

A Primary Burn Wound Does Not Slow the Contraction Rate of an Adjacent Excisional Wound

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The response to major burn injury includes systemic release of mediators that may have an effect on wound healing. The authors evaluated the effect of a burn injury on the contraction of an excisional wound adjacent to the burn, and the effect of plasma derived from burn-injured animals on the contraction of the fibroblast-populated collagen matrix (FPCM). Nine rats (90–100 days old) under anesthesia received a standardized 40% total body surface area burn to the dorsum, and eight rats (controls) were sham burned. Immediately thereafter all animals had a square (2.25 cm²) of unburned dermis excised from the dorsum, superior to the burn wound. The excisional wound area was measured at 2 to 3-day intervals postoperatively. Plasma was collected from some animals on postburn day 15; the contraction-stimulating ability of burn vs. control plasma was measured in the FPCM. All animals remained free of sepsis. The excisional wound area in all animals decreased to 50% and then 25% of the initial area after approximately 4 and 8 days respectively. The rate of wound contraction (i.e., wound area reduction) did not differ between burn and control animals. Contraction stimulated by 5% plasma in the FPCM (expressed as a percentage of the original matrix area) was 70.2 ± 6.4 (standard deviation) mm² vs. 62.4 ± 3.9 mm² for burn vs. control rats respectively ($p > 0.05$). Burn injury in this model did not alter the contraction of an excisional wound at an unburned site. There was no significant difference in the contraction-stimulating ability (FPCM model) of plasma from the burned rats compared with plasma from unburned control rats. Burn injury appears to have an inconsequential effect on the contraction of an adjacent wound.

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onary perfusion pressure and vascular resistance, and increased cardiomyocyte apoptosis.^{1–6} In addition, cutaneous burn injury alters pulmonary permeability and increases alveolar macrophage cytokine production.^{7–12} Gut permeability can also be altered by a burn, resulting in bacterial translocation.^{13–15} In other models, granulation tissue formation in subcutaneous polyvinyl alcohol implants is decreased in animals with intra-abdominal abscess. This effect is partially reversed with the administration of a tumor necrosis factor antagonist.¹⁶ In a model of femur fracture, hepatic oxygen consumption increased in the isolated liver 2 and 48 hours after femur fracture. Kupffer cells from this preparation had an altered synthetic response to phorbol ester or lipopolysaccharide stimulation.¹⁷ From these studies, it is apparent that cytokines and other biochemical mediators produced at a site of trauma, infection, or other inflammatory process enter the systemic circulation, producing remote disturbances in cellular and organ function.^{18,19}

Because there are numerous examples of metabolic processes that are affected by major burn injury, we wanted to determine whether such an injury would affect the healing process (specifically, wound contraction) in a wound adjacent to the burn injury. This would have clinical relevance, because burn patients often must heal wounds remote from their burns (e.g., skin graft harvest sites). Additionally, we were anticipating that if an effect of burn injury on wound contraction was observed, then we might be able to isolate a plasma mediator of this effect, which could have therapeutic potential. We hypothesized that major burn injury would release mediators that would decrease contraction both in vivo (in an adjacent excisional wound) and in vitro (in the collagen matrix). We used a rat model in which sequential scald burn injury (the primary inflammatory event) and unburned skin excision (the adjacent secondary wound) were

A localized inflammatory process may influence events remote from the inflammatory process. In this regard, a 40% total burn surface area (TBSA) burn in adult rats, rabbits, and guinea pigs produces cardiac dysfunction, including impaired contraction and relaxation, decreased cor-

performed. The rate of contraction of the excisional wound was measured, and the contraction-stimulating effect of the animals' plasma was assessed using an *in vitro* wound model.

