wild-type (n=24) and ICAM-1 null (n=24) mice. Wound area was calculated using daily photographs. Histologic samples were harvested on post-operative Days 1,3,7, and 14. Wound margins were evaluated for mRNA expression of 13 inflammatory cytokines. A separate group of wild-type and ICAM-1 null mice (n=24) received full-thickness incisions with tensiometry measured at Day 14. Separately, complete blood counts were measured in unwounded wild-type (n=4) and ICAM-1 null mice (n=4).

Results—Wound-closure was significantly delayed in ICAM-1 null mice through Day 7 by gross and histologic measurement. mRNA expression of VEGF-A was increased in ICAM-1 null mice on Day 3, although no increase in VEGF-A was observed in the wound bed by immunohistochemistry. ICAM-1 null wounds demonstrated higher stiffness upon Day 14 tensiometry compared to the wild-type (1880 ± 926 kPa vs. 478 ± 117 kPa; p<0.01), and had higher counts of white blood cells (10,009 vs. 5,720 cells/microliter, p<0.05), neutrophils (2,130 vs. 630 cells/microliter, p<0.01), and lymphocytes (7,130 vs. 4,740 cells/microliter, p<0.05).

Conclusions—ICAM-1 null mice demonstrate delayed wound-healing and decreased wound elasticity compared to wild-type controls. This lag, however, was less than observed in earlier membrane-bound ICAM-1 knockouts, suggesting that other ICAM-1 isoforms may promote delayed wound-healing.

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Keywords

ICAM-1; Wound Healing; LFA-1; sICAM-1; Mouse Model; Tensiometry; Leukocyte Adhesion

INTRODUCTION

Wound healing is a dynamic and interactive process involving soluble mediators, the extracellular matrix, and hematopoietic and parenchymal cells. The normal wound repair process follows a specific time sequence and can be temporally categorized into four broad overlapping phases: hemostasis, inflammation, tissue formation, and tissue remodeling. Each phase is associated with distinct cellular and soluble mediators, and characterizing these pathways is an important and dynamic area of research.

Intercellular Adhesion Molecule-1 (ICAM-1, CD54) is central to the inflammatory process and wound healing in humans and mice. A member of the immunoglobulin super family, ICAM-1 is present on endothelial cell membranes and is essential to leukocyte adhesion and migration during inflammation via interactions with its natural ligand located on leukocyte cell membranes, lymphocyte function-associated antigen-1 (LFA-1). ICAM-1 has been implicated in B and T cell activation and function [1–6], T cell co-stimulation [7], and induction of apoptosis [8]. ICAM-1 deficient mice demonstrate impaired wound healing, displaying reduced infiltration of phagocytic cells, inhibited formation of granulation tissue, and decreased susceptibility to lipopolysaccharide induced septic shock [9, 10]. Although previous ICAM-1 deficient models lacked expression of membrane bound ICAM-1, these knockouts were capable of expressing alternative isoforms of ICAM-1 that could have impacted the results and conclusions of earlier wound-healing studies [11]. Therefore, we aimed to study dermal wound healing in the absence of all isoforms of ICAM-1 using a true ICAM-1 null mouse.

METHODS

ICAM-1 null mice (Jackson Laboratories, Bar Harbor, ME) were from C57/BL6 male colonies and generated by replacing the entire coding region of the ICAM-1 gene with a puromycin cassette as previously described [12]. Genotypes of all mice were confirmed by PCR.

Wounding

Male 8–12 week old wild-type C57/BL6 and ICAM-1 null mice (back crossed to C57/BL6) were anesthetized using isoflurane 2–3% in 70% O₂. Animals received a preoperative dose of penicillin 1×10⁶ U/kg IM and buprenorphine 0.1 mg/kg IM. All mice underwent depilation of the surgical site and topical antiseptics with povidone/iodine scrub. For tensiometry measurements two 1.5 cm incisions were made 1 cm lateral to the spine on each side of wild-type (n=6) and ICAM-1 null (n=6) mice. For all other measurements, including realtime PCR, a 6 mm circular punch was used to create two wounds lateral to the spine in 24 additional mice per group.

Mice were housed with 2 wounded cage mates and allowed to feed ad libitum. Standardized photographs were taken daily for 14 days and wound area of the circular punch wounds assessed using OPTIMAS 6.0 digital analysis software (Optimas Corp., Del Mar, CA). All photographs were taken perpendicular to the wound, with a ruler placed adjacent to the wounds to ensure accurate measurement.

Tissue Harvest

Wound tissue was harvested on days 1, 3, 7 and 14 after wounding, with 6 mice in each group per time point. Animals were euthanized after wound harvest. Under dissecting microscope, a cross-section of wounded tissue was taken through the center of the wound for further analysis. One of the two open wounds in each animal was processed for histology and the other snap frozen for molecular analysis.

Histology and Morphometric Analysis

Tissue from the circular punch wounds was fixed using Histochoice® synthetic fixative (Amresco Inc., Parkway Solon, OH), paraffin embedded, and sectioned (5µm). Sections were stained with either hematoxylin and eosin for wound size measurements, or Verhoeff/Van Geison stain for elastic tissue. Sections were examined and photographed (SuperCoolScan, Nikon Inc., USA) under 10×, 40×, and 60× magnification. Measurements of epithelial gap and granulation tissue height were assessed at each time point using OPTIMAS 6.0 digital analysis software (Optimas Corp., Del Mar, CA). Initial wound margins were defined by the region devoid of hair follicles and the wound gap defined as the distance between the initial wound margins. Epithelial gap was defined as the distance between the migrating epithelial front beneath the clot. Granulation tissue height was defined as the width of tissue exhibiting granular vessel formation, and was calculated as previously described [13]. Black-stained elastic tissue on Verhoeff/Van Geison staining was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). All slides were analyzed by at least two blinded observers. A random number generator was used to designate slides for blinding of histological analysis.

Immunohistochemistry

Tissues were sectioned (5µm), blocked in rabbit serum (Vectastain ABC kit, Vector Laboratories, USA), and incubated in anti-mouse F4/80 (1:50 Clone CI:A3-1, Serotec, USA), anti-neutrophil primary (1:100 clone RB6-8C5, Serotec, USA), or anti-VEGF (1:20, Santa Cruz biotech, USA) for 1 hr at room temperature. Endogenous peroxide was blocked in 3% H₂O₂ for 10 minutes. Incubation with biotinylated HRP-streptavidin secondary antibody occurred for 30 minutes (1:200 Vectastain kit, Vector Laboratories, USA). Incubation with chromagen (3,3-diaminobenzidine, Vector Laboratories, USA) produced brown reaction product positive cells. Control sections were stained with rat serum and without primary antibody. For each tissue section, reaction-positive cells were counted in 5 high-power fields at 60× magnification.

Complete Blood Count

To further determine the effect of ICAM-1 deficiency on the leukocyte infiltration into the wounds, we sought to compare baseline blood leukocyte count in unwounded animals. Blood was obtained from the tail veins of healthy 8–12 week old male wild-type and ICAM null mice. Complete blood counts were determined using an automated murine-specific cell counter. Measurements included white blood cell count with differential (cells/µl), red blood cell count (cells/µl), hemoglobin (g/dl), hematocrit (%), mean corpuscular hemoglobin (pg), mean corpuscular hematocrit (g/dL), red cell distribution (%), and platelets (cells/µl).

Tensiometry

Incisional wounds were harvested in 1 cm × 4 cm sections perpendicular to the original incisions. Tissues were handled carefully to avoid damage and submerged in PBS (pH 7.4) on ice to prevent desiccation. Prior to analysis, width, length and depth of tissue strips were measured with a digital caliper. Tensiometry measurements were performed within 1 hour of

harvest using the Instron 4301 universal materials testing instrument (Instron, High Wycombe, UK) set at a cross-head speed of 5 mm/min.