Materials and Methods

Materials

Sprague–Dawley rats, 90 to 100 days old (weight, 350–400 g), were used. Dulbecco's modified Eagle medium (DMEM), minimal essential medium at a 10-fold concentration (MEM 10×), antibiotic–antimycotic solution, and trypsin/ethylenediamine tetraacetic acid (EDTA) solution were obtained from Life Technologies (Rockville, MD). Fetal bovine serum (FBS; Rehatuin) was obtained from Intergen (Purchase, NY). Vitrogen (3 mg per milliliter bovine dermal collagen solubilized in 0.012 N hydrochloride) was obtained from Cohesion Technologies (Palo Alto, CA). Trypsin inhibitor, fatty acid-free albumin, HEPES, and routine chemicals were obtained from Sigma (St. Louis, MO). Methoxyflurane was obtained from Schering–Plough Animal Health (Union, NJ). Spectra/Por dialysis membranes were obtained from Spectrum Laboratories (Laguna Hills, CA). Vacutainers were obtained from Becton, Dickinson (Franklin Lakes, NJ).

Rat Burn Model

The animal component of this study was reviewed and approved by the University of Texas Institutional Animal Care & Research Advisory Committee, and was in accordance with guidelines from the National Institutes of Health. The rats were housed individually and allowed food and water *ad libitum*. Under methoxyflurane inhalation anesthesia, a 40% TBSA full-thickness scald burn was induced on the posterior aspect of the rat by immersion in 100°C water for 10 seconds. This is the Walker burn model²⁰ (modified at our institution to produce a 40% TBSA burn²¹). The aperture of the burn template was adjusted based on calculations of body weight and TBSA. A 40% burn is the largest burn that can be obtained reasonably with this model. Control rats under anesthesia were immersed in room temperature water (sham burn). All rats received 4 ml per kilogram per percent TBSA of

lactated Ringer's solution by intraperitoneal injection immediately after burn or sham burn.^{22,23}

Excisional Wounding

After water immersion, all rats were dried and then underwent excision of a 1.5 × 1.5-cm (marked with a template) full-thickness (dermis and panniculus carnosus) square of unburned skin from the nape, superior to the burn wound.²⁴ This 2.25-cm² excisional wound was the largest that could be obtained in conjunction with the burn in this model. The superior edge of the burn wound and the inferior edge of the excisional wound were separated by a minimum of 1.5 cm. The initial area of the excisional wound was measured by tracing the wound margin onto a plastic transparent sheet, digitally scanning the sheet, and calculating the area of the tracing with NIH Image (public-domain software, National Institute of Mental Health). The excisional wound area of all animals was measured subsequently with the rat under a light anesthetic (methoxyflurane) every 2 to 3 days until the excisional wound was closed.

Plasma Preparation

After the last wound area measurement was taken on day 15 in the first rat experiment (see Results), blood was collected quickly from euthanized rats by aspirating the aorta. Opened Vacutainers (4.5 ml draw blue top with sodium citrate; final concentration, 13 mmol per liter) were filled carefully with 4.5 ml of blood and mixed gently. The tubes were centrifuged at 1,240 g for 15 minutes at 22°C. The supernatant was recentrifuged at 22,000 g for 30 minutes at 4°C. The supernatant from the second centrifugation was dialyzed against a 100× volume of Dulbecco's phosphate-buffered saline (150 mmol per liter sodium chloride, 3 mmol per liter potassium chloride, 6 mmol per liter sodium phosphate, 1 mmol per liter potassium phosphate, 1 mmol per liter calcium chloride, and 0.5 mmol per liter magnesium chloride) for 24 hours at 4°C (dialysate changed once) using a membrane with a molecular weight cutoff of 12,000 to 14,000 (Spectra/Por 2). The dialyzed plasma was centrifuged again at 22,000 g for 30 minutes at 4°C, and stored at –80°C.

Fibroblast Culture

The use of human cells for this study was reviewed and approved by the institutional review board of the University of Texas. Human fibroblasts from neonatal foreskin were cultured in medium (DMEM buffered with 20 mmol per liter HEPES and 44 mmol per liter sodium bicarbonate with 1% antibiotic-antimycotic solution; pH, 7.2) supplemented with 10% FBS. Cells were maintained in 75-cm² flasks, were not allowed to become overconfluent, and were used before the 10th passage.