Real time PCR

Wounds were harvested with a 2 mm margin of uninjured tissue to ensure capture of the proliferating wound margins and snap frozen in liquid nitrogen. RNA was isolated using Trizol reagent (GIBCO, BRL) according to the manufacturer's instructions. Rneasy Mini kit (Qiagen, Valencia, CA) was used to isolate total RNA, and isolated RNA concentrations were determined by spectrophotometry. Reverse transcription of RNA (200 ng) was performed in combination with 4 μ l AMV buffer, 4 μ l $MgCl_2$, 2 μ l dNTP, 2 μ l random primer, 1 μ l RNase inhibitor, 0.8 μ l AMV reverse transcriptase, and DEPC H_2O for final volume. PCR cycling was as follows: 25°C for 10 minutes, 42°C for 60 minutes, 95°C for 5 minutes, 94°C for 5 minutes, followed by 35 cycles 94°C for 30 seconds, 67°C for 1 minutes, 74°C for 1 minute. PCR samples were electrophoresed on 1% agarose gel for 1 hr at 80 volts, stained with ethidium bromide, and viewed under ultraviolet light to assess integrity. Quantitative analysis of cDNA was performed after reverse transcription of mRNA. Detection of SYBR Green I fluorescence product was used in the LightCycler (Roche) system for amplification detection. Commercially available primers for IL-1 β , IL-6, IL-10, IP-10, TGF β 1, TGF β 3, TNF α , bFGF, PDGF, VEGF-A, IFN γ , MIP-2, KC, and GAPDH were used. Each reaction well contained a total volume of 20 μ l consisting of 1 μ l of cDNA, 0.5 μ l of forward primer (10 μ M), 0.5 μ l of reverse primer (10 μ M), 10 μ l of SYBR Green ReadyMix (SYBR Green I dye, Taq polymerase, $MgCl_2$), and 7.8 μ l of DEPC H_2O . Temperature cycles were: 10 minutes at 95°C followed by 45 cycles of denaturation for 15 seconds at 95°C, annealing for 10 seconds at 60°C, and elongation for 15 seconds at 72°C. After amplification, melting curve analysis was performed by heating samples from 60°C to 95°C for 1 minute. cDNA from the wounds of both wild-type and ICAM-1 null mice were analyzed for each cytokine with GAPDH serving as an internal control.

Statistical Analysis

Values are reported as mean $\pm$ standard deviation. Student's t-tests were performed for continuous variables with a p-value < 0.05 indicating statistical significance. Real time PCR data was used to express mRNA level with a denominator of C-value for GAPDH (internal control).

RESULTS

Gross Wound Area

Daily wound photographs of healing back punch wounds showed a significant difference in wound closure rates (Figure 1). Dorsal wounds in both groups expanded initially after wounding. Wound area was significantly larger in ICAM-1 null mice on Days 1–8 post-op when compared to wild-type mice (n=6 per time point; p<0.05) (Figure 2). Beyond post-op Day 8, however, no significant difference was observed.

Microscopic Analysis

Histologic measurement on Days 1 and 3 revealed a larger wound gap in ICAM-1 null mice as compared to wild-type (Day 1: 9.95 ± 0.62 mm vs. 8.25 ± 0.34 mm; p<0.05, Day 3: 8.73 ± 0.49 mm vs. 5.68 ± 0.53 mm; p<0.05) (Figure 3). By Day 7, however, no significant difference in wound gap between the two strains was observed. The epithelial gap of ICAM-1 null wounds was larger than that of wild-type wounds on Day 1 (8.25 ± 0.39 mm vs. 7.46 ± 0.33 mm; p<0.05), however, beyond this time point no significant difference in epithelial gap was noted. Elastin staining was increased in wild type mice as compared to

ICAM-1 null mice, although this difference did not reach statistical significance (463 ± 249 pixels/hpf vs. 362 ± 173 pixels/hpf; $p=0.47$) (Figure 4). No significant differences in granulation tissue height were noted at any time point.

Immunohistochemistry

No significant difference was noted between the two strains for Day 1 wound margin sections stained for Gr-1 neutrophil antigen (Figure 5). Similarly, Day 3 wounds displayed no significant difference between ICAM-1 null and wild-type mice in the number of F4/80 (macrophage antigen) or VEGF-A positive cells.

Complete Blood Counts

To further explain the similar leukocyte infiltration noted in wild-type and ICAM-1 null wounds we sought to assess for inherent differences or similarities in blood leukocyte counts. Results are presented in Table I. Note that values for red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hematocrit, and platelets did not differ between the two strains.