Fibroblast-Populated Collagen Matrix (FPCM)

The FPCM model has been described previously.^{25,26} Fibroblasts were harvested from 75-cm² flasks using 0.25% trypsin/1 mmol per liter EDTA in Hank's balanced salt solution (without divalent cations). Trypsinization was quenched with crude soybean trypsin inhibitor (10 mg per milliliter in medium; 1 ml of trypsin inhibitor solution per milliliter of trypsin/EDTA). Cells were washed once with 10 ml of medium and resuspended in medium to a concentration of 5×10^6 cells per milliliter (fibroblast solution). The matrix solution was made by quickly combining on ice the following fractions in order from first to last: medium (0.175), MEM 10 \times (0.0625), 0.1 M sodium hydroxide (0.0625), Vitrogen (0.5), and fibroblast solution (0.2). The final concentration of cells and collagen in the matrix solution (and hence the FPCM) was 1×10^6 cells per milliliter and 1.5 mg per milliliter respectively. Immediately after addition of the fibroblast solution, the matrix solution was incubated with gentle mixing in a 37°C water bath for 4 minutes. An aliquot (200 μ l) of the warmed matrix solution was placed into each well of a 24-well plate (Costar, Corning, NY). Each well had a 12-mm circular inscription on the bottom, and the matrix solution spread out and filled this circle. The "aliquoted" matrix solution polymerized into a gel during an incubation for 1 hour at 37°C, and then the contraction assay was performed.

FPCM Contraction Assay

The events of this assay are illustrated in Figure 1. After the matrix solution polymerized into a gel, 1.0 ml of medium with fatty acid-free albumin (5 mg per milliliter) was added to each well

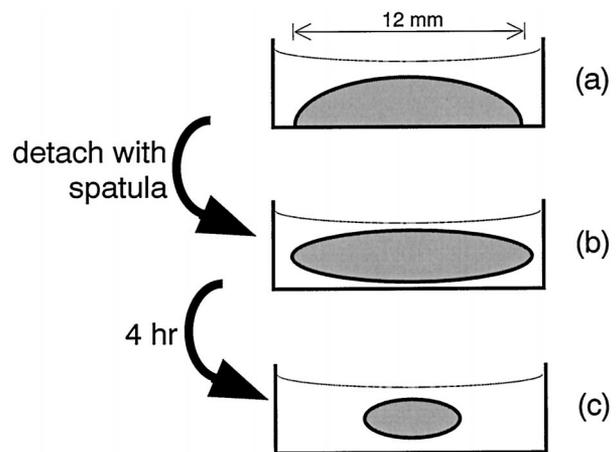


Fig 1. (A–C) Contraction assay for the fibroblast-populated collagen matrix, a side view. (Contrast with Figure 2; see text for details.)

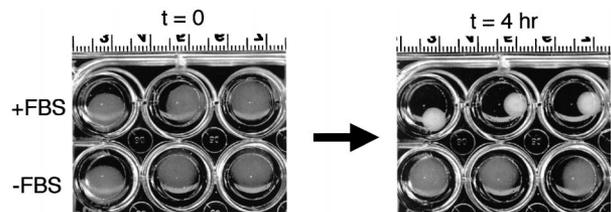


Fig 2. Contraction assay using the positive and negative control (plus and minus fetal bovine serum [FBS] respectively), performed in triplicate. Matrices (2×10^5 cells per matrix, 0.2 ml volume, 1.5 mg per milliliter collagen) were treated with Dulbecco's modified Eagle medium and fatty acid-free albumin (5 mg per milliliter) in the presence or absence of FBS (5%) for 4 hours at 37°C. A scan was obtained at $t = 0$ hours and $t = 4$ hours. This yielded an overhead view of the 24-well plate (six wells visible here). Inside each well is a floating matrix. The ruler is in centimeters.