Real time PCR

There was no significant difference between the two strains ($n=6$ per group) in mRNA expression for the genes studied in wounds harvested on Day 1 (Figure 6). On Day 3, ICAM-1 null wounds expressed significantly higher levels of KC and VEGF-A relative to wild-type.

Tensiometry

Healed incisional wounds of ICAM-1 null demonstrated decreased elasticity compared to wild-type wounds. The Young's modulus (a measure of stiffness) was significantly higher in the ICAM-1 null wounds than wild-type wounds (1880 ± 926 kPa vs. 478 ± 117 kPa; $p<0.01$). Both body weight and the cross-sectional area of wounds are potential confounders to wound breaking strength. There was no significant difference in body weight, cross sectional area or thickness of the skin samples analyzed.

DISCUSSION

Endothelial adhesion molecules are critical in controlling the migration of circulating leukocytes into peripheral tissue following injury. Elimination or alteration of these adhesion molecules delays wound healing by reducing leukocyte infiltration and attenuating the inflammatory response [9].

This study demonstrates the impact of a complete absence of ICAM-1 on wound-healing in mice. While others have examined the inflammatory consequences of ICAM-1 deficient mice [10], the specific mutations that were used target either domain 4 or 5 [10, 14] of the 7 exon ICAM-1 gene. Targeting these domains resulted in mice that were presumed to lack total expression of ICAM-1. However, subsequent studies have shown these animals may express alternatively spliced isoforms of ICAM-1 that maintain the ability to bind ICAM-1's major ligand, LFA-1 [11]. Our findings suggest that these alternatively spliced isoforms of ICAM-1 may have influenced the wound healing response seen in previous knockout models.

Utilizing mice with the exon five mutation, Nagaoka et al demonstrated a significant increase in both epithelial gap and wound gap that persisted until at least Day 7 [9]. Our findings of a comparable epithelial gap by Day 3 and wound gap by Day 7 between ICAM-1

null and wild-type mice differ from earlier studies, and suggest that the current ICAM-1 null model exhibits a distinct wound-healing profile as compared to previous partial KO models.

ICAM-1 is important in leukocyte firm adhesion and subsequent transmigration. However by immunohistochemistry in our study, comparable amounts of neutrophils and macrophages were identified in wounds of wild-type and ICAM-1 null mice at Days 1 and 3 post wounding, respectively. In contrast, ICAM-1 domain 5 knockout mice demonstrated attenuated neutrophil concentration at 1 and 4 hours postoperatively and decreased macrophage concentration on Day 3 [9]. These differences exist between knockout strains despite the 1 domain 5 knock-out mice having a higher blood leukocyte count (15,000 cells/uL) than our ICAM null mice [10]. This suggests that other confounding factors are responsible for the more impaired leukocyte trafficking in that the ICAM-1 domain 5 knockout model. Alternative spliced isoforms of ICAM-1 bind to LFA-1, and competitive binding by these isoforms may abrogate the compensatory mechanisms typically noted when a certain molecule is knocked-out. Clearly these variations in leukocyte trafficking suggest important differences that underline the potential function of alternate ICAM-1 isoforms.

The mechanism behind the wound healing differences between our strain of ICAM-1 null mice and ICAM-1 domain 5 knock-out mice is unclear, though a few hypotheses exist. ICAM-1 is one of many molecules involved in promoting leukocyte adhesion to vascular endothelium. Therefore it is possible that, in an effort to compensate for the paucity of LFA-1 interactions, a mouse completely devoid of ICAM-1 will up-regulate other adhesion molecules, such as Mac-1. These compensatory mechanisms may serve to “normalize” leukocyte adhesion and ultimately wound healing parameters. When alternative isoforms of ICAM-1 are present that retain the ability to bind LFA-1, such compensation may not be triggered. Furthermore, the isoforms may compete for binding with other adhesion-promoting molecules on the endothelial surface and worsen the ability of leukocytes to traffic to the site of injury or inflammation. One such isoform that may be found in the ICAM-1 domain 4 or 5 knockout mice is the soluble form of ICAM-1 (sICAM-1). sICAM-1 is a 453 acid residue glycosylated peptide is composed of 5 distinct domains, stabilized by disulphide bonds. It is found in serum, cerebrospinal fluid, synovial fluid, and respiratory tract secretions [15]. sICAM may result from shedding of surface ICAM-1 or as a consequence of its own genetic coding [16, 17]. Importantly, sICAM-1 has been proposed to inhibit leukocyte transmigration, possibly by promoting de-adhesion through competitively binding to LFA-1 [17]. sICAM-1 could thus have contributed to delayed wound healing in previous ICAM-1 knockouts by exerting either of the effects mentioned above.