(see Fig 1A). This medium and albumin was supplemented with 5% (final concentration) FBS (positive control), nothing (negative control), or 5% test plasma. Preliminary experiments revealed that a maximal contraction response was obtained with 5% FBS (data not shown). Immediately after the addition of medium, the gels were lifted gently off the bottom of the well with a spatula so that they floated freely in the medium (see Fig 1B), and were then scanned so that their initial ($t = 0$) area (in the horizontal plane) could be calculated using NIH Image. After the $t = 0$ scan, the gels were incubated at 37°C, and serum-stimulated contraction ensued (see Fig 1C). The gels were rescanned at $t = 4$ hours for final area calculation.

A typical result for this contraction assay using the positive and negative control is shown in Figure 2. There was an approximate 75% reduction in gel area (i.e., contraction to 25% of original area) in the presence of FBS; minimal contraction occurred in the absence of FBS. No contraction occurred in cell-free matrices (data not shown). Contraction was quantified with the term “% original area” (Fig 4), which is defined in the following equation:

$$\% \text{ Original Area} = (A_f/A_o) \times 100$$

where A_o is the gel area (in square millimeters) at $t = 0$ and A_f is the area at $t = 4$ hours.

Statistical Methods

Unpaired data were compared with the unpaired t -test. Three or more groups of unpaired data were compared with analysis of variance (ANOVA).

Results

Two experiments comparing the rate of contraction of the secondary excisional wound between burned and sham-burned rats were performed (Fig 3). In the first experiment (Fig 3A), the excisional wound area measurements were taken every 3 days for 15 days. The initial (day 0) excisional wound area for the burned ($N = 5$) and sham-burned ($N = 4$) animals was 4.85 ± 1.12 (standard deviation) cm^2 and $3.60 \pm 0.83 \text{ cm}^2$ ($p = 0.11$, unpaired t -test), and was greater than the area of the excised skin ($1.5 \times 1.5 \text{ cm} = 2.25 \text{ cm}^2$). This initial expansion of the excisional wound was secondary to retraction of the excisional wound margin, which was secondary to tension within the dermis. The initial area of the excisional wound appeared to be greater in the burned compared with the sham-burned rats, but this was not significant. When the excisional wound area data are plotted as relative area (that is, each wound area is expressed as a fraction of the initial wound area; see inset Fig 3A), then it is apparent that there is no difference in the rate of excisional wound closure between the burned and sham-burned animals ($p > 0.05$, ANOVA).

The rat experiment was repeated with eight additional animals (four burn, four sham burn), as shown in Figure 3B. Because approximately 80% to 90% of the excisional wound contraction