Regulatory mechanisms in expression of other genes may also explain the differences seen in this complete ICAM-1 null model. VEGF-A mRNA, for example, is slightly increased in this model compared to wild-type mice. An increase of VEGF may promote the formation of new leaky vessels [18], thereby allowing cells to migrate into the wound bed and may have contributed to the higher than expected neutrophil content in the wounds. Despite this small increase in VEGF-A mRNA, however, immunohistochemical staining for VEGF-A showed no difference in expression between ICAM-1 null and wild-type mice.

Interestingly, our study indicated that ICAM-1 null wounds are significantly less elastic than wild-type wounds on Day 14, although a decrease in elastic tissue could not be confirmed on histology. To our knowledge there have not been any other studies that evaluate elasticity, as measured by Young’s modulus, in ICAM-1 KO mice, and therefore no comparisons exist. It is clear that despite normal leukocyte counts in the wound bed and wound areas that do not differ from wild-type after Day 8, ICAM-1 null mice heal with wounds that display very different tensile properties from wild-type mice.

In summary, mice completely devoid of ICAM-1 have delayed healing of open skin wounds. This delay in wound healing is not as severe as previously reported in ICAM-1 domain 5 knockout mice, and these data thereby support the premise that alternatively spliced isoforms of ICAM-1 may have a deleterious effect on the wound healing response.

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Figure 1. Side-by-side daily photographs of back punch wounds of wild-type (left) and ICAM-1 null mice (right) are shown.

Figure 2. Wound area per Optimas analysis of gross daily photographs. Wound area was significantly larger in ICAM-1 null mice on days 1–8 post-op ($p < 0.05$).

Figure 3.

Parameters of wound healing in ICAM-1 null and wild-type mice. Wound gap was significantly lower in ICAM-1 null groups on Days 1 and 3 ($p < 0.05$), while epithelial gap was significantly lower on Day 1 ($p < 0.05$). No difference was observed in granulation tissue height at any timepoint.

Figure 4. Representative Verhoeff/Van Geison staining of wild-type (left) and ICAM-1 null (right) wounds for elastic tissue (black) and collagen (red-purple). Elastin staining was increased in wild type mice as compared to ICAM-1 null mice, although this difference did not reach statistical significance (40× magnification).

Figure 5.
Representative immunohistologic slides stained for the Gr-1 neutrophil antigen on day 1 and for F4/80 macrophage antigen on day 3 (10× magnification).
a. Gr-1 stain of wild-type Day 1 b. Gr-1 stain of ICAM-1 null Day 1 c. F4/80 of wild-type Day 3 d. F4/80 of ICAM-1 null Day 3

Figure 6. mRNA expression of wild-type and ICAM-1 null mice. ICAM-1 null mice showed significantly higher expression of KC and VEGF-A mRNA relative to wild-type ($p < 0.05$) on Day 3. No statistically significant differences were noted between groups on Day 1.

Table 1

Complete Blood Count Results

	Complete ICAM-1 Null (n=4)	Wild-Type (n=4)
Total leukocytes (cells/μl)	10,009 [*]	5,720
Absolute Neutrophils (cells/μl)	2,130 ^{Δ}	630
Absolute Lymphocytes (cells/μl)	7,130 ^{Δ}	4,740
Neutrophil Proportion	21% ^{Δ}	11%
Lymphocyte Proportion	71% [*]	83%

^{*}
p < 0.05

^{Δ}
p < 0.01