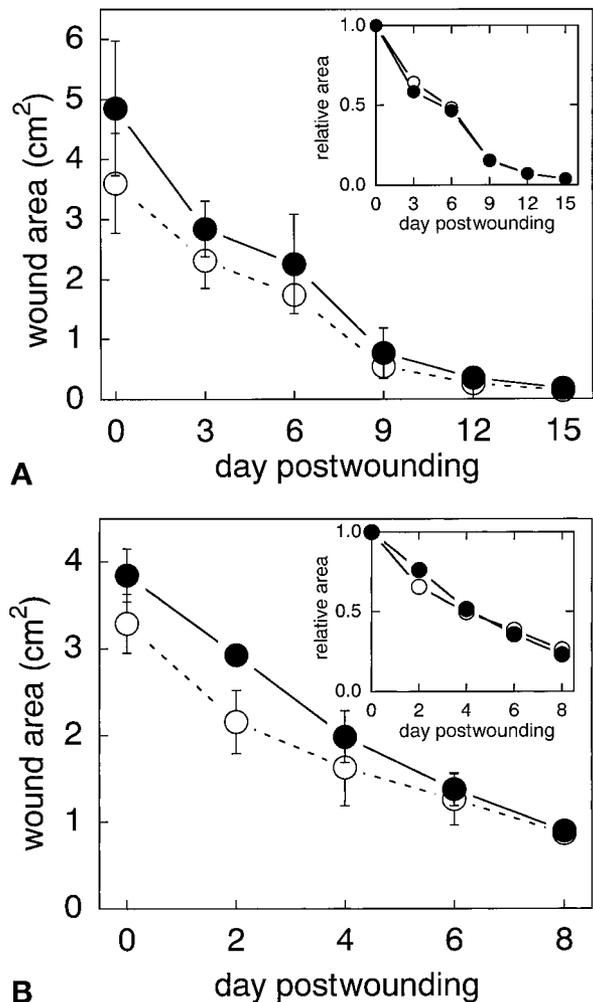


Fig 3. (A) Decrease in excisional wound area with respect to postwounding day in the burned (filled circles) and sham-burned (open circles) rats ($N = 5$ for burned rats; $N = 4$ for sham-burned rats). Error bars represent standard deviation. Inset: Same data, but relative excisional wound area (i.e., contracted wound area/initial wound area) is plotted. (B) Same type of experiment as in A, but with another group of rats and with earlier times of area measurement.

occurred during the first 9 days (see Fig 3A) during the first experiment, we chose to take area measurements more frequently (every 2 days) during the second. The initial excisional wound area was greater in the burned ($3.84 \pm 0.30 \text{ cm}^2$) vs. the sham-burned ($3.29 \pm 0.34 \text{ cm}^2$) animals (see Fig 3B; $p = 0.05$, unpaired t -test). When the relative area of the excisional wound is plotted (see inset Fig 3B), it can be determined that, as in the first experiment (see Fig 3A), there is no difference in the rate of excisional wound closure between the burned and the sham-burned animals ($p > 0.05$, ANOVA).

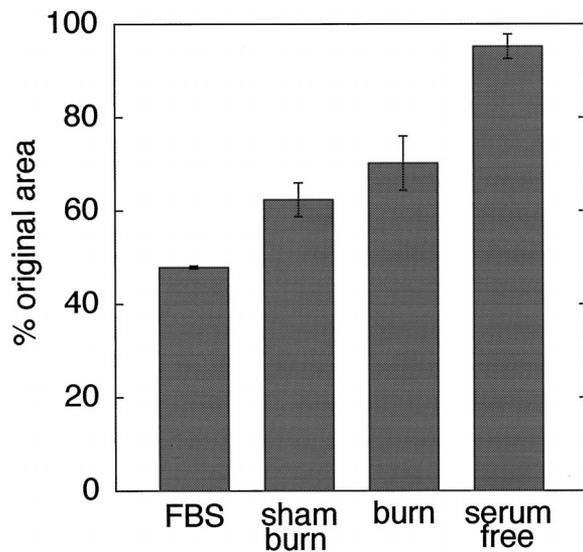


Fig 4. Contraction (expressed as a percentage of the original gel area) of the fibroblast-populated collagen matrix stimulated by 5% fetal bovine serum (FBS) vs. 5% plasma from burned and sham-burned animals. Each bar represents an average of six matrices; error bars represent standard deviation. A shorter bar means more contraction.

In addition to determining the direct effect of burn wound injury on excisional wound contraction in an animal model, we also wanted to test the efficacy of plasma from the control vs. experimental animals to stimulate contraction in the FPCM. This would be a functional test for circulating mediators/inhibitors of contraction. If we could identify a difference in contraction-stimulating efficacy in sham burn plasma vs. burn plasma, then the contribution of specific mediators could be pursued. Matrices were treated with 5% plasma solutions during the FPCM contraction assay (see Fig 4). The gel contraction (expressed as percent original area) for FBS, sham-burn plasma, burn plasma, and no serum addition was $47.9 \pm 0.4\%$, $62.4 \pm 3.9\%$, $70.2 \pm 6.2\%$, and $95.2 \pm 2.4\%$ respectively ($p < 0.05$, ANOVA). The percent original area obtained with the sham-burn plasma was 12% less than that obtained with the burn plasma, but this did not reach significance ($p > 0.05$, unpaired *t*-test).

Discussion

The rate of excisional wound contraction in this rat model is comparable with contraction rates

described for wounds in similar models.^{24,27-29} In the current study, a 40% TBSA full-thickness skin burn in the rat failed to alter the rate of wound contraction of an adjacent excisional wound in unburned skin. There was no significant difference in FPCM contraction stimulated by burn plasma compared with sham-burn plasma. Our data suggest that major burn injury does not have a biologically important effect on the contraction of an adjacent excisional wound.

Our finding of negative results draws attention to method issues such as test sensitivity or sampling error/bias—that is, whether a false-negative result was obtained because the experimental design was inadequate, the test sensitivity was too low, or sampling was performed at the wrong time. To address these items, several method-related issues should be discussed. The distance between the burn injury and the excisional wound was at least 1.5 cm. This relatively short distance would have been a greater issue if the rates of wound contraction were different between the burned and sham-burned animals, because the proximity of the burn could then have been argued to have a physical and/or paracrine effect on the excisional wound. In fact, the burn wound did seem to induce more retraction of the excisional wound margin (as indicated by a notably greater initial wound area in one of the two animal experiments), but nevertheless the wounds contracted at the same rate. In all likelihood, the loss of compliance in the burned skin caused a greater distraction of the edges of the excisional wound in the burned animals. If this local distracting effect were to influence the rate of wound contraction, it would seem logical that wound contraction would be slowed, but we did not observe this.

It could be argued that a paracrine effect of the burn wound actually accelerated the contraction rate of the nearby excisional wound, but this hypothetical paracrine effect was masked by the distracting effect of the burn wound. This theory, however, is not supported by the *in vitro* wound model, in that contraction of the FPCM stimulated by burn plasma was not different from that stimulated by sham-burn plasma (see Fig 4). Therefore, it is unlikely that the proximity of the burn wound to the excisional wound masked a

biologically important difference in wound contraction rates.

Another issue is the choice of serum vs. plasma. Serum is the liquid portion present after the clotting of whole blood. Activation of the clotting cascade and platelet degranulation releases a variety of soluble factors into serum with wide-ranging biological activities. Plasma is the liquid portion of unclotted whole blood. Because clotting has not occurred in plasma, it contains a different profile of mediators compared with serum. In dose-response assays in the FPCM, serum has 50% to 100% greater efficacy of stimulating contraction than plasma (see Fig 4; other data not shown). In most of our experiments with the FPCM outside of this project, we have used serum because of its greater efficacy. We chose to use plasma in the current study because, according to our hypothesis, the burn wound was believed to secrete factors that would enter the bloodstream (i.e., the plasma), travel to the excisional wound, and modulate contraction. Clotted blood is not a component of this hypothesis, so assays with serum only would confound the investigation.

A related issue is the time of plasma collection (postburn day 15). It is possible and even probable that the use of plasma collected on postburn day 1 would have produced a greater difference in matrix contraction than we observed with plasma from postburn day 15. The systemic inflammatory response that is prominent early on with a burn of this magnitude might have increased the chance for observing a large difference in the FPCM contraction assay. What we were trying to detect, however, was an effect during the prolonged healing phase of the burn, not a brief effect immediately postburn. Noting an effect on contraction stimulation in the FPCM immediately postburn would be difficult to interpret, and would have questionable clinical correlation because contraction *in vivo* does not engage fully until several days after wounding.

It cannot be disputed that major burn injury affects the function of a variety of tissues and metabolic processes. We had proposed originally that burn injury would moderate the rate of contraction in an adjacent excisional wound and in our *in vitro* wound model. We interpret our findings as indicating that a major burn injury

does not have a biologically important effect on the contraction of a secondary excisional wound during the prolonged healing phase of a burn. The relevance of these findings in a rodent model to human physiology remains unclear.

